After patients and relatives had given informed consent, genomic DNA was extracted from EDTA peripheral blood using standard methods, and G6PD gene exons 2 to 13 were amplified by polymerase chain reaction (PCR) as described elsewhere.[6] PCR products were screened by single strand conformation polymorphism (SSCP) analysis and sequenced with the automatic genetic analyzer ABI Prism 310.

The G6PD gene from patient 1 showed a SSCP mobility shift in the fragment spanning exon 10 and sequencing revealed a previously undescribed mutation 1205C→A, predicting the amino acid change 402 Thr→Asn. T402 is a poorly conserved residue, located at the β-sheet M of the polypeptide chain; crystal structure analysis of human G6PD protein[4] showed that T402 is located at the dimer interface (Figure 1). The T402 side chains of the two monomers do not make any intersubunit contacts and are 5.4Å apart, across the dimer interface. Instead, they are within a distance compatible with a van der Waals effect of the L420 side chain in β strand N within the same monomer. Furthermore, β strand N harbors D421, a residue interacting with the nicotinamide ring of the structural $NADP^+$. The T402N mutation would introduce steric hindrance forcing the two dimers apart. In summary, as suggested for amino acid substitutions mapping close to this region,[7-9] T402N will both affect the dimer interface and interfere with the structural $NADP^+$ binding site,[4] severely affecting protein stability. DNA sequencing of the G6PD gene from patients 2 and 3 revealed a previously undescribed mutation in exon 12, a 1366G→A substitution, predicting the amino acid change 456Asp→His. D456 is located in the beginning of the α-helix N, further down the polypeptide carboxylic terminus. Structural analysis showed D456 at the protein surface, far away from the dimer interface (Figure 1). D456 forms a strong salt bridge with R454 within helix N which is further stabilized by interactions with D282 and D286 in helix J. Replacing D456 by His would partially disrupt this network of salt bridges. Furthermore, the positively charged His side chain at this position will certainly introduce unfavorable electrostatic interactions with R459, R454 and K293, affecting protein structure and stability. Although not located in the immediate vicinity of the active site, D456 is highly conserved from bacteria to humans reflecting important functional and/or structural features; hence D456H is in accordance with the general concept that the clinically more severe G6PD deficiencies are mainly associated with mutations at conserved amino acids.[5]

Both mutations were confirmed by restriction enzyme digestion and no other mutations were detected in the remaining exons or adjacent regions of the G6PD gene. The screening of 100 alleles from a control group failed to detect these mutations. The new variants T402N and D456H were named G6PD *Covão do Lobo* and G6PD *Figueira da Foz*, respectively, after the patients' place of birth.

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Key words: G6PD deficiency, Portugal, G6PD class I mutations, dimer interface, G6PD structure and amino acid conservation.

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References

1. Mehta A, Mason PJ, Vulliamy TJ. Glucose-6-phosphate dehydrogenase deficiency. Baillieres Best Pract Res Clin Haematol 2000;13:21-38.
2. Fiorelli G, Martinez di Montemuros F, Cappellini MD. Chronic non-spherocytic haemolytic disorders associated with glucose-6-phosphate dehydrogenase variants. Baillieres Best Pract Res Clin Haematol 2000;13:39-55.
3. Naylor CE, Rowland P, Basak AK, Gover S, Mason PJ, Bautista JM, et al. Glucose 6-phosphate dehydrogenase mutations causing enzyme deficiency in a model of the tertiary structure of the human enzyme. Blood 1996;87:2974-82.
4. Au SWN, Gover S, Lam VMS, Adams MJ. Human glucose-6-phosphate dehydrogenase: the crystal structure reveals a structural NADP(+) molecule and provides insights into enzyme deficiency. Structure Fold Des 2000;8:293-303.
5. Cheng YS, Tang TK, Hwang M. Amino acid conservation and clinical severity of human glucose-6-phosphate dehydrogenase mutations. J Biomed Sci 1999;6:106-14.
6. Calabro V, Mason PJ, Filosa S, Civitelli D, Cittadella R, Tagarelli G, et al. Genetic heterogeneity of glucose-6-phosphate dehydrogenase deficiency revealed by single-strand conformation and sequence analysis. Am J Hum Genet 1993; 52:527-36.
7. Costa E, Cabeda JM, Vieira E, Pinto R, Pereira SA, Ferraz L, et al. Glucose-6-phosphate dehydrogenase aveiro: a de novo mutation associated with chronic nonspherocytic hemolytic anemia. Blood 2000;95:1499-501.
8. Grabowska D, Jablonska-Skwiecinska E, Plochocka D, Chelstowska A, Lewandowska I, Witos I, et al. A novel mutation in the glucose-6-phosphate dehydrogenase gene in a subject with chronic nonspherocytic hemolytic anemia-characterization of enzyme using yeast expression system and molecular modeling. Blood Cells Mol Dis 2004;32:124-30.
9. van Wijk R, Huizinga EG, Prins I, Kors A, Rijksen G, Bierings M, et al. Distinct phenotypic expression of two de novo missense mutations affecting the dimer interface of glucose-6-phosphate dehydrogenase. Blood Cells Mol Dis 2004;32:112-7.
10. De Lano WL. The PyMOL Molecular Graphics System (2002) on World Wide Web (*http://www.pymol.org*).

Red Cell Disorders

Prevalence and severity of liver disease in patients with β thalassemia major. A single-institution fifteen-year experience

During the last years, liver disease has emerged as a major cause of mortality in patients with β thalassemia major (TM).[1,2] In spite of its clinical relevance, TM-associated liver damage has been insufficiently characterized.[1,2-5] We therefore retrospectively analyzed all TM patients of our Department who underwent liver biopsy since 1990.

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All patients were being regularly transfused in order to maintain the pretransfusion hemoglobin level at approximately 9.5 g/dL. Chelation therapy with desferrioxamine 40–60 mg/kg/day for 5–6 days/week was initiated as soon

Table 1. Iron overload score in liver biopsies and associated factors.

Iron overload score	Biopsies (%)	Ferritin (μg/L)	ALT (IU/L)	LIC (mg/g dry weight)	Age (years)	Fibrosis score	Necro -inflammatory score
Grade 0	8 (10.1)	1968 (918.5-3541.5)	63.5 (14-270)	ND*	23.5 (17-35)	0 (0-4)	1 (0-3)
Grade 1	23 (29.1)	1663 (1285.5-3022)	22 (10-600)	10.2 (3.2-35.0)	14 (6-20)	0 (0-2)	1 (0-3)
Grade 2	17 (21.5)	1720 (622.5-3500)	42 (11-365)	12.7 (4.1-22.3)	14 (9-31)	0 (0-2)	2 (0-4)
Grade 3	17 (21.5)	2265 (1300-4452.5)	50 (16-245)	25.5 (15.5-48.6)	18 (10-47)	0 (0-4)	2 (0-4)
Grade 4	14 (17.7)	2629.5 (1230-6707)	62.5 (23-130)	32.1 (28.9-45.0)	17.5 (11-33)	2 (0-2)	2 (0-3)
		p<0.01	p<0.05	p<0.005	p=0.001	p<0.05	p=0.154

*Results are expressed as median (range). *ND: not determined in any patient.*

Table 2. Fibrosis score in liver biopsies and associated factors.

Fibrosis score	Biopsies (%)	Ferritin (mg/L)	ALT (IU/L)	LIC (mg/g dry weight)	Age (years)	Iron overload score	Necro -inflammatory score
Grade 0	48 (60.8)	1850. (918.5-4452.5)	44 (10-600)	12.95 (3.2-48.6)	15 (6-34)	1.5 (0-4)	1 (0-4)
Grade 1	15 (19.0)	2410 (622.5-4929.5)	19 (11-365)	19.8 (4.1-35.0)	16 (9-21)	2 (1-4)	2 (1-4)
Grade 2	13 (16.5)	2438 (1300-6707)	47 (26-130)	29.2 (6.4-32.1)	18 (11-33)	4 (1-4)	2 (2-4)
Grade 3	1 (1.3)	2737	31	ND*	25	3	2
Grade 4	2 (2.5)	1572 (985-2159.5)	104 (100-108)	ND*	41 (35-47)	1.5 (0-3)	1.5 (1-2)
		p=0.122	p=0.312	p=0.357	p<0.05	p<0.005	p<0.01

*Results are expressed as median (range). *ND: not determined in any patient.*

as ferritin levels rose above approximately 1000 μg/L.

A single pathologist who was unaware of the patients' data evaluated all biopsies. Iron overload was graded according to the method of Rowe, fibrosis according to the Scheuer staging score, and necroinflammatory reaction according to the Scheuer's grading system. Liver iron concentration (LIC) was also determined on paraffin-embedded biopsy specimens by atomic absorption spectrophotometry.[6]

Results are expressed as median and range. The χ^2 and Kruskal-Wallis tests were used for comparisons of categorical and continuous variables between groups respectively. Associations between continuous variables were explored using Spearman's Rank order correlation coefficient.

Seventy-nine TM patients (43 males and 36 females) were studied; at time of liver biopsy, their median age was 16 years (range, 6 to 47). The median ferritin in the year preceding liver biopsy was 2001.5 μg/L (range, 622.5 to 6707). The median alanine transferase (ALT) levels in the six-month period before the biopsy were 41.5 IU/L (range, 10 to 600); 41 patients (53.9%) had ALT levels above the upper limit of normal. Serum ferritin was significantly associated with increased ALT concentrations (p=0.001).

Thirty-one patients (39.2%) were positive for hepatitis C virus (HCV) antibody; 13 (41.9%) of them were positive for HCV RNA (median level, 170,000 IU/mL; range, 110,000 to 2,200,000) and the infecting genotype was 1a, 1b, 3a and 4 in 3 (23.1%), 4 (30.8%), 5 (38.5%) and 1 (7.7%) of the patients, respectively. HCV antibody positive patients had higher ALT levels and necroinflammatory score than had seronegative patients (p<0.001 for both comparisons).

Median iron overload score was 2 (range, 0 to 4). Seventy-one liver specimens (89.9%) showed at least grade 1 iron overload; age, ferritin, ALT, LIC and fibrosis score were significantly different among the five grades of iron overload (Table 1).

Median fibrosis score was 0 (range, 0 to 4) and fibrosis was present in 31 biopsies (39.2%). Age, iron overload and necroinflammatory score were significantly different among the five grades of fibrosis (Table 2). Cirrhosis was present in two biopsies (2%); both patients were HCV antibody-positive. Median necroinflammatory score was 2 (range, 0 to 4). ALT and fibrosis score were significantly different among the five grades of necroinflammation (*data not shown*). Thirty-one samples were analyzed for LIC. The median LIC was 15.5 mg/g dry weight (range, 3.2 to 48.6). Sixteen samples (51.6%) had an LIC over 15 mg/g dry weight, a concentration associated with a high risk for cardiac disease (4). LIC was also significantly correlated with ALT levels (p<0.005). No complications occurred during the biopsies.

The major consequence of chronic transfusion therapy in TM patients is a progressive accumulation of iron. The liver, as a major storage depot for iron, frequently shows excessive hemosiderosis.[2,7] In our study, we found a prevalence of 39.2% grade 3–4 iron overload; this is similar to results of a recent study, as well as to that reported approximately 10 years ago and could reflect the rather unsatisfactory compliance rate with desferoxamine therapy observed in many patients.[2,7]

Significant fibrosis is frequent in TM patients, and is mostly influenced by iron overload (2,8,9). Indeed, the severity of fibrosis in our study was related to iron overload score. The 20.3% prevalence of significant hepatic fibrosis is lower than that reported previously.[2,7] Furthermore, only 2% of our patients had cirrhosis, which is in accordance to recent studies.[2]

TM patients have rather mild liver necroinflammation, mainly attributable to HCV infection; in contrast, liver iron accumulation per se seems to induce only a low degree of inflammation.[2,8] In the present study as well, the necroinflammatory score of HCV antibody positive patients was significantly higher than that of seronega-

tive patients. Furthermore, we found no evidence of increased iron overload in patients with more prominent inflammatory changes.

HCV infection is an independent risk factor for liver fibrosis.[10] Nevertheless, fibrosis progression was unrelated to the presence of HCV RNA in a recent study.[2] In our study as well, HCV antibody and HCV RNA positive patients did not significantly differ from seronegative patients in fibrosis score.

In conclusion, liver disease is common in TM patients and severe hepatic iron overload is still observed in approximately 40% of them. In this context, increased implementation of the direct assessment of liver iron content to monitor hemosiderosis is of special importance in order to individualize chelation therapy. In parallel, the maximum possible efforts must be concentrated on improving compliance to chelation therapy.

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References

1. Zurlo MG, De Stefano P, Borgna-Pignatti C, Di Palma A, Piga A, Melevendi C, et al. Survival and causes of death in thalassaemia major. Lancet 1989; 2:27-30.
2. Prati D, Maggioni M, Milani S, Cerino M, Cianciulli P, Coggi G, et al. Cooleycare Cooperative Group. Clinical and histological characterization of liver disease in patients with transfusion-dependent beta-thalassemia. A multicenter study of 117 cases. Haematologica 2004; 89:1179-86.
3. Prati D, Zanella A, Farma E, De Mattei C, Bosoni P, Zappa M, et al. A multicentre prospective study on the risk of acquiring liver disease in anti-HCV negative patients affected from homozygous β-thalassemia. The Cooleycare Cooperative Group. Blood 1998;92:3460-4.
4. Prati D. Benefits and complications of regular blood transfusion in patients with β-thalassaemia major. Vox Sang 2000; 79:129-37.
5. Prati D. Transmission of viral hepatitis by blood and blood derivatives: current risks, past heritage. Dig Liver Dis 2002; 34:812-7.
6. Angelucci E, Baronciani D, Lucarelli G, Baldassarri M, Galimberti M, Giardini C, et al. Needle liver biopsy in thalassaemia: analyses of diagnostic accuracy and safety in 1184 consecutive biopsies. Br J Haematol 1995;89:757-61.
7. Angelucci E, Baronciani D, Lucarelli G, Giardini C, Galimberti M, Polchi P, et al. Liver iron overload and liver fibrosis in thalassemia. Bone Marrow Transplant 1993;12 Suppl 1:29-31.
8. Poli G. Pathogenesis of liver fibrosis: role of oxidative stress. Mol Aspects Med 2000;21:49-98.
9. Pietrangelo A. Iron, oxidative stress and liver fibrogenesis. J Hepatol 1998; 28 Suppl 1:8-13.
10. Angelucci E, Muretto P, Nicolucci A, Baronciani D, Erer B, Gaziev J, et al. Effects of iron overload and hepatitis C virus positivity in determining progression of liver fibrosis in thalassemia following bone marrow transplantation. Blood 2002; 100:17-21.

Disorders of iron Metabolism

Hemoglobinopathies, body iron stores and gestational diabetes mellitus

Higher iron stores, reflected by an elevated ferritin concentration and elevated transferrin saturation, can affect glucose intolerance during pregnancy. We determined the incidence of gestational diabetes mellitus in patients with heterozygous form of hemoglobinopathies and in a healthy control group.

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The association between β thalassemia major and intermedia and an increased risk of diabetes mellitus (DM) has been shown by various studies.[1-3] Glucose intolerance correlated significantly with the number of transfusions received by subjects with β thalassemia major.[1] Interestingly, patients with heterozygous sickle cell anemia had higher values of glycosylated hemoglobin than did homozygous patients.[4]

In a retrospective case-control study at the Department of Obstetrics, University Hospital Zurich we compared the incidence of gestational diabetes (GDM) in a set of pregnancies among women heterozygous for various hemoglobinopathies and in a normal, matched control group. The matching criteria were the known diabetes risk factors, i.e. maternal age, parity, ethnicity, weight and body mass index. Between 1998 and 2001 we identified 29 patients presenting heterozygous hemoglobinopathies and anemia. Hematologic work-up, including high performance liquid chromatography (HPLC), plasma iron status and erythrocyte indices, showed the presence of α thalassemia trait in 2, hemoglobinopathy E in 2, β thalassemia in 20 and sickle cell trait in 5 pregnancies. Before measuring plasma ferritin concentration, any inflammation was excluded. Treatment of anemia in the patients with a hemoglobin concentration less than 9.0 g/dL was individualized on the basis of hematologic parameters and iron status, and consisted of recombinant human erythropoietin (rhEPO; 10,000 U) with or without parenteral iron (iron-III-saccharate 100 mg).[5] Screening for GDM and the determination of iron status were conducted before therapy. Screening tests for genetic hemochromatosis were not done. All patients and controls were examined for GDM between 24 and 28 weeks of gestation as proposed by Perucchini *et al.*[6]

The incidence of diabetes mellitus was 20.7% (6/29) in the study group versus 0% in the control group (Fisher's exact test; p=0.0009). When all cases with impaired glucose tolerance were included the incidence was 31% (9/29) in the study group vs. 6.9% (4/58) in the control group (Fisher's exact test; p=0.008). The demographic data and iron status of the study and the control group are listed in Table 1. The median ferritin values were statistically different in the patients with GDM in the study group (76 vs. 35 ng/mL; Mann-Whitney test, p=0.004). There is little information on the relation between heterozygous forms of hemoglobinopathies and the impairment of glucose regulation. Only one study has examined the association between heterozygous α thalassemia trait