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Cardiac involvement by CMR in different genotypic groups of thalassemia major patients

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ABSTRACT

Beta thalassemia major (β-TM) displays a great deal of phenotypic heterogeneity, not fully investigated in terms of cause-effect. We aimed to detect if different genotypic groups could be related to different levels of cardiac impairment, evaluated by cardiovascular magnetic resonance (CMR).

We considered 671 β-TM patients (age 30.1 years, 52.9% females) consecutively enrolled in the Myocardial Iron Overload (MIO) in Thalassemia network. MIO was assessed by T2* technique. Biventricular function was quantified by cine images. Myocardial fibrosis was evaluated by late gadolinium enhancement (LGE) technique. Three groups of patients were identified: heterozygotes β^+/β° ($N = 279$), homozygotes β^+ ($N = 154$), homozygotes β° ($N = 238$).

Transfusional needs resulted significantly lower in homozygous β^+ TM patients when compared to the other groups. The homozygous β^+ group *versus* the heterozygous and homozygous β° groups showed higher global heart T2* values ($P < 0.0001$) and a lower number of patients with a global heart T2* value < 20 ms ($P < 0.001$). The homozygotes β^+ showed a lower number of patients with a pathological left ventricular ejection fraction (LVEF) than the other two groups ($P < 0.05$).

The β^+/β^+ TM patients showed less MIO and a concordant better systolic heart function. These data support the knowledge of different genotypic groups in the management of β-TM patients.

1. Introduction

Beta thalassemia major (β-TM) is the commonest genetic disorder worldwide [1]. It is characterized by the absence (β°) or reduced output (β^+) of the β chains of haemoglobin. Subjects with β-TM are homozygotes or compound heterozygotes for β° or β^+ genes, except for rare dominant forms. β-TM is the most severe form of β-thalassemia, characterized by a transfusion-dependent anemia, secondary to a severe state of alpha-non alpha globin chain imbalance. The degree of imbalance between the alpha and non-alpha globin chains is the main determinant of the clinical severity of the disease and it is caused by the type of gene mutation [2]. More than 200 different mutations affecting the beta globin gene have been identified, whose relative frequency varies in different countries. Most of them are single base substitutions or the loss/insertion of a few basis within the gene or its immediate flanking sequences [3]. Among β-TM patients a spectrum of different

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genotypes has been found [4,5]. Because of the wide heterogeneity at the molecular level, it is possible to observe a variability in clinical manifestations. Nevertheless, the phenotype to genotype correlation is complex and it is related to the complicated interaction of the environment and other genetic factors with the different allelic variants [6].

β-TM patients require regular life-long transfusions to survive, resulting in iron overload. Heart complications secondary to iron overload remain the main cause of morbidity and mortality in β-TM, although the survival of patients has improved in the last decade [7]. T2-star (T2*) cardiac magnetic resonance (CMR) represents a key technique to quantify cardiac iron loading non-invasively and with a high reproducibility [8]. This technique is important for the management of β-TM patients, allowing an early diagnosis [9,10] and a constant monitoring of cardiac iron overload to evaluate the effectiveness of iron chelation therapy [11,12]. Moreover, CMR is considered the gold-standard method to assess biventricular size and function parameters with excellent reproducibility [13] and to detect the concordant positive effect in removing iron from the heart [11]. Among β-TM patients different levels of myocardial iron overload have been observed, related to different degrees of heart dysfunction [14]. In light of the variability observed at both molecular and phenotypic levels, considering the importance of T2* CMR in the clinical management of β-TM patients, we aimed to detect if different genotypic groups of β-TM patients, homozygotes or compound heterozygotes for β° or β^{+} [15], could be related to different levels of cardiac impairment, evaluated by CMR.

2. Material and methods

2.1. Study population

Six hundred seventy-one patients with thalassemia major (316 M/355 F, mean age 30.1 ± 9.4 years), consecutively enrolled in the MIOT (Myocardial Iron Overload in Thalassemia) network were studied retrospectively. The MIOT network is constituted by nine MRI sites and 61 thalassemia centres where CMR exams are performed using homogeneous, standard and validated procedures and where patients' clinical-instrumental data are put in a centralized database via web [8,16]. All the subjects were of Italian ancestry and because of their family different regional origin, they are a representative sample of the Italian population. All clinical and laboratory investigations were done at the thalassemia centres where the patients were treated. All patients were regularly transfused to maintain a pre-transfusion haemoglobin concentration above 9-10 g/dl. MRI scanning was performed within one week before regular scheduled blood transfusion. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study.

2.2. Collection and analysis of samples

Blood samples of all 671 TM patients were collected in EDTA for DNA extraction. Genomic DNA was extracted from peripheral blood leucocytes using the salting-out method [17]. All coding and noncoding regions of β globin gene were amplified by PCR in different fragments ranging from 200 bp to 13.4 kb and partially overlapped. The PCR conditions were different, depending on the protocol used by the laboratory of the thalassemia centre where the patient was treated.

2.3. Genotype characterization

β thalassemia mutations were identified by reverse hybridization assay (β-globin strip assay, Nuclear Laser, Vienna Lab, Austria).

2.4. MRI

MR exams were performed using a 1.5 T scanner (GE, Milwaukee, USA).

An eight-element cardiac phased-array receiver surface coil with breath holding in end-expiration and ECG-gating were used.

For iron overload assessment, T2* gradient-echo multiecho sequences were acquired. For the heart a multislice approach was used: basal, medium, and apical slices were acquired [18,19]. A mid-hepatic slice was obtained [20]. T2* images analysis was performed using a custom-written, previously validated software (HIPPOMIOT®) [21]. The software provided the T2* value for all the 16 segments of the left ventricle (LV). Global heart T2* value was obtained by averaging all segmental values. A T2* measurement of 20 ms was taken as "conservative" normal value for segmental and global values [21,22]. Hepatic T2* values were calculated in a circular region of interest [23] and were converted into liver iron concentration (LIC) using the Wood's calibration curve [24,25].

For the quantification of biventricular function parameters, cine images were acquired in sequential 8 mm short-axis slices. Images were analysed using MASS® software (Medis, Leiden, The Netherlands). Atrial areas were measured from the four chamber view projection in ventricular end-systolic phase. End-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV) and ejection fraction (EF) were measured for both the ventricles. The mass of the left ventricle (LV) was also evaluated. Except for EF, indices of these parameters were normalized for the body surface area (EDVI, ESVI, SVI, mass index). Heart dysfunction was diagnosed in presence of LV and/or RV EF < 2 standard deviations (SD) from the mean values normalized to age and gender [26].

To detect the presence of macroscopic myocardial fibrosis, late gadolinium enhancement (LGE) short-axis images were acquired 10–18 min after Gadobutrol (Gadovist®; Bayer Schering Pharma; Berlin, Germany) intravenous administration at the standard dose of 0.2 mmoL/kg. Also, vertical, horizontal, and oblique long-axis views were acquired. LGE images were not acquired in patients with a glomerular filtration rate < 30mL/min/1.73 m^2 and in patients who refused. LGE was considered present when visualized in two different views [27,28]. The extent of LGE areas was quantified using a validated software [29].

2.5. Statistical analysis

All data were analyzed using SPSS version 13.0. Continuous variables were described as median (interquartile range) and categorical variables were expressed as frequencies and percentages. The normality of distribution of the continuous parameters was assessed by using the Kolmogorov - Smirnov test. Comparisons among the 3 groups were made by Kruskal-Wallis test for continuous variables, as all of them showed a non normal distribution. $\chi 2$ testing was performed for non-continuous variables. The Bonferroni adjustment was used in all pairwise comparisons. We did not apply a correction model for the variables significantly different among the groups because they were not associated to dependent variable or were correlated with a weak (between -0.3 and 0.3) Spearman's correlation coefficient. In all tests, a 2 - tailed probability value of 0.05 was considered statistically significant.

3. Results

The overall frequencies of the β genotypes in TM are summarized in Table 1, where for each allele belonging to genotype is indicated the corresponding phenotypic expression (β° or β^{+}). We recorded 32 different genotypes among TM patients.

Three groups of patients were identified: heterozygotes β^{+}/β° (N = 279), homozygotes β^{+} (N = 154), homozygotes β° (N = 238). No significant differences for sex, age and haemato-chemical parameters

Table 1
Beta genotypes.

Genotypes	HGVS nomenclature[a]	Type	Genotype n°	Frequency %
CD39/CD39	HBB:c.118C > T/HBB:c.118C > T	$\beta^{\circ}/\beta^{\circ}$	163	24.3
CD39/IVS-1,110	HBB:c.118C > T/HBB:c.93-21G > A	β°/β^{+}	123	18.3
IVS-1,6/CD39	HBB:c.92 + 6 T > C/HBB:c.118C > T	β^{+}/β°	68	10.1
IVS-1,110/IVS-1,110	HBB:c.93-21G > A/HBB:c.93-21G > A	β^{+}/β^{+}	63	9.4
IVS-1,6/IVS-1,110	HBB:c.92 + 6 T > C/HBB:c.93-21G > A	β^{+}/β^{+}	48	7.2
CD39/IVS-1,1	HBB:c.118C > T/HBB:c.92 + 1G > A	$\beta^{\circ}/\beta^{\circ}$	39	5.8
IVS-1,1/IVS-1,110	HBB:c.92 + 1G > A/HBB:c.93-21G > A	β°/β^{+}	23	3.4
CD39/IVS-2,745	HBB:c.118C > T/HBB:c.316-106C > G	β°/β^{+}	18	2.7
IVS-1,110/IVS-2,745	HBB:c.93-21G > A/HBB:c.316-106C > G	β^{+}/β^{+}	15	2.2
IVS-1,6/IVS-1,1	HBB:c.92 + 6 T > C/HBB:c.92 + 1G > A	β^{+}/β°	15	2.2
IVS-1,6/IVS-2,745	HBB:c.92 + 6 T > C/HBB:c.316-106C > G	β^{+}/β^{+}	13	1.9
CD39/IVS-2,1	HBB:c.118C > T/HBB:c.315 + 1G > A	$\beta^{\circ}/\beta^{\circ}$	12	1.8
IVS-1,6/IVS-1,6	HBB:c.92 + 6 T > C/HBB:c.92 + 6 T > C	β^{+}/β^{+}	11	1.6
IVS-1,1/IVS-1,1	HBB:c.92 + 1G > A/HBB:c.92 + 1G > A	$\beta^{\circ}/\beta^{\circ}$	10	1.5
IVS-2,1/IVS-1,110	HBB:c.315 + 1G > A/HBB:c.93-21G > A	β°/β^{+}	10	1.5
−87/CD39	HBB:c.-137C > G/HBB:c.118C > T	β^{+}/β°	9	1.3
CD39/CD6	HBB:c.118C > T/HBB:c.20delA	$\beta^{\circ}/\beta^{\circ}$	5	0.7
Others		β°/β^{+}	12	1.8
		$\beta^{\circ}/\beta^{\circ}$	10	1.5
		β^{+}/β^{+}	4	0.6

Beta genotypes in β TM.
[a] HGVS: Human Genome Variation Society.

were found among the groups.

The frequency of transfusions, intended as the number of transfusional units in the 12 months before the MRI scan, was significantly lower in the homozygous β^{+} patients than the heterozygous (36.0 U vs 38.0 U, $P < 0.05$) and the homozygous β° patients (36.0 U vs 40.0, $P < 0.0001$) (Fig 1).

No significant differences were found among the three groups in the frequency of chelation therapy with desferrioxamine (DFO), deferasirox (DFX) and combined/sequential therapy carried out for more than 12 months. The frequency of deferiprone (DFP) therapy was significantly higher in the homozygous β^{+} group than the heterozygous (21.4% vs 10.8%, $P < 0.01$) and homozygous β° groups (21.4% vs 10.9%, $P < 0.05$). The median dose of DFP therapy in the homozygous β^{+}, heterozygous and homozygous β° groups was 75 (9.5), 75 (12.4), 75 (5.0) mg/kg/day, respectively.

The clinically relevant findings in the three groups are summarized in Table 2.

The homozygous β^{+} group showed significantly higher global heart T2* values than the heterozygous group (35.0 ms vs 32.0 ms, $P < 0.01$) and homozygous β° group (35.0 ms vs 28.5 ms, $P < 0.0001$) (Fig 2a). The homozygous β^{+} group *versus* the heterozygous and the homozygous β° group showed a lower number of segments with a pathological T2* value (1.0 vs 1.0 vs 2.0; $P < 0.01$ and $P < 0.0001$ respectively) (Fig 2b). The percentage of patients with a global heart T2* value < 20 ms was lower in the homozygous β^{+} group than the heterozygous (14.3% vs 26.9%, $P < 0.01$) and homozygous β° groups (26.9% vs 34%, $P < 0.0001$) (Fig 2c). There were not significant differences among groups in bi-atrial areas or LV and RV end volume indexes.

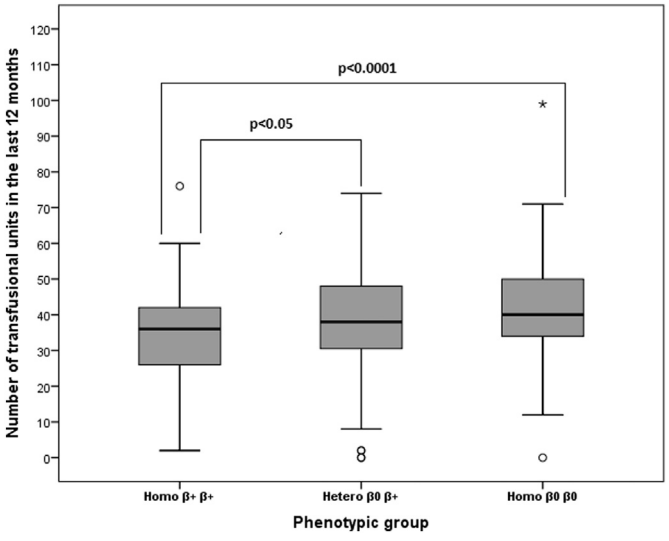

Fig. 1. Number of transfusional units.
Number of transfusional units in the last 12 months in the 3 genotypic groups. The P-value for each significant pairwise comparison is indicated.

The homozygous β^{+} and the heterozygous groups *versus* the homozygous β° group showed a significantly higher LV SVI (54.0 vs 51.3, $P < 0.01$ and 53.5 vs 51.3, $P < 0.05$, respectively).

The homozygous β^{+} group showed a significantly lower number of patients with a pathological left ventricular ejection fraction (LVEF) than the homozygous β° group (14.3% vs 27.2% $P < 0.05$). The homozygous β^{+} group showed a lower number of patients with a pathological LVEF than the heterozygous group at the limit of significance (14.3% vs 24.2%, $P = 0.057$) (Fig 2d).

There were not significant differences among groups in the prevalence of macroscopic myocardial fibrosis and MRI LIC. The MRI findings in the three groups are summarized in Table 3.

4. Discussion

The wide spread of molecular analysis techniques for studying the globin genes has allowed to explore the genotypic heterogeneity of β-thalassemic patients. The understanding of the molecular background of β-thalassemia is becoming an important step in the comprehension of the influencing factors of the diverse clinical manifestations. Despite the extensively studied complex relationship between phenotype and genotype [2,6], there is an increasing interest in the identification of genetic factors allowing to predict patients' phenotype [30–32]. Among β-TM patients a wide variability of genotypes was previously found [4,5]. Nevertheless, in countries in which β-thalassemia is prevalent, including Italy, only a few mutations have reached high frequencies because of the protection they provide against malaria [6]. In the present study we analyzed a representative sample of the Italian β-TM population, in which we identified 32 different genotypes. Our results reconfirmed that the commonest genotypes are homozigosity CD39 (β°)

Table 2
Clinical findings.

Parameters/variables	β^+/β^+ (N = 154)	β°/β^+ (N = 279)	β°/β° (N = 238)	P
Age (years)	31.6 (14.9)	30.3 (12.8)	33.0 (11.4)	0.073
Sex (M/F)	78/76	133/146	105/133	0.435
Hb pre-transfusion (g/dl)	9.6 (0.6)	9.6 (0.7)	9.5 (0.7)	0.394
Ferritin levels (ng/l)	1150.0 (1599.0)	1090.0 (1297.0)	1013.0 (1359.3)	0.728
Age at first transfusion (years)	1.0 (1.0)	1.0 (1.0)	1.0 (0.0)	0.079
Transfusional units (U) in the last 12 months	36.0 (16.5)	38.0 (18.0)	40.0 (16.0)	< 0.0001
ALT (u/l)	30.5 (27.0)	32.0 (34.0)	30.0 (30.0)	0.954
AST (u/l)	30.0 (35.0)	28.0 (26.0)	28.0 (23.0)	0.469
Positive HCV RNA (%)	29.9	29.7	30.4	0.986
Diabetes mellitus (%)	7.9	7.8	9.2	0.847
Chelation therapy, none, N, (%)	0/150 (0.0)	1/272 (0.4)	1/231 (0.4)	0.735
Chelation therapy, DFO, N, (%)	47/150 (31.3)	94/272 (34.6)	75/231 (32.5)	0.773
Chelation therapy, DFP, N, (%)	33/150 (21.4)	30/272 (10.8)	26/231 (10.9)	< 0.005
Chelation therapy, DFX, N, (%)	40/150 (26.7)	70/272 (25.7)	53/231 (22.9)	0.663
Chelation therapy, combined DFP + DFP, N, (%)	19/150 (12.7)	49/272 (18.0)	47/231 (20.3)	0.153
Chelation therapy, sequential DFO/DFP, N, (%)	6/150 (4.0)	16/272 (5.9)	15/231 (6.5)	0.577
Chelation therapy, sequential DFO/DFX, N, (%)	1/150 (0.7)	1/272 (0.4)	0/231 (0.0)	0.501
Excellent/good compliance to chelation therapy (%)	93.0	89.2	93.4	0.200

Clinical findings in the 3 groups identified on the basis of the β-globin gene phenotypic expression.

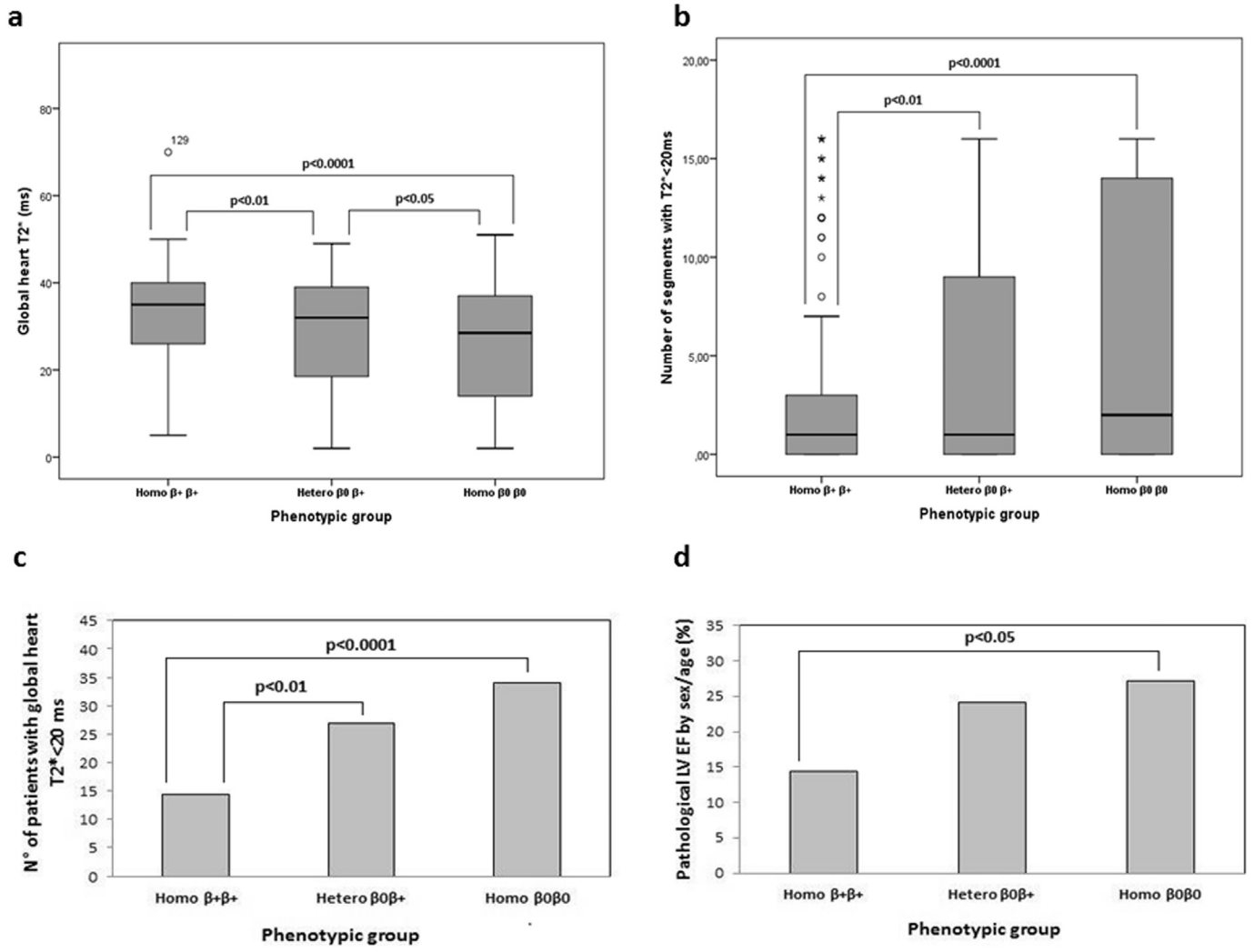

Fig. 2. Iron overload and left ventricular function.
a) Global heart T2* values in the 3 genotypic groups; b) Number of segments with T2* < 20 ms in the 3 genotypic groups; c) Number of patients with global heart T2* < 20 ms in the 3 genotypic groups; d) Percentage of patients with a pathological LV EF in the 3 genotypic groups. The *P*-value for each significant pairwise comparison is indicated.

Table 3
MRI findings.

Parameters/variables	β^+/β^+ (N = 154)	β°/β^+ (N = 279)	β°/β° (N = 238)	P
Global heart T2* (ms)	35.0 (14.0)	32.0 (21.0)	28.5 (23.5)	< 0.0001
Global heart T2* < 20 ms (%)	14.3	26.9	34.0	< 0.0001
Number of segments with T2* < 20 ms	1.0 (3.0)	1.0 (9.0)	2.0 (14.0)	< 0.0001
Left atrial area (cm^2/m^2)	13.0 (3.0)	12.8 (3.1)	13.1 (3.3)	0.370
Right Atrial Area (cm^2/m^2)	12.2 (2.8)	12.1 (3.0)	12.1 (3.3)	0.647
LV EDVI (ml/m^2)	88.0 (22.0)	87.5 (24.1)	84.0 (21.0)	0.153
LV ESVI (ml/m^2)	32.3 (14.8)	33.0 (13.8)	31.0 (15.0)	0.448
LV SVI (ml/m^2)	54.0 (12.0)	53.5 (14.0)	51.3 (12.0)	< 0.05
LV mass index (g/m^2)	55.0 (16.0)	57.0 (18.8)	56.0 (17.0)	0.168
LV EF (%)	62.0 (8.1)	60.9 (8.8)	61.5 (9.1)	0.087
Pathological LV EF (%) by age/sex	14.3 (*N* = 140)	24.2 (*N* = 264)	27.2 (*N* = 228)	< 0.05
Cardiac index ($l/min/m^2$)	3.9 (1.1)	3.7 (1.5)	3.6 (1.3)	0.150
RV EDVI (ml/m^2)	81.6 (20.9)	81.2 (24.0)	80.0 (22.8)	0.432
RV ESVI (ml/m^2)	30.0 (10.8)	31.0 (13.7)	31.0 (14.0)	0.713
RV SVI (ml/m^2)	52.0 (16.5)	51.0 (16.2)	49.2 (13.7)	0.092
RV EF (%)	63.0 (10.0)	62.0 (9.0)	61.0 (11.0)	0.110
Pathological RV EF (%) by age/sex	15.7 (N = 140)	19.7 (N = 264)	21.9 (N = 228)	0.345
Myocardial fibrosis by LGE (%)	14.7 (*N* = 102)	19.0 (*N* = 205)	20.0 (*N* = 190)	0.524
MRI LIC (mg/g dw)	5.6 (10.1)	5.3 (8.2)	4.9 (8.6)	0.319

MRI findings in the 3 groups identified on the basis of the β-globin gene phenotypic expression.

and compound heterozigosity CD39/IVS-1,110 (β°/β^+) as reported in previous studies [4,5].

We identified three groups of β-TM patients, homozygous β^+, homozygous β° and heterozygous β^+/β°, homogeneous for sex, age and haemato-chemical parameters, but different as regards the ability to produce adequate amounts of beta globin chains. In particular, homozygous β° group included patients with two severe mutations and consequently a high alpha-non alpha globin chain imbalance; heterozygous group included patients with a combination of mild/severe mutations and homozygous β^+ group consisted of patients with two mild mutations and, thus, a lower imbalance between alpha and beta globin chains.

In the most severe form of β-thalassemia (β^0/β^0) the complete absence of beta globin chains and the resulting ineffective erythropoiesis, lead to a marked anemia when compared to the milder forms.

To our knowledge there are a few studies that compare β^0 and β^+ thalassemia. Nevertheless, a previous report by Sagar et al. [32] shows that iron-induced toxicity, as indicated by DNA damage, seems to vary in β-thalassemia patients according to the type of beta globin gene mutation. They observed a higher extent of DNA damage in the nucleated red blood cells (RBCs) of homozygous β° patients as compared to the β^0 hetero- or β^+ homozygotes, which may be due to a higher alpha to non-alpha chain ratio in the former.

Among β-TM patients different patterns of cardiac iron overload have been previously found, related to different risks of heart dysfunction [14].

In the present study, comparing cardiac iron overload by T2* MRI in different genotypic groups of β-TM patients, we found a significantly lower global and segmental heart iron burden in β^+ homozygotes than in heterozygotes and β° homozygotes. This result supports the hypothesis that in β° homozygotes the complete absence of β Hb chains increases the degree of hemolysis and blood requests, leading to a higher iron overload. In fact, the homozygous β^+ group showed a significantly lower transfusion demand when compared to the other two groups. We could speculate that the genotype group could be proposed as a predictor of myocardial iron overload. Moreover, DFP therapy was significantly more frequent in β^+ homozygotes when compared to the other two groups and it is known DFP is more effective in removing iron from the heart [12].

According to the severity of the genotype, we observed that cardiac T2* falls in concert with cardiac function, as we observed a lower number of patients with a pathological LV EF in the β^+ homozygotes when compared to the other two groups. This is in agreement with previous studies showing that the probability of a reduced left ventricular function increments as cardiac iron increases [7,14]. Nevertheless, patients with decreased cardiac T2* levels and a normal cardiac function have been found, suggesting that T2* is able to identify preclinical cardiac dysfunction [9,33].

Conversely, we did not find significant differences in LIC and serum ferritin levels between the groups. This finding could be explained by the fact that all patients in our study have received chelation therapy since early childhood. It is known that iron clearance from the hearth is slower than from the liver, as there are organ-specific mechanisms of iron uptake/release [34]. For this reason several studies have demonstrated poor correlation between hepatic and cardiac iron [35,36]. Thus, in all groups of patients we observed similar LIC and ferritin levels, but significantly different cardiac iron overload, probably because of the capacity of the chelation therapy to remove iron from the liver in a faster way than from the heart. Moreover, the milder genotype group homozygous β^+ group is more frequently treated with low doses of DFP, which seems to be less effective in removing iron from the liver [11]. Unfortunately, in the clinical practice was not possible to test the degree of ineffective erythropoiesis, differences in factors like hepcidin and genetic modifiers that could have explained better the differences among the three groups in terms of total body iron burden and global heart iron overload.

It is known that age [27], diabetes mellitus (DM) and chronic hepatitis C (CHC) [37,38] are risk predictors for macroscopic myocardial fibrosis. In this study, we did not find significant differences in macroscopic myocardial fibrosis between the three genotypic groups, probably because the groups were homogeneous for age, prevalence for DM and HCV RNA positivity.

5. Conclusions

In conclusion, the homozygous β^+ group of β-TM patients showed less myocardial iron overload and a concordant better global heart function when compared to the more severe groups. Our data suggest that the knowledge of different genotypic groups in relation to the seriousness of the alpha-non alpha chain imbalance can be useful in helping the clinical and instrumental management of β-TM patients. Despite the complexity of phenotype to genotype correlation, the identification of genetic factors that can help to predict patients' phenotypic features, like cardiac iron levels and the degree of cardiac

dysfunction, could help to prevent the development of complications, like in this case heart failure secondary to iron overload, which remains the leading cause of mortality in β-TM.

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Authorship contributions

LP and AP conceived the study and wrote the paper. AS, RL, RR, AM, DGD, LC, AV, GR and MM collected the data. AP and MM were responsible for data collection. AM and SS contributed to the interpretation of the results and data analysis. All authors contributed to critical revision and final approval of the version to be published.

Declarations of interest

None.

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