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Primary *HBB* gene mutation severity and long-term outcomes in a global cohort of β-thalassaemia

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Summary

In β-thalassaemia, the severity of inherited β-globin gene mutations determines the severity of the clinical phenotype at presentation and subsequent transfusion requirements. However, data on associated long-term outcomes remain limited. We analysed data from 2109 β-thalassaemia patients with available genotypes in a global database. Genotype severity was grouped as β^0/β^0, β^0/β^+, β^+/β^+, β^0/β^{++}, β^+/β^{++}, and β^{++}/β^{++}. Patients were followed from birth until death or loss to follow-up. The median follow-up time was 34·1 years. Mortality and multiple morbidity outcomes were analyzed through five different stratification models of genotype severity groups. Interestingly, β^0 and β^+ mutations showed similar risk profiles. Upon adjustment for demographics and receipt of conventional therapy, patients with β^0/β^0, β^0/β^+, or β^+/β^+ had a 2·104-increased risk of death [95% confidence interval (CI): 1·176–3·763, $P = 0·011$] and 2·956-increased odds of multiple morbidity (95% CI: 2·310–3·784, $P < 0·001$) compared to patients in lower genotype severity groups. Cumulative survival estimates by age 65 years were 36·8% for this subgroup compared with 90·2% for patients in lower genotype severity groups ($P < 0·001$). Our study identified mortality and morbidity risk estimates across various genotype severity groups in patients with β-thalassaemia and suggests inclusion of both β^+ and β^0 mutations in strata of greatest severity.

Keywords: genotype, phenotype, morbidity, mortality, survival.

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Introduction

β-Thalassaemia comprises a diverse group of recessively inherited disorders of haemoglobin synthesis characterized by ineffective erythropoiesis and chronic anaemia of varying severity that may necessitate lifelong blood transfusion. It is caused by mutations resulting in a single nucleotide substitution, small deletions or insertions within the β-globin (*HBB*) gene or its immediate flanking sequence, or less commonly, large deletions. This results in reduced or absent production of β-globin chains and adult haemoglobin. Although the various phenotypes of β-thalassaemia are defined based on clinical observation, genotype–phenotype associations have been established. Patients with clinically symptomatic disease are those who are homozygous or compound heterozygous for *HBB* gene mutations, although some may only be heterozygous for *HBB* gene mutations while also harbouring α-globin gene triplications. Phenotype severity is closely tied to the degree of imbalance between α-globin and β-globin chains. Thus, the primary modifier of phenotype severity remains the severity of the inherited *HBB* gene mutation itself, with more than 350 mutations currently described and assigned various levels of severity based on the amount of β-globin chain product.[1,2] Co-inheritance of α-thalassaemia and genetic variation in the expression of transcription regulators like BCL11A with persistent fetal haemoglobin production have also been identified as secondary modifiers of phenotype severity.[3,4]

Several predictive models have established the role of *HBB* gene mutation severity in determining the degree of anaemia and clinical symptoms at diagnosis, and the need and timing of initiation of blood transfusion therapy[5–7]; although in some studies of transfused patients, stratification by genotype failed to accurately provide an evaluation of residual erythropoietic activity and mean annual transfusion volume.[8] More

importantly, data on the association between *HBB* gene mutation severity and long-term mortality and morbidity outcomes remain limited.[9,10] Such information can prove essential for various reasons: (i) the different degrees of mutation severity and their combinations in homozygous and compound heterozygous states present some level of complexity for the treating physician in predicting the long-term phenotype course, which makes outcomes-based genotype severity grouping more practical for tailoring management; (ii) although the impact of *HBB* gene mutation severity on long-term outcomes is primarily mediated by consequent treatment choices, a broader impact through yet undefined pathology cannot be fully excluded; and (iii) novel and gene therapy trials in β-thalassaemia are commonly defining eligibility or subgroup analyses based on different *HBB* gene mutation strata; thus, determining or standardizing strata of highest clinical interest would be essential to allow proper interpretation of benefit. The latter remains essential for regions with limited resources, where informed prioritization of patients for novel treatment strategies is likely to be needed.

With this background, the aim of this study was to explore the association between *HBB* gene mutation severity and mortality and morbidity outcomes in a large, global cohort of patients with β-thalassaemia.

Materials and methods

Data were retrieved from an International Health Repository (IHR) established and approved on 25 May 2017 by the Italian Ethical Committee (EudraCT and Sponsor's Protocol Code Numbers: 2017-004457-17 and 143AOR2017). All data were anonymized and added to the repository following informed consent by patients or their legal representatives in case of death. The database included all β-thalassaemia patients attending participating centres from 1 January 1997 onwards, and historic data were retrieved on all those patients from birth up to 31 December 2020, death, or loss to follow-up. The database included 13 international thalassaemia reference centres from eight countries: Italy, Iran, Pakistan, USA, Oman, Egypt, Greece, and Saudi Arabia.

For the current analysis, we retrieved data on 2109 β-thalassaemia patients with available information on *HBB* gene mutations [*n*/*N* (%) of total patients included in the database: Italy, 1460/4347 (33·5%); Pakistan, 266/293 (90·7%); Oman, 200/224 [(89·2%); USA, 64/132 (48·5%); Greece, 91/108 (84·3%); Saudi Arabia, 28/30 (93·3%); Egypt, 0/930; Iran, 0/1949]. Patients had homozygous or compound heterozygous *HBB* gene mutations, or heterozygous *HBB* gene mutations combined with α-globin gene triplications. *HBB* gene mutation severity was determined with guidance from publicly available mutation databases and resources,[2,11–13] and was classified as β^0, where no functional β-globin chains can be produced; β^+, where β-globin chain production is severely reduced; and β^{++}, where β-globin chain production is mildly reduced.[14] For the avoidance of doubt, it should be noted that other scholars label these as β^0, severe β^+, and β^+, respectively; but we elected to use the more common designation labels of β^0, β^+, and β^{++}. Patients were subsequently assigned to one of six genotype severity groups: β^0/β^0, β^0/β^+, β^+/β^+, β^0/β^{++}, β^+/β^{++}, and β^{++}/β^{++} (including patients heterozygous for *HBB* gene mutation with α-globin gene triplications). Genotypes with ≥1% occurrence in the study cohort are summarized in Table I.

For each patient, data were also retrieved for gender, country where the patient primarily received treatment [grouped as 'US or Europe' and 'Asia or Middle East and North Africa [(MENA)'], year of birth (grouped as <1970 and ≥1970, to reflect the period when regular transfusion and iron chelation became available in participating countries), age at last observation, status (alive or dead) at last observation, and whether the patient was splenectomized, or had received regular transfusion and/or iron chelation therapy. The age at which regular transfusion and/or iron chelation therapy were started was also retrieved. Data were also collected for history of complications of interest including heart disease (arrythmias or health failure), liver disease (fibrosis, cirrhosis, or hepatocellular carcinoma), and endocrinopathy (diabetes mellitus, hypothyroidism, hypoparathyroidism, hypogonadism, osteoporosis); diagnosed per local standards. For each patient, we also retrieved the predicted complication risk score developed and calculated in previous work from this group.[15] The score was derived and validated from logistic regression formulae to predict clinical complications using a set of indicators of phenotype severity (age, age at diagnosis, age at first transfusion, age at first chelation, haemoglobin level, serum ferritin level, aspartate aminotransferase level, alanine aminotransferase level, and left ventricular ejection fraction).

Table I. Genotypes with ≥1% occurrence in the study cohort.

Genotype	Genotype severity group	*n* (%)
Codon 39/Codon 39	β^0/β^0	273 (12·9)
IVS I-5/IVS I-5	β^+/β^+	212 (10·1)
Codon 39/IVS I-110	β^0/β^+	198 (9·4)
Codon 39/IVS I-6	β^0/β^{++}	154 (7·3)
IVS I-110/IVS I-110	β^+/β^+	123 (5·8)
IVS I-110/IVS I-6	β^+/β^{++}	121 (5·7)
IVS I-6/IVS I-6	β^{++}/β^{++}	80 (3·8)
Codon 39/ IVS I-1	β^0/β^0	79 (3·7)
IVS I-1/IVS I-1	β^0/β^0	47 (2·2)
IVS I-1/IVS I-110	β^0/β^+	43 (2·0)
Codon 39/-87	β^0/β^{++}	33 (1·6)
IVS I-1/IVS I-6	β^0/β^{++}	33 (1·6)
Codon 39/IVS II-745	β^0/β^+	31 (1·5)
IVS I-110/IVS II-745	β^+/β^+	30 (1·4)
IVS II-745/IVS I-6	β^+/β^{++}	27 (1·3)
IVS II-1/IVS II-1	β^0/β^0	26 (1·2)
Codon 44/Codon 44	β^0/β^0	24 (1·1)
Codon 39/IVS II-1	β^0/β^0	23 (1·1)
Codon 30/Codon 30	β^0/β^0	21 (1·0)

Statistical analysis

Descriptive statistics are presented as median and interquartile range (IQR) or percentages. Kaplan–Meier survival curves were constructed to estimate cumulative survival, and the log-rank test was used for comparisons of survival curves. Cox and logistic regression analyses were used to estimate hazard ratios (HR) and 95% confidence intervals (CI) of death and odds ratios (OR) and 95% CI of multiple (≥2) morbidity respectively. Multivariate modelling was used to test for confounding or mediating effects between variables. All *P* values were two-sided with the level of significance set at <0·05.

Results

A total of 2109 patients were included in this analysis. The median (IQR) age at the last observation (corresponding to follow-up time from birth) was 34·1 years (21·1–43·8). The majority of patients (n = 1784, 84·6%) were born in 1970 or afterwards. There was an equal male to female distribution with 1080 patients (51·2%) being female. A total of 494 (23·4%) patients were primarily treated in Asia or MENA and 1615 (76·6%) in the US or Europe. Most patients received regular transfusion [n = 1954, 92·7%; starting at a median (IQR) age of 17 months (12–48)] and/or iron chelation therapy [n = 1964, 93·1%; starting at a median (IQR) age of 60 months (36–120)], and 910 (43·1%) were splenectomized during childhood.

Genotype severity group distribution was as follows: β^0/β^0 (n = 643, 30·5%), β^0/β^+ (n = 379, 18·0%), β^+/β^+ (n = 381, 18·1%), β^0/β^{++} (n = 327, 15·5%), β^+/β^{++} (n = 209, 9·9%), and β^{++}/β^{++} [n = 170, 8·1%; including 37 patients heterozygous for *HBB* gene mutation (32 β^0, 2 β^+, 3 β^{++}) with α-globin gene triplications]. Characteristics of patients in each group are summarized in Table II. The majority of patients across groups were from the US or Europe, except for patients with β^+/β^+ which were predominantly from Asia or MENA. There was a trend towards more common and earlier use of regular transfusion and/or iron chelation therapy and less frequent use of splenectomy, with ascending genotype severity. There was no clear trend in differences of the previously established complication risk score[15] between the various genotype severity groups.

Mortality

Overall, 101 (4·8%) patients died during the observation period. Crude mortality rates were: β^0/β^0 (29/643, 4·5%), β^0/β^+ (16/379, 4·2%), β^+/β^+ (36/381, 9·4%), β^0/β^{++} (8/327, 2·4%), β^+/β^{++} (8/209, 3·8%), and β^{++}/β^{++} (4/170, 2·4%).

We constructed five univariate Cox regression models to estimate HR and 95% CI of death by genotype severity group using various stratifications of clinical interest: Model 1, individual genotype severity group comparisons; Model 2,

Table II. Characteristics of patients in the six genotype severity groups.

Parameter	β^0/β^0 (n = 643)	β^0/β^+ (n = 379)	β^+/β^+ (n = 381)	β^0/β^{++} (n = 327)	β^+/β^{++} (n = 209)	β^{++}/β^{++} (n = 170)
Age at last observation in years, median (IQR)	30·6 (16·4–42·9)	35·2 (24·9–41·9)	25·4 (15·8–36·8)	38·7 (28·9–47·6)	37·3 (26·8–46·5)	41·9 (34·1–53)
Year of birth, *n* (%)						
<1970	77 (12·0)	35 (9·2)	19 (5·0)	86 (26·3)	44 (21·1)	64 (37·6)
≥1970	566 (88·0)	344 (90·8)	362 (95·0)	241 (73·7)	165 (78·9)	106 (62·4)
Female, *n* (%)	338 (52·6)	193 (50·9)	190 (49·9)	167 (51·1)	103 (49·3)	89 (52·4)
Region, *n* (%)						
US or Europe	450 (70·0)	317 (83·6)	164 (43·0)	313 (95·7)	207 (99·0)	164 (96·5)
Asia or MENA	193 (30·0)	62 (16·4)	217 (57·0)	14 (4·3)	2 (1·0)	6 (3·5)
Splenectomized, *n* (%)	234 (36·4)	142 (37·5)	153 (40·2)	165 (50·5)	116 (55·5)	100 (58·8)
Regularly transfused, *n* (%)	600 (93·3)	367 (96·8)	362 (95·0)	301 (92·0)	192 (91·9)	132 (77·6)
Age at start of regular transfusion in months, median (IQR)	12 (12–36)	12 (12–36)	12 (7–24)	24 (12–60)	36 (12–60)	144 (48–339)
Iron chelated, *n* (%)	592 (92·1)	361 (95·3)	359 (94·2)	309 (94·5)	199 (95·2)	144 (84·7)
Age at start of iron chelation in months, median (IQR)	48 (24–84)	48 (36–84)	60 (36–96)	84 (36–204)	72 (36–156)	264 (72–372)
CoRS*, median (IQR)	0·75 (0·41–0·91)	0·82 (0·57–0·93)	0·66 (0·38–0·88)	0·83 (0·59–0·92)	0·84 (0·62–0·93)	0·77 (0·53–0·91)

IQR, interquartile range; MENA, Middle East and North Africa; CoRS, complication risk score.
*The CoRS was calculated as described previously,[15] using a set of indicators of phenotype severity (age, age at diagnosis, age at first transfusion, age at first chelation, haemoglobin level, serum ferritin level, aspartate aminotransferase level, alanine aminotransferase level, and left ventricular ejection fraction).

β^0/β^0 *versus* non-β^0/β^0 (reflecting common eligibility and subgroup analysis stratification used in novel therapy trials); Model 3, homozygous or compound heterozygous for β^0 and β^+ *versus* lower genotype severity groups (reflecting common eligibility and subgroup analysis stratification used in novel therapy trials); Model 4, homozygous for β^0 or compound heterozygous for β^0 and β^+ *versus* homozygous for β^+ *versus* lower genotype severity groups (reflecting a recent survival analysis from Cyprus[9]); Model 5, patients who have both *HBB* genes *versus* a single gene *versus* no genes affected by β^0 or β^+ mutations (reflecting a common clinical consideration scenario; Table III). There was evident that, irrespective of stratification, patients with β^0/β^0, β^0/β^+, or β^+/β^+ had a significantly higher risk of mortality compared with patients in lower genotype severity groups. Surprisingly, it was also noted that patients with β^+/β^+ had a considerably higher risk of mortality than patients with β^0/β^0 or β^0/β^+ (Model 4). The latter was no longer the case when multivariate Cox regression models were constructed to evaluate the same associations, while adjusting for year of birth (<1970 vs. ≥1970), gender, region (US or Europe *versus* Asia or MENA), splenectomy status, regular transfusion status, and iron chelation status (Table III, Table SI). Taking Model 4 as an example, it was clear that there was a mediating effect for the association between β^+/β^+ genotype severity and an increased risk of mortality, primarily driven by region (adjusted HR of death in Asia or MENA *versus* US or Europe: 17·043; 95% CI: 9·864–29·449, $P < 0·001$; Table SI). Nonetheless, after adjustment, the HR of death in patients with β^+/β^+ and that for β^0/β^0 or β^0/β^+ compared with those in lower genotype severity groups become similar (~twofold increased risk). Notably, the increased risk of mortality in patients with β^0/β^0, β^0/β^+, or β^+/β^+ compared with patients in lower genotype severity groups was also independent of receipt of conventional management. Per Model 5, there was also an increased risk of mortality for patients with β^0/β^{++} or β^+/β^{++} compared with patients with β^{++}/β^{++} but this did not reach statistical significance (Table III). The Kaplan–Meier survival curves for Model 3 and Model 5 are illustrated in Fig 1. Cumulative survival estimates by age of 65 years per Model 3 were 36·8% (β^0/β^0 or β^0/β^+ or β^+/β^+) and 90·2% (β^0/β^{++} or β^+/β^{++} or β^{++}/β^{++}); log-rank χ^2: 29·376, $P < 0·001$ (Fig 1A); and differences between genotype severity groups were similarly observed when evaluated separately for patients in US or Europe and Asia or MENA. Cumulative survival estimates by age of 65 years per Model 5 were 36·8% (β^0/β^0 or β^0/β^+ or β^+/β^+), 84·1% (β^0/β^{++} or β^+/β^{++}), and 95·9% (β^{++}/β^{++}); log-rank χ^2: 30·541, $P < 0·001$ (Fig 1B).

Morbidity

The distributions of morbidity rates in different genotype severity groups are illustrated in Fig 2. The crude multiple (≥2) morbidity rate was higher with ascending genotype severity (Table IV). On multivariate logistic regression analysis adjusting for year of birth (<1970 vs. ≥1970), gender,

Table III. Risk of mortality by genotype severity in various stratification models.

Model	*N*	Death, *n* (%)	Unadjusted HR (95% CI)	*P* value	Adjusted HR (95% CI)*	*P* value
Model 1						
β^{++}/β^{++}	170	4 (2·4)	Referent	—	Referent	—
β^+/β^{++}	209	8 (3·8)	3·339 (0·968–11·512)	0·056	3·112 (0·904–10·716)	0·072
β^0/β^{++}	327	8 (2·4)	1·886 (0·549–6·485)	0·314	1·748 (0·508–6·011)	0·375
β^+/β^+	381	36 (9·4)	17·453 (5·669–53·734)	<0·001	4·401 (1·399–14·460)	0·015
β^0/β^+	379	16 (4·2)	4·860 (1·513–15·608)	0·008	3·287 (1·001–10·795)	0·050
β^0/β^0	643	29 (4·5)	5·958 (1·941–18·289)	0·002	4·270 (1·367–13·335)	0·012
Model 2						
β^{++}/β^{++} or β^+/β^{++} or β^0/β^{++} or β^+/β^+ or β^0/β^+	1466	72 (4·9)	Referent	—	Referent	—
β^0/β^0	643	29 (4·5)	1·179 (0·763–1·821)	0·459	1·393 (0·895–2·169)	0·142
Model 3						
β^{++}/β^{++} or β^+/β^{++} or β^0/β^{++}	706	20 (2·8)	Referent	—	Referent	—
β^+/β^+ or β^0/β^+ or β^0/β^0	1403	81 (5·8)	3·863 (2·296–6·500)	<0·001	2·104 (1·176–3·763)	0·012
Model 4						
β^{++}/β^{++} or β^+/β^{++} or β^0/β^{++}	706	20 (2·8)	Referent	—	Referent	—
β^+/β^+	381	36 (9·4)	8·768 (4·848–15·859)	<0·001	2·324 (1·152–4·688)	0·018
β^0/β^+ or β^0/β^0	1022	45 (4·4)	2·795 (1·601–4·879)	<0·001	2·051 (1·134–3·708)	<0·001
Model 5						
β^{++}/β^{++}	170	4 (2·4)	Referent	—	Referent	—
β^+/β^{++} or β^0/β^{++}	536	16 (3·0)	2·312 (0·744–7·187)	0·147	2·226 (0·717–6·912)	0·166
β^+/β^+ or β^0/β^+ or β^0/β^0	1403	81 (5·8)	7·371 (2·505–21·690)	<0·001	3·943 (1·292–12·031)	0·016

HR, hazard ratio; CI, confidence interval; MENA, Middle East and North Africa.

*Adjusted for year of birth (<1970 vs. ≥1970), gender, region (US or Europe *versus* Asia or MENA), splenectomy status, regular transfusion status, iron chelation status.

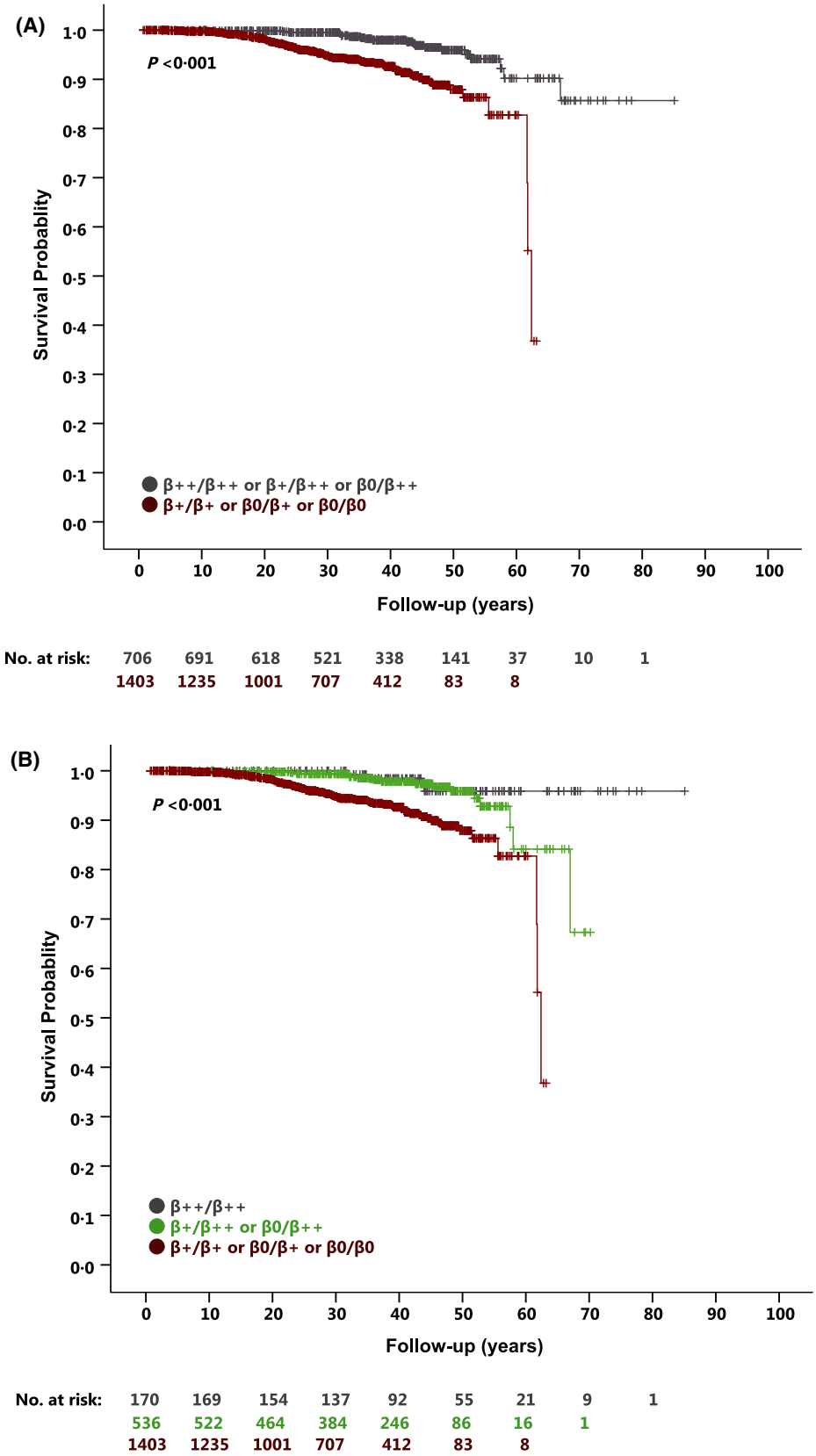

Fig 1. Kaplan–Meier survival curves for: (A) Model 3, and (B) Model 5 stratification of genotype severity groups. [Colour figure can be viewed at wileyonlinelibrary.com]

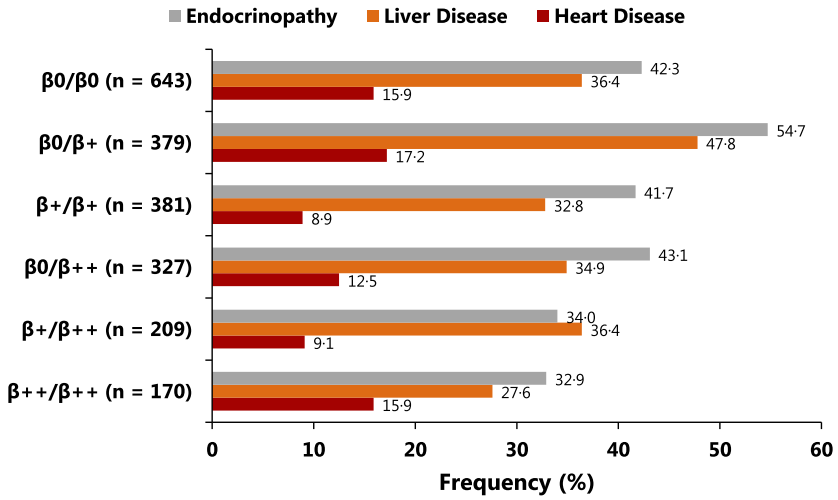

Fig 2. Frequency of complications of interest in different genotype severity groups. [Colour figure can be viewed at wileyonlinelibrary.com]

Table IV. Risk of multiple morbidity by genotype severity in various stratification models.

Model	*N*	Multiple morbidity*, *n* (%)	Unadjusted OR (95% CI)	*P* value	Adjusted OR (95% CI)†	*P* value
Model 1						
β^{++}/β^{++}	170	34 (20·0)	Referent	–	Referent	–
β^{+}/β^{++}	209	49 (23·4)	1·225 (0·748–2·007)	0·420	1·370 (0·806–2·328)	0·245
β^{0}/β^{++}	327	81 (24·8)	1·317 (0·838–2·070)	0·232	1·534 (0·943–2·497)	0·085
β^{+}/β^{+}	381	99 (26·0)	1·404 (0·904–2·181)	0·131	3·148 (1·898–5·224)	<0·001
β^{0}/β^{+}	379	156 (41·2)	2·798 (1·824–4·292)	<0·001	5·412 (3·346–8·753)	<0·001
β^{0}/β^{0}	643	201 (31·3)	1·819 (1·206–2·744)	0·004	3·583 (2·264–5·670)	<0·001
Model 2						
β^{++}/β^{++} or β^{+}/β^{++} or β^{0}/β^{++} or β^{+}/β^{+} or β^{0}/β^{+}	1466	419 (28·6)	Referent	–	Referent	–
β^{0}/β^{0}	643	201 (31·3)	1·136 (0·929–1·390)	0·214	1·413 (1·131 −1·765)	0·002
Model 3						
β^{++}/β^{++} or β^{+}/β^{++} or β^{0}/β^{++}	706	164 (23·2)	Referent	–	Referent	–
β^{+}/β^{+} or β^{0}/β^{+} or β^{0}/β^{0}	1403	456 (32·5)	1·591 (1·293–1·958)	<0·001	2·956 (2·310–3·784)	<0·001
Model 4						
β^{++}/β^{++} or β^{+}/β^{++} or β^{0}/β^{++}	706	164 (23·2)	Referent	–	Referent	–
β^{+}/β^{+}	381	99 (26·0)	1·160 (0·870–1·547)	0·312	2·355 (1·658–3·346)	<0·001
β^{0}/β^{+} or β^{0}/β^{0}	1022	357 (34·9)	1·774 (1·428–2·204)	<0·001	3·117 (2·419–4·018)	<0·001
Model 5						
β^{++}/β^{++}	170	34 (20·0)	Referent	–	Referent	–
β^{+}/β^{++} or β^{0}/β^{++}	536	130 (24·3)	1·281 (0·838–1·958)	0·253	1·460 (0·925–2·305)	0·104
β^{+}/β^{+} or β^{0}/β^{+} or β^{0}/β^{0}	1403	456 (32·5)	1·926 (1·301–2·851)	0·001	3·980 (2·563–6·181)	<0·001

OR, odds ratio; CI, confidence interval; MENA, Middle East and North Africa.

*Two or more morbidities.

†Adjusted for year of birth (<1970 vs. ≥1970), gender, region (US or Europe *versus* Asia or MENA), splenectomy status, regular transfusion status, iron chelation status.

region (US or Europe *versus* Asia or MENA), splenectomy status, regular transfusion status, and iron chelation status (Table IV, Table SII), similar observations were made with regards to patients with β^{0}/β^{0}, β^{0}/β^{+}, or β^{+}/β^{+} (Model 3) having significantly increased odds of multiple morbidity (OR: 2·956; 95% CI: 2·310–3·784, $P < 0·001$) compared with patients in lower genotype severity groups. Per Model 5, there was also an increased risk of multiple morbidity for patients with β^{0}/β^{++} or β^{+}/β^{++} compared with patients with β^{++}/β^{++}, but this did not reach statistical significance (Table IV).

Discussion

Our study established mortality and morbidity outcomes for various genotype severity groups that could further guide

clinical decision making and research. The key observation is that patients who harbour β^+ *HBB* gene mutations show a similar risk of mortality and morbidity to patients with β^0 mutations. Thus, any stratification for clinical or research purposes aiming to delineate a group with 'severe' genotype and outcomes should include β^0/β^0, β^0/β^+ as well as β^+/β^+ and not only β^0/β^0 and β^0/β^+. The most common β^+ mutations in our cohort, comprised mostly of patients from Italy, were IVS I-5 (East Asian, Asian Indian), IVS I-110 (Mediterranean), and IVS II-745 (Mediterranean); followed by IVS II-654 (Chinese) and IVS II-848 (Mediterranean, African-American). Efforts that establish global databases of *HBB* gene mutations are highly commended, as these allow standardization and uniform reporting of mutation severity.[11,12,16]

At the moment, stratification by genotype severity in novel therapy trials remains variable. For instance, subgroup analysis of the phase 3 trial of luspatercept, an erythroid maturation agent, in transfusion-dependent β-thalassaemia stratified patients as β^0/β^0 and non-β^0/β^0; and showed a positive yet relatively lower effect of transfusion reduction in patients with a β^0/β^0 genotype.[17] Efficacy data from the phase 1/2 gene therapy trials with betibeglogene autotemcel (LentiGlobin BB305) in transfusion-dependent β-thalassaemia have also been stratified for patients with β^0/β^0 and non-β^0/β^0, although in this programme IVS I-110 was also grouped with β^0 mutations.[18] Benefit was more pronounced in the non-β^0/β^0 subgroup, for which gene therapy was approved in Europe. The two ongoing phase 3 trials of betibeglogene autotemcel in children and adults with β^0/β^0 or non-β^0/β^0 transfusion-dependent β-thalassaemia have also used similar genotype severity group considerations (ClinicalTrials.gov numbers, NCT02906202 and NCT03207009). Studies with other gene therapy approaches using the lentiviral vector GLOBE have solely focused on patients with β^0 and β^+ mutations.[19] Clinical trials of gene editing in transfusion-dependent β-thalassaemia with engineered nucleases such as zinc finger nucleases (ST-400) and clustered regularly interspaced short palindromic repeats linked to Cas9 nucleases (CRISPR-Cas9; CTX001) are also under way and preliminary results of transfusion reduction are further evaluated according to genotype severity combinations of β^0 and β^+.[20,21] We strongly support such consideration of the underlying molecular profile of patients recruited in novel therapy trials, considering that severe mutations are associated with very low intrinsic capacity for haemoglobin and red-cell synthesis; thus, understanding efficacy specific to this subgroup will be essential to quantify benefit and inform future clinical use. Our data further promotes this by highlighting poor outcomes and persistent unmet need for patients with severe genotypes, despite receiving transfusion and iron chelation therapy.[22] Reliance on transfusion burden alone to determine eligibility and interpret benefit may mask such differences at the molecular level, since transfusion in itself can suppress endogenous erythropoiesis.[23,24]

In this study, higher risks of mortality and morbidity were observed in patients with severe genotypes despite early use of conventional management. This is in contrast to a recent study from Cyprus, which showed that survival was worse in milder genotypes, primarily attributed to late initiation of adequate transfusion therapy.[9] Despite advances in transfusion and iron chelation therapy and improved survival of β-thalassaemia patients over the past few decades, a considerable proportion of patients continue to live with high iron burden and associated morbidity.[25,26] It should also be noted that our study, with decades of follow-up, was only able to evaluate for 'use' and 'timing' of transfusion and iron chelation therapy, but effective management is also dependent on 'adequate' use, dosing, and adherence; among other patient- and disease-related factors.[13] Geographical variations in management standards continue to be observed,[27] and suboptimal access to adequate therapy continues to be a challenge in resource-poor countries. Nonetheless, our study still found an association of poor outcomes in severe genotypes even after adjustment for region of care. Taken together, our study calls for adequate and optimal management for patients with severe genotypes including those with β+ mutations. Adequate management of patients in lower genotype severity groups is also needed. Despite being at lower risk of poor outcomes compared to severe genotypes, the crude rates of morbidity and mortality remain high. Even in non-transfused patients, high rates of morbidity and mortality can be attributed to primary iron overload (increased intestinal absorption), anaemia, and hypercoagulability that can lead to early death from hepatic and cardiovascular disease; the latter not being necessarily iron-mediated but more likely due to thromboembolic events and pulmonary hypertension.[28]

Secondary genetic modifiers such as co-inheritance of α-thalassaemia or variations that can lead to more effective synthesis of γ-globin chains and fetal haemoglobin after birth were not evaluated in this study but could also affect disease severity, especially the differences in mortality and morbidity outcomes between patients in lower genotype severity groups. Moreover, the impact of genotype on pathophysiologic mechanisms beyond anaemia and iron overload (typically addressed by transfusion and iron chelation) cannot be fully dismissed and may explain variations in outcomes despite conventional management.

The fact that we did not see a clear trend in differences of the previously established complication risk score (based on variables of age, timing of management initiation, iron overload, anaemia, hepatic and cardiac function)[15] between the various genotype severity groups is probably attributed to relative similarity in use of intervention and the observation that even milder severity groups remain at risk of morbidity. It also reflects inability of such spot/random measurements of laboratory and imaging measures to explain the association between genotype and long-term outcomes. As previously mentioned, this also further supports that variations in outcomes likely extend beyond basic metrics of intervention ('use' *versus* 'no use' and 'timing') to elements of adequacy and adherence to treatment, and that they may also rely on

pathophysiologic mechanisms beyond those directly attributed to anaemia and iron overload; all of which should be considered in future risk assessment models.

Our study may be limited by the retrospective cohort design which can lead to missing information and loss to follow-up bias typical of long-term retrospective studies. This could lead to under- or overestimation of mortality and morbidity risks. Prospective validation, however, may not be feasible, considering the long observation period required for morbidity and mortality outcomes to manifest. However, we do encourage further research to explore disease- and management-related reasons for variation in outcomes across β-thalassaemia genotypes and targeting expanded patient cohorts including haemoglobin E/β-thalassaemia and α-thalassaemia. Lastly, we only analyzed patients with available genotype data in our database. Whether the availability of molecular testing for included patients was random or rather selects patients receiving more comprehensive treatment cannot be determined, but this calls for a need to establish collaborations that aim for wider testing and support for centres without adequate resources. The majority of our current sample was also from the US and Europe (specifically, Italy) and we encourage future collaborations to aim at including other geographies with high prevalence of thalassaemia.

In conclusion, our study identified mortality and morbidity risk estimates across various genotype severity groups in patients with β-thalassaemia and suggests inclusion of both β^{+} and β^{0} mutations in strata of greatest severity. Such understanding of outcomes and unmet needs should help with modifications to current management regimens and the design and interpretation of novel therapy trials targeting the underlying pathophysiology in patients with β-thalassaemia. This also highlights the need for routine determination of genotype to support clinical management decisions, a practice that is unfortunately not yet widely applied.

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Data sharing

Data were collected and stored on the IHR electronic platform (www.sanitasicilia.eu/IWG), and can be made available upon request to the corresponding author.

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Author contributions

Study design: KMM, AV, AMa. Data collection: AV, AMe, SAP, VDM, SHA, AF, PR, AC, SD, EVl, STS, ZAN, EVi. Data analysis: KMM, AV. Manuscript drafting: KMM, AV, AMa. Data interpretation and manuscript review for intellectual content: all authors. Final approval for submission: all authors.

Conflict of interest

KMM has been or is a consultant for Novartis, Celgene Corp (Bristol Myers Squibb), Agios Pharmaceuticals, CRISPR Therapeutics and Vifor Pharma. AMe received speakers' honoraria from Chiesi Farmaceutici S.p.A. EVl received hononaria from DEMO S.A. Pharmaceutical Industry and Novartis. AP is the principal investigator of the MIOT project that receives 'non-profit support' from industrial sponsorships (Chiesi Farmaceutici S.p.A. and Bayer) and she received speakers' honoraria from Chiesi Farmaceutici S.p.A. ATT has been or is consultant for Novartis, Celgene Corp (Bristol Myers Squibb), Vifor Pharma, Silence Therapeutics and Ionis Pharmaceuticals; and received research funding from Novartis, Celgene Corp (Bristol Myers Squibb), La Jolla Pharmaceutical Company, Roche, Protagonist Therapeutics and Agios Pharmaceuticals. VGS serves as an advisor to and/or has equity in Novartis, Forma, Cellarity, Ensoma, and Branch Biosciences. AMa has been or is a member of advisory boards for Novartis, Celgene Corp (Bristol Meyers Squibb) and Bluebird Bio. The remaining authors have no conflicts of interest to disclose.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table SI. Effect estimates using Cox regression for the outcome of mortality with genotype severity considered in different stratification models.

Table SII. Effect estimates using Cox regression for the outcome of multiple morbidity with genotype severity considered in different stratification models.

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