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Effect of *HBB* genotype on survival in a cohort of transfusion-dependent thalassemia patients in Cyprus

Petros Kountouris^{1,2,*}, Kyriaki Michailidou^{1,2}, Soteroula Christou³, Michael Hadjigavriel⁴, Maria Sitarou⁵, Anita Kolnagou⁶, Marina Kleanthous^{1,2}, Paul Telfer⁷

Affiliations:

¹The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus

²Cyprus School of Molecular Medicine, Nicosia, Cyprus

³Thalassemia Centre, Archbishop Makarios Hospital, Nicosia, Cyprus

⁴Thalassemia Centre, Limassol General Hospital, Limassol, Cyprus

⁵Thalassemia Centre, Larnaca General Hospital, Larnaca, Cyprus

⁶Thalassemia Centre, Paphos General Hospital, Paphos, Cyprus

⁷Centre for Genomics and Child Health, Blizard Institute, Queen Mary University of London, UK

*Correspondence should be addressed to:

Petros Kountouris,
The Cyprus Institute of Neurology and Genetics,
6 International Airport Avenue,
1683 Nicosia, Cyprus.
e-mail: petrosk@cing.ac.cy
Tel.: 0035722392623

Abstract

Initiation of regular transfusion in transfusion-dependent thalassemia (TDT) is based on the assessment of clinical phenotype. Pathogenic *HBB* variants causing β -thalassemia are important determinants of phenotype and could be used to aid decision making. We investigated the association of *HBB* genotype with survival in a cohort study in the four thalassemia centres in Cyprus. *HBB* genotype was classified as severe (β^0/β^0 or β^+/ β^0), moderate (β^+/β^+), or mild (β^0/β^{++} or β^+/β^{++}). Risk factors for mortality were evaluated using multivariate Cox proportional-hazards regression.

537 subjects were followed for a total of 20,963 person years. 80.4% (95% CI 76.4–84.7) of individuals survived to 50 years of age with increasing rates of liver, infection and malignancy-related deaths observed during recent follow-up. We evaluated non-modifiable risk factors and found worse outcomes associated with male sex (Hazard ratio 1.9, 95% CI 1.1–3.0, $p=0.01$) and milder genotype (Hazard ratio 1.6, 95% CI 1.1–2.3, $p=0.02$). The effect of genotype was confirmed in a second model, which included treatment effects. Patients with a milder genotype initiated transfusion significantly

later and had reduced blood requirements compared to those with moderate or severe genotypes, although pre-transfusion hemoglobin levels did not differ between genotypes. Our results suggest that early treatment decisions to delay transfusion and different long-term treatment strategies in milder genotypes have led to adverse long-term effects of under-treated thalassemia and worse survival. We propose that *HBB* genotype determination and use of this information to aid in decision making can improve long-term outcomes of thalassaemia patients.

Introduction

Standard care of transfusion-dependent thalassemia (TDT) has improved dramatically over the past five decades with the introduction of regular transfusion, a variety of iron chelation drugs, improved evaluation of transfusion iron overload with MRI modalities, and development of specialist centres and regional networks(1-7). There has been a consensus on standard care disseminated in national and international guidelines, which are periodically updated(8-10), resulting in a progressive improvement in survival in successive birth cohorts(2,5,11).

One aspect of care which is not standardised concerns the decision to start regular transfusion. Those at the most severe end of the phenotypic spectrum require regular transfusion before the age of 2 years for survival. At the other end of the spectrum are non-transfusion dependent thalassemia (NTDT) patients who do not require regular transfusion to survive, are less anaemic, and have less bone marrow expansion. Clinicians have traditionally avoided regular transfusion in these cases because of concerns about the consequences of long-term transfusion, notably transfusion iron overload. The result of not correcting the thalassemia phenotype in earlier life includes a variety of chronic complications which can become apparent later. These include liver disease, pulmonary hypertension, endocrine dysfunction, bone disease and splenomegaly leading to splenectomy. Quality of life is significantly impaired and life expectancy may be reduced(12-15). In the middle of the phenotypic spectrum are TDT patients who are not absolutely transfusion-dependent early in their life, and regular transfusion may be delayed in the age range 2–10 years(16,17), during which interval they may be at risk of developing complications of untreated thalassemia.

Over 400 *HBB* variants causing β -thalassemia have been reported to date(18) and are generally classified as β^0 , where no functional β -globin chains can be produced; β^+ , where a β -globin chain production is severely reduced; and β^{++} , where β -globin chain production is mildly reduced(19). A previous study has evaluated the influence of *HBB* pathogenic variants and other genetic modifiers on time to initiate regular transfusion, and developed a predictive model derived from a composite score. *HBB* genotype was found to be the most powerful predictor of severity(16,17). In routine practice, genetic diagnosis is often not available to the clinician and is not currently used for clinical decision making. Further evidence of different outcomes associated with different genotypes is needed to recommend routine determination of genotype at birth and to make use of genotypic information in clinical decision making about initiation of transfusion and changing other aspects of standard practice.

Cyprus is a Mediterranean island with a relatively stable indigenous population at high risk of thalassemia. Following introduction of a national carrier screening programme in 1974, all new diagnoses of infants on the government-controlled southern part of the island have been documented

and standard care is given in four treatment centres(20), based on shared protocols for transfusion and chelation developed over the past 30 years. The decision for the start of transfusion has been purely based on clinical observation according to best standard practice. The patients have remained stable within each clinic with very little immigration or emigration. The Cyprus thalassemia cohort consists of TDT patients born between 1960 and 2000, and follow-up to 2004 was published previously(1). Herein, we present updated information with longer follow-up from this cohort, in which we generate a more robust estimate of survival of thalassemia with standard treatment, we confirm changing causes of mortality, and we explore the association of genotype with long-term outcomes and survival.

Methods

Patients and data preparation

This study is a follow-up of a previously described patient cohort(1). Briefly, we included all patients that have been transfusion-dependent at the time of the first study, born in Cyprus between 1960 and 2000, and treated using standard care in the four thalassemia centres. Data were collected and validated for each treatment centre up to 30th September 2018. Causes of death were classified as cardiac, liver (including hepatocellular carcinoma), malignancy, infection and other, using standard clinical criteria. Considering the introduction of the Cyprus thalassemia screening and prevention programme(20), which resulted in identification soon after birth of all affected children from 1974 onwards, year of birth was categorised as pre-1974 or 1974 onwards. Based on commonly agreed categories of pathogenic variant severity(21), *HBB* genotype was classified as severe, moderate and mild, as shown in Table 1, assuming equally spaced severity across categories.

Chelation treatment was categorized based on the predominant chelator used during follow-up from year 2000 onwards: i) deferoxamine (DFO) only, ii) deferiprone (DFP) alone or in combination with DFO, and iii) deferasirox (DFX). DFO was given by subcutaneous or intravenous infusion, whereas DFP and DFX were administered orally. For splenectomy, the effect on survival was assumed to be dependent on whether and when it had been performed, and was categorized as i) before 16 years of age, and ii) after 16 years of age or not done.

To evaluate genotype association with the transfusion regimens, we used data previously collected for 336 cohort patients, as part of the THALAMOSS project(22). These data comprised the age of the first regular transfusion, and annual blood usage and pre-transfusion hemoglobin levels, between 2014 and 2016.

Statistical analysis

A univariate analysis was performed to estimate the overall survival and the association of different risk factors with mortality using Kaplan Meier analysis. The Log-Rank test was used to evaluate the statistical significance of the risk factors on survival. For the multivariate analysis, we used the Cox proportional-hazards (CoxPH) regression with a backwards approach in which all potential factors that were significant in the univariate analysis were included in the initial regression model and, sequentially, removed to obtain the best-fit model. All models were tested whether they meet the proportional hazards assumption for a CoxPH model.

Proportions were compared in contingency tables using the Pearson's χ^2 test. Mortality rates and confidence intervals were calculated overall and for periods 1980-89, 1990-99, 2000-09, and 2010-18. The Kruskal-Wallis test and the Wilcoxon rank-sum test for post-hoc analyses were utilised to study the association between the HBB genotype with transfusion data. All statistical analyses were performed using R (version 3.6.1) and R packages survival and survminer.

Ethical considerations

The study was approved by the Cyprus National Bioethics Committee and was initiated by PT as part of a project commissioned by the Cyprus Ministry of Health. Subsequently, informed consent was obtained for clinical follow-up of the cohort as part of the THALAMOSS project (EU FP7 grant agreement 306201), which included collection of transfusion data.

Results

A summary of the clinical characteristics of the 537 cohort patients are documented in Table 2. There were two fewer patients than in the original study. Both of these patients were found to have been ineligible, one because of birth prior to 1960, and the other with a diagnosis of Hb H disease. Eight patients had been treated with sibling allogeneic stem cell transplant, at a mean age of 12.7 years. Follow-up on these eight patients was censored on the date of transplant.

Total patient years of follow up was 20,963. The majority of the patients were born between 1960 and 1979 (453 patients, 84.4%). Splenectomy status was significantly associated with clinic ($p=0.02$), with 21.8% splenectomized during childhood in Nicosia, compared to 36.7%, 29.3% and 34.9%, respectively, in Larnaca, Limassol and Paphos, while there was no significant treatment difference in choice of iron chelation therapy between clinics. Thirty-eight (7.1%) patients had evidence of hepatitis C infection (HCV antibody positive), of whom 9 (1.7%) were HCV RNA positive at the end of the study and 29 (7.3%) were HCV RNA negative. Three patients (0.6%) were hepatitis B surface antigen positive.

A summary of the genotypes of the study patients are shown in Table 1. *HBB* genotype data were not available for 24 of patients, who had died before genotyping was introduced in Cyprus. An additional

patient was removed from the genotype analysis because thalassemia was caused by α -locus duplications co-inherited with β -thalassemia trait(23). Homozygous IVS I-110 G>A (β^+) was the commonest genotype (59.8%) and there was a large proportion of patients with a combination of IVS I-110 G>A (β^+) and IVS I-6 T>C (β^{++}). There was a significant trend on performing splenectomy in childhood with decreasing severity of genotype, specifically 19% in the severe genotype group, 27.8% in the moderate genotype group, and 38.1% in the mild genotype group ($p=0.02$). There was also a significant trend across the genotypic spectrum in chelation, with increasing proportions of patients with a milder phenotype remaining on DFO rather than switching to oral chelation, specifically 14.3% with severe genotype, 17.5% with moderate genotype; and 28.9% with mild genotype ($p=0.02$). Genotyping for the α -globin locus and the XmnI C/T polymorphism was almost complete for the study population (92.4% and 89.4%, respectively). The XmnI T allele, which is associated with increased Hb F production, has a low prevalence and, thus, limited influence on clinical outcomes in the study population. In contrast, α^+ -thalassemia trait is present in about 19% of the population, whereas α^0 -thalassemia trait has lower prevalence (1.6%)(24).

Mortality rates and causes of death

By September 2018, 94 (17.5%) patients had died. Crude comparisons of proportions are shown in Table 2, indicating that survival is significantly associated with sex, splenectomy in childhood, and receiving oral chelation compared to those chelated primarily with DFO. However, 44 of 70 (57.1%) of the patients with DFO chelation died before the year 2000, and these patients would not have had the opportunity to receive prolonged oral chelation. Those with more severe *HBB* genotypes were also more likely to survive ($p=0.03$ for trend). The mean age of death in the severe genotype category was higher (39.2 years, 95% CI 28.4–50.1) compared to the moderate (mean age 31.4, 95% CI 28.3–34.4) and mild genotype category (35.0 years, 95% CI 30.0–40.1). There were no significant differences in surviving versus non-surviving proportions with different α -globin gene numbers or with the XmnI polymorphism.

Causes of death are shown in Table 3. Liver disease included 6 cases of liver failure and 4 cases of hepatocellular carcinoma; other malignancies comprised acute myeloid leukaemia (2), T cell lymphoma (1), metastatic melanoma (1), renal carcinoma (1), carcinoma of colon (1), carcinoma of skull (1); infection (15) ; other (18). Other causes of death are categorized as: not known (7), accident (5), stroke (2), hypoglycaemia (1), myasthenia (1), acute graft versus host disease (1), pulmonary hypertension (1). Table 3 also shows mortality rates per 10,000 years from different causes during sequential time periods of follow-up. There was a lower incidence of overall mortality in the period 1980-89, but no significant differences in mortality rates in subsequent time periods and no overall trend. There was a significant decrease in cardiac mortality over the last three time periods, and an increased incidence of deaths due to liver disease, cancer and infective causes during successive

decades. However, there were insufficient numbers of deaths from these causes in each individual decade to evaluate statistically. The proportion of deaths due to heart disease was lower in those with a mild genotype (6 out of 20, 30%) compared to those with moderate (23 out of 44, 52%) and severe genotypes (4 out of 8, 50%). These differences did not reach statistical significance. Of the 15 who died due to infectious causes, 6 of 9 with known splenectomy status had been splenectomised. Five of 94 (5.3%) patients who died were HCV RNA positive at time of death. Three of these died of heart failure and two from liver disease. HCV infection could be a contributing factor for these deaths, but likely to be a primary cause in only 2 (2.1%) of cases.

Survival analysis

We constructed two models to explore risk factors associated with mortality. In both models, data were right censored on the 30th September 2018, at death or bone marrow transplantation (BMT). In the first model, we considered the entire follow-up period and evaluated non-modifiable risk factors. These comprised sex, year of birth, treatment clinic and genotype. To assess the impact of treatment factors (iron chelation therapy and splenectomy), a second model was constructed. This takes into account the licensing of the oral iron chelator DFP in 1999 and general availability for prescription from 2000. DFP was sometimes used as single therapy and frequently in combination with DFO, depending on the clinician's assessment of iron overload severity. A second oral chelator, DFX, was licensed and available for prescription in 2006 and there was a switching between different chelator regimes over time after 2006 to optimise therapy for each individual. Since it would be biased to compare chelator efficacy during the period 1980-2000, when the only option available was DFO, follow-up was restricted to the period 2000-2018, when at least one option for oral chelation was available, and data was left truncated at 1/1/2000.

Survival analysis results are shown in Table 4 and Figure 1. Survival by age 50 is estimated at 80.4% (95% CI 76.4-84.7). In the first model of survival, univariate analysis showed that male sex, milder *HBB* genotype and birth <1974 were significantly associated with worse survival, while there was no significant effect of treatment clinic, α -globin gene number or XmnI polymorphism. When the survival analysis was applied separately to cardiac and non-cardiac deaths, the genotype had no significant effect on cardiac deaths, but a significant effect on non-cardiac deaths ($p=0.02$). In the multivariate model, independent predictors of survival were male sex (HR 1.9, 95% CI 1.2–3.0, $p=0.01$) and genotype, where each decrement in severity was associated a 1.6-fold increased risk of mortality (HR 1.6, 95%CI 1.1–2.3, $p=0.02$).

In the second model, 489 evaluable patients were followed for a total of 8644 person years. Estimated survival was 89.9% (95% CI 87.3–92.6) as of 30th September 2018. Univariate and multivariate analysis of risk factors for survival over this time period is shown in Table 5 and Figure 2. In the univariate analysis, male sex, birth <1974, milder genotype, splenectomy in childhood and DFO-only

chelation treatment were significantly associated with worse survival, with iron chelation treatment being the most significant factor. In multivariate analysis, the best fit model included chelation, sex, splenectomy in childhood and *HBB* genotype. Importantly, the significant effects of male sex and milder genotype on survival were confirmed in the second multivariate model, while DFO-only treatment and splenectomy in childhood were also associated with worse survival.

Association of genotype with transfusion parameters

Transfusion data were available for 336 cohort patients (62.6%) and were used to evaluate possible associations between the *HBB* genotype and the transfusion regimens. The mean age of first regular transfusion was 29.4 months, the mean transfusion frequency per year was 28.7 times, the mean blood volume transfused was 179 ml/kg/year and the mean pre-transfusion hemoglobin level was 9.9 g/dL.

The *HBB* genotype was significantly associated with the age of first regular transfusion ($p=0.001$), the annual transfusion frequency ($p=0.04$) and the blood volume transfused ($p=0.01$), whereas no association was found for pre-transfusion hemoglobin levels ($p=0.8$).

Post-hoc statistical analysis was subsequently performed to demonstrate differences in transfusion between different genotypic severity groups, shown in Figure 3. Statistically significant differences were identified between the severe and mild groups for the age of first transfusion ($p=0.004$) and the annual transfusion frequency ($p=0.024$). In addition, statistically significant differences were found between the moderate and mild groups for the age of first regular transfusion ($p=0.0006$), the annual transfusion frequency ($p=0.025$) and the blood volume transfused ($p=0.003$). There was no significant association between the severe and moderate groups for any of the transfusion parameters studied.

Discussion

The first report on this cohort was right-censored at 31st December 2004(1). The current data cut-off allows a further 14 years of follow-up, and more robust estimates of overall survival, trends in mortality and risk factors for mortality.

In a study of health outcomes and healthcare costs in the UK(11), survival of thalassemia patients treated according to standard guidelines was forward-estimated at 63% by the age of 50 years. This was based on a review of available data in which clinical follow-up was only reported up to 30 years of age. The estimate of 80.4% in this study is more robust and significantly more optimistic. Life expectancy in the general population of Cyprus has been reported recently using national statistics over the period 1986–2012(25). About 90–95% are expected to survive to 50 years, and our data would suggest that a diagnosis of thalassemia reduces the percentage surviving to this age by about 15%. These data should be helpful in discussing outcomes of thalassemia with new parents, in refining health economic models, comparing outcomes of new therapies with standard care, and in

making treatment decisions about use of alternative donor transplantation, where overall survival with unrelated, or mismatched family donors are currently less than 80%(26).

Causes of death appear to be changing with longer term follow-up of TDT patients. We have confirmed reduction in deaths due to cardiac causes over the past 20 years, as reported previously in this cohort(1) and in other studies(5). We also noted increasing mortality related to liver disease and infection in this cohort, a finding that has also been reported in recent registry studies from Sicily and Greece(6,27). The increasing numbers of deaths due to malignancies has also previously been noted(28). Although the incidence of cancer naturally increases with age, and it has not been established that the incidence is significantly different from the general population, the types of malignancy appear to be different, notably with a higher representation of hepatocellular carcinoma. Further follow-up of the cohort and evaluation in other similar studies will be needed to confirm this finding.

The distribution of *HBB* genotypes in this study are consistent with the known distribution of thalassemia alleles in the Cypriot population(29,30). There is a predominance of homozygosity for IVS I-110 G>A, a severe β^+ variant causing a cryptic splice site in the first intron of *HBB* which reduces functional β -globin mRNA to about 20% of normal(31). Next in prevalence is IVS 1-6 T>C, causing aberrant splicing at the first intronic junction of *HBB*, with a less severe reduction in β -globin mRNA. Being a milder variant, it has been classified as β^{++} or β^+ on different studies. We have selected the former annotation for this study(21). The most severe category (β^0/β^0) is rare in the Cyprus TDT population (0.8%), and this contrasts with some other TDT populations, such as in Sardinia where 92% of TDT patients have homozygous or compound heterozygous β^0 mutations(16). Nevertheless, the two commonest variants in this cohort are common in the Mediterranean and the Middle East, and are also observed in many immigrant populations(18). For risk factor analysis, we introduced a genotypic severity categorization, with the assumption that severity increases linearly across the spectrum mild/moderate/severe. This categorization could be explored in studies of severity, treatment and outcomes in other populations, even if the specific variants are different.

The variability of risk factors during follow-up present problems for statistical modelling. We addressed this by evaluating two different models, the first of which evaluated non-modifiable factors only and did not include treatment factors, but assumed that standard care was applied uniformly to all subjects. The worse survival associated with male sex has been previously reported(1,2) and could be linked to worse adherence to the recommended chelation therapy and higher prevalence of specific complications, such as heart disease, in male patients. Moreover, the significantly worse survival associated with milder *HBB* genotype would suggest that standard care is not sufficiently effective to cancel the pathological effects of milder genotype. One recent confirmatory study has shown a convergence in mortality in their population of thalassemia major and thalassemia intermedia (TI)

patients in Sicily with similar length of follow up(27). The definition of TI in the study from Sicily is regular transfusion after the age of two. Based on the transfusion data of the current study, the above definition of TI encompasses 44.4% (122 out of 275) of patients with moderate or mild genotypes in the Cyprus TDT patient cohort.

The second model, which included treatment effects, confirmed that male sex and milder *HBB* genotype was significantly associated with mortality. Here, chelation therapy for each individual was categorized according to the predominant agent used. We confirmed that oral iron chelation therapy had a strong independent protective effect on survival compared to continuing with DFO therapy. The positive effect on survival on switching from DFO to DFP-containing therapy has been previously reported from this cohort and in other studies(3,32). The survival benefit of DFX compared to DFO has not previously been reported, and may be related to improved adherence to an oral rather than injected agent and its once-daily administration. It is important to note that the categorization of chelation therapy is a simplification, and the assumptions of the CoxPH model are not fully met with regard to chelation. Therefore, the significant protective effects observed in this study should be interpreted with caution.

We did not have comprehensive data on other genetic modifiers(33), while some genotype data were missing, particularly α -globin genotype (44/537, 8.2%) and XmnI polymorphism (57/537, 10.6%). However, the prevalence of severity-alleviating factors, such as α^0 deletions and minor allele of the XmnI polymorphism, is low in this population and is unlikely to impact overall analysis of survival. In contrast, *HBB* genotype appears to be a significant determinant of severity, a finding confirmed in a study of likelihood of initiation of transfusion in β -thalassemia, in which transfusion was 30 times more likely in severe genotype compared to mild genotype(15,16). The effect of genotype on survival could potentially be biased due to deaths of patients with severe genotypes at an earlier age and exclusion of patients who died early in the follow-up, before genotyping was introduced in Cyprus. Nevertheless, this is unlikely to have a major influence on our conclusions, because there was no significant difference between genotype frequencies and age at death in patients with available genotypes, while the year of birth was used in a multivariate survival analysis (data not shown) without materially changing the significant effect of genotype in survival.

Survival in TDT is associated with a variety of risk factors, not all of which are quantifiable, and these risk factors are likely to differ according to the cause of mortality. The cause of mortality appears to be changing from cardiac iron overload to other etiologies more closely associated with the long-term effects of inadequate suppression of the underlying thalassemia phenotype. Our results suggest that *HBB* genotype could affect mortality through its causative effect on disease phenotype, which is now more apparent because of the reduction of cardiac deaths. The analysis of transfusion data shows that patients with mild genotypes are beginning transfusion significantly later in life and require less

blood. However, once started on transfusion, there is no difference in pre-transfusion hemoglobin level between the genotypes, indicating that lower volumes of blood are required to suppress ineffective erythropoiesis in the mild genotype group. These observations in this study suggest that the delay in starting transfusion, along with the long-term transfusion strategy, are likely to negatively impact the survival of patients with mild genotypes. Notably, current treatment guidelines for TDT recommend that the decision about starting transfusion is based on clinical assessment. Our results suggest that determining the *HBB* genotype and using this information to aid in decision making could improve long-term outcomes.

The effect of genotype on mortality could be examined in other previously described thalassaemia cohorts(2,6,7) and in combined datasets, thus allowing the study of a more diverse spectrum of genotypes. This represents a future direction of our work. If confirmed, the effect of genotype could have significant implications for management of TDT. Firstly, it would provide evidence that patients diagnosed with β -thalassemia should initiate regular transfusion early, using a transfusion regimen which effectively suppresses erythropoiesis, to avoid long-term complications of uncontrolled anaemia and marrow expansion. Secondly, it would contribute to the decision of instituting emerging strategies for treating TDT including enhancement of endogenous erythropoiesis(34), as well as lentiviral and gene editing strategies(35,36), both of which appear to be more effective in patients with milder genotypes and higher residual β -globin chain production.

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Authorship Contributions

PK, MK and PT designed the study protocol, wrote and edited the manuscript, SC, MH, MS and AK managed the patients according to national and international guidelines and collected clinical data, PK and MK performed the DNA studies, PK, KM and PT performed statistical analysis.

Conflict of Interest Disclosures

PT: Celgene Corporation: PI in Phase 3 study of Luspatercept (Celgene); Bluebird Bio, Inc: Research funding and acted on advisory boards; Novartis: Participated on advisory boards; Apopharma: Participated on advisory boards.

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Table 1. Pathogenic *HBB* variations in TDT cohort patients

Allele 1	Allele 2	Allele phenotypes	Genotype category	Number (%)
IVS I-110 G>A	IVS I-1 G>A	β^+/β^0	Severe	48 (9.4)
IVS I-110 G>A	CD 39 CAG>TAG	β^+/β^0	Severe	23 (4.5)
IVS I-1 G>A	IVS II-745 C>G	β^0/β^+	Severe	5 (1.0)
IVS I-1 G>A	IVS I-1 G>A	β^0/β^0	Severe	2 (0.4)
IVS I-1 G>A	CD 44 -C	β^0/β^0	Severe	2 (0.4)
IVS I-110 G>A	IVS II-1 G>A	β^+/β^0	Severe	2 (0.4)
IVS I-110 G>A	CD 44 -C	β^+/β^0	Severe	1 (0.2)
IVS I-110 G>A	CD 5 -CT	β^+/β^0	Severe	1 (0.2)
IVS II-745 C>G	CD 39 CAG>TAG	β^+/β^0	Severe	1 (0.2)
IVS I-110 G>A	IVS I-110 G>A	β^+/β^+	Moderate	306 (59.8)
IVS I-110 G>A	IVS II-745 C>G	β^+/β^+	Moderate	29 (5.7)
IVS I-110 G>A	IVS II-848 C>A	β^+/β^+	Moderate	1 (0.2)
IVS II-745 C>G	IVS II-745 C>G	β^+/β^+	Moderate	1 (0.2)
IVS I-110 G>A	IVS I-6 T>C	β^+/β^{++}	Mild	81 (15.8)
IVS II-745 C>G	IVS I-6 T>C	β^+/β^{++}	Mild	4 (0.8)
IVS I-110 G>A	Hb Knossos	β^+/β^{++}	Mild	1 (0.2)
IVS I-110 G>A	-87 C>G	β^+/β^{++}	Mild	1 (0.2)
IVS I-1 G>A	IVS I-6 T>C	β^0/β^{++}	Mild	1 (0.2)
IVS I-6 T>C	CD 39 CAG>TAG	β^{++}/β^0	Mild	1 (0.2)
CD 39 CAG>TAG	Hb Lepore Boston-Washington	β^0/β^{++}	Mild	1 (0.2)
Total				512

Table 2: Demographic and clinical features of cohort patients

¹Analysis restricted to population where variable was known. d.f.: degrees of freedom, ²One patient was removed from the genotype analysis because thalassemia was caused by α -locus duplications co-inherited with β -thalassemia trait(23).

	Alive N=443 (%)	Died N=94 (%)	Total N=537	Comparison of proportion alive and died (<i>p</i> value)
Sex				
Male	214 (77.0)	64 (23.0)	278	$\chi^2 = 11.4, 1 \text{ d.f.}, p < 0.001$
Female	229 (88.4)	30 (11.6)	259	
Year of birth				
< 1974	186 (73.5)	67 (26.5)	253	$\chi^2 = 25.5, 1 \text{ d.f.}, p < 0.001$
≥ 1974	257 (90.5)	27 (9.5)	284	
Age At end of study period or death (Yrs, Mean, SD)				
	43.1 (8.1)	30.4 (11.0)		
Clinic				
Larnaca	99 (81.8)	22 (18.2)	121	$\chi^2 = 2.9, 3 \text{ d.f.}, p = 0.401$
Limassol	115 (82.1)	25 (17.9)	140	
Nicosia	186 (81.2)	43 (18.8)	229	
Paphos	43 (91.5)	4 (8.5)	47	
HBB genotype²				
Severe	77 (90.6)	8 (9.4)	85	$\chi^2 = 6.8, 2 \text{ d.f.}, p = 0.034^1$
Moderate	293 (86.9)	44 (13.1)	337	
Mild	70 (77.8)	20 (22.2)	90	
Not known	2 (8.3)	22 (91.7)	24	
α-globin gene number				
1	2 (100)	0 (0)	2	$\chi^2 = 1.8, 5 \text{ d.f.}, p = 0.878^1$
2	14 (93.3)	1 (6.7)	15	
3	84 (89.4)	10 (10.6)	94	
4	328 (86.5)	51 (13.5)	379	
5	2 (100)	0 (0)	2	
6	1 (100)	0 (0)	1	
Not known	12 (27.3)	32 (72.7)	44	
XmnI polymorphism				
CC	407 (88.3)	54 (11.7)	461	$\chi^2 = 1.5, 1 \text{ d.f.}, p = 0.225^1$
CT	19 (100)	0 (0)	19	
Not known	17 (29.8)	40 (70.2)	57	
Splenectomy				
Childhood	108 (74.5)	37 (25.5)	145	$\chi^2 = 22.7, 1 \text{ d.f.}, p < 0.001^1$
Adulthood or not splenectomised	334 (91.0)	33 (9.0)	367	
Not known	1 (4.2)	24 (95.8)	25	
Iron chelation therapy				
DFO only	52 (42.6)	70 (57.4)	122	$\chi^2 = 176, 2 \text{ d.f.}, p < 0.001$
DFO switched to DFP- containing	311 (93.7)	21 (6.3)	332	
DFO switched to DFX	80 (96.4)	3 (3.6)	83	

Table 3. Incidence rates for mortality by cause and period of follow-up

Incidence presented as events per 10,000 patient-years; CI: confidence interval

	Heart		Liver		Cancer		Infection		Other		Overall		Patient years follow-up
	Number	Incidence (95% CI)	Number	Incidence (95% CI)	Number	Incidence (95% CI)	Nun	Incidence (95% CI)	Number	Incidence (95% CI)	Number	Incidence (95% CI)	
1980-1989	4	7.5 (2.8–20.1)	1	1.9 (0.27–13.3)	0	0.00	2	3.77 (0.9–15.0)	4	7.5 (2.8–20.1)	11	20.7 (11.5–37.4)	5310.7
1990-1999	21	41.3 (26.9–63.3)	2	3.9	0	0.00	4	7.9 (3.0–20.9)	6	11.8 (5.3–26.2)	33	64.9 (46.2–91.2)	5085.7
2000-2009	13	27.4 (15.9–47.1)	2	4.2 (1.1–16.8)	3	6.3 (2.0–19.6)	4	8.4 (3.2–22.4)	4	8.4 (3.2–22.4)	26	54.8 (37.3–80.3)	4747.9
2010-2018	6	15.3 (6.9–34.1)	5	12.8 (5.3–30.7)	4	10.2 (3.8–27.2)	5	12.8 (5.3–30.7)	4	10.2 (3.8–27.2)	24	61.3 (41.1–91.3)	3915.8
1980-2018	44	23.1 (17.2–31.0)	10	5.3 (2.0–8.5)	7	3.7 (1.0–6.4)	15	7.9 (4.7–13.1)	18	9.4 (6.0–15.0)	94	49.3 (40.3–60.3)	19060.0

Table 4. Univariate and multivariate analysis of risk factors for survival in primary analysis.

¹The vector of trend weight for the three categories was 0, 1 and 2 for severe, moderate and mild genotype, respectively. ²Analysis was based on cases for which data were available (see Table 2 for missing data). d.f.: degrees of freedom, CI: confidence interval, HR: hazard ratio

	Univariate		Multivariate
	% survival at age 50 (95% CI)	Comparison of survival estimate	
Sex			
Female	87.3 (82.9–91.8)	$\chi^2 = 13.62$, 1 d.f., $p < 0.001$	HR 1.9, 95% CI 1.2–3.0, $p = 0.011$
Male	73.6 (66.9–81.0)		
Year of birth			
<1974	75.4 (70.1–81.1)	$\chi^2 = 10.2$, 1 d.f., $p = 0.001$	
≥1974	Not yet reached		
Clinic			
Larnaca	79.4 (71.6–88.0)	$\chi^2 = 2.7$, 3 d.f., $p = 0.437$	
Limassol	82.0 (75.1–89.5)		
Nicosia	78.7 (72.0–86.0)		
Paphos	84.5 (68.9–100.0)		
HBB genotype¹			
Severe	85.7 (75.4–97.5)	$\chi^2 = 4.4$, 1 d.f., $p = 0.037$	HR 1.6, 95% CI 1.1–2.3, $p = 0.023$
Moderate	85.3 (80.6–90.2)		
Mild	77.9 (68.7–88.3)		
α-globin gene number²			
< 4	90.9 (84.5–97.7)	$\chi^2 = 2.01$, 1 d.f., $p = 0.157$	
≥4	83.6 (78.8–88.8)		
XmnI polymorphism²			
CC	86.2 (82.1–90.5)	$\chi^2 = 2.8$, 1 d.f., $p = 0.097$	
CT	100		

Table 5. Univariate and multivariate analysis of risk factors for survival in secondary analysis
D.f.: degrees of freedom, CI: confidence interval, HR: hazard ratio

		Univariate		Multivariate
	Number	% survival at R censorship at 30/9/18 (95% CI)	Comparison of survival estimate (Log Rank)	
Sex				
Female	245	93.0 (89.9–96.3)	$\chi^2 = 5.3$, 1 d.f., $p=0.021$	HR 2.34, 95% CI 1.26–4.34, $p=0.007$
Male	244	86.7 (82.6–91.1)		
Year of birth				
<1974	215	86.0 (81.5–90.8)	$\chi^2 = 6.3$, 1 d.f., $p=0.012$	
≥1974	274	93.0 (90.0–96.1)		
Clinic				
Larnaca	107	90.3 (84.8–96.2)	$\chi^2 = 2.0$, 3 d.f., $p=0.578$	
Limassol	128	89.0 (83.7–94.6)		
Nicosia	209	89.0 (84.9–93.3)		
Paphos	45	95.6 (89.7–100)		
HBB genotype				
Severe	81	93.8 (88.7–99.2)	$\chi^2 = 6.4$, 1 d.f., $p=0.011$	HR 2.04, 95% CI 1.05–3.98, $p=0.036$
Moderate	321	90.9 (87.7–94.1)		
Mild	84	82.1 (74.3–90.8)		
α-globin gene number				
<4	108	92.5 (87.7–97.7)	$\chi^2 = 1.1$, 1 d.f., $p=0.287$	
≥4	369	89.1 (86.0–93.2)		
XmnI polymorphism				
CC	452	89.5 (86.7–92.4)	$\chi^2 = 2.0$, 1 d.f., $p=0.158$	
CT	19	100		
Iron chelation				
DFO only	74	64.5 (54.2–76.8)	$\chi^2 = 75.1$, 2 d.f., $p<0.001$	HR 0.10, 95% CI 0.05–0.19, $p<0.001$ HR 0.08, 95% CI 0.025–0.028, $p<0.001$
DFO switched to DFP-containing	332	93.7 (91.1–96.3)		
DFO switched to DFX	83	96.4 (92.4–100)		
Splenectomy				
Childhood	130	82.2 (75.8–89.1)	$\chi^2 = 14.7$, 1 d.f., $p<0.001$	HR 7.92, 95% CI 2.03–30.75, $p=0.003$
Adulthood or not splenectomised	354	93.7 (91.2–96.3)		

Figure legends

Figure 1. Kaplan Meier curves for survival from birth

(A.) Overall survival, and categorised by (B) sex, (C) *HBB* genotype, and (D) year of birth

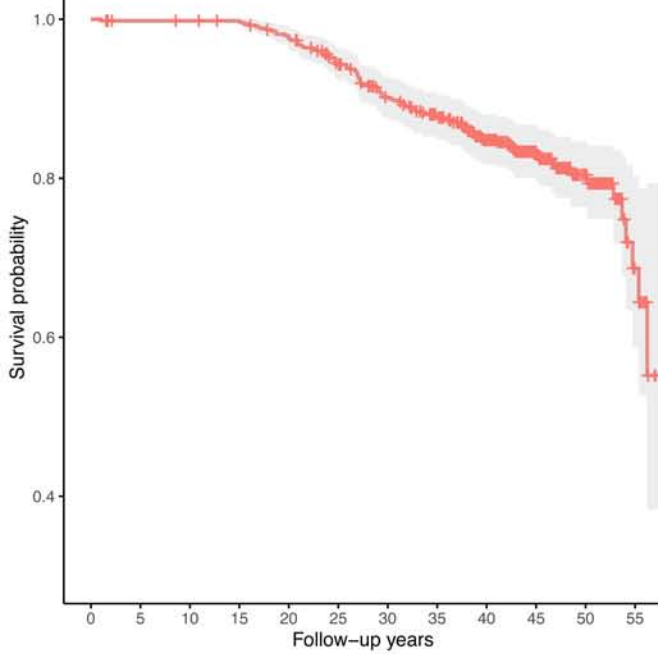
Figure 2. Kaplan Meier curves for survival during follow-up 2000 to 2018. Overall survival of cohort and effects of significant risk factors in univariate analysis

(A) Overall survival, and categorised by (B) sex, (C) *HBB* genotype, (D) year of birth, (E) splenectomy during childhood, and (F) chelation

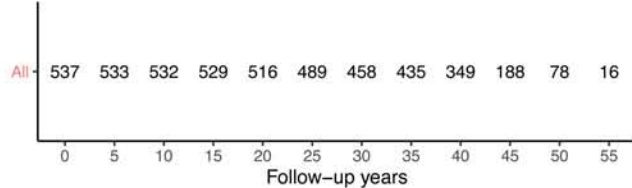
Figure 3. Boxplots illustrating the distribution of transfusion data among different *HBB* genotype severity groups. Statistically significant associations are shown, using the Wilcoxon rank-sum non-parametric test.

Figure 1

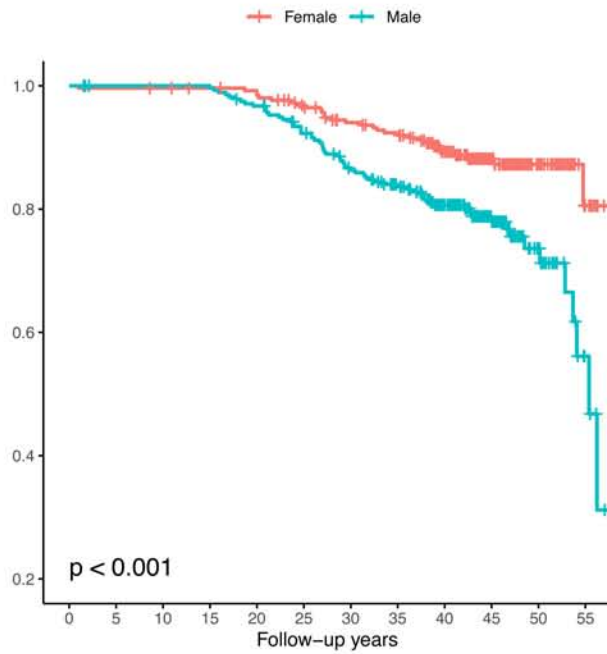
A. Overall Survival



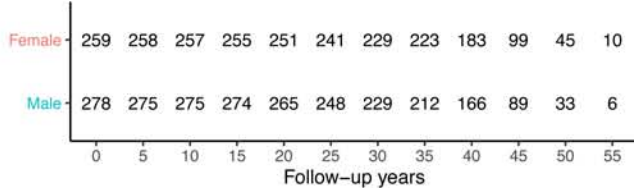
Number at risk



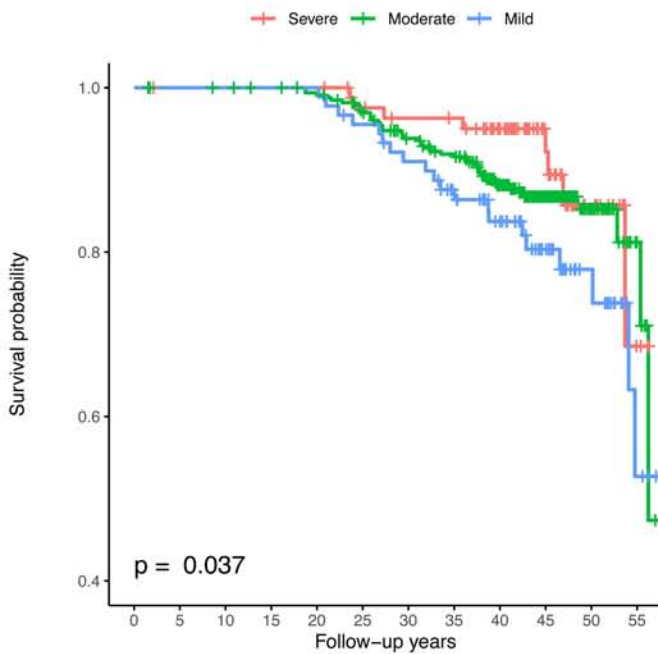
B. Sex



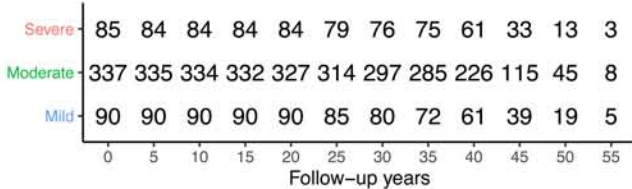
Number at risk



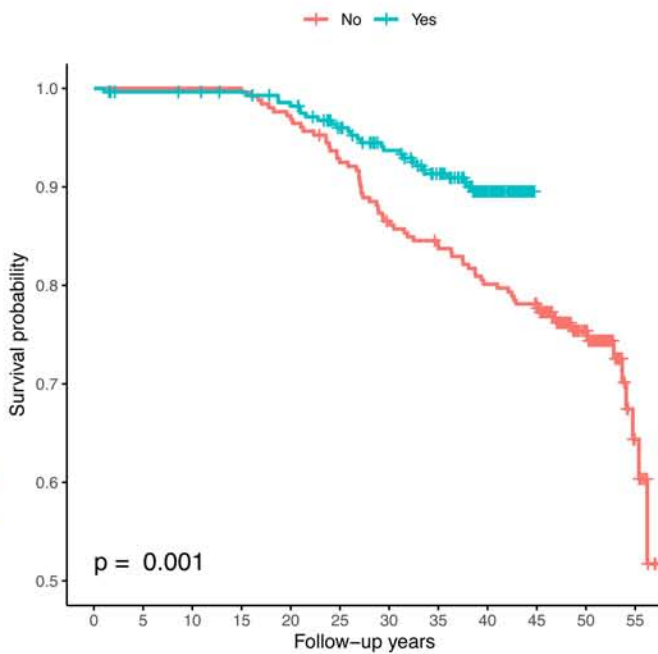
C. HBB genotype



Number at risk



D. Year of birth \geq 1974



Number at risk

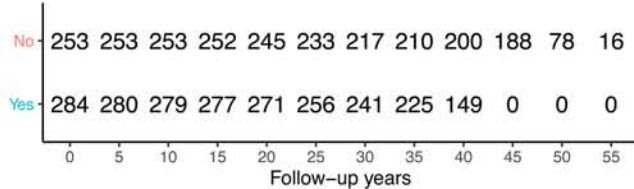
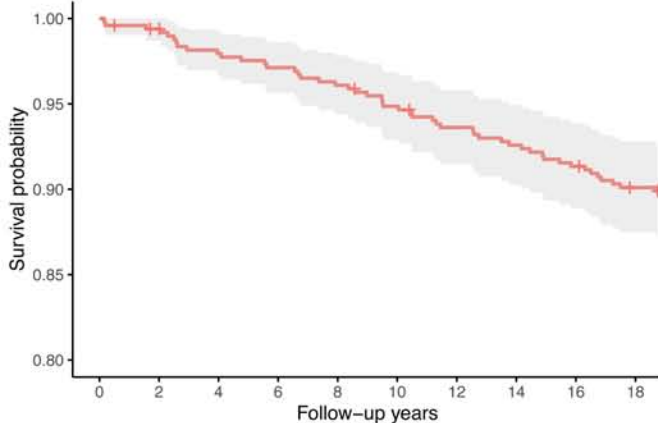


Figure 2

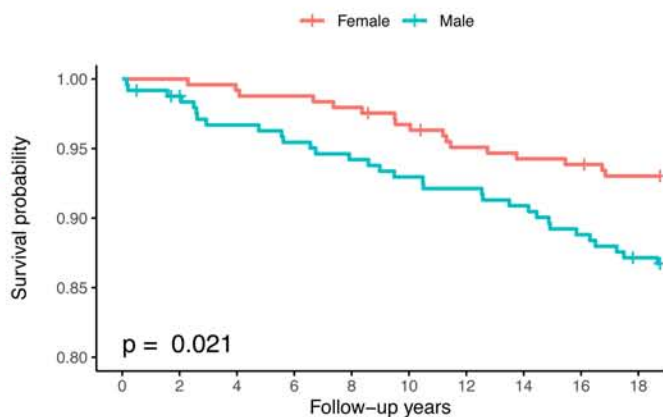
A. Overall survival



Number at risk

All	489	484	476	472	467	460	453	448	442	434
	0	2	4	6	8	10	12	14	16	18

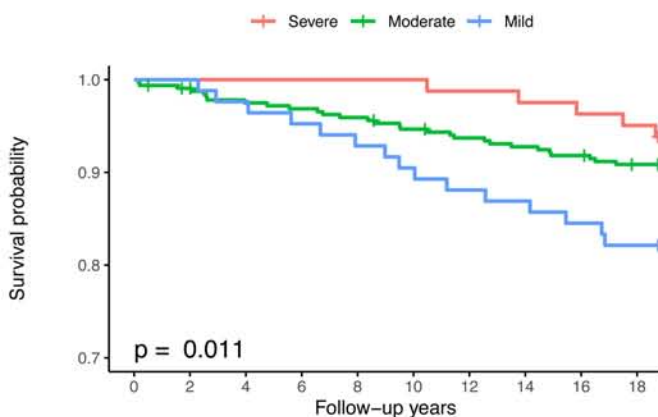
B. Sex



Number at risk

Female	245	245	243	242	240	236	231	229	228	225
Male	244	239	233	230	227	224	222	219	214	209
	0	2	4	6	8	10	12	14	16	18

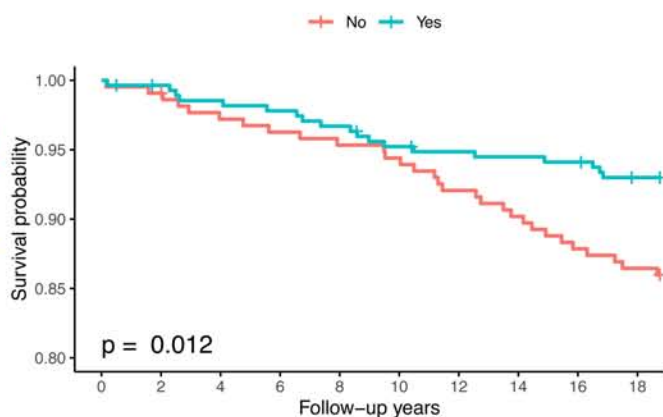
C. HBB genotype



Number at risk

Severe	81	81	81	81	81	81	80	79	78	77
Moderate	321	316	310	308	305	300	296	293	290	285
Mild	84	84	82	80	78	76	74	73	71	69
	0	2	4	6	8	10	12	14	16	18

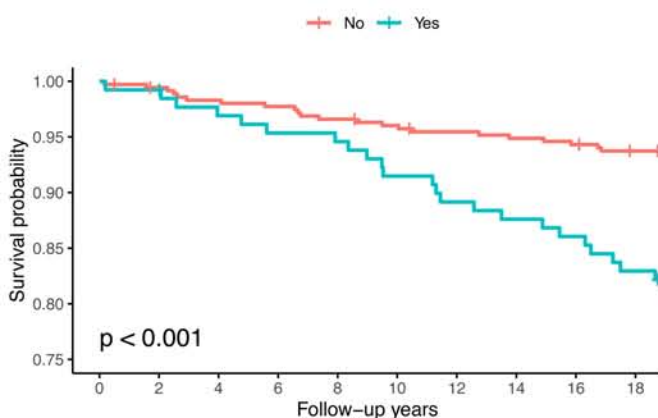
D. Year of birth \geq 1974



Number at risk

No	215	213	208	206	204	202	197	193	188	185
Yes	274	271	268	266	263	258	256	255	254	249
	0	2	4	6	8	10	12	14	16	18

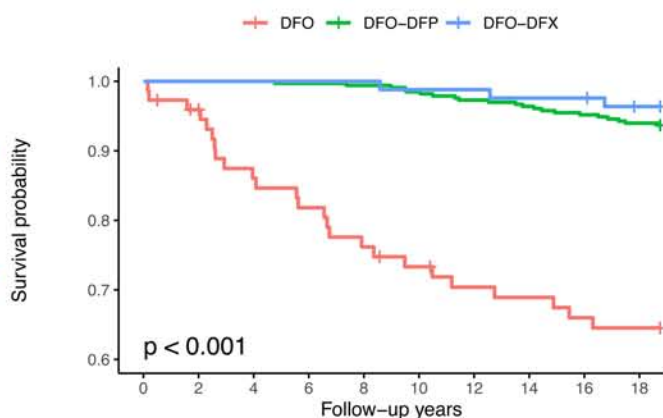
E. Splenectomy in childhood



Number at risk

No	354	350	346	344	340	337	334	332	330	326
Yes	130	129	125	123	122	118	115	113	111	107
	0	2	4	6	8	10	12	14	16	18

F. Chelation



Number at risk

DFO	74	69	61	58	54	51	48	47	45	44
DFO-DFP	332	332	332	331	330	327	323	320	316	312
DFO-DFX	83	83	83	83	83	82	82	81	81	78
	0	2	4	6	8	10	12	14	16	18

Figure 3

