Research Article

Serum Bilirubin Levels and Promoter Variations in *HMOX1* and *UGT1A1* Genes in Patients with Fabry Disease

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The aim of our study was to assess the possible relationships among heme oxygenase (HMOX), bilirubin UDP-glucuronosyl transferase (UGT1A1) promoter gene variations, serum bilirubin levels, and Fabry disease (FD). The study included 56 patients with FD (M : F ratio = 0.65) and 185 healthy individuals. Complete standard laboratory and clinical work-up was performed on all subjects, together with the determination of total peroxyl radical-scavenging capacity. The (GT)n and (TA)n dinucleotide variations in the *HMOX1* and *UGT1A1* gene promoters, respectively, were determined by DNA fragment analysis. Compared to controls, patients with FD had substantially lower serum bilirubin levels (12.0 versus 8.85 μmol/L, $p = 0.003$) and also total antioxidant capacity ($p < 0.05$), which showed a close positive relationship with serum bilirubin levels ($p = 0.067$) and the use of enzyme replacement therapy ($p = 0.036$). There was no association between *HMOX1* gene promoter polymorphism and manifestation of FD. However, the presence of the TA$_7$ allele *UGT1A1* gene promoter, responsible for higher systemic bilirubin levels, was associated with a twofold lower risk of manifestation of FD (OR = 0.51, 95% CI = 0.27–0.97, $p = 0.038$). Markedly lower serum bilirubin levels in FD patients seem to be due to bilirubin consumption during increased oxidative stress, although *UGT1A1* promoter gene polymorphism may modify the manifestation of FD as well.

1. Introduction

Fabry disease (FD, OMIM 301500), an X-linked metabolic disorder, is caused by a deficiency of the lysosomal enzyme α-galactosidase A, resulting in the accumulation of glyco-sphingolipids (in particular globotriaosylceramide (GB3)) in endothelial cells and other tissues [1, 2]. The majority of FD complications results from progressive damage to the central and peripheral nervous system, kidney, and heart due to severe vasculopathy [2, 3]. Indeed, α-galactosidase A deficiency was shown to accelerate atherosclerosis in apolipoprotein E-deficient mice [4]. Moreover, endothelial dysfunction was also reported in a clinical study on FD patients [5], but the vascular damage in FD differs from classical atherosclerosis [6–8]. The storage in FD, predominantly found in endothelial and smooth muscle cells as well as in stenotic lesions, is characterized by intimal and subintimal proliferation [6]. Increasing clinical evidence suggest that FD is a rather heterogeneous disease differing in the age of manifestation, severity, and organ involvement depending on specific genetic background [9]. There is also an increasing evidence that FD prevalence may be largely underestimated [9, 10], which strongly supports the importance of known and reasons for additional putative genetic factors influencing its clinical manifestation.

The pathological processes in FD are associated with increased oxidative, nitrosative, and carbonyl stress as evidenced by excessive production of reactive oxygen species (ROS) [11–13]; increased plasma and urinary carbonylated proteins [14]; and nitrotyrosine in plasma, cardiac, and

vascular tissues [15–17], resulting in DNA oxidative damage in the cells and tissues [12, 17].

The oxidative stress defense system consists of antioxidative enzymes and antioxidative substrates. Among the latter, bilirubin, the heme catabolic product, belongs among the most potent endogenous antioxidants [18, 19]. In fact, serum bilirubin positively correlates with total antioxidant status (TAS) in newborns with neonatal jaundice, as well as adult subjects (for review, see [20]). Bilirubin has been reported to be a strong negative predictor/biomarker of oxidative stress-mediated diseases such as atherosclerosis [21]. On the other hand, decreased systemic antioxidant defense mechanisms have been reported in patients with FD [13, 14].

By regulating bilirubin production and its biotransformation in the liver, heme oxygenase-1 (encoded by *HMOX1*, OMIM*141250) and bilirubin UDP-glucuronosyl transferase (*UGT1A1*, OMIM*191740) enzymes play an important role in oxidative stress defense. Microsatellite variations in the promoter regions modulate *HMOX1* and *UGT1A1* gene expression and have been associated with oxidative stress-mediated diseases such as atherosclerosis [22, 23]. Indeed, this is true for subjects with a high number of (GT)n repetitions in the *HMOX1* gene promoter, resulting in decreased expression of HMOX1 (categorized as class L allele carriers) [22]. On the other hand, those with congenitally decreased expression of *UGT1A1* (which is caused in the majority of Caucasians by homozygosity of the so-called UGT1A1*28 allele) [24] results in mild unconjugated hyperbilirubinemia (Gilbert's syndrome), a condition associated with a decreased risk of cardiovascular diseases [21].

The objective of this study was to determine whether genetic variations in *HMOX1* and *UGT1A1* genes, as well as systemic levels of bilirubin, might affect the risk of the development of FD, a metabolic disorder accompanied by increased oxidative stress.

2. Materials and Methods

2.1. Patients. The study was performed on 56 FD patients (M : F ratio = 0.70) diagnosed in our center from 2000 to 2012. The diagnosis of FD was based on a demonstrated reduction of α-galactosidase A activity in leukocytes or plasma and confirmed by DNA mutation analysis. Of those, 45% suffered from cardiovascular disease ($n = 25$), 48% from neurological complications ($n = 27$), 30% from renal disease ($n = 17$), 27% from cutaneous disease ($n = 15$), and 18% from ocular complications ($n = 10$). Out of the whole FD group, 55% of patients ($n = 31$) were treated with ERT according to international recommendations [25].

The control group was based upon age- and sex-matched 185 clinically healthy subjects without any chronic medication, representing a general population sample from the same geographical region. These subjects were recruited from healthy blood donors and employees of the General University Hospital. All subjects in both cohorts were of Caucasian ancestry.

The study protocol and all procedures conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as

TABLE 1: Serum bilirubin concentrations in patients with FD.

	FD	Controls	p value
	Males + females		
Age [years]	**43 ± 11.5** ($n = 56$)	**41.7 ± 9.7** ($n = 185$)	0.303
Bilirubin [μmol/L]	**9.0** [7.6–13.5]	**12.0** [9–16.4]	**0.006**
	Males		
Age [years]	**42 ± 11.6** ($n = 22$)	**41.6 ± 9.9** ($n = 108$)	0.77
Bilirubin [μmol/L]	**13.3** [8.7–16.7]	**12.6** [9.8–17.7]	0.719
	Females		
Age [years]	**43.6 ± 17.1** ($n = 34$)	**41.9 ± 9.7** ($n = 77$)	0.313
Bilirubin [μmol/L]	**8.1** [6.9–9.6]	**10.6** [8.5–14.9]	**0.002**

Data expressed as mean ± SD, or median and IQ range, depending on their normality. FD: Fabry disease.

reflected in a priori approval by the institution's Ethics Committee, and all subjects signed informed-consent forms.

2.2. Laboratory Investigation. Peripheral venous blood was obtained from all participants after overnight fasting. The serum was immediately processed, and bilirubin was determined on an automatic analyzer (modular analyzer, Roche Diagnostics GmbH, Germany), using routine laboratory assay.

Total antioxidant status (TAS) was determined fluorometrically as the serum peroxyl radical-scavenging capacity, based on the relative proportion of chain-breaking antioxidant consumption present in the serum compared to that of Trolox (a reference and calibration antioxidant compound) [26].

The (GT)$_n$ (dbSNP rs1805173) and (TA)$_n$ (dbSNP rs81753472) dinucleotide variations in *HMOX1* and *UGT1A1* gene promoters, respectively, were determined by fragment analysis using an automated capillary DNA sequencer, as previously described [27]. The length variations of *HMOX1* (GT)$_n$ repeats were classified into short S ($n < 27$), medium M ($n = 27$–32), and long L ($n \geq 33$) subgroups.

2.3. Statistical Analyses. The data are presented as either the mean ± SD or the median and 25–75% interquartile range. Data were evaluated by a t-test or the Rank Sum test depending on their normality. Allele frequency was analyzed by a chi-square test. Linear regression analyses were used to assess the association between serum bilirubin and TAS. The impact of individual genetic variations on the risk of FD was analyzed by logistic regression. All tests were made at the 0.05 significance level. All statistical analyses were performed using SigmaPlot software, version 11.0 (Systat Software Inc., USA).

3. Results

3.1. Serum Bilirubin Concentrations and Total Antioxidant Status in Patients with FD. Compared to age-matched control subjects, patients with FD displayed significantly lower systemic bilirubin concentrations (Table 1). The difference

TABLE 2: The effect of ERT on bilirubin and total antioxidant status in patients with FD.

	ERT+ ($n = 31$)	ERT− ($n = 25$)	Controls ($n = 185$)	p value
		Males + females		
Bilirubin [μmol/L]	**9.7** [8.6–15.5]	**7.2** [5.6–8.7]		**0.004**
	7.14 ± 1.9	**6.10 ± 1.2**		**0.036**
TAS		**6.10 ± 1.2**	**6.92 ± 2**	**0.059**
	7.14 ± 1.9		**6.92 ± 2**	0.503
		Males*		
TAS	**7.3 ± 2** ($n = 21$)	($n = 2$)	**7.43 ± 1.9** ($n = 108$)	0.794
		Females		
Bilirubin [μmol/L]	**8.9 ± 0.8** ($n = 10$)	**7.5 ± 1.9** ($n = 23$)		0.186
TAS	**6.5** [5.5–7.6]	**5.8** [5.2–7.4]		0.201
	6.5 [5.5–7.6]		**6.0** [4.8–7.2] ($n = 77$)	0.235

Out of the entire group, 55% of patients were treated with ERT (enzyme replacement therapy) (95% men and 30% women). TAS, total antioxidant status, was determined according to [26]. FD: Fabry disease. *Only two male FD patients were untreated with ERT, making comparison with the untreated group impossible.

was more evident when subjects were gender split; in fact, only FD-affected females showed significantly lower systemic bilirubin concentrations compared to healthy controls (Table 1). On the contrary, the difference was not evident in male subjects, probably due to the positive effect of ERT (predominantly used in male FD patients), which was associated with significantly higher serum bilirubin levels (Table 2). Similarly, the total serum peroxyl-scavenging activity, depressed in FD patients, was also significantly improved in ERT-treated patients (Table 2). Both variables (i.e., TAS and serum bilirubin levels) were positively correlated with borderline statistical significance ($p = 0.067$, Figure 1). No differences in serum bilirubin levels were observed when analyzing FD patient subgroups according to organ involvement (data not shown).

3.2. The Association between HMOX1 and UGT1A1 Promoter Gene Variants and FD Manifestation.

As expected, significantly lower serum bilirubin concentrations between FD patients and control subjects were observed within all *UGT1A1* genotypes, with the lowest values in *UGT1A1* [TA]$_{6/6}$ wild types and the highest in *UGT1A1* [TA]$_{7/7}$ Gilbert's syndrome genotypes (Table 3). The *HMOX1* promoter gene status had no effect on serum bilirubin concentrations (data not shown).

No significant differences in frequencies of the L-allele in the *HMOX1* gene (associated with lower enzyme activity) between the FD and control groups were found (OR = 1.69; 95% CI = 0.54–5.24, $p = 0.46$) indicating no modifying role of *HMOX1* promoter gene variation in FD manifestation (Table 4). In contrast, frequency of the TA$_7$ allele of the *UGT1A1* gene, responsible for higher serum bilirubin levels, was associated with a decreased risk of FD manifestation (OR = 0.51, 95% CI = 0.27–0.97, $p = 0.038$) (Table 4).

4. Discussion

Bilirubin has been reported to be a strong negative predictor/biomarker of oxidative stress-mediated diseases. This
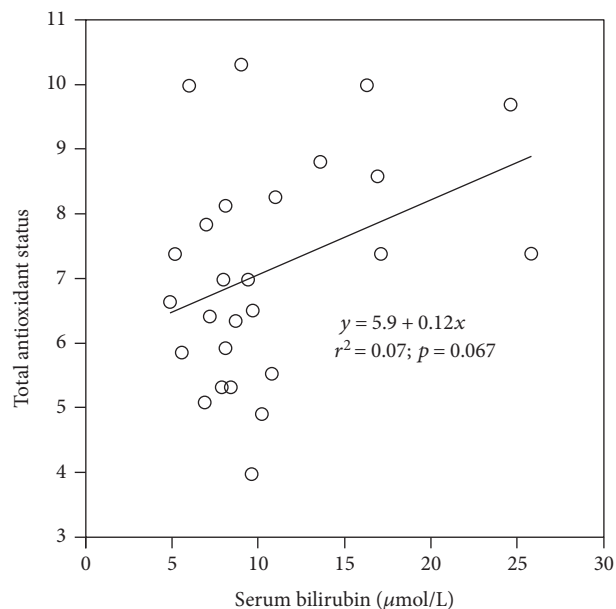

FIGURE 1: The relationship between serum bilirubin and total antioxidant status in patients with FD. Each dot represents a single subject. FD, Fabry disease.

association has been reported for atherosclerosis and cancer, as well as metabolic, autoimmune, and neurodegenerative diseases [20, 24, 28].

With respect to oxidative stress-mediated damage, FD is not the exception, and the role of increased oxidative, nitrosative, and carbonyl stress in pathogenesis of cardiovascular complications of FD is indisputable [11, 14, 15, 17].

Indeed, increased protein nitration and oxidative DNA damage leading to accelerated apoptosis were reported in cardiomyocytes of FD patients [12, 17]. Moreover, increased lipoperoxidation and oxidative protein damage in the plasma of FD patients were reported by Biancini et al. [14]. The severity of FD correlated well with exaggeration of oxidative

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ROS: Reactive oxygen species
TAS: Total antioxidant status
UGT1A1: Bilirubin UDP-glucuronosyl transferase.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Alena Jirásková and Giulia Bortolussi performed all the genetic analyses. Gabriela Dostálová, Lenka Eremiášová, Lubor Golaň, and Vilém Danzig performed all the clinical work-up. Alena Jirásková and Libor Vítek planned the whole study. All authors contributed to the data analysis and interpretation, and they read and approved the final manuscript.

Acknowledgments

References

[1] Y. A. Zarate and R. J. Hopkin, "Fabry's disease," Lancet, vol. 372, no. 9647, pp. 1427–1435, 2008.

[2] D. P. Germain, "Fabry disease," Orphanet Journal of Rare Diseases, vol. 5, p. 30, 2010.

[3] M. N. Sheppard, "The heart in Fabry's disease," Cardiovascular Pathology, vol. 20, no. 1, pp. 8–14, 2011.

[4] P. F. Bodary, Y. Shen, F. B. Vargas et al., "Alpha-galactosidase A deficiency accelerates atherosclerosis in mice with apolipoprotein E deficiency," Circulation, vol. 111, no. 5, pp. 629–632, 2005.

[5] J. M. Lorenzen, B. Dietrich, J. Fiedler et al., "Pathologic endothelial response and impaired function of circulating angiogenic cells in patients with Fabry disease," Basic Research in Cardiology, vol. 108, no. 1, p. 311, 2013.

[6] R. Schiffmann, A. Rapkiewicz, M. Abu-Asab et al., "Pathological findings in a patient with Fabry disease who died after 2.5 years of enzyme replacement," Virchows Archives, vol. 448, no. 3, pp. 337–343, 2006.

[7] T. Kovarnik, G. S. Mintz, D. Karetova et al., "Intravascular ultrasound assessment of coronary artery involvement in Fabry disease," Journal of Inherited Metabolic Disease, vol. 31, no. 6, pp. 753–760, 2008.

[8] B. Tomberli, F. Cecchi, R. Sciagra et al., "Coronary microvascular dysfunction is an early feature of cardiac involvement in patients with Anderson-Fabry disease," European Journal of Heart Failure, vol. 15, no. 12, pp. 1363–1373, 2013.

[9] M. Spada, S. Pagliardini, M. Yasuda et al., "High incidence of later-onset Fabry disease revealed by newborn screening," American Journal of Human Genetics, vol. 79, no. 1, pp. 31–40, 2006.

[10] A. Rolfs, T. Bottcher, M. Zschiesche et al., "Prevalence of Fabry disease in patients with cryptogenic stroke: a prospective study," Lancet, vol. 366, no. 9499, pp. 1794–1796, 2005.

[11] J. S. Shen, X. L. Meng, D. F. Moore et al., "Globotriaosylceramide induces oxidative stress and up-regulates cell adhesion molecule expression in Fabry disease endothelial cells," Molecular Genetics and Metabolism, vol. 95, no. 3, pp. 163–168, 2008.

[12] G. B. Biancini, D. J. Moura, P. R. Manini et al., "DNA damage in Fabry patients: an investigation of oxidative damage and repair," Mutatation Research - Genetic Toxicology and Environmental Mutagenesis, vol. 784-785, pp. 31–36, 2015.

[13] G. B. Biancini, C. E. Jacques, T. Hammerschmidt et al., "Biomolecules damage and redox status abnormalities in Fabry patients before and during enzyme replacement therapy," Clinica Chimica Acta, vol. 461, pp. 41–46, 2016.

[14] G. B. Biancini, C. S. Vanzin, D. B. Rodrigues et al., "Globotriaosylceramide is correlated with oxidative stress and inflammation in Fabry patients treated with enzyme replacement therapy," Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, vol. 1822, no. 2, pp. 226–232, 2012.

[15] D. F. Moore, L. T. Scott, M. T. Gladwin et al., "Regional cerebral hyperperfusion and nitric oxide pathway dysregulation in Fabry disease: reversal by enzyme replacement therapy," Circulation, vol. 104, no. 13, pp. 1506–1512, 2001.

[16] L. Shu, A. Vivekanandan-Giri, S. Pennathur et al., "Establishing 3-nitrotyrosine as a biomarker for the vasculopathy of Fabry disease," Kidney International, vol. 86, no. 1, pp. 58–66, 2014.

[17] C. Chimenti, F. Scopelliti, E. Vulpis et al., "Increased oxidative stress contributes to cardiomyocyte dysfunction and death in patients with Fabry disease cardiomyopathy," Human Pathology, vol. 46, no. 11, pp. 1760–1768, 2015.

[18] R. Stocker, Y. Yamamoto, A. F. McDonagh, A. N. Glazer, and B. N. Ames, "Bilirubin is an antioxidant of possible physiological importance," Science, vol. 235, no. 4792, pp. 1043–1046, 1987.

[19] B. Frei, R. Stocker, and B. N. Ames, "Antioxidant defenses and lipid peroxidation in human blood plasma," Proceedings of the National Academy of Sciences of the United States of America, vol. 85, pp. 9748–9752, 1988.

[20] L. Vitek and H. A. Schwertner, "The heme catabolic pathway and its protective effects on oxidative stress-mediated diseases," Advances in Clinical Chemistry, vol. 43, pp. 1–57, 2007.

[21] L. Vitek, M. Jirsa, M. Brodanova et al., "Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels," Atherosclerosis, vol. 160, no. 2, pp. 449–456, 2002.

[22] M. Exner, E. Minar, O. Wagner, and M. Schillinger, "The role of heme oxygenase-1 promoter polymorphisms in human disease," Free Radical Biology and Medicine, vol. 37, no. 8, pp. 1097–1104, 2004.

[23] H. A. Schwertner and L. Vitek, "Gilbert syndrome, UGT1A1*28 allele, and cardiovascular disease risk: possible protective effects and therapeutic applications of bilirubin," Atherosclerosis, vol. 198, no. 1, pp. 1–11, 2008.

[24] L. Vitek and J. D. Ostrow, "Bilirubin chemistry and metabolism; harmful and protective aspects," Current Pharmaceutical Design, vol. 15, no. 25, pp. 2869–2883, 2009.

[25] M. Biegstraaten, R. Arngrimsson, F. Barbey et al., "Recommendations for initiation and cessation of enzyme replacement therapy in patients with Fabry disease: the European

Fabry Working Group consensus document," *Orphanet Journal of Rare Diseases*, vol. 10, p. 36, 2015.

[26] L. Iuliano, C. Piccheri, I. Coppola, D. Pratico, F. Micheletta, and F. Violi, "Fluorescence quenching of dipyridamole associated to peroxyl radical scavenging: a versatile probe to measure the chain breaking antioxidant activity of biomolecules," *Biochimica et Biophysica Acta (BBA)-General Subjects*, vol. 1474, no. 2, pp. 177–182, 2000.

[27] A. Jiraskova, M. Lenicek, and L. Vitek, "Simultaneous genotyping of microsatellite variations in HMOX1 and UGT1A1 genes using multicolored capillary electrophoresis," *Clinical Biochemistry*, vol. 43, no. 7-8, pp. 697–699, 2010.

[28] K. H. Wagner, M. Wallner, C. Molzer et al., "Looking to the horizon: the role of bilirubin in the development and prevention of age-related chronic diseases," *Clinical Science (London)*, vol. 129, no. 1, pp. 1–25, 2015.

[29] D. F. Moore, F. Ye, M. L. Brennan et al., "Ascorbate decreases Fabry cerebral hyperperfusion suggesting a reactive oxygen species abnormality: an arterial spin tagging study," *Journal of Magnetic Resonance and Imaging*, vol. 20, no. 4, pp. 674–683, 2004.

[30] L. Arning, "The search for modifier genes in Huntington disease - multifactorial aspects of a monogenic disorder," *Molecular and Cellular Probes*, vol. 30, no. 6, pp. 404–409, 2016.

[31] G. Altarescu, D. F. Moore, and R. Schiffmann, "Effect of genetic modifiers on cerebral lesions in Fabry disease," *Neurology*, vol. 64, no. 12, pp. 2148–2150, 2005.

[32] C. Simoncini, L. Chico, D. Concolino et al., "Mitochondrial DNA haplogroups may influence Fabry disease phenotype," *Neuroscience Letters*, vol. 629, pp. 58–61, 2016.

[33] D. F. Moore, M. P. Gelderman, P. A. Ferreira et al., "Genomic abnormalities of the murine model of Fabry disease after disease-related perturbation, a systems biology approach," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 19, pp. 8065–8070, 2007.

[34] G. L. Mazzone, I. Rigato, and C. Tiribelli, "Unconjugated bilirubin modulates nitric oxide production via iNOS regulation," *Bioscience Trends*, vol. 4, no. 5, pp. 244–248, 2010.

[35] G. L. Mazzone, I. Rigato, J. D. Ostrow et al., "Bilirubin inhibits the TNF alpha-related induction of three endothelial adhesion molecules," *Biochemical and Biophysical Research Communications*, vol. 386, no. 2, pp. 338–344, 2009.

[36] G. L. Mazzone, I. Rigato, J. D. Ostrow, and C. Tiribelli, "Bilirubin effect on endothelial adhesion molecules expression is mediated by the NF-kappaB signaling pathway," *Bioscience Trends*, vol. 3, no. 4, pp. 151–157, 2009.

[37] P. N. De Francesco, J. M. Mucci, R. Ceci, C. A. Fossati, and P. A. Rozenfeld, "Fabry disease peripheral blood immune cells release inflammatory cytokines: role of globotriaosylceramide," *Molecular Genetics and Metabolism*, vol. 109, no. 1, pp. 93–99, 2013.

[38] A. Kadl, J. Pontiller, M. Exner, and N. Leitinger, "Single bolus injection of bilirubin improves the clinical outcome in a mouse model of endotoxemia," *Shock*, vol. 28, no. 5, pp. 582–588, 2007.

[39] M. Wallner, A. C. Bulmer, C. Molzer et al., "Haem catabolism: a novel modulator of inflammation in Gilbert's syndrome," *European Journal of Clinical Investigation*, vol. 43, no. 9, pp. 912–919, 2013.

[40] D. Rob, J. Marek, G. Dostalova, L. Golan, and A. Linhart, "Uric acid as a marker of mortality and morbidity in Fabry disease," *PLoS One*, vol. 11, no. 11, article e0166290, 2016.

[41] M. F. McCarty, ""Iatrogenic Gilbert syndrome" - a strategy for reducing vascular and cancer risk by increasing plasma unconjugated bilirubin," *Medical Hypotheses*, vol. 69, no. 5, pp. 974–994, 2007.