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The Role of Inflammatory and Cell-Death Biomarkers in the Progression of Liver Fibrosis in Patients with β-Thalassaemia Major and Iron Overload

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ABSTRACT: Repeated blood transfusion and the inability to regulate iron levels in the liver of patients with β-thalassaemia major (BTM) cause chronic oxidative stress, which can trigger inflammatory and apoptotic pathways and, eventually, hepatic fibrogenesis. Five markers along this axis were measured by commercial sandwich ELISA kits in an analytical case–control study of 96 individuals (60 BTM patients and 36 healthy controls): tumour necrosis factor-α (TNF-α), interleukin-20 (IL-20) and nuclear factor-ϰB (NF-ϰB) for inflammation, caspase-3 for apoptosis and Mac-2 binding protein glycosylation isomer (M2BPGi) as a fibrosis-associated marker. Patients had significantly higher levels of all five markers than controls (2.1–3.0-fold; all Holm-adjusted $p<0.001$; Hedges' g 2.1–3.2). Within patients, TNF-α, IL-20, caspase-3 and NF-ϰB showed moderate-to-strong positive inter-correlations (r 0.60–0.90), indicating correlated inflammatory–apoptotic activation. M2BPGi showed only weak correlations with these inflammatory/apoptotic markers (r −0.11 to 0.03), while its largest observed association in the study matrix was an inverse correlation with LDH ($r=-0.33$). Ferritin showed weak positive correlations with the inflammatory markers (r 0.19–0.27). Thus, inflammation and apoptosis increase in parallel in iron-overloaded BTM, whereas the M2BPGi signal is only weakly coupled to the inflammatory–apoptotic markers cross-sectionally. The cross-sectional design does not allow causal inference or direct staging of fibrosis

1. INTRODUCTION

1.1 Background

Amongst the most prevalent inherited disorders globally, β-thalassaemia major (BTM) was estimated to affect >1.3 million people worldwide in 2021, with the highest burden observed in the Mediterranean basin, the Middle East and South-East Asia [1, 2]. The disease results from decreased or absent synthesis of the β-globin chain, which causes an imbalance in globin-chain synthesis, leading to the accumulation of excess α-chains in erythroid precursors, ineffective erythropoiesis and chronic haemolytic anaemia [1, 3]. Severe, early-onset, transfusion-dependent cases require lifelong regular transfusion, which is the primary treatment, together with iron-chelation therapy and multidisciplinary follow-up [1, 3, 4]. These treatments have improved survival but also cause a chronic iron burden, which is the major determinant of long-term organ complications [2, 4].

There is no physiological mechanism for excreting excess iron from the human body, and each transfusion delivers about 200–250 mg of iron, leading to a gradual increase in systemic iron over time in the absence of chelation [3, 4, 5]. The liver is an important early target because a substantial portion of the iron load is deposited in the liver, and hepatic injury is sustained

before clinical effects appear in other organs [4, 5, 8]. The most commonly used surrogate marker of body iron is serum ferritin; however, ferritin is an acute-phase reactant that may be affected by inflammation and hepatocellular injury, so it does not necessarily correspond to tissue iron [8, 9]. Chronic haemolysis, transfusion and gastrointestinal iron absorption yield a particularly high hepatic iron burden in BTM [5, 34].

The toxicity of excess iron is mainly redox-related. Once transferrin is saturated, non-transferrin-bound iron and labile plasma iron appear in the circulation; this redox-active iron penetrates hepatocytes, expands the labile intracellular iron pool and catalyses the Fenton and Haber–Weiss reactions that produce hydroxyl radicals and other reactive oxygen species (ROS) [5, 6, 7]. The resulting oxidative stress exhausts antioxidant defences, such as NADPH-dependent reducing systems, superoxide dismutase and reduced glutathione, and leaves oxidative footprints such as lipid-peroxidation products (isoprostanes) and oxidised DNA bases (8-hydroxy-2'-deoxyguanosine, 8-OHdG) [5, 7]. Repeated oxidative damage to hepatocytes triggers inflammatory signalling, hepatocyte apoptosis and the fibrogenic programme of hepatic stellate cells (HSCs), converting them into collagen-producing myofibroblasts [8, 9, 10, 11]. This suggests a mechanistic sequence (iron overload → oxidative stress/hepatocellular injury → inflammation → apoptosis → fibrogenesis) that underpins the marker panel examined here, together with the iron-status and oxidative-stress markers that surround it.

Each marker in the panel occupies a specific position on this axis. Nuclear factor-κB (NF-κB) is a key transcription factor in hepatic inflammation; it activates a cascade of pro-inflammatory cytokines and chemokines in injured hepatocytes and promotes the survival of activated stellate cells [10, 11, 19]. Its main effectors include tumour necrosis factor-α (TNF-α), which is transcriptionally regulated by NF-κB, both mirrors and enhances the inflammatory status, and links inflammation to hepatocyte death and stellate-cell activation [17, 18, 20]. Interleukin-20 (IL-20), a pro-inflammatory member of the IL-10 cytokine family, is over-expressed in fibrotic and cirrhotic liver, activates quiescent stellate cells and up-regulates transforming growth factor-β1 (TGF-β1), TNF-α and type-I collagen, forming an auto-amplifying fibrogenic loop [23, 24, 25]. Caspase-3 is the execution-phase cysteine protease of apoptosis; hepatocyte death is sufficient to initiate and sustain fibrogenesis, and the engulfment of apoptotic bodies by stellate cells increases their collagen production [12, 13, 16]. Lastly, the Mac-2 binding protein glycosylation isomer (M2BPGi), a glyco-biomarker of stellate-cell activation, is released into the circulation; its serum concentration is proportional to the stage of fibrosis in various chronic liver diseases, providing a simple, non-invasive fibrosis readout [27, 28, 31]. Together, these markers represent the entire inflammatory–apoptotic–fibrogenic pathway rather than five unrelated analytes.

In regions such as the Middle East, where β-thalassaemia is relatively prevalent and access to the complete range of transfusion, chelation and specialist monitoring is only partial, this question has special clinical relevance, as liver complications may build up before they are detected [2, 4]. In this context, markers that can be measured in a small blood sample and that directly interrogate the biology of liver injury are appealing both for research and, potentially, for future risk stratification. Analysing inflammatory, apoptotic and fibrosis-associated markers in combination (rather than separately) also makes it possible to test whether inflammatory and fibrotic processes occur as a coordinated whole or as partially separable processes, with implications for the potential use of these markers.

1.2 Knowledge gap

Although each of these processes has been studied in BTM, they are typically investigated in isolation, and most clinical studies rely on serum ferritin and liver enzymes, which do not directly interrogate the underlying biology [8, 9, 33]. Many uncertainties remain regarding non-invasive fibrosis assessment in BTM, and blood-based candidate markers have not been well studied in this unique context, where hepatic iron and inflammation can mask biochemical and imaging-based markers of fibrosis. It therefore remains unknown whether the activation of inflammatory signalling (TNF-α, IL-20, NF-κB) and apoptosis (caspase-3) in iron-overloaded BTM is associated, and quantitatively correlated, with circulating levels of a fibrosis-associated biomarker (M2BPGi), and how these relationships coexist with iron-status and oxidative-stress markers measured in the same patients. Addressing this requires the markers to be assessed together within the same cohort, in the context of their mutual relationships, rather than individually.

1.3 Aim, objectives and hypotheses

This study aimed to assess inflammatory and apoptotic markers and one circulating fibrosis-associated marker in patients with β-thalassaemia major and iron overload. The specific objectives were (i) to compare serum levels of TNF-α, IL-20, NF-ϰB, caspase-3 and M2BPGi between patients and healthy controls and to quantify the magnitude of the differences; and (ii) to examine, within the patient group, the correlations of these five markers with ten iron-status (ferritin, transferrin, hepcidin, bilirubin, lactate dehydrogenase) and oxidative-stress (isoprostane, NADPH, glutathione, superoxide dismutase, 8-OHdG) variables measured in the same individuals. We hypothesised that the inflammatory and apoptotic markers would be increased in patients and would parallel the increase in the fibrosis marker, suggesting a coordinated iron–inflammation–apoptosis–fibrosis response. Given the descriptive, cross-sectional design, all associations are regarded as observational and are not claimed to reflect cause and effect or a temporal order.

2. MATERIALS AND METHODS

2.1 Study design and setting

This was a cross-sectional, analytical case–control study based on 96 serum samples collected from 23 December 2025 to 15 April 2026. Patients were recruited at Al-Karamah Teaching Hospital/Blood Diseases Center in Baghdad (Karkh), Iraq, while healthy controls were recruited from several local sites during the same period. A case–control design was chosen because it allows the five biomarkers and the associated iron-status and oxidative-stress variables to be compared with a healthy reference population and is feasible in a single-centre study. Because each participant was assessed only once, the study does not examine progression of individual patients over time, but rather the inflammatory–apoptotic–fibrogenic axis at a single time point.

2.2 Participants and eligibility

A total of 96 participants aged 15–25 years were enrolled and divided into two groups: 60 patients with transfusion-dependent β-thalassaemia major (case group) and 36 apparently healthy controls. The diagnosis of β-thalassaemia major had to be confirmed by haemoglobin electrophoresis and/or molecular testing, together with documented transfusion dependence. A study record was completed for each participant at recruitment; medical and family history were documented, and participants were screened for other diseases that could confound the biomarker measurements. Patients with acute infection, an active autoimmune/inflammatory condition, malignancy, significant renal disease or any other identifiable cause of chronic liver disease were excluded to avoid confounding, as these conditions independently affect inflammatory, apoptotic and fibrosis-associated markers. The controls had no personal history of haemoglobinopathy, liver disease or chronic systemic illness. Baseline clinical information, including sex, transfusion history, chelation history and viral hepatitis status, was recorded at recruitment.

2.3 Blood sampling and pre-analytical handling

Blood was drawn from each participant under standard conditions, allowed to clot and centrifuged to separate the serum, which was then aliquoted and frozen for later batch analysis to avoid repeated freeze–thaw cycles. Haemolysed or lipaemic samples were excluded, as recommended by the manufacturers, because they may interfere with the colorimetric readings. Serum was the sample matrix for all five immunoassays, and all samples were brought to room temperature and mixed before analysis.

2.4 Biomarker measurement

Levels of the five target markers were measured using commercial human double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kits (Table I): human tumour necrosis factor-α (TNF-α; FineTest, Wuhan Fine Biotech, Wuhan, China; cat. no. EH0302), human nuclear factor-ϰB (NF-ϰB; FineTest EH3422), human caspase-3 (FineTest EH0546), human M2BPGi (FineTest EH4477) and human interleukin-20 (IL-20; ELK Biotechnology, Wuhan, China; cat. no. ELK1291). In each assay, the analyte binds to the pre-coated capture antibody and then to a biotinylated detection antibody, followed by a streptavidin–horseradish peroxidase conjugate, tetramethylbenzidine substrate and stop solution; optical density is read at 450 nm, and concentrations are interpolated from a standard curve prepared from the kit calibrator by two-fold serial dilution (four-parameter logistic fit). For the FineTest kits, 100 µL of standard or sample was incubated for

90 min at 37 °C, followed by 60 min with biotinylated antibody, 30 min with streptavidin–HRP and 10–20 min with TMB substrate, with washing between steps; for the ELK1291 kit, the corresponding incubations were 80, 50, 50 and 20 min at 37 °C. Samples were analysed in duplicate, and serum samples were diluted two-fold in sample-dilution buffer for the FineTest assays and assayed undiluted for the IL-20 assay, according to the manufacturer's instructions. The kit catalogue numbers, measurement ranges, sensitivities, serum volumes and manufacturer precision and recovery data are summarised in Table I. In addition to the five ELISA markers, iron-status (ferritin, transferrin, hepcidin, total serum bilirubin and lactate dehydrogenase) and oxidative-stress (isoprostane, NADPH, glutathione, superoxide dismutase and 8-OHdG) variables were measured in the same patients and included in the within-patient correlation analysis; these variables were not part of the patient–control comparison and are therefore reported only through the correlation analysis (Section 3.3).

TABLE I. Analytical characteristics of the five sandwich-ELISA assays (verified from the manufacturers' kit inserts).

Biomarker	Kit (manufacturer, cat. no.)	Range	Sensitivity	Serum	CV (intra / inter)	Recovery (serum)
TNF-α	FineTest EH0302	15.625–1000 pg/mL	9.375 pg/mL	50 µL	5.6–6.0% / 5.5–5.9%	92–103%
IL-20	ELK Biotechnology ELK1291	15.63–1000 pg/mL	6 pg/mL	100 µL	<8% / <10%	87–99%
NF-κB	FineTest EH3422	0.313–20 ng/mL	0.188 ng/mL	50 µL	4.7–5.4% / 5.0–5.9%	90–101%
Caspase-3	FineTest EH0546	0.313–20 ng/mL	0.188 ng/mL	50 µL	5.0–5.9% / 5.3–6.7%	95–100%
M2BPGi	FineTest EH4477	0.0156–1.0 ng/mL	0.0094 ng/mL	10 µL	3.7–5.1% / 5.4–5.5%	90–105%

2.5 Statistical analysis

Continuous variables are summarised as mean ± standard deviation (SD). Distributions were examined before comparison, and patient–control differences were assessed by two-group testing in GraphPad Prism 10.4.1. To quantify the differences more fully, each comparison is reported with Welch's t statistic, its degrees of freedom, a two-sided p value, the fold change (ratio of patient to control mean) and the Hedges' g standardised effect size with its 95% confidence interval (CI), all calculated from the group means, SDs and sample sizes. Because five related comparisons were made, the p values were corrected with the Holm–Bonferroni procedure to control the family-wise error rate. Within the patient group, associations among all measured variables were examined by Pearson correlation analysis in GraphPad Prism 10.4.1 and are displayed as the study correlation matrix (Fig. 3); selected principal coefficients, with Fisher-z 95% CIs and two-sided p values, are tabulated in Table IV. A two-sided $p<0.05$ was considered statistically significant.

3. RESULTS

3.1 Participant characteristics

The cohort comprised 60 patients with transfusion-dependent β-thalassaemia major and 36 healthy controls (96 serum samples in total). Patients were 15–25 years old; 35/60 (58%) were male and 25/60 (42%) were female, while the control group comprised 18 males and 18 females. Fifty-six patients (93%) were receiving iron-chelation therapy (38 deferasirox, 9 deferoxamine and 9 deferiprone), while 4 (7%) were not. Clinical records documented splenomegaly in 25 patients (42%), hepatosplenomegaly in 18 (30%) and previous splenectomy in 6 (10%); 11 (18%) had none of these complications. All 60 patients had a recorded family history of thalassaemia. All participants satisfied the eligibility criteria described in Section 2.2, and no sample was excluded from the biomarker analysis.

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3.2 Biomarker levels and comparison between patients and controls

Compared with controls, all five biomarkers were significantly increased (Table II); all differences were large and remained significant after Holm correction (Table III; Fig. 1). In absolute terms, TNF-α rose from 19.75±4.71 to 58.57±14.54 pg/mL, IL-20 from 47.54±22.19 to 128.99±27.20 pg/mL, NF-κB from 2.23±0.69 to 4.64±0.88 ng/mL, caspase-3 from 1.91±0.64 to 5.12±1.19 ng/mL, and M2BPGi from 0.527±0.274 to 1.438±0.506 ng/mL. The relative increases ranged from 108% (NF-κB) to 197% (TNF-α).

The degree of separation was marked. Hedges' g ranged from 2.08 (M2BPGi) to 3.24 (TNF-α), far exceeding the conventional threshold for a large effect (0.8; Fig. 2), and the lower bound of every 95% CI exceeded 1.5. TNF-α showed the largest standardised effect, followed by IL-20, caspase-3 and NF-κB, whereas the effect for M2BPGi, although still very large, was the smallest of the five, reflecting the wider relative dispersion of this marker in both groups. These results indicate that the entire inflammation–apoptosis–fibrosis panel was consistently and substantially elevated in patients.

TABLE II. Serum concentrations of the five biomarkers in healthy controls and β-thalassaemia major patients (mean ± SD).

Parameter	Unit	Healthy controls (n = 36)	Thalassaemia patients (n = 60)	Sig.*
M2BPGi	ng/mL	0.527 ± 0.274	1.438 ± 0.506	p<0.001
TNF-α	pg/mL	19.750 ± 4.705	58.574 ± 14.543	p<0.001
IL-20	pg/mL	47.538 ± 22.191	128.992 ± 27.195	p<0.001
NF-κB	ng/mL	2.228 ± 0.693	4.643 ± 0.882	p<0.001
Caspase-3	ng/mL	1.911 ± 0.642	5.119 ± 1.192	p<0.001

*Values are mean ± standard deviation. Patient–control comparisons were assessed by Welch's t test; *Holm-adjusted p value across the five biomarker comparisons (Table III).*

TABLE III. Group comparison and effect sizes of the five biomarkers between β-thalassaemia major patients and healthy controls.

Biomarker	Controls mean±SD	Patients mean±SD	Mean diff. [95% CI]	Fold change	Hedges' g [95% CI]	Welch t (df); p (Holm)
TNF-α, pg/mL	19.75±4.71	58.57±14.54	38.82 [34.77, 42.88]	2.97	3.24 [2.63, 3.86]	19.08 (77); <0.001
IL-20, pg/mL	47.54±22.19	128.99±27.20	81.45 [71.32, 91.59]	2.71	3.18 [2.57, 3.79]	15.97 (85); <0.001
NF-κB, ng/mL	2.23±0.69	4.64±0.88	2.41 [2.09, 2.74]	2.08	2.93 [2.35, 3.52]	14.89 (87); <0.001
Caspase-3, ng/mL	1.91±0.64	5.12±1.19	3.21 [2.84, 3.58]	2.68	3.11 [2.51, 3.72]	17.12 (93); <0.001

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Biomarker	Controls mean±SD	Patients mean±SD	Mean diff. [95% CI]	Fold change	Hedges' g [95% CI]	Welch t (df); p (Holm)
M2BPGi, ng/mL	0.527±0.274	1.438±0.506	0.91 [0.75, 1.07]	2.73	2.08 [1.57, 2.59]	11.43 (93); <0.001

Mean ± SD are the study's measured values. Mean difference 95% CI from Welch's method; Hedges' g with 95% CI calculated from the standardised mean difference (small-sample corrected); fold change = patient mean / control mean; df, Welch–Satterthwaite degrees of freedom (rounded); p values Holm-corrected across the five markers. All raw and Holm-adjusted p<0.001.

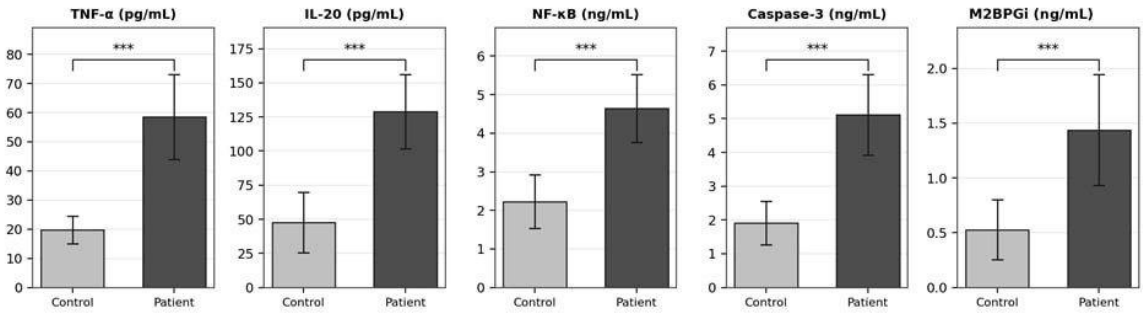


Fig. 1. Serum TNF-α, IL-20, NF-κB, caspase-3 and M2BPGi in patients and controls (bars = mean, whiskers = SD). ***Holm-adjusted p<0.001.

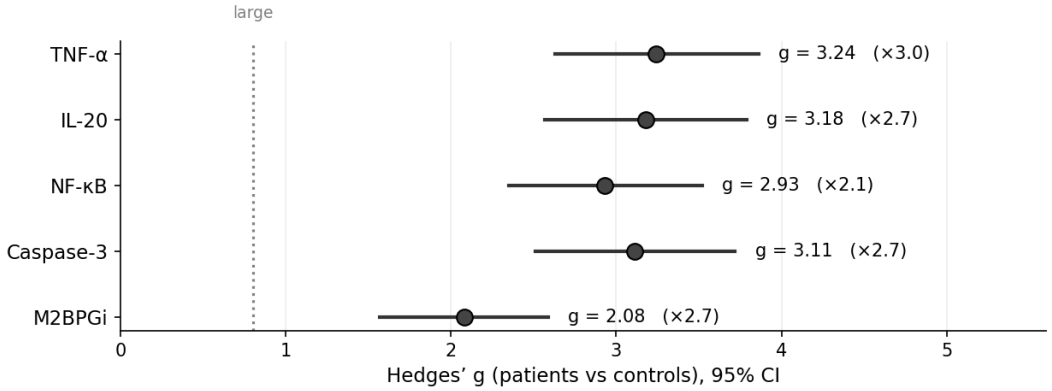


Fig. 2. Effect sizes (Hedges' g, 95% CI) and fold change (×) for each biomarker; all effects far exceed the large-effect threshold (dotted line, g = 0.8).

3.3 Correlation structure within the patient group

Within the patient group (n = 60), the strongest correlations occurred among the inflammatory and apoptotic markers. TNF-α correlated strongly with IL-20 (r=0.90), caspase-3 (r=0.78) and NF-κB (r=0.77); caspase-3 correlated with IL-20 (r=0.75) and NF-κB (r=0.60); and IL-20 correlated with NF-κB (r=0.63), all p<0.001. Strong relationships were also observed among oxidative-stress variables, including NADPH–glutathione (r=0.90), isoprostane–glutathione (r=−0.80) and isoprostane–NADPH (r=−0.75), all p<0.001. Among iron/haemolysis variables, ferritin and transferrin were inversely correlated (r=−0.83, p<0.001), whereas total bilirubin and LDH were positively correlated (r=0.70, p<0.001). These are correlation patterns in the study matrix and are not interpreted as formal clusters.

Ferritin showed weak positive correlations with TNF-α (r=0.25, p=0.054), IL-20 (r=0.27, p=0.037), caspase-3 (r=0.19, p=0.15) and NF-κB (r=0.21, p=0.11); ferritin also correlated weakly with SOD (r=0.26, p=0.045), and hepcidin with caspase-

3 (r=0.26, p=0.045). M2BPGi was only weakly correlated with the inflammatory/apoptotic markers (TNF-α r=−0.03, caspase-3 r=0.03, IL-20 r=−0.07, NF-κB r=−0.11; all p>0.05), but it showed a modest inverse correlation with LDH (r=−0.33, p=0.010). Thus, M2BPGi was largely uncoupled from the inflammatory–apoptotic markers cross-sectionally, but it was not unrelated to every measured variable.

TABLE IV. Principal correlations among measured variables in patients, with 95% confidence intervals and significance (n = 60; Fisher-z CI, t-test p).

Variable pair	r	95% CI	p	Interpretation
TNF-α – IL-20	0.90	0.84 to 0.94	<0.001	Inflammatory/apoptotic
TNF-α – Caspase-3	0.78	0.66 to 0.86	<0.001	Inflammatory/apoptotic
TNF-α – NF-κB	0.77	0.64 to 0.86	<0.001	Inflammatory/apoptotic
Caspase-3 – IL-20	0.75	0.61 to 0.84	<0.001	Inflammatory/apoptotic
Caspase-3 – NF-κB	0.60	0.41 to 0.74	<0.001	Inflammatory/apoptotic
IL-20 – NF-κB	0.63	0.45 to 0.76	<0.001	Inflammatory/apoptotic
NADPH – Glutathione	0.90	0.84 to 0.94	<0.001	Oxidative-stress relationship
Isoprostane – Glutathione	−0.80	−0.88 to −0.69	<0.001	Oxidative-stress relationship
Isoprostane – NADPH	−0.75	−0.84 to −0.61	<0.001	Oxidative-stress relationship
FER – Transferrin	−0.83	−0.90 to −0.73	<0.001	Iron transport (inverse)
TSB – LDH	0.70	0.54 to 0.81	<0.001	Haemolysis
M2BPGi – LDH	−0.33	−0.54 to −0.08	0.010	Fibrosis-associated marker–LDH
M2BPGi – TNF-α	−0.03	−0.28 to 0.23	0.820	Weak, NS
M2BPGi – Caspase-3	0.03	−0.23 to 0.28	0.820	Weak, NS
M2BPGi – IL-20	−0.07	−0.32 to 0.19	0.595	Weak, NS
M2BPGi – NF-κB	−0.11	−0.35 to 0.15	0.403	Weak, NS

NS, not significant; FER, ferritin; TSB, total serum bilirubin. Coefficients are taken from the GraphPad Prism correlation matrix from the study records (Fig. 3); CIs and p values were derived from r and n = 60. The full 17-variable matrix, including NF-κB, MCV and MCH, is shown in Fig. 3.

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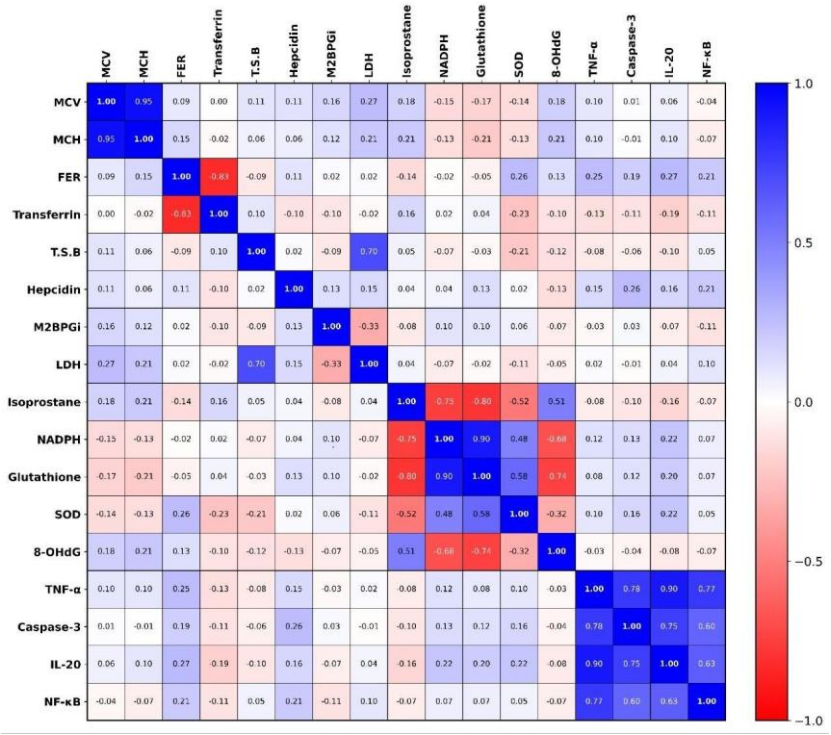


Fig. 3. Pearson correlation matrix (GraphPad Prism 10.4.1) from the study records for the patient group (n = 60), including MCV, MCH, iron-status, oxidative-stress, inflammatory, apoptotic and fibrosis-associated variables. Blue = positive, red = negative; values are Pearson r.

4. DISCUSSION

4.1 Principal findings

The aim of this study was to assess, in a single cohort, a panel of markers covering the inflammatory–apoptotic–fibrogenic axis in β-thalassaemia major with iron overload, together with the surrounding iron-status and oxidative-stress variables. Two main findings emerged. First, all five serum markers (TNF-α, IL-20, NF-κB, caspase-3 and M2BPGi) were significantly higher in patients than controls, with very large between-group effect sizes (Hedges' g 2.1–3.2). Second, the within-patient correlation pattern was not uniform: TNF-α, IL-20, caspase-3 and NF-κB were moderately to strongly inter-correlated, whereas M2BPGi showed only weak correlations with these inflammatory/apoptotic markers. M2BPGi did show a modest inverse association with LDH (r=−0.33), so it should not be described as independent of every measured variable. Overall, the data support coordinated inflammatory–apoptotic activation in iron-overloaded BTM while also showing that the relationship between the circulating fibrosis-associated signal and inflammation is not linear at a single time point.

4.2 Coupled inflammatory and apoptotic activation

The positive relationships among TNF-α, IL-20, caspase-3 and NF-κB are consistent with known hepatic biology and suggest concurrent inflammatory and apoptotic activation. In injured hepatocytes and activated Kupffer cells, NF-κB activation promotes inflammatory cytokine signalling and influences activated stellate-cell survival [10, 11, 14, 19]. In the present matrix, NF-κB correlated with TNF-α (r=0.77), IL-20 (r=0.63) and caspase-3 (r=0.60), while TNF-α correlated strongly with IL-20 (r=0.90) and caspase-3 (r=0.78). TNF-α links inflammation to hepatocyte injury and stellate-cell responses [17, 18, 20], and IL-20 can promote profibrogenic signalling in experimental liver disease [23–25]. The association of caspase-3 with these inflammatory markers is consistent with evidence that hepatocyte apoptosis and hepatocyte–stellate-cell interactions can contribute to fibrogenesis [12, 13, 15, 16]. The weak positive correlations of ferritin with the inflammatory markers are also compatible with oxidative stress and inflammation described in iron-overloaded β-thalassaemia [5, 6, 21, 26], although the cross-sectional design does not establish directionality or causation.

4.3 M2BPGi: elevated but weakly coupled

In patients, M2BPGi was higher than in controls, consistent with evidence that this molecule is associated with liver fibrosis severity in several chronic liver diseases [27–31]. However, this study did not include liver histology, elastography or another reference standard for fibrosis stage. The observed increase in M2BPGi should therefore be interpreted as an elevated fibrosis-associated biomarker signal, not as proof of active fibrosis or of a particular fibrosis stage in these patients. Its weak cross-sectional correlations with TNF-α, IL-20, caspase-3 and NF-ϰB may reflect the different biological time scales captured by circulating inflammatory/apoptotic markers and a fibrosis-associated marker, as well as unmeasured profibrogenic pathways. The modest inverse correlation between M2BPGi and LDH ($r=-0.33$) also shows that M2BPGi was not completely unrelated to the surrounding biological variables. Taken together, these findings indicate that systemic inflammatory or apoptotic markers should not be used as surrogates for M2BPGi in BTM on the basis of this cross-sectional dataset.

4.4 Iron status and oxidative stress

Weak positive correlations were observed between ferritin and several inflammatory markers, consistent with a relationship between iron burden and inflammatory activity and with the acute-phase behaviour of ferritin [4, 8, 21, 22]. The negative correlation between ferritin and transferrin ($r=-0.83$) is physiologically plausible. The oxidative-stress variables showed several strong pairwise relationships: NADPH and glutathione were strongly positively correlated ($r=0.90$), whereas isoprostane was inversely correlated with glutathione ($r=-0.80$) and NADPH ($r=-0.75$), and positively correlated with 8-OHdG ($r=0.51$). These patterns are compatible with altered redox balance and oxidative damage in iron-loaded β-thalassaemia [5, 7, 26, 35]. The positive correlation between bilirubin and LDH ($r=0.70$) is consistent with haemolysis. Overall, the matrix shows several biologically plausible relationships, but the present analysis is descriptive and should not be interpreted as proving discrete biological clusters or a fixed causal pathway.

4.5 Comparison with previous studies

Previous BTM studies have mostly correlated ferritin and conventional liver measures with liver injury and have used elastography or other non-invasive approaches to assess fibrosis [8, 9, 32–34]. In children and adolescents, significant hepatic fibrosis has been associated with older age, longer disease duration, chelation non-adherence and elevated ferritin levels [33]; hepcidin and conventional iron markers have been investigated in relation to liver damage [9]; and emerging candidates, such as circulating microRNAs, are being examined for minimally invasive staging of fibrosis in this population [32] [27–31]

4.6 Clinical and research implications

These findings have several implications, all of which are preliminary given the study design. If replicated against a fibrosis reference standard (histology, elastography or validated imaging), the panel could be used to stratify iron-loaded patients by inflammatory–apoptotic phenotype, offering an opportunity to monitor hepatic function more closely or to consider more intensive chelation in the subset of iron-loaded patients with more active inflammatory and apoptotic signalling. However, because the inflammatory/apoptotic and fibrosis signals were poorly coupled cross-sectionally, systemic cytokine and/or caspase-3 levels should not, at present, be used as surrogate markers of the fibrosis signal; the two reflect related but distinct aspects of the disease process. The ELISA kits are research-use-only reagents, and the panel is not a validated clinical diagnostic; its translation into a diagnostic tool would require standardisation of assays and thresholds. together with external and prospective validation.

4.7 Strengths and limitations

This study has several strengths. It measures a biologically coherent panel covering inflammation, apoptosis and a fibrosis-associated marker at a single time point; it includes a healthy control group; it reports group differences with effect sizes and multiplicity correction rather than p values alone; and it places the markers in the context of iron-status and oxidative-stress variables using the study correlation matrix. Several caveats must, however, be considered. The design is cross-sectional; therefore, the ‘progression’ described in the title and mechanistic framework is not demonstrated longitudinally. Fibrosis was indexed only by M2BPGi, without liver histology or elastography; M2BPGi is not equivalent to a measured fibrosis stage. M2BPGi is reported in ng/mL,because many clinical studies report M2BPGi as a cut-off index (COI) from automated lectin–

antibody immunoassays, the absolute values reported here are not directly comparable with published COI thresholds. Although baseline clinical descriptors were recorded and are reported descriptively, they were not included in multivariable analyses of the biomarkers, and the timing of sampling relative to the most recent transfusion was not analysed. The sample size did not permit more complex multivariable modelling, and the single-centre patient recruitment and research-use-only reagents limit generalisability and clinical inference. Prospective studies with a validated fibrosis reference standard are needed to determine temporal and prognostic relationships among iron status, inflammation, apoptosis and fibrosis.

5. CONCLUSION

The inflammatory markers TNF-α, IL-20 and NF-κB, the apoptotic marker caspase-3 and the fibrosis-associated marker M2BPGi were all significantly higher in patients with β-thalassaemia major and iron overload than in healthy controls. TNF-α, IL-20, caspase-3 and NF-κB were moderately to strongly inter-correlated, whereas M2BPGi was only weakly correlated with these inflammatory/apoptotic markers; M2BPGi showed a modest inverse association with LDH. These findings support coordinated inflammatory–apoptotic activation in iron-overloaded BTM but do not establish active fibrosis, fibrosis stage, temporal progression or causality. Prospective studies with a validated fibrosis reference standard are required before these biomarkers can be used for clinical fibrosis assessment.

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