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ORIGINAL ARTICLE

Alterations in iron status predict cardiac response to blood transfusion in β-thalassemia major

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Background and aim: Despite significant advancements in the management of thalassemia, cardiac complications still represent a leading cause of disability and death. Heart dysfunction, although mainly related to myocardial iron overload (IO), might already manifest when the homeostasis of circulating iron species is altered. This study aimed to investigate the presence of heart function changes in relation to scheduled blood transfusions (BT) in transfusion-dependent thalassemic patients, to identify alterations in cardiac function early after BT or within a 7-10 days interval. **Population and methods:** Twenty patients (8 females; average age 41.65 years), followed at the Center for Hereditary Anemias, University Hospital of Modena, were enrolled to perform an echocardiographic evaluation (ECE) before scheduled BT (T_0), a targeted ECE immediately after the transfusion (T_{early}), and a targeted ECE 7-10 days thereafter (T_{late}). Medical history, biochemical data, and parameters related to iron status including serum levels of labile plasma iron (LPI), non-transferrin-bound iron (NTBI), and 3 year-average serum ferritin, were collected to assess predictors of transfusion-related cardiac changes. **Results:** Global longitudinal strain (GLS) at baseline was worse, on average, in patients with higher ferritin or lower serum calcium; early post-transfusion GLS improved significantly in patients with ferritin>1500 ng/mL or albumin-corrected calcium <8.4 mg/dL, whereas it remained stable in control groups. Notably, several early post-transfusion changes could be consistently predicted by variables related to iron homeostasis or transfusion status. Cardiac MRI T2* showed moderate IO in only one patient. **Conclusion:** β-thalassemic patients with hyperferritinemia or hypocalcemia are likely those who benefit most from BT in terms of systolic function. Even in the absence of overt myocardial IO, alterations in circulating iron status predict early dysfunctions in cardiac response after scheduled blood transfusion.

KEYWORDS
thalassemia, anemia, iron, ferritin, blood tranfusion, systolic function, diastolic function, labile plasma iron, non-transferrin bound iron

Abbreviations

2C/3C/4C, 2/3/4 (apical) chamber view; AF, atrial fibrillation; AV, atrioventricular; BMI, body mass index; BT, blood transfusion; Hb, hemoglobin; EF, ejection fraction; GLS, global longitudinal strain; IO, iron overload; IVS, interventricular septal thickness; LA, left atrium; LPI, labile plasma iron; LV, left ventricle; LVEDD/V, left ventricle end-diastolic diameter/volume; LVESD/V, left ventricle end-systolic diameter/volume; MRI, magnetic resonance imaging; NTBI, non transferrin-bound iron; PAPs, pulmonary artery systolic pressure; PRBC, packed red blood cell unit; PW-TDI, pulsed wave tissue Doppler imaging; PWT, posterior wall thickness; STJ, sino-tubular junction; SV, sinus of Valsalva; SVPT, supraventricular paroxysmal tachicardia; TAA, toracic aortic arch; TAPSE, tricuspid annular plane systolic excursion; TDI, tissue doppler imaging; TDT, transfusion dependent thalassemia; TI, thalassemia intermedia; TM, β-thalassemia major; TRV, tricuspid regurgitation velocity.

1 | INTRODUCTION

In the last few decades, the prognosis and management of patients with β-thalassemia major (TM) have significantly improved, mainly thanks to novel treatment strategies such as optimized transfusion schedules, iron chelation and bone marrow transplantation [1, 2, 3, 4]. However, cardiac complications remain one of the leading causes of death in TM [1, 3, 5].

After the introduction of chronic transfusion therapy, myocardial iron overload (IO) has become the main mechanism of cardiovascular damage in transfusion-dependent thalassemic (TDT) patients. Cardiac IO results in heart failure, arrythmias like atrial fibrillation (AF), and pulmonary hypertension [6, 7]. Therefore, evaluation of IO and cardiac dysfunction by magnetic resonance imaging (MRI) as well as echocardiography is essential in the management of thalassemic patients [1, 8, 9]. Although MRI T2* measurement of iron deposits is the most established imaging technique for measuring cardiac IO, also echocardiographic tissue doppler imaging (TDI) parameters have shown significant correlations [10]. However, the clinical usefulness and applications of echocardiography in identifying IO-related cardiac abnormalities in patients with TM is still a matter of active investigation [11].

Chronic iron overload diseases, like β-thalassemia major, are characterized by the presence of circulating forms of non transferrin-bound iron (NTBI) with potentially toxic effects [12, 13]. In this regard, labile plasma iron (LPI), a redox active and chelatable component of NTBI, seems to be more accurate than ferritin in evaluating the efficacy of chelation therapy [14, 15]. Furthermore, some studies have started to investigate the role of serum NTBI/LPI in predicting heart disease in thalassemic patients [16, 17, 18]. Chronic blood transfusion schedules, in turn, represent both an iron- and volume- overload challenge, the combined effect of which could best be investigated through point-of-care assessments such as cardiac ultrasound. It should be noted that, even if temporary, the functional stress imposed by repeated transfusions might induce long-term alterations across years or decades.

With this pilot study, we sought to investigate through consecutive echocardiographic evaluations the presence of cardiac function changes in relation to scheduled blood transfusion (BT) in TDT patients and to explore the significance of possible clinical/biochemical predictors of such changes.

2 | MATERIALS AND METHODS

This monocentric, prospective, cohort study included 20 patients (12 males, 8 females; average age 41.65 years), followed at the Centre for Hereditary Anemias, University Hospital of Modena, with transfusion-dependent β-thalassemia major and older than 18 years. Patients underwent a physical examination and an echocardiographic evaluation before scheduled transfusion of packed red blood cell (PRBC), a targeted echocardiofocus evaluation immediately after the transfusion, and a targeted echocardiofocus evaluation 7-10 days thereafter (no other PRBC transfusions were performed within this interval).

For each patient, a complete medical history was recorded, with particular attention to chelation therapy, transfusion needs, previous diseases, comorbidities, and complications of thalassemia. Laboratory tests closest to the time of PRBC transfusion were recorded: blood chemistry included a complete blood count with formula, liver and kidney function tests, iron status (i.e. ferritin, iron and transferrin saturation), lipid panel, glycemia, glycated hemoglobin, fructosamine, TSH reflex, albumin-adjusted serum calcium, uric acid, vitamin D, vitamin B12, folic acid, and BNP; additionally, an average value of serum ferritin was calculated, considering a minimum period of three years. MRI T2* measurements of hepatic and cardiac iron content performed within 6 months before the inclusion in the study were collected for correlation studies; when not available, MRI estimations which were closest to the echocardiographic evaluations were retrieved to assess the presence of myocardial IO.

The following cardiac events were recorded, if present in the patients' medical history: heart failure, arrhythmic cardiopalm episodes resulting in hospitalisation or medical examination, arrhythmias such as atrial fibrillation (AF) or supraventricular paroxysmal tachicardia (SVPT).

2.1 | Echocardiographic evaluation

All examinations were performed with ESAOTE MyLabTM Omega ultrasound by a single operator, certified at the echocardiography school of the Italian Society of Echocardiography and Cardiovascular Imaging (SIECVI).

Evaluation of cardiac function was made using 2D-echocardiography, pulsed wave tissue Doppler imaging (PW-TDI) and Doppler color flow imaging, used to assess the intracardiac blood flow. All values were indexed for body surface area and included: left ventricle end-diastolic diameter (LVEDD, mm/m^2); interventricular septal thickness (IVS, mm) and posterior wall thickness (PWT, mm); left ventricle mass (gr/m^2); right and left atrial volumes (mL/m^2); ejection fraction (EF, %) calculated with Simpson's method; global longitudinal strain (GLS%), evaluated with speckle tracking technique in 2, 3, and 4 chamber views (2/3/4C) whenever technically feasible; left ventricular systolic speed curve calculated with TDI (s'); aortic and tricuspid flow velocity (m/s); pulmonary artery systolic pressure (PAPs, mmHg), calculated from tricuspid regurgitation peak; excursion of the tricuspid annular plane in M-mode (TAPSE, mm); right ventricle median diameter (mm); aortic diameter at the level of Valsalva sinuses (mm); aortic diameter at sino-tubular junction (mm); tubular ascending aortic diameter (mm); tele-inspiratory and tele-expiratory inferior vena cava diameter (mm); inferior vena cava collapsibility index (%). Left ventricle diastolic dysfunction was was classified according to 2016 ASE recommendations [9] and diagnosed by integrating multiple echocardiographic parameters such as maximum early diastolic wave speed (E wave, cm/s), maximum late diastolic wave speed (A wave, cm/s), E wave to A wave (E/A) ratio, maximum medial and lateral e' waves speeds (cm/s) calculated by TDI and E wave to average e' wave ratio (E/e' average).

2.2 | NTBI and LPI measurement

Serum non-transferrin bound iron (NTBI) and labile plasma iron (LPI) were dosed with a protocol modified from that of Esposito et al. [19]; all samples were stored at -80°C after collection, and, once thawed, were analysed in triplicate at 20 μL/well.

2.3 | Statistical analysis

Data were expressed as means (± standard deviation) for continuous variables, or percentages for nominal or ordinal variables. Volume variations ($\Delta_{T_{early}\text{-}T_0}$) were expressed as relative changes (e.g. $[LVEDV_{T_{early}}\text{-}LVEDV_{T_0}]/LVEDV_{T_0}$), so percentage change can

be obtained by multiplying the relative change by 100.

Comparison of continuous variables between groups was made using Student's t-test or Wilcoxon test when appropriate. Categorical variables were compared between groups using the Chi-square test. Due to the nature of the study and the limited sample size, values of $p < 0.099$ and ≥ 0.05 were reported as a trend.

Associations between variables were evaluated by considering either Pearson's or Spearman's r correlation coefficient, depending whether both variables complied to Shapiro-Wilk normality assumption or not. In all statistical evaluations a p value <0.05 was considered statistically significant.

Due to the exploratory nature of the study and the small sample size, only univariate linear regressions were performed, as the statistical power was considered insufficient to reliably support multivariable analyses. Therefore, the impact of confounding factors such as age, sex, and others could not be assessed. Similarly, adjustments for multiple comparisons were not applied, allowing a comprehensive exploration of correlations between clinical, biochemical, and functional data for each patient.

Data were collected on Microsoft Excel spreadsheets. Statistical analysis was performed with R [20].

2.4 | Ethical Approval

The protocol of the study was approved by the local Institutional Ethics Committee (Comitato Etico Area Vasta Emilia Nord - AVEN) and all patients gave their written informed consent. The study was performed in accordance with the Declaration of Helsinki.

3 | RESULTS

3.1 | Baseline characteristics

Table 1 reports the biochemical characteristics of the study population. INR, total cholesterol, GOT, serum calcium and uric acid were significantly higher in males. Average pre-transfusion Hb was 9.81±0.67 g/dL. Average serum ferritin was 1006.15±685.41 ng/mL. Average serum glucose, Hb1Ac, and fructosamine were 107.65±29.63 mg/dL, 57.41±12.02 mmol/mol and 288.83±52.92 μmol/mL, respectively.

BMI was 24.79±4.16 for females, 21.99±1.72 for males. Eighty percent of the total population was affected by type-II diabetes mellitus, 55% had hypogonadotropic hypogonadism. No significant differences in endocrinopathies were observed between sexes (Table S1). Sixty percent of patients was splenectomized (8 males; 4 females).

3.2 | Iron status and transfusion regimen

Most patients (60%) were under iron chelation therapy with deferasirox (one patient was under deferiprone-deferasirox association), 20% were under desferoxamine, 10% were under deferiprone, and 15% were under a deferiprone-desferoxamine association (Table 2). When available, cardiac MRI T2* measurements were negative for IO in all but one patient, who displayed a relaxation time compatible with moderate IO (Table S2).

All patients were regularly transfused with PRBC units (Table S3). For each patient, pre-transfusion serum ferritin levels averaged over either 1 or ≥ 3 years were substantially comparable.

3.3 | Echocardiography

Basal ecocardiography parameters are reported in Table S4 . Basal LVEDD, PWT, medial and lateral e' maximal velocity, LVED volume, and median RV diameter were significantly higher in males. LA volume was on average 31.92±13.65

mL/m^2 in males and 22.71±5.95 mL/m^2 in females. EF was normal on average (59.18%±6.84%), while GLS was at the lower limit of normal across all patient groups.. Most patients (85%) had at least mild diastolic dysfunction according to 2016 ASE guidelines [9]. Mild mitral, aortic, and tricuspidalic regurgitations were observed in 40%, 10%, and 30% of the patients, respectively. Average PAPs was 28.96±3.38 mmHg.

3.4 | Cardiac events

Several parameters differed significantly between patients who had experienced at least one cardiac event (n=12) and those who did not (n=8) (Table 3). Fructosamine and BNP were significantly higher in patients with cardiac events; likewise, Hb1Ac showed a non-significant trend. Echocardiographic parameters differed significantly in LVEDV and TAPSE, which were both reduced in patients with cardiac events. Right AV gradient and PAPs were non-significantly increased in patients with cardiac events.

3.5 | Comparison between pre- and post-transfusion echocardiographic parameters

LA volume and PAPs showed a statistical trend towards increasing after blood transfusion. Peak medial e' wave, end-expiratory and end-inspiratory VCI diameter increased significantly. At targeted evaluation performed 7-10 days after transfusion, the ejection fraction was significantly reduced compared to baseline (Table 4).

End-diastolic and end-systolic left ventricle volumes increased in patients with LPI or NTBI levels lower than 0.4 μmol/L or 1 μmol/L, respectively, but decreased in patients with LPI/NTBI values higher than those thresholds. Patients with higher ferritin or lower serum calcium had worse baseline GLS values (due to low sample sizes, no statistical test was deemed feasible). Notably, early after transfusion patients with ferritin >1500 ng/mL or serum calcium <8.4 mg /dL significantly improved in systolic function, as compared to control groups in whom GLS was substantially unchanged (Figure 1).

3.6 | Correlations between basal echocardiographic and clinical parameters

Basal echocardiographic parameters and absolute/relative changes between T_{early} and T_0 were correlated with several clinical and biochemical variables. Due to low sample size, correlations with T_{late} parameters were not considered. Overall, several significant correlations were identified (Table S5). The variables which most consistently predicted basal echocardiographic characteristic, as well as early post-transfusion changes were: age, BMI, monthly PRBC transfused, average pretransfusion Hb, total and conjugated bilirubin, serum calcium, folic acid, BNP, and variables related to iron homeostasis/iron overload status. A selection of scatter plots is displayed in Figure 2. LPI correlated positively with basal LVEDD, while both LPI and NTBI correlated negatively with $\Delta_{T_{early}\text{-}T_0}$ LVEDV and LVESV (% change). Higher levels of serum calcium and folic acid correlated positively with ΔGLS.

3.7 | Tables and Figures

TABLE 1 Biochemical characteristics of the study population at baseline; ns, not significant.

Basal blood chemistry parameters	Total (n=20)	Males (n=12)	Females (n=8)	p*	Reference range
White blood cells (10^3/mm^3)	9.98±5.38	9.06±3.69	11.35±7.32	ns	(4.0–10.9)
Red blood cells (10^6/mm^3)	3.47±0.26	3.54±0.23	3.36±0.28	ns	(M: 4.5–6.0, F: 4.0–5.4)
Hb (g/dL)	9.81±0.67	9.87±0.72	9.72±0.63	ns	(M: 13–17, F: 12–16)
Hematocrit (%)	30.39±2.76	30.85±2.67	29.71±2.94	ns	(M: 40–54, F: 36–48)
MCV (fL)	87.6±3.82	87.11±4.02	88.32±3.65	ns	(80–100)
MCH (pg)	28.32±1.31	27.88±1.17	28.98±1.29	0.06	(27–33)
MCHC (g/dL)	32.57±1.55	32.06±1.14	33.33±1.85	0.07	(32–36)
RDW (cv%)	15.91±2.00	16.60±2.11	14.87±1.33	0.05	(12.6–15.8)
PLT (10^3/mm^3)	486.85±230.81	505.08±258.23	459.50±195.91	ns	(150–400)
INR	1.11±0.12	1.16±0.12	1.04±0.07	0.02	(0.8–1.2)
aPTT	1.1±0.21	1.13±0.21	1.07±0.22	ns	(0.8–1.2)
Serum glucose (mg/dL)	107.65±29.63	101.00±19.90	117.62±39.63	ns	(70–110)
Serum creatinine (mg/dL)	0.73±0.20	0.77±0.20	0.68±0.18	ns	(M: 0.7–1.3, F: 0.6–1.1)
eGFR Cockcroft-Gault (mL/min)	110.89±33.49	120.57±35.10	96.37±26.58	ns	(>60)
Uric acid (mg/dL)	5.02±1.52	5.84±1.21	3.53±0.59	0.01	(M: 3.5–7.2, F: 2.6–6.0)
Total cholesterol (mg/dL)	110.47±26.13	99.54±26.28	125.5±17.99	0.02	(<200)
HDL (mg/dL)	46.47±13.7	43.36±12.88	50.75±14.47	ns	(M: >40, F: >50)
LDL (mg/dL)	56.73±19.84	49.81±19.41	66.25±17.20	0.07	(<115)
TG (mg/dL)	80.22±31.91	80.40±40.9	80.00±17.92	ns	(<150)
Tot. Bil. (mg/dL)	2.15±1.32	2.52±1.57	1.60±0.52	ns	(0.2–1.2)
Albumin (g/dL)	4.51±0.35	4.60±0.38	4.37±0.29	ns	(3.5–5.0)
GOT (U/L)	23.47±9.70	27.18±10.94	18.37±4.43	0.04	(M: <40, F: <32)
GPT (U/L)	23.3±18.94	28.25±22.85	15.87±6.93	ns	(M: <41, F: <31)
GGT (U/L)	28.00±27.07	34.66±32.73	18.00±10.69	ns	(M: <60, F: <40)
ALP (U/L)	75.8±21.97	78.25±23.79	72.12±19.88	ns	(30–120)
LDH (U/L)	344.00±89.41	337.90±73.84	351.62±110.84	ns	(125–220)
Na (mEq/L)	138.20±2.04	138.00±1.85	138.50±2.39	ns	(135–145)
K (mEq/L)	4.05±0.27	4.10±0.26	3.96±0.29	ns	(3.5–5.0)
Ca (mg/dL)	9.00±0.63	9.23±0.44	8.66±0.73	0.04	(8.5–10.5)
P (mg/dL)	3.22±0.63	3.11±0.58	3.37±0.70	ns	(2.5–4.5)
Fe (mcg/dL)	259.63±76.89	258.27±44.85	261.50±111.00	ns	(M: 65–176, F: 50–170)
Transferrin (mg/dL)	176.5±30.67	181.10±34.89	170.75±25.51	ns	(200–360)
Transferrin saturation (%)	105.22±29.73	103.10±9.14	107.87±45.00	ns	(20–50)
Ferritin (ng/mL)	1006.15±685.41	989.08±672.73	1031.75±50.14	ns	(M: 30–400, F: 15–150)
Fructosamine (mmol/L)	288.83±52.92	275.30±40.28	305.75±64.24	ns	(205–285)
TSH (μIU/mL)	2.01±0.98	2.16±0.84	1.82±1.16	ns	(0.4–4.0)
Vitamin D (ng/mL)	28.43±13.47	26.26±16.26	31.84±7.17	ns	(>30)
HbA1c (mmol/mol)	57.41±12.02	55.40±7.01	60.28±17.18	ns	(<48)
BNP (pg/mL)	100.55±92.38	105.25±115.53	93.50±45.75	ns	(<100)
Vitamin B12 (pg/mL)	349.72±157.63	364.27±157.34	326.85±167.76	ns	(200–900)
Folic acid (ng/mL)	6.68±4.83	7.40±5.81	5.55±2.74	ns	(3.0–20.0)

TABLE 2 Iron chelating and cardiovascular therapies in the study population. ARBs, angiotensin receptor blockers; DOACs, direct oral anticoagulants.

Therapy	Total patients (n=20)	Males (n=12)	Females (n=8)ns
Iron chelation therapy			
DFO (desferrioxamine)	4 (20%)	2 (16.6%)	2 (25%)
DFP (deferiprone)	2 (10%)	1 (8.3%)	1 (12.5%)
DFX (deferasirox)	12 (60%)	9 (75%)	3 (37.5%)
DFO+DFP	3 (15%)	1 (8.3%)	2 (25%)
Previous iron chelation therapy			
DFO	13 (65%)	6 (50%)	7 (87.5%)
DFP	11 (55%)	7 (58.3%)	3 (37.5%)
DFX	7 (35%)	4 (33.3%)	3 (37.5%)
DFO+DFP	5 (25%)	3 (25%)	2 (25%)
Cardiovascular therapy			
β-blockers	5 (25%)	4 (33.3%)	1 (12.5%)
ARBs	4 (20%)	2 (16.6%)	2 (25%)
Calcium antagonists	1 (5%)	1 (8.3%)	0 (0%)
DOACs	4 (20%)	4 (33.3%)	0 (0%)
Anti-platelet agents	4 (20%)	2 (16.6 %)	2 (25%)
Diuretics	1 (5%)	1 (8.3%)	0 (0%)

TABLE 3 Clinical, biochemical, and echocardiographic parameters in patients with or without previous cardiac events (abbreviations explained in the specific section at the beginning of the paper; ns, not significant).

Patient characteristics	Previous cardiac events (n=12, 60%)	No previous cardiac events (n=8, 40%)	p	Normal range
Glucose metabolism				
Basal Hb1Ac (mmol/mol)	61.30±14.49	51.85±2.96	ns	(< 38)
Fructosamine (μmol/L)	312.09±54.04	252.28±22.74	0.01	(200–285)
BNP (pg/mL)	135.66±104.71	47.87±25.45	0.01	(< 100)
Basal echocardiographic parameters				
IVS thickness (cm)	1.04±0.14	0.96±0.12	ns	(M: <1.1, F: <1.0)
LVEDD (cm)	4.42±0.56	4.66±0.41	ns	(M: 4.2–5.9, F: 3.9–5.3)
PWT (cm)	0.84±0.09	0.83±0.11	ns	(M: <1.1, F: <1.0)
LV mass (g/m^2)	89.46±21.28	84.62±12.37	ns	(M: 49–115, F: 43–95)
STJ diameter (cm)	2.52±0.18	2.72±0.39	ns	(M: 2.0–3.6, F: 1.7–3.1)
SV diameter (cm)	2.88±0.24	3.19±0.39	0.05	(M: 2.5–3.6, F: 2.2–3.4)
TAA diameter (cm)	2.89±0.23	3.09±0.34	ns	(< 3.8)
LA volume (mL/m^2)	29.96±13.99	25.66±8.14	ns	(M: <40, F: <38)
E peak velocity (cm/s)	97.75±19.13	93.87±16.10	ns	(50–90)
E/A ratio	1.77±0.74	1.37±0.37	ns	(1–2)
Medial e' peak velocity (cm/s)	11.75±2.70	12.62±3.92	ns	(> 7 cm/s)
Lateral e' peak velocity (cm/s)	16.00±4.63	19.37±5.95	ns	(> 10 cm/s)
Average E/e' ratio	7.07±2.99	5.99±1.62	ns	(< 14)
LV s' peak velocity (cm/s)	11.00±1.85	9.41±2.15	ns	(> 8)
LVEDV (mL/m^2)	56.65±11.66	68.79±10.30	0.02	(M: 60–105, F: 55–90)
LVESV (mL/m^2)	23.70±6.60	26.41±5.96	ns	(M: 20–40, F: 15–35)
EF (%)	58.08±7.41	61.25±5.87	ns	(M: >52, F: >54)
GLS (%)	-18.11±3.44	-19.75±3.44	ns	(< -20)
Diastolic dysfunction	9	8	ns	(Absent)
Grade 1	7	7	ns	-
Grade 2	-	-	-	-
Grade 3	2	0	ns	-
Grade 4	0	1	ns	-
Right AV gradient (mmHg)	24.90±3.67	21.6±1.51	0.07	(< 25)
PAPs (mmHg)	29.92±3.71	26.6±1.51	0.07	(< 35)
TRV (m/s)	2.49±0.19	2.33±0.08	ns	(< 2.8)
TAPSE (mm)	21.59±3.28	27.38±2.09	0.00	(> 17)
Transfusion regime				
Monthly PRBC units	2.84±0.62	3.26±0.89	ns	(n/a)
Average PRBC units per transfusion	1.50±0.46	1.80±0.35	ns	(n/a)
Average pre-transfusion Hb	9.65±0.26	9.56±0.55	ns	(M: >13, F: >12 g/dL)
Average between-transfusion interval (days)	16.22±5.03	17.37±4.13	ns	(n/a)
Average pre-transfusion ferritin	1003.76±598.44	962.75±552.52	ns	(M: 30–400, F: 15–150 ng/mL)
Iron status				
LPI (μmol/L)	0.88±1.18	0.99±1.53	ns	(< 0.4)
NTBI (μmol/L)	4.68±4.24	2.94±2.21	ns	(< 0.9)

TABLE 4 Comparison between pre-transfusion (T_0), early post-transfusion (T_{early}), and late post-transfusion (T_{late}) echocardiographic parameters.* T_0 vs T_{early}; ** T_0 vs T_{late}.
Abbreviations: EF, ejection fraction; GLS, global longitudinal strain; IVC, inferior vena cava; PAPs, systolic pulmonary artery pressure; LA, left atrium; LV, left ventricle; EDV, end-diastolic volume; ESV, end-systolic volume; TAPSE, tricuspid annular plane systolic excursion; TRV, triscuspid regurgitation velocity.

	T_0	T_{early}	T_{late}	p^*	p^{**}
LA volume	28.24±11.93	30.79±10.70	28.76±9.70	.06	ns
E peak velocity	96.73±18.02	99.15±16.42	85.54±14.05	ns	ns
E/A ratio	1.58±0.63	1.63±0.67	1.54±0.60	ns	ns
Medial e' peak velocity	12.10±3.17	13.50±3.81	11.31±3.15	**.01**	ns
Lateral e' peak velocity	17.35±5.32	17.80±4.36	16.31±4.13	ns	ns
Average E/e' ratio	6.84±2.59	6.66±2.07	6.31±2.07	ns	ns
LV s' peak velocity	10.05±2.13	10.05±2.41	9.96±1.61	ns	ns
LV EDV	61.80±12.72	60.89±11.49	55.66±13.78	ns	ns
LV ESV	24.80±6.50	24.15±4.69	23.70±5.57	ns	.091
EF	59.35±6.86	59.35±6.41	57.62±5.72	ns	**.047**
GLS	-18.92±3.49	-19.36±3.53	-18.67±11.33	ns	ns
Right AV gradient	24.20±3.55	25.75±3.80	24.77±5.03	ns	ns
PAPs	28.87±3.20	31.13±3.97	28.83±4.80	.08	ns
TRV	2.46±0.18	2.52±0.19	2.48±.25	ns	ns
TAPSE	23.14±4.21	23.36±4.90	24.39±3.72	ns	ns
IVC diameter (end-exp.)	1.61±0.29	1.93±0.19	1.65±0.50	**.00**	ns
IVC diameter (end-insp.)	0.75±0.38	1.08±0.44	0.83±0.46	**.00**	ns
IVC collapsibility index	0.54±0.19	0.43±0.23	0.54±0.23	.06	ns

FIGURE 1 **A-D:** LVEDV and LVESV increased in patients with LPI or NTBI levels lower than 0.4 or 1 μmol/L, respectively, but decreased in patients with LPI/NTBI values higher than those thresholds (y-axis shows relative change). **E-F:** patients with ferritin >1500 ng/mL or serum calcium <8.4 mg /dL significantly improved in systolic function at T_{early}, as compared to control groups in whom GLS was substantially unchanged. **G-H:** patients with higher ferritin or lower serum calcium had worse baseline GLS values (no statistical test was feasible due to low sample sizes).

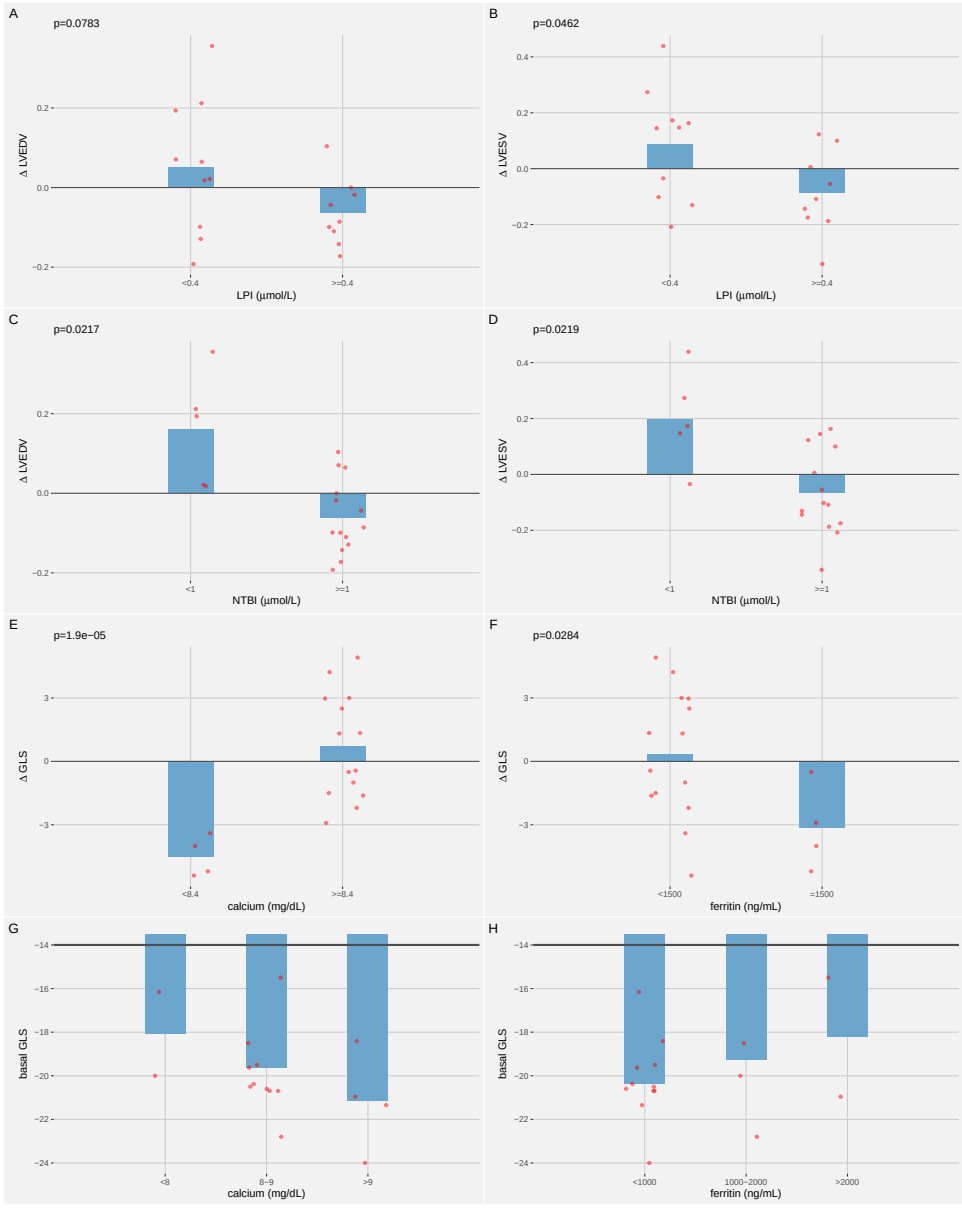
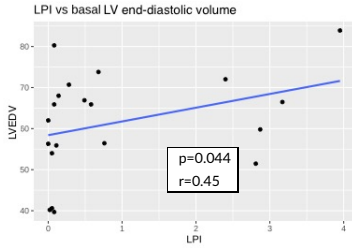
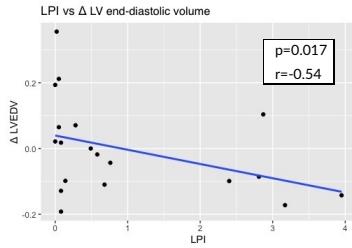


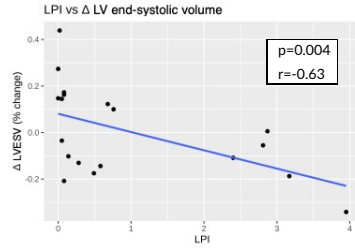
FIGURE 2 Correlation scatter plots between transfusion-related echocardiographic parameters and clinical/biochemical variables. Several early post-transfusional changes could be consistently predicted by variables related to iron homeostasis (LPI, NTBI), transfusion status (average pre-transfusion Hb, total weight of transfused PRBC units), or others. Overall, alterations in the status of circulating iron species correlated with a decrease in end-diastolic and end-systolic volumes after transfusion; transfusion status may have an effect on both basal and post-transfusion diastolic function. Other significant correlations are reported in Table **??**.



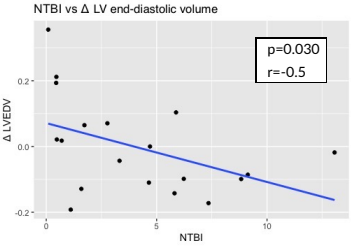
(a) LPI vs basal LVEDV



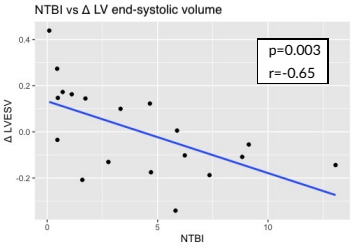
(b) LPI vs ΔLVEDV



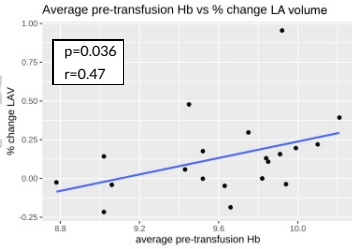
(c) LPI vs ΔLVESV



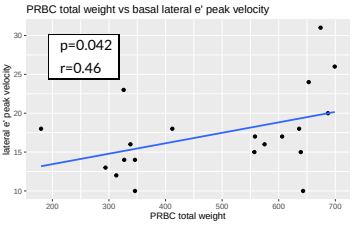
(d) NTBI vs ΔLVEDV



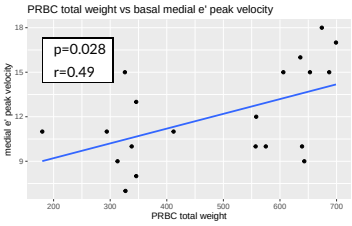
(e) NTBI vs ΔLVESV



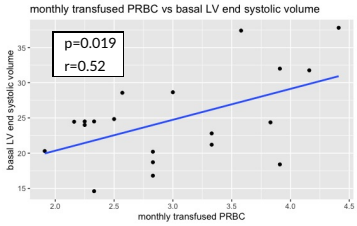
(f) Avg. $Hb_{pre\text{-}transfusion}$ vs %LAV



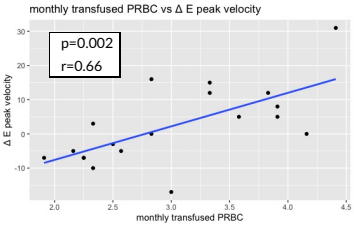
(g) PRBC total weight vs basal peak lateral e' velocity



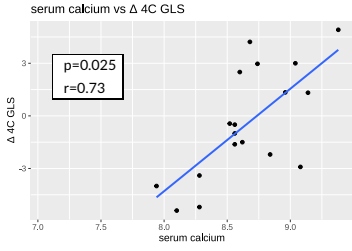
(h) PRBC total weight vs basal peak medial e' peak velocity



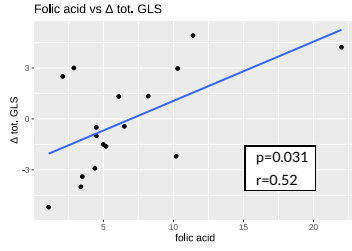
(i) monthly PRBC units vs basal LVESV



(n) monthly PRBC vs Δ(peak E wave)



(p) Serum calcium vs ΔGLS



(s) Folic acid vs $\Delta_{tot.\ GLS_{early}}$-$tot.\ GLS_0$

4 | DISCUSSION

Despite the advancements in the management of thalassemia, patients with transfusion-dependent β-TM are still burdened by adherence to a precise transfusion schedule, which poses them at risk of iron overload (IO) disorders. Among the latter, myocardial IO still represents a leading cause of mortality in this patients. Furthermore, chronic blood transfusion (BT) represents a repeated functional stress in terms of both iron and volume overload. Be that as it may, transfusion-related heart dysfunction might already manifest when the homeostasis of circulating iron species is altered, even in the absence of overt IO [16, 17, 18]. In fact, alterations in LPI/NTBI may be clinically relevant as they could herald the development of initial heart dysfunction with greater sensitivity than MRI iron quantification. In this regard, ultrasonography assessment of heart function could play an essential role in monitoring and orienting clinical decisions, as it is usually easier to access for patients and quicker to perform in bedside as well as walking clinic settings. As a matter of fact, several studies have started to investigate the potential of echocardiography in the management of patients with thalassemia [21, 22, 23, 24, 11]. In the context of clinical research, ultrasound studies are ideal for seriate assessments, allowing to detect early and/or transitory morphofunctional changes that may not be captured with other methods.

Aiming to add further evidence to these fields of investigations, with this study we show that several post-transfusion alterations in myocardial function could be identified in a population of regularly transfused β-TM patients. In this setting, several clinical and biochemical variables have been identified as predictors of functional alterations. In particular, variables related to alterations in circulating iron species have been consistently associated to a worsening of cardiac function.

Remarkably, our analysis disclosed that post-transfusion morphofunctional changes could also reverse *in sign* (e.g. *decreasing/worsening* instead of *increasing/improving*) depending on the level of the correlated variable. In fact, Figure 1 highlights that patients with higher LPI/NTBI levels did not show an increase in LV volumes after blood transfusion. In this context, LVESV increase can be interpreted as a direct consequence of LVEDV increase - likely a physiologic adaptation to volume overload. An impaired volume response in patients with high LPI/NTBI levels could be due to subclinical alterations in LV compliance, potentially heralding overt diastolic dysfunction.

While linear regressions from Figure 2 confirm that higher LPI/NTBI levels predict lower post-transfusion compliance of the LV, they additionally show that higher LPI levels significantly correlated with larger basal LV end-diastolic volumes, perhaps as a reflection of irreversible structural changes in a context of chronic alterations in circulating iron.

Overall, these results could indicate that heart adaptation to volume variation may be better in patients with lower NTBI/LPI levels. In fact, NTBI and LPI levels have already been proposed as predictors of systolic dysfunction [16].

In a previous work, our group showed that LPI is associated with cardiac events in adult TM and TI patients [18]. Instead, in this study NTBI levels were higher in patients with previous cardiac events, whereas no significant differences were remarked in LPI levels. Further investigation are warranted, with wider patients cohorts and longer follow-up periods, better to clarify the cardiovascular role of LPI/NTBI species in IO conditions.

Intriguingly, patients with ferritin levels higher than 1500 ng/mL exhibited a significant improvement in systolic function after BT. Although no statistical test could be performed due to the small sample size, a trend toward worse basal systolic function could be observed in patients with higher ferritin. In this regard, Abtahi et al. reported a significant correlation between GLS and MRI T2* cardiac iron deposits (p value < 0.01), and a statistical trend between GLS and ferritin (p value = 0.06), on a cohort of 52 asymptomatic thalassemic patients [22]: in that study, the authors proposed that GLS could be used as a screening exam to diagnose early cardiac IO. Crucially, only one patient in our population displayed T2* MRI relaxation times compatible with moderate cardiac iron deposits (Table S2) whereas all

the other patients for whom this measurement was available had no signs of heart IO. These findings may imply that a condition of altered circulation of iron species, however within the normal range of iron content as measured by cardiac MRI T2*, could negatively impact on basal systolic function. At the same time it may be hypothesised that the subclinical impairment in systolic function related to intracellular IO can be effectively rescued through BT, at least in its initial phases, perhaps due to enhanced transfusion-related oxygen delivery to myocardial tissue. In this regard, a cutoff of serum ferritin >1500 ng/mL could be proposed to identify those patients whose cardiac systolic function might improve effectively through transfusion therapy.

In contrast to ferritin, higher albumin-adjusted serum calcium levels seem to be associated with better systolic function. The latter significantly improves after blood transfusion in patients with hypocalcemia, while remaining substantially unchanged in those with normal serum calcium levels. Since β-TM patients are commonly affected by endocrinopathies and bone metabolism alterations, these findings are of great interest as they prompt a link between β-TM mineral alterations in bone metabolism and calcium homeostasis, transfusion needs, and cardiac function.

Quite unexpectedly, folic acid levels showed a correlation similar to calcium. Further studies are warranted to confirm whether folic acid levels, perhaps through their hematopoietic effect, effectively impact on systolic function under condition of volume/iron overload in chronic anemias.

As to diastolic dysfunction, average pre-transfusion Hb significantly correlated with preload-dependent parameters such as E wave peak velocity and E/A ratio, whereas no significant correlation was observed with parameters which are associated with intrinsic diastolic dysfunction (i.e. e' velocity and E/e' ratio). Notably, higher average pre-transfusion Hb also predicted a greater percent change in LA volume, similarly to LPI/NTBI for LV volumes: figure 2f reports that no substantial changes in LA volume are predicted at Hb levels ~9.0 g/dL. Overall, these findings seem compatible with those reported by Burns et al. [25], who observed that in heart failure with preserved ejection fraction, anemia is associated with volume/preload-dependent markers of diastolic dysfunction, but not with markers of intrinsic cardiac dysfunction. On the other hand, the weight of PRBCs transfused at T_0 significantly correlated with functional descriptors of diastolic function, including intrinsic markers such as e' peak velocities (Table **??**). In this regard, it is interesting to note that basal e' velocities positively correlated with monthly PRBC (septal e') and PRBC total weight (septal and lateral e'), thus suggesting that morphologic modifications may take place to adapt to repeated volume overload challenges. At T_{early}, both E and e' peak velocities seem to significantly increase according to monthly PRBCs or the number of PRBCs transfused at T_0, respectively, perhaps as a physiologic adaptation to volume loading.

Additionally, significant correlations were disclosed between BNP and several T_0 as well as post-transfusion parameters (Table S5): such findings warrant further investigation, since studies on BNP role in evaluating diastolic dysfunction in thalassemic patients have yielded contrasting results [26, 27]. Finally, our study confirmed a correlation between diastolic dysfunction parameters (particularly e' peak velocity and E/e' ratio) and cardiac MRI T2* iron levels [28] (not shown).

Several other significant correlations have been disclosed in our analysis and are reported in Table S5 in Supplementary files. Overall, most correlations are consistent with the results here discussed.

Among the limitations of this study, it should be mentioned the relatively small number of patients, some of whom could not be followed until the T_{late} timepoint. At the same time, several studies on β-TM have been carried on comparable patient numbers [24], as this is a rare disease for which large cohorts are usually difficult to gather. As a consequence of suboptimal patient numbers for such a rare disease, statistical analysis is unavoidably underpowered and may not disclose significant associations. On the other hand, the sample size did not allow for multivariable analyses, and therefore the impact of adjusting for variables such as age, sex, and others could not be evaluated. Furthermore, the large number of correlation tests performed warrants a cautious interpretation of the results. As the study did not incorporate adjustments for multiple comparisons in order to comprehensively collect clinical, biochem-

ical, and functional data for each patient, it is possible that some of the statistically significant results may not be entirely accurate. Overall, these limitations warrant cautious interpretation of the findings from our study.

Be that as it may, it must be remarked that most predictions in our study were consistent between them, which strongly suggests that they may be the meaningful representation of a pathophysiological phenomenon. Further studies are warranted to investigate in more detail the findings of this exploratory work. In fact, larger population cohorts and/or case/control studies would be instrumental in confirming the results of this pilot investigation, as well as disconfirming some possibly spurious correlations that may have occurred due to the mentioned limitations.

To the best of our knowledge, this is the first study which comprehensively investigates the early cardiac morpho-functional changes related to scheduled blood transfusion in β-TM. In this regard, cardiac ultrasound represents the most appropriate tool for rapid, seriate evaluations. Notably, the observed alterations in both systolic and diastolic function, however temporary, may have a long-term impact as they repeat themselves. Taken together, our findings point toward an impairment in post-transfusion cardiac function under condition of altered circulation of iron species in β-TM, even in the absence of overt myocardial iron overload. At the same time, TM patients with hyperferritinemia or hypocalcemia may benefit the most from blood transfusion in terms of systolic function, likely due to a worse baseline compared to patients with normal ferritin and calcium levels. Due to the exploratory nature of this study, further research is warranted to fully corroborate its findings.

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Conflict of interest The authors report no conflicts of interest.

Data availability The datasets analysed during the current study are not publicly available due to privacy restrictions, but are available from the corresponding author on reasonable request.

Ethics approval The protocol of the study was approved by the local Institutional Ethics Committee (Comitato Etico Area Vasta Emilia Nord - AVEN) and all patients gave their written informed consent. The study was performed in accordance with the Declaration of Helsinki.

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