Comparative effectiveness of adding omega-3 and Manuka honey combination to conventional therapy in preventing and treating oxidative stress in pediatric β-thalassemia major – a randomized clinical trial

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Abstract. – **OBJECTIVE:** β-thalassemia major is an inherited hematological disorder with significant oxidative stress and iron overload. Oxidative stress results in several pathological complications, including cell death, tissue injury, organ dysfunction, and thyroid dysfunction. The present study examined the effectiveness of omega-3 and Manuka honey combination or Manuka honey alone to the conventional therapy (deferasirox, blood transfusion, and L-carnitine) used for preventing and managing oxidative stress or iron overload-induced oxidative stress conditions in pediatric β-thalassemic patients (type major).

PATIENTS AND METHODS: 165 patients participated in this randomized, double-blind, standard therapy-controlled, parallel-design multisite trial. The patients were randomly allocated into three groups, receiving either 1,000 mg omega-3 fish oil [350 mg eicosapentaenoic acid (EPA) and 250 mg docosahexaenoic acid (DHA)] combined with Manuka honey lozenge (344 mg) daily or Manuka honey alone plus the conventional therapy for ten months. Plasma 8-iso-prostaglandin F2α (8-iso-PGF2α), Lactate dehydrogenase (LDH), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), CRP (C-reactive protein), ferritin level, and serum iron were determined at baseline and month 10.

RESULTS: Omega-3 and Manuka honey combination were a significant add-on to conventional therapy of β-thalassemia in reducing the oxidative stress condition. The combination of Omega-3 and Manuka honey reduced the level of F2-isoprostane(8-iso-PGF2α) significantly compared to the Manuka alone and the control groups. Additionally, they showed an antihemolytic action measured by reduced LDH level. The combination restored the patient's lipid profile (LDL-C and HDL-C) significantly compared to the control group. Manuka honey enhanced the action of omega-3 in reducing oxidative stress by reducing serum iron significantly compared to the control group.

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CONCLUSIONS: Results showed that omega-3 + Manuka honey was more effective than Manuka alone or the conventional treatment alone in managing oxidative stress of β-thalassemic patients.

Key Words:
Omega-3, Oxidative stress, Thalassemia, Reactive oxygen species, Lipid peroxidation, Manuka Honey.

Abbreviations

ANOVA : two-way Analysis of Variance; CBC: complete blood count; CRP: C-reactive protein; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; GC-MS: gas chromatography-mass spectrometry; HbA: adult hemoglobin; HbA2: Hemoglobin A2; HDL-C: high-density lipoprotein cholesterol; HP: Hewlett-Packard; HPLC: high-performance liquid chromatography; I.D.: identification; LDL-C: low-density lipoprotein cholesterol; MGO: methylglyoxal; Post hoc LSD: after the fact least significant difference test; RBC: red blood cells; ROS: roxygen species; SPSS: Statistical Software Package for the Social Sciences; UMF: Unique Manuka Factor; ηp^2: partial effect size; 8-iso-PGF2α: 8-iso-prostaglandin F2α.

Introduction

Thalassemia major is a recessive disorder that results from a mutation in the β-globin locus, which results in a quantitative defect of hemoglobin synthesis and relative excess in α-globin. The excessive α-globin leads to the formation of insoluble aggerates that denote ineffective erythropoiesis. Accordingly, red blood cells cannot survive, and functional hemoglobin is reduced[1,2]. As such, reduced hemoglobin synthesis results in microcytic hypochromic anemia and reduced amount of adult hemoglobin (HbA) in patients' complete blood count (CBC)[3].

β-thalassemia is the most common type of hemoglobin disorder in Egypt. Among Egyptians, 9% carry β-thalassemia, and around 1,000 per 1.5 million per year live birth will suffer from β-thalassemia[4,5]. As evident, it is essential to understand and treat this condition effectively.

Iron overload is one of the significant consequences of oxidative stress, frequent blood transfusion, ineffective erythropoiesis, and increased iron gastrointestinal absorption. Iron overload alters the immune system and increases the risks of infection and severe illness[1,6]. Precisely, iron overload leads to iron deposition in visceral organs like the liver and heart, causing organ dysfunction[7]. Iron intake is typically integrated into heme units to form hemoglobin molecules. In excess iron (frequent blood transfusion and increased iron absorption), free iron remains unbounded and triggers the formation of reactive oxygen species (ROS)[8,9].

Hemoglobin instability results from the excess of α-chain globin, which is unstable and is oxidized into methemoglobin, then into hemichrome, followed by heme and free iron release. Thereby, ROS formation is activated[8,9].

In line with that, oxidative stress results from ineffective erythropoiesis inside the bone marrow. Oxidative stress due to ineffective erythropoiesis leads to hemolysis and the release of free hemoglobin that produces (ROS). The ROS are responsible for the damage of vital organs[7,10]. Primarily, ROS promote lipid peroxidation and leads to cell death. ROS leads to several pathological complications, including thyroid dysfunction, red blood cell (RBC) hemolysis, vascular inflammation, and ineffective erythropoiesis[11,12]. Of those ROS, F2-isoprostanes concentration levels were high in thalassemic patients, which was an indicative biomarker for oxidative stress[13,14]. Moreover, F2-isoprostane is the best and most accurate biomarker for lipid peroxidation and iron-induced oxidative stress[13-15]. Earlier studies[13,15-17] have investigated oxidative stress pathogenesis in various diseases, but little confirmed the oxidative stress biomarker associated with β-thalassemia major. Besides, most clinical studies[8,18,19] shed light on malondialdehyde (MDA) as the most common final product of polyunsaturated fatty acids peroxidation in the cells. Although, different types of F2-isoprostanes concentration levels were high in thalassemia patients[14,20]. Accordingly, further studies are needed to confirm which type of F2-isoprostanes were associated with β-thalassemia major disease.

Treating iron overload and oxidative stress is crucial as both are considered severe β-thalassemia complications.

The current therapies for β-thalassemia are pharmacological therapies (hydroxyurea medication, the gamma-globin chain inducer), gene therapy, iron chelation therapy, and blood transfusion[10,21]. None of those therapies have focused on treating oxidative stress as a significant complication of β-thalassemia. Previous clinical studies[22-24] have investigated the impact of antioxidants on β-thalassemia. In addition, antioxidants have improved the antioxidant defense system, reducing iron overload.

Previous clinical studies[25,26] have reported the antioxidant and antihemolytic effects of omega-3 supplements precisely against RBC hemolysis and lipid peroxidation. In line with that, previous clinical studies[27-29] have implied Manuka honey's chelation, antioxidant, and anti-inflammatory effects against iron overload and oxidative stress. Conversely, neither study has investigated the antioxidant combinations' effect in treating oxidative stress of β-thalassemia major disease[30-32].

The present study targeted pediatric patients with β-thalassemia major and clinical presentation of iron overload and ineffective erythropoiesis. The current study aims to investigate the effectiveness of the omega-3 and Manuka honey combination plus the conventional therapy (Deferasirox, blood transfusion, and L-Carnitine) against the conventional therapy alone in treating oxidative stress.

Patients and Methods

Study Design

A parallel-double blinded multisite randomized controlled trial (1:1 allocation ratio) was conducted from 2019-11-05 to 2020-11-05. The randomization process involved two independent researchers. The first researcher conducted random sampling and allocation using a 'random allocation software', version 1.00 (Isfahan University of Medical Sciences, Isfahan, Iran)[33]. While the second researcher implemented the randomized list using Microsoft Excel software version 2016. The randomized list generated a unique I.D. number for each patient to label each drug container delivered to the patients.

Based on the treatment plan, an independent nurse (blinded from the study) is delivered the medication container upon request by the treating physician. Patients and clinicians involved in the study were blinded to the assigned groups. The experimental and conventional treatments were packed in identical drug containers with the label generated by the randomized list to secure the blinding process.

Study Population and Participants

The pediatric patients attending the Beni-Suef and Giza governmental hospitals in Egypt for regular care at the pediatric hematology clinic were the target population for this study. The present research article demonstrated Egyptian pediatric patients aged 7-18 who presented with a β-thalassemia major type (HbA2 and HbF levels determination with hemoglobin electrophoresis), ineffective erythropoiesis, and iron overload were included. Clinical and laboratory investigations were performed during regular care at the pediatric hematology clinic to confirm the patient's condition.

The minimum sample size estimation for this present study was 150 patients. However, we recruited extra five (5) patients in each group to count future dropouts. All patients voluntarily participated in the trial through informed consent signed by the patient's parents or legal guardian before randomization. Patients were allocated in equal numbers into two study groups. Fifteen (15) patients were excluded from the final statistical analysis for compliance and irregular follow-up visits with the treating physician (Figure 1).

The allocated groups were the conventional therapy alone (deferasirox +/- blood transfusion and L-carnitine) that was the control group (55 patients), the Manuka honey supplements alone (55 patients), and the Omega-3 + Manuka honey supplements (55 patients), represented the experimental groups. All groups received conventional therapy (deferasirox +/- blood transfusion and L-carnitine). Patients were excluded when aged >18 years old and had any other type of anemia and renal or hepatic insufficiency that might alter the effectiveness of the medications.

According to the Helsinki Declaration, the present study was conducted as a multisite trial. It was approved by the Research Ethics Committee, Faculty of Medicine Beni-Suef University, Beni-Suef, Egypt, under FMBSUREC/07072019/Gamaleldin. The study was registered on clinicaltrials.gov, NCT04292314. In addition, biomedical ethics approval (HAPO-02-K-012-2020-02-359) was granted from the Faculty of Medicine, University of Umm Al-Qura in MAKKAH, Saudi Arabia, for study sites abroad within its faculty facilities and regional research centers.

Study Outcomes

The present study investigated the impact of the experimental interventions and treatment duration on oxidative stress (elevated F2-isoprostane) status, RBC's membrane stability, the severity level of anemia, and iron status of pediatric thalassemic patients. Oxidative stress was measured by the lipid peroxidation biomarker (8-iso-PGF2α), low-density lipoprotein cholesterol (LDL-C) levels, and high-density lipoprotein (HDL-C) levels. The correlated biomark-
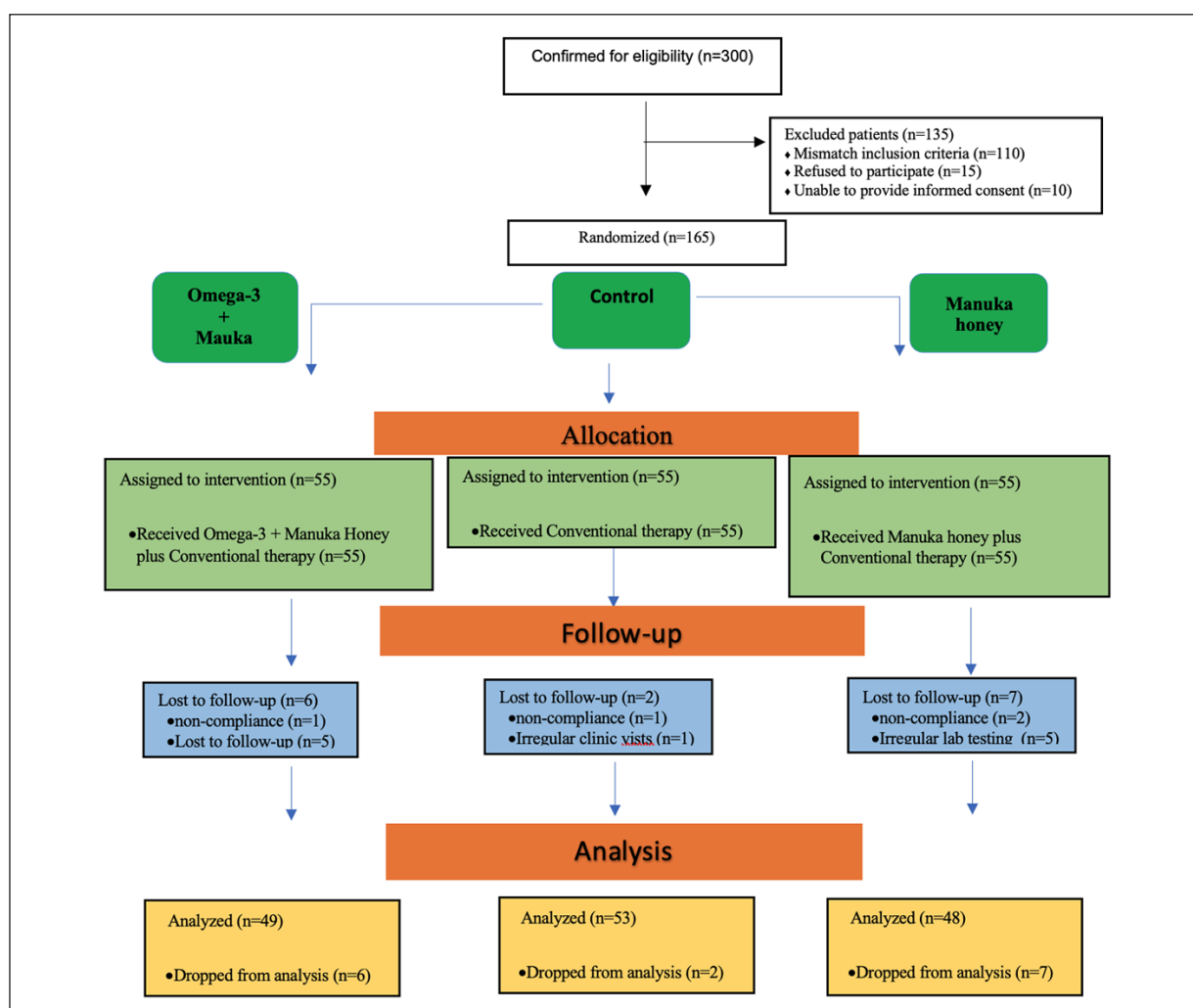
Figure 1. The study flow chart depicts the entire process of the clinical trial.

er C-reactive protein (CRP) measured RBC's membrane stability, and lactate dehydrogenase (LDH) measured RBC hemolysis. Iron status was measured by serum iron and ferritin level. Anemia severity level was measured by hemoglobin level (Hgb) and frequency of blood transfusion. The biochemical tests were performed at baseline (month zero) and then every month until the end of the study (month ten) to evaluate the effects of the interventions. The level of F2-isoprostanes was the primary outcome measure (measured every 100 days in all groups) in this study.

Moreover, the secondary outcome measures were LDL-C, HDL-C, CRP, serum iron, and serum ferritin. A 5-mL of venous blood was drawn into vacutainer tubes to analyze the hematological parameters using a hematology analyzer (LH-se-

ries and AU-series Beckman coulter commercial kits, Beckman Coulter Inc., Fullerton, CA, USA).

The clinical chemistry laboratory used automated procedures to measure the lipid profile on the biochemical auto-analyzer (Hitachi 912 auto-analyzer, Roche Boehringer Mannheim Diagnostics, Indianapolis, IN, USA). Concurrently, the F2-isoprostane levels were measured by a quantitative analysis using High-performance liquid chromatography [HPLC, Hewlett Packard (H.P.)], 1100 system (1100 series photodiode array detector, Wilmington, DE) coupled with Gas chromatography-mass spectrometry (GC-MS system, HP 5890 Series II GC and Trio 1000 MS, operated under negative chemical ionization, Fisons instruments, Manchester, United Kingdom) based on a protocol adapted from the Walter protocol[31].

A standard stock solution (500 pg/mL) was prepared, and the calibration curve was calculated as peak area[31].

Experimental and Conventional Therapy Interventions

The conventional therapy of β-thalassemia major was delivered to all patients in the study. Per treatment protocol, the standard therapy was deferasirox 21 mg/kg/day[35] for ten months (maximum dose of 28 mg/kg/days) until a definite hematologic response. In addition, patients in all groups received blood transfusions, when necessary, by the treating clinicians[36]. The two experimental therapies added to standard therapy were: omega-3 supplementation [350 mg (12 mg/kg/day) eicosapentaenoic acid (EPA) and 250 mg (6 mg/kg/day) docosahexaenoic acid (DHA)][26,37] for ten consecutive months or Manuka honey lozenge MGO-400 (Methylglyoxal-400)=344 mg (12 mg/kg/day), Unique Manuka Factor-13 (UMF-13) per day[29,38,39] for ten consecutive months. The adherence to study treatment was assessed every month at each follow-up visit by receiving the used treatment bottles from the patients.

Statistical Analysis

The statistical software SPSS 25 (IBM Corp., Armonk, NY, USA) was used for data analysis. A two-way analysis of variance (ANOVA) test was used to analyze the mean differences among groups for the continuous normally distributed primary outcome (changes of F2-isoprostanes levels) and the secondary outcomes (LDL-C, HDL-C levels, CRP, LDH, Ferritin, and serum iron). Kruskal-Wallis's H test was used to compare the median between the groups for continuous non-normally distributed variables (blood transfusion sessions) with a significance level adjusted to $p<0.05$. Statistical results from that test were presented as median [quartile-1 (percentile 25), quartile-3 (percentile 75)].

In addition, Pearson's Product-Moment Correlation analysis was done between the F2-isoprostane level and the serum ferritin level to identify the association's strength and direction.

The Shapiro-Wilk test was used to measure the normality of all outcomes. ANOVA two-way and Post-hoc LSD tests were used to analyze the main and interaction effects among the groups for continuous normally distributed variables. The two-way ANOVA test's assumptions were checked and met. The significance level was adjusted to $p<0.05$. Means and standard deviations (S.D.) values were presented to summarize the data analysis output.

To resolve the multiplicity of the present trial, the analysis of secondary outcomes was performed as a separate sub-trial from the primary trial measurement (analysis of F2 isoprostanes). The sample size for the primary and secondary outcomes was calculated using the power analysis procedures (*a priori* statistical test with ANOVA main/interaction effects). G-Power software, version 3.1[40], was used for the power analysis.

The sample size was calculated, taking into account primary and secondary outcomes. It was considered that 50 patients are the minimum sample size estimation for each group to detect a significant difference in the means value of the F2-isoprostanes with a pooled standard deviation=23, obtained from previous studies[14,41]. However, 165 patients were allocated, considering the dropout cases. The allocation was two experimental groups (every 55 patients) in the omega-3 + Manuka honey group, Manuka honey alone group, and 55 patients in the control group. The study powered at 80% with an 0.05 alpha level and a medium-size effect (partial η2 = 0.06).

Results

A total of 165 participants who met the inclusion criteria were allocated into three groups. Of those, 150 patients were subjected to the final analysis as follows: 49 (mean age 8.5±4, 60% male) were in the omega-3+Manuka group, 48 (mean age 10.5±3.5, 50% male) in the Manuka alone group, and 53 (mean age 11±5.5, 70% male) in the control group were analyzed. The flow chart of the study is shown in Figure 1. Table I shows the baseline characteristics of participants in the month zero study period. Figure 2 shows the GC-MS chromatographic quantitative analysis of 8-iso-PGF2α in months zero (before treatment) and ten (end of the study).

Results showed that the omega-3 plus Manuka honey combination significantly reduced the level of 8-iso-PGF2α after ten months of treatment compared to Manuka alone and control groups. Analysis of variance between the three groups investigating the main and interaction effects (Table II, Figures 3-6) showed that adding a combination of omega-3+Manuka to the conventional therapy was more significant than Manuka honey alone and control groups.

Table I. Baseline[b] characteristics of patients in month zero.

Variable	Control group	Manuka honey alone group	Omega-3 + Omega-3 + Mauka honey group
Age (y)	11 ± 3.5	9 ± 4.0[a]	12.5 ± 5.5
Gender (n)			
Male	29 (58%)	27 (54%)	30 (60%)
Female	21 (42%)	23 (46%)	20 (40%)
Plasma-8-iso-PGF2α (pg/ml)	541 ± 19	544 ± 15.6	538 ± 16.2
HDL cholesterol (mg/dL)	37 ± 7.3	35 ± 5.8	32± 6.7
LDL cholesterol (mg/dL)	50 ± 15.4	54 ± 10.5	52 ± 11.5
Lactic acid dehydrogenase (U/L)	519 ± 139	522 ± 145	513 ± 122
C-reactive protein (mg/dL)	3.1 ± 2.8	3.2 ± 3.9	2.9 ± 2.6
Ferritin (ng/mL)	1690 ± 202	1623 ± 189	1,700 ± 230
Serum iron (μg/dL)	136 ± 11.5	132± 12	135 ± 9.5
Hemoglobin level (g/dL)	6.5 ±0.89	7.2 ± 0.50	6.9 ± 0.67
Blood transfusion frequency[c] (number of sessions)	8.5 (8.1,9.1)[c]	8.9 (8.2,9.4)[c]	7.4 (6.9,8.1)[c]

S.D.: standard deviation. [a]Mean ± S.D. (all such values), [b]baseline data of the prior ten months of conventional therapy alone, reported at month zero, [c]Median (Quartile 1, Quartile 3).

Manuka honey supplementation reduced CRP levels significantly (p=0.048) compared to the control group, which is linked with endothelial dysfunction during oxidative stress of β-thalassemic patients, as reported in previous clinical studies[17,42]. At the same time, the analysis showed that Manuka honey supplementation significantly reduced the Ferritin level (p=0.043) and serum iron (p=0.045), which are linked to iron overload-induced oxidative stress and hemolysis process in β-thalassemic patients.
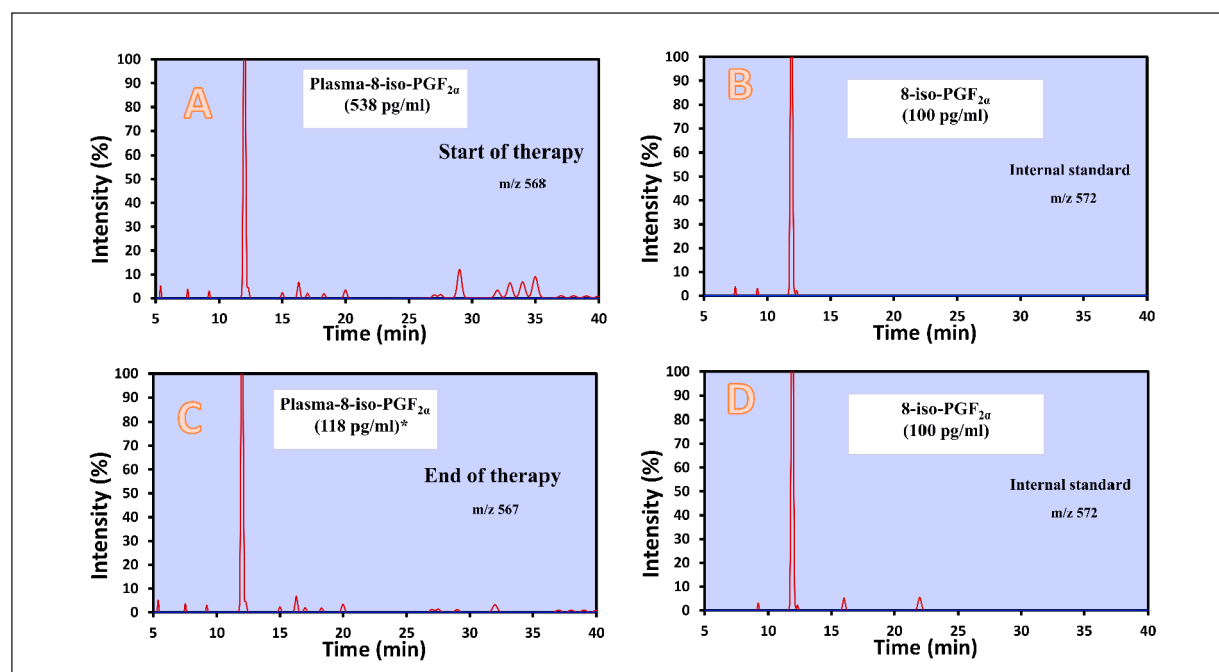

Figure 2. GC-MS chromatograms of Plasma-8-iso-PGF2α in month zero (before treatment) and month 10 (after ten months of treatment). **A**, mean of 8-iso-PGF2α in month zero at mass to charge ratio (m/z)=568 is 538 pg/mL. **B**, the mean of 8-iso-PGF2α in month zero at a mass-to-charge ratio (m/z)=567 is 118 pg/mL. **C-D**, are the internal standards detection of 8-iso-PGF2α. A combination of omega-e + Manuka honey significantly reduced the F2-isoprostane (8-iso-PGF2α), $p < 0.05$, compared to the manuka alone and control groups.

Table II. Mean changes for the evaluated outcomes after ten months of treatment compared to baseline values (before treatment).

Variable	Control group	Manuka honey alone group	Omega-3 + Mauka honey group	F-Statistics Kruskal-Wallis H Statistics
Plasma-8-iso-PGF2αc (pg/ml)	480 ± 4.04	156 ± 3.03*	**118 ± 4.53***,a	Drug factor, $F_{2,1617} = 47.08$ Treatment duration factor, $F_{10,1617} = 4.93$ Interaction, $F_{20,1617} = 3.39$
HDL cholesterol (mg/dL)	39 ± 5.6	40 ± 6.8*	**44 ± 4.2***,a	Drug factor, $F_{2,1617} = 12.11$ Treatment duration factor, $F_{10,1617} = 8.22$ Interaction, $F_{20,1617} = 4.60$
LDL cholesterol (mg/dL)	58 ± .10.5	76 ± 12.3*	**90 ± 11.6***,a	Drug factor, $F_{2,1617} = 245.7$ Treatment duration factor, $F_{10,1617} = 52.2$ Interaction, $F_{20,1617} = 24.38$
Lactic acid dehydrogenase (U/L)	498 ± 123	486 ± 133	**301 ± 118***,a	Drug factor, $F_{2,1617} = 126.3$ Treatment duration factor, $F_{10,1617} = 6.48$ Interaction, $F_{20,1617} = 3.36$
C-reactive protein (mg/dL)	2.6 ± 1.8	**1.2 ± 0.95***	1.9 ± 0.89*	Drug factor, $F_{2,1617} = 3.52$ Treatment duration factor, $F_{10,1617} = 4.48$ Interaction, $F20,1617 = 3.54$
Ferritin (ng/mL)	1310 ± 195	**659 ± 146***	670 ± 129*	Drug factor, $F2,1617 = 236.11$ Treatment duration factor, $F10,1617 = 43.7$ Interaction, $F_{20,1617} = 26.18$
Serum iron (ug/dL)	125 ± 9.8	**79 ± 8.3***	84 ± .7.6*	Drug factor, $F_{2,1617} = 223.11$ Treatment duration factor, $F_{10,1617} = 49.1$ Interaction, $F_{20,1617} = 21.36$
Hemoglobin level (g/dL)	7.1 ± 0.33	8.5 ± 0.45*	8.9 ± 0.86*	Drug factor, $F_{2,1617} = 15.46$ Treatment duration factor, $F_{10,1617} = 330$ Interaction, $F_{20,1617} = 119.12$
Blood transfusion frequencyb (number of sessions)	7.3 (6.7,7.8)	4.5 (4.5,1)*,c	3 (3.5,4.1)*,c	Kruskal-Wallis H = 7.674409

N.A., not applicable. *Significantly different from the control group at $p < 0.05$, aSignificantly different from all groups, bKruskal-Willis test, cMedian (Quartile 1, Quartile 3).

Omega-3 plus Manuka honey combination significantly reduced the level of F2-isoprostane ($p=0.036$) and LDH level ($p=0.041$). In line with this, the omega-3 plus Manuka honey combination increased the LDL-C ($p=0.032$) and HDL-C ($p=0.026$) significantly compared to the Manuka honey alone and control groups (Table II). Both are linked with lipid peroxidation, ROS, and hemoglobin instability in β-thalassemic patients.

Concurrently, blood transfusion frequency was significantly reduced in month ten with the omega-3 plus Manuka group ($p=0.046$) and Manuka alone group ($p=0.044$) compared to the control group. Hemoglobin level was significantly in-creased with the omega-3 plus Manuka group ($p=0.039$) and Manuka alone group ($p=0.041$) compared to the control group. All results are reported in Table II.

Pearson's correlation showed a high, positive correlation ($r=0 .804$, $n=1,650$, $p=0.038$) between 8-iso-PGF2α and ferritin levels. Kruskal-Willis test showed that there was a statistically significant difference in the frequency of blood transfusion between the study's groups, $H=7.674409$, $p=0.039$, with a mean rank frequency of blood transfusion of 809 for omega-3 plus Manuka honey combination group, 816 for Manuka honey alone group, and 851 for the control group.
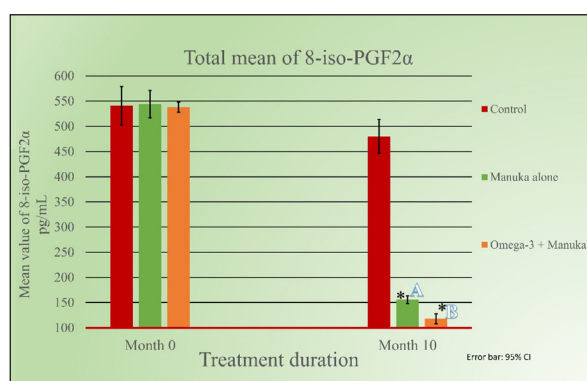
Figure 3. The therapeutic effects of the study's interventions on 8-iso-PGF2α level after ten months of treatments. The study interventions' effect on 8-iso-PGF2α level during ten months of treatment was measured as the total mean change of 8-iso-PGF2α from baseline value and the intervention effect size (partial eta squared, ηp^2). Manuka honey alone and omega-3 + Manuka honey significantly decreased the level of 8-iso-PGF2α (mean of 156 pg/mL, mean of 118 pg/mL, respectively) compared to the standard treatment (mean of 480 pg/mL). *Significantly different from the control group; A, $\eta p^2 = 0.62$; B, $\eta p^2 = 0.91$.
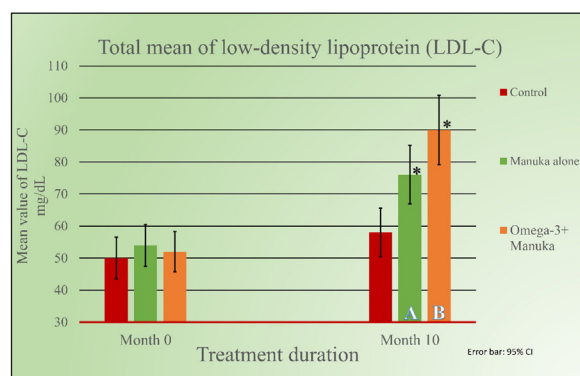

Figure 5. The therapeutic effects of the study's interventions on the low-density lipoprotein cholesterol (LDL-C) level after ten months of treatments. The study interventions' effect on the level of LDL-C during ten months of treatment was measured as the total mean change of LDL-C from baseline value and the intervention effect size (partial eta squared, ηp^2). Manuka honey alone and Omega-3 + Manuka honey significantly increased the level of LDL-C (mean of 76 mg/dL, mean of 90 mg/dL, respectively) compared to the standard treatment (mean of 58 mg/dL). *Significantly different from the control group; A, $\eta p^2 = 0.76$; B, $\eta p^2 = 0.89$.

Discussion

The present study indicated that omega-3 and Manuka honey was an effective add-on to the conventional therapy of pediatric β-thalassemia major disease. Omega-3 and Manuka honey supplementation reduced the 8-iso-PGF2α (biomarker of oxidative stress) significantly after ten months of treatment. In addition, lipid biomarkers (LDL-C and HDL-C) were restored significantly under the effect of the combination compared to the control treatment group.
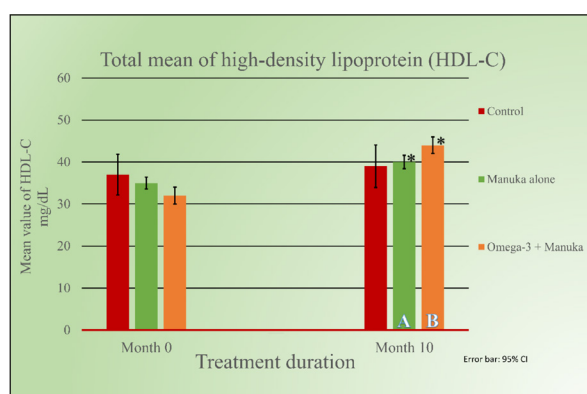

Figure 4. The therapeutic effects of the study's interventions on the high-density lipoprotein cholesterol (HDL-C) level after ten months of treatments. The study interventions' effect on the level of HDL-C during ten months of treatment was measured as the total mean change of HDL-C from baseline value and the intervention effect size (partial eta squared, ηp^2). Omega-3 + Manuka honey and Manuka honey alone significantly increased the level of HDL-C (mean of 44 mg/dL, mean of 40 mg/dL, respectively) compared to the standard treatment (mean of 39 mg/dL). *Significantly different from the control group; A, $\eta p^2 = 0.84$; B, $\eta p^2 = 0.91$.
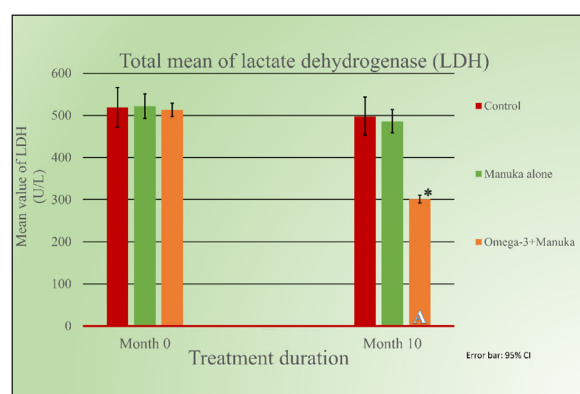

Figure 6. The therapeutic effects of the study's interventions on lactate dehydrogenase (LDH) levels after ten months of treatments. The study interventions' effect on the level of LDH during ten months of treatment was measured as the total mean change of LDH from baseline value and the intervention effect size (partial eta squared, ηp^2). Omega-3 + Manuka honey significantly reduced the level of LDH (mean of 301 U/L) compared to the manuka honey alone (mean of 486 U/L) standard treatment (mean of 498 U/L). *Significantly different from the control group; A, $\eta p^2 = 0.85$.

Omega-3 supplementation denoted a significant reduction in LDH level (antihemolytic effect) comparted to the Manuka alone and control groups. Oxidative hemolysis is caused secondary to the oxidative condition in β-thalassemia[19].

Manuka supplementation denoted a significant reduction in serum iron compared to the control group. Elevated iron has contributed to oxidative conditions in β-thalassemia[18].

Previous studies[14,43] have concluded that F2-isoprostane, the esterified eicosanoids found in tissue by radical oxidation, is correlated with several diseases. Similarly, it was reported that several diseases could be classified according to the level and type of F2-isoprostanes measured in those diseases[16]. Nevertheless, no study has confirmed the type of F2-isoprostane correlated with thalassemia disease, precisely β-thalassemia. Conversely, our findings concluded that 8-iso-PGF2α was significantly elevated in β-thalassemic patients, precisely β-thalassemia major.

Earlier research findings[44] have indicated that F2-isoprostane, malondialdehyde (MDA), and hydroperoxides are considered biomarkers of lipid peroxidation owing to oxidative stress. In addition, hypocholesterolemia has been reported in patients with β-thalassemia[45,46]. However, no convincing evidence exists on the correlation between 8-iso-PGF2α as a lipid peroxidation biomarker and hypocholesterolemia in pediatric β-thalassemia. Our findings indicated that the elevated 8-iso-PGF2α was specifically elevated and significantly positively correlated with hypocholesterolemia in pediatric β-thalassemia patients.

Previous studies[47,48] have reported that omega-3 supplementation affects oxidative stress by replacing the arachidonic acid (AA) in the RBC membrane, which is the precursor for the formation of F2-isoprostanes. In addition, omega-3 supplementation restores the lipid profile of the patient by restoring the RBC membrane's stability[25,49].

The effect of Manuka honey in treating oxidative stress has been proposed in earlier studies[23,28,50,51]. The phenolic and flavonoid components of Manuka honey counteract against lipid peroxyl and reactive oxygen species[50]. Additionally, Manuka honey exhibits free radical sequestration and metallic iron chelation.

Our findings indicated that adding omega-3 + Manuka honey lozenge for ten months to conventional therapy significantly reduced oxidative stress in thalassemia patients. Combining omega-3 and Manuka honey reduces oxidative stress by reducing lipid peroxidation and enhancing RBC's membrane lipid composition. As a result, they increase the HDL and LDL levels by interaction effect in thalassemic patients who demonstrated hypocholesteremia.

Indeed, hypocholesteremia and lipid peroxidation have been correlated to RBC hemolysis and ineffective erythropoiesis in oxidative stress[46]. Previous clinical studies[52,53] have investigated the various treatment modalities on the lipid profile of the thalassemic patient. However, they have not submitted sufficient evidence on the oxidative stress of those patients.

The combination of omega-3 and Manuka honey supplementation significantly reduces the elevated plasma-8-iso-PGF2α in patients. Previous clinical studies[24,54,55] have investigated the antioxidant effect of omega-3 and Manuka honey on the level of F2-isoprostane and lipid peroxidation. Nevertheless, they have not introduced convincing evidence of the antioxidant effect on 8-iso-PGF2α in thalassemic patients.

In line with that, our findings denoted that the omega-3 + Manuka honey combination significantly reduces the level of LDH. In agreement, earlier research findings[56,57] indicated that omega-3 supplementation showed an anti-hemolytic effect in several diseases.

For the Manuka honey alone group, Manuka significantly affects the iron status and endothelial dysfunction biomarkers by interaction effect measured as reduced ferritin and CRP levels, reflecting the patients' chelation activity and the inflammatory status of RBC.

Previous clinical studies[28,29,54] confirmed the antioxidant and chelation effects of Manuka honey in several medical conditions. Nevertheless, they have not introduced conceiving evidence of the Manuka honey effect on β-thalassemic patients. In addition, research findings from an earlier study[58] concluded that different ferritin levels and serum iron were not statistically significantly reduced after six months of supplementation with Greek honey. Conversely, our findings significantly reduced the ferritin, serum iron, and CRP levels compared to the control group.

Previous clinical studies[25,59] concluded that omega-3 supplementation improved the RBC's membrane lipid composition. At the same time, Methylglyoxal components in Manuka honey improved the hemoglobin stability of RBC. Our findings indicated that hemoglobin levels were significantly increased with the omega-3 and Manuka honey combination compared to the control group.

In addition, the frequency of blood transfusion was significantly reduced. Results indicated that cumulative responses for the experimental interventions were reported in reducing oxidative stress. Better outcomes could be accomplished over a more extended treatment duration than the ten months studied. Using IAC (immunoaffinity chromatography) instead of HPLC with GG-MS could be helpful in the detection of 8-iso-PGF2α in thalassemic patients.

Limitations

The present study denoted some limitations. First, the duration of treatment with the study's interventions shall be extended to a longer period to assess the tolerability of the combination dose on the pediatric population. Second, thalassemic patients with comorbid diseases should be included in further studies to assess the efficacy of the combination in treating oxidative stress in those patients.

Conclusions

Key findings from the study were that the omega-3 and Manuka honey combination was generally more effective in managing iron overload-induced oxidative stress than the conventional therapy alone or Manuka honey alone. The antioxidant and chelation effects of Manuka honey supplementation were confirmed. Although, the better outcome was confirmed when combined with omega-3 supplements.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

The present study was approved by the Beni-Suef University Institutional Review Board (IRB) (FMBSU-REC/07072019/Gamaleldin) and was registered on Clin-icalTrials.gov (NCT04292314). The study was conducted following the principles of the Declaration of Helsinki. The present article represents part two (Comparative Effectiveness of Adding Omega-3 and Manuka Honey combination to conventional therapy in preventing and treating oxidative stress in Pediatric β-thalassemia Major) and apart from a large clinical trial that targets β-thalassemia Major disease in the pediatric population. Part one of the clinical trial investigated the effectiveness of adding the Nigella sativa and Manuka honey combination to the conventional therapy in treating iron overload. Each part is independent of the other and has a different research hypothesis, results, and measuring outcomes.

Informed Consent

All participants were recruited voluntarily, and their legal guardians (parents) signed informed consent.

Availability of Data and Materials

The datasets generated during and/(or) analyzed during the current study are available from the corresponding author upon reasonable request. The data are secured and not publicly available due to their containing information that could compromise the privacy of the patients.

Authors' Contribution

The corresponding author, on behalf of all authors, states that all authors have contributed substantially to the design of the work. Conceptualization, Material preparation, data collection, and analysis were performed by [Mohamed M. Gamaleldin]. The first draft of the manuscript was written by [Mohamed M. Gamaleldin], and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Study design, supervision, review, and editing: [Ivo L Abraham], [Mohamed Hussein Meabed]; Review and supervision: [Ahmed A. Elberry]; Data interpretation, supervision, and review: [Raghda R.S. Hussein]; Data interpretation and statistical analysis: [Shaimaa M. Abdelhalim], [Dania saad waggas]. Dr. Raghda R.S. Hussein and Prof. Ivo L Abraham, respectively, were the chair and co-chair of the Ph.D. dissertation that generated the present article.

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References

1) Khandros E, Kwiatkowski JL. Beta thalassemia: Monitoring and new treatment approaches. Hematol Oncol Clin North Am 2019; 33: 339-353.

2) Nienhuis AW, Nathan DG. Pathophysiology and clinical manifestations of the beta-thalassemias. Cold Spring Harb Perspect Med 2012; 2: a011726.

3) Origa R. Beta-thalassemia. In: Adam MP, Mirzaa GM, Pagon RA, Wallace SE, Bean LG, Gripp KW, Amemiya A, ed. Genereviews® [internet]. Seattle (WA): University of Washington, Seattle; 2021.

4) El-Beshlawy A, Youssry I. Prevention of hemoglobinopathies in egypt. Hemoglobin 2009; 33 Suppl 1: S14-20.

5) El-Shanshory MR, Sherief LM, Hassab HM, Ragab SM, Yahia S, Mansour AK, Ahmed AS, Abdou SH, Helmy AM, Watany MM, Gad AAM, Guindy MA, Mourad ZI, Soliman MA, El-Farahaty RM, El-Dahtory F, Darwish A, Elmabood SA, Kabbash IA, Saied SM. Prevalence of iron deficiency anemia and beta thalassemia carriers among relatives of beta thalassemia patients in nile delta region, egypt: A multicenter study. J Egypt Public Health Assoc 2021; 96: 27.

6) Mishra AK, Tiwari A. Iron overload in beta thalassaemia major and intermedia patients. Maedica 2013; 8: 328-332.

7) Maria Sposi N. Oxidative stress and iron overload in β-thalassemia: An overview. In: IntechOpen; 2020.

8) Fibach E, Dana M. Oxidative stress in beta-thalassemia. Mol Diagn Ther 2019; 23: 245-261.

9) Bou-Fakhredin R, De Franceschi L, Motta I, Eid AA, Taher AT, Cappellini MD. Redox balance in beta-thalassemia and sickle cell disease: A love and hate relationship. Antioxidants (Basel) 2022; 11.

10) Ali S, Mumtaz S, Shakir HA, Khan M, Tahir HM, Mumtaz S, Mughal TA, Hassan A, Kazmi SAR, Sadia, Irfan M, Khan MA. Current status of beta-thalassemia and its treatment strategies. Molecular Genetics & Genomic Medicine 2021; 9.

11) Voskou S, Aslan M, Fanis P, Phylactides M, Kleanthous M. Oxidative stress in β-thalassaemia and sickle cell disease. Redox Biology 2015; 6: 226-239.

12) De Sanctis V, Soliman AT, Canatan D, Yassin MA, Daar S, Elsedfy H. Thyroid disorders in homozygous β-thalassemia: Current knowledge, emerging issues and open problems. Mediterr J Hematol Infect Dis 2019; 11: e2019029.

13) Ariningrum D, Adhipireno P, Suromo LB. Hyperferritinemia and oxidative stress in the kidney of beta thalassemia major. Bali Medical Journal 2019; 8.

14) Matayatsuk C, Lee C-YJ, Kalpravidh RW, Sirankapracha P, Wilairat P, Fucharoen S, Halliwell B. Elevated f2-isoprostanes in thalassemic patients. Free Radical Biology and Medicine 2007; 43: 1649-1655.

15) Chandra M, Panchatcharam M, Miriyala S. Biomarkers in ros and role of isoprostanes in oxidative stress. In: Ahmad R, ed. Free radicals and diseases [internet]. IntechOpen; 2016.

16) van 't Erve TJ, Kadiiska MB, London SJ, Mason RP. Classifying oxidative stress by f2-isoprostane levels across human diseases: A meta-analysis. Redox Biol 2017; 12: 582-599.

17) Cottone S, Mule G, Nardi E, Vadala A, Guarneri M, Briolotta C, Arsena R, Palermo A, Riccobene R, Cerasola G. Relation of c-reactive protein to oxidative stress and to endothelial activation in essential hypertension. Am J Hypertens 2006; 19: 313-318.

18) Sposi NM. Oxidative stress and iron overload in β-thalassemia: An overview. In: Marwa Z, Tamer H, eds. Beta thalassemia. Rijeka: IntechOpen; 2019:Ch. 6.

19) Hanan F. Abd-El Rasoul MDNAMPD. Evaluation of oxidative stress and antioxidant status in beta thalassemia major patients: A single-center study. The Medical Journal of Cairo University 2020; 88: 2147-2155.

20) P W, S A, P W. B-thalassemia - call for restoration of normal vitamin e status. Annals of Hematology & Oncology 2022; 9: 9-10.

21) de Dreuzy E, Bhukhai K, Leboulch P, Payen E. Current and future alternative therapies for beta-thalassemia major. Biomed J 2016; 39: 24-38.

22) Yanpanitch OU, Hatairaktham S, Charoensakdi R, Panichkul N, Fucharoen S, Srichairatanakool S, Siritanaratkul N, Kalpravidh RW. Treatment of beta-thalassemia/hemoglobin e with antioxidant cocktails results in decreased oxidative stress, increased hemoglobin concentration, and improvement of the hypercoagulable state. Oxid Med Cell Longev 2015; 2015: 537954.

23) Imam M, Zhang S, Ma J, Wang H, Wang F. Antioxidants mediate both iron homeostasis and oxidative stress. Nutrients 2017; 9: 671.

24) Petrosino T, Serafini M. Antioxidant modulation of f2-isoprostanes in humans: A systematic review. Crit Rev Food Sci Nutr 2014; 54: 1202-1221.

25) Tc K, Wnb D, Marlyne M, Ac P, Kansci G, Fokou E. Antisickling and antioxidant properties of omega-3 fatty acids epa / dha. 2019; 9: 1-6.

26) Katrenčíková B, Vaváková M, Paduchová Z, Nagyová Z, Garaiova I, Muchová J, Ďuračková Z, Trebatická J. Oxidative stress markers and antioxidant enzymes in children and adolescents with depressive disorder and impact of omega-3 fatty acids in randomised clinical trial. Antioxidants 2021; 10: 1256.

27) Blasa M, Candiracci M, Accorsi A, Piacentini M, Piatti E. Honey flavonoids as protection agents against oxidative damage to human red blood cells. Food Chemistry 2007; 104: 1635-1640.

28) Ankley LM, Monteiro MP, Camp KM, O'Quinn R, Castillo AR. Manuka honey chelates iron and impacts iron regulation in key bacterial pathogens. J Appl Microbiol 2020; 128: 1015-1024.

29) Alvarez-Suarez J, Gasparrini M, Forbes-Hernández T, Mazzoni L, Giampieri F. The composition and biological activity of honey: A focus on manuka honey. Foods 2014; 3: 420-432.

30) Sovira N, Lubis M, Wahidiyat PA, Suyatna FD, Gatot D, Bardosono S, Sadikin M. Effects of α-tocopherol on hemolysis and oxidative stress markers on red blood cells in β-thalassemia major. Clinical and Experimental Pediatrics 2020; 63: 314-320.

31) Srichairatanakool S, Fucharoen S. Antioxidants as complementary medication in thalassemia. In: InTech; 2014.

32) Bou-Fakhredin R, De Franceschi L, Motta I, Eid AA, Taher AT, Cappellini MD. Redox balance in β-thalassemia and sickle cell disease: A love and hate relationship. Antioxidants 2022; 11: 967.

33) Saghaei M. Random allocation software for parallel group randomized trials. BMC Med Res Methodol 2004; 4.

34) Walter MF, Blumberg JB, Dolnikowski GG, Handelman GJ. Streamlined f2-isoprostane analysis in plasma and urine with high-performance liquid chromatography and gas chromatography/mass spectroscopy. Anal Biochem 2000; 280: 73-79.

35) Wei Z, Yang G, Huang Y, Peng P, Long L, Long Y, Huang X, Zhou X, Lai Y, Liu R. A 15-years follow-up of deferasirox in beta-thalassaemia major patients with iron overload. Br J Haematol 2020; 191: e81-e83.

36. Farmakis D, Porter J, Taher A, Domenica Cappellini M, Angastiniotis M, Eleftheriou A. 2021 thalassaemia international federation guidelines for the management of transfusion-dependent thalassemia. Hemasphere 2022; 6: e732.

37) Woo J, Couturier J, Pindiprolu B, Picard L, Maertens C, Leclerc A, Findlay S, Johnson N, Grant C, Kimber M. Acceptability and tolerability of omega-3 fatty acids as adjunctive treatment for children and adolescents with eating disorders. Eating Disorders 2017; 25: 114-121.

38) Cohen HA, Varsano I, Kahan E, Sarrell EM, Uziel Y. Effectiveness of an herbal preparation containing echinacea, propolis, and vitamin c in preventing respiratory tract infections in children. Arch Pediatr Adolesc Med 2004; 158: 217-221.

39) El-Seedi HR, Eid N, Abd El-Wahed AA, Rateb ME, Afifi HS, Algethami AF, Zhao C, Al Naggar Y, Alsharif SM, Tahir HE, Xu B, Wang K, Khalifa SAM. Honey bee products: Preclinical and clinical studies of their anti-inflammatory and immunomodulatory properties. Front Nutr 2021; 8: 761267.

40) Faul F, Erdfelder E, Buchner A, Lang AG. Statistical power analyses using g*power 3.1: Tests for correlation and regression analyses. Behav Res Methods 2009; 41: 1149-1160.

41) Al-Azzawie HF. Association of vitamin profile with oxidant / antioxidant status in iraqi b -thalassemic major children. JPCR 2017; 4: 555643.

42) Medina-Leyte DJ, Zepeda-García O, Domínguez-Pérez M, González-Garrido A, Villarreal-Molina T, Jacobo-Albavera L. Endothelial dysfunction, inflammation and coronary artery disease: Potential biomarkers and promising therapeutical approaches. International Journal of Molecular Sciences 2021; 22: 3850.

43) Schwedhelm E, Benndorf R, Boger R, Tsikas D. Mass spectrometric analysis of f2-isoprostanes: Markers and mediators in human disease. Current Pharmaceutical Analysis 2007; 3: 39-51.

44) Janicka M, Kot-Wasik A, Kot J, Namieśnik J. Isoprostanes-biomarkers of lipid peroxidation: Their utility in evaluating oxidative stress and analysis. International Journal of Molecular Sciences 2010; 11: 4631-4659.

45) Haghpanah S, Davani M, Samadi B, Ashrafi A, Karimi M. Serum lipid profiles in patients with beta-thalassemia major and intermedia in southern iran. Journal of Research in Medical Sciences 2010; 15: 150-154.

46) Samal P, Mohanty J, Das S, Padma S. Serum lipid profiles of patients having beta-thalassemia in a tertiary care hospital. Ann Rom Soc Cell Biol 2021; 25: 14250-14257.

47) Yang J, Fernández-Galilea M, Martínez-Fernández L, González-Muniesa P, Pérez-Chávez A, Martínez JA, Moreno-Aliaga MJ. Oxidative stress and non-alcoholic fatty liver disease: Effects of omega-3 fatty acid supplementation. Nutrients 2019; 11: 872.

48) Mas E, Woodman RJ, Burke V, Puddey IANB, Beilin LJ, Durand T, Mori TA. The omega-3 fatty acids epa and dha decrease plasma f 2 -isoprostanes: Results from two placebo-controlled interventions. Free Radic Res 2010; 44: 983-990.

49) Daak AA, Lopez-Toledano MA, Heeney MM. Biochemical and therapeutic effects of omega-3 fatty acids in sickle cell disease. Complement Ther Med 2020; 52: 102482.

50) Ahmed S, Sulaiman SA, Baig AA, Ibrahim M, Liaqat S, Fatima S, Jabeen S, Shamim N, Othman NH. Honey as a potential natural antioxidant medicine: An insight into its molecular mechanisms of action. Oxid Med Cell Longev 2018; 2018: 8367846.

51) Wang X, Li Y, Han L, Li J, Liu C, Sun C. Role of flavonoids in the treatment of iron overload. Front Cell Dev Biol 2021; 9: 685364.

52) Tamaddoni A, Nasseri E, Mohammadi E, Qujeq D, Zayeri F, Zand H, Mir SM, Gholami M. A double-blind randomized controlled trial of curcumin for improvement in glycemic status, lipid profile and systemic inflammation in β-thalassemia major. Journal of Herbal Medicine 2020; 21.

53) Petelin A, Kenig S, Kopinč R, Deželak M, Černelič Bizjak M, Jenko Pražnikar Z. Effects of royal jelly administration on lipid profile, satiety, inflam-

mation, and antioxidant capacity in asymptomatic overweight adults. Evidence-based Complementary and Alternative Medicine 2019; 2019.

54) Martinello M, Mutinelli F. Antioxidant activity in bee products: A review. Antioxidants 2021; 10: 71.

55) Ogluszka M, Lipinski P, Starzynski RR. Interaction between iron and omega-3 fatty acids metabolisms: Where is the cross-link? Crit Rev Food Sci Nutr 2022; 62: 3002-3022.

56) Xin G, Eshaghi H. Effect of omega-3 fatty acids supplementation on indirect blood markers of exercise-induced muscle damage: Systematic review and meta-analysis of randomized controlled trials. Food Science & Nutrition 2021; 9: 6429-6442.

57) Kim YG, Lee JH, Raorane CJ, Oh ST, Park JG, Lee J. Herring oil and omega fatty acids inhibit staphylococcus aureus biofilm formation and virulence. Front Microbiol 2018; 9: 1241.

58) Tsamesidis I, Egwu CO, Samara D, Vogiatzi D, Lettas A, Lymperaki E. Effects of greek honey and propolis on oxidative stress and biochemical parameters in regular blood donors. Journal of Xenobiotics 2022; 12: 13-20.

59) Banerjee S, Chakraborti AS. Methylglyoxal modification enhances the stability of hemoglobin and lowers its iron-mediated oxidation reactions: An in vitro study. International Journal of Biological Macromolecules 2017; 95: 1159-1168.