

tion was performed by adding 1  $\mu$ L of the post-PER to 9  $\mu$ L of formamide. We loaded each sample on the ABI 310 genetic analyzer and analyzed the samples using Pop4 polymer, a 47-cm capillary column, and the ABI GeneScan E Run Module. The results obtained from the single primer-extension analyses (data not shown) were in complete concordance with the known genotypes of the samples.

During development of the multiplex assay, we discovered that sequencing primers that have a 2–3 bp difference cannot be clearly resolved (overlapping peaks) on the ABI Genotyper electropherogram. This effect was attributable to the primer size and the specific dyes (causing mobility shifts) attached to the ddNTPs. For the ABI 310 genetic analyzer to clearly resolve overlapping peaks, the 3120+1G→A singleplex sequencing primer was increased in length by 10 bp on the 5' end (and named the 3120+1G→A multiplex sequencing primer). This enabled distinct peak separations on the ABI Genotyper electropherogram (Fig. 1, A–C). Multiplexing the singleplex assays was then achieved by combining both sequencing primers and the diluted PCR amplified products into a single PER. Each multiplex PER was in a total volume of 10  $\mu$ L containing 5  $\mu$ L of SNaPshot Ready Reaction Mix, 1  $\mu$ L of the 3120+1G→A multiplex sequencing primer (0.15 pmol/ $\mu$ L; 5'-CTTCTGCCTCTTACCATATTGACTTCATCCAG-3'), 1  $\mu$ L of the I148T sequencing primer (0.15 pmol/ $\mu$ L; 5'-CATTTTGGCCTTCATCACA3'), 1  $\mu$ L (0.15 pmol/ $\mu$ L) of each of the 3120+1G→A and I148T diluted PCR-amplified products, and 1  $\mu$ L of water. The conditions for thermal cycling and post-primer-extension treatment was performed as previously stated in the singleplex PER. The multiplex samples were prepared for analysis on the ABI genetic analyzer by adding 2  $\mu$ L of the post-PER to 9  $\mu$ L of formamide. Multiplex analysis performed on the ABI 310 capillary electrophoresis unit detected five wild-type samples (Fig. 1C) and two heterozygote samples (Fig. 1, A and B). The I148T heterozygote sample exhibited one peak for the two wild-type 3120+1G alleles, one peak for the wild-type I148 allele, and one peak for the mutated I148T allele (Fig. 1A). The 3120+1G→A heterozygote sample exhibited one peak for the two wild-type I148 alleles, one peak for the wild-type 3120+1G allele, and one peak for the mutated 3120+1G→A allele (Fig. 1B). The five wild-type samples all showed one peak for the two wild-type I148 alleles and one peak for the two wild-type 3120+1G alleles (representative sample; Fig. 1C). The negative water control sample showed no peaks (Fig. 1D). Multiplexing with the ABI Prism SNaPshot ddNTP primer-extension assay allows rapid identification of both wild-type and mutated alleles at 3120+1G and I148 in the CFTR gene. The successful development of this multiplex assay has enabled us to speculate that further manipulation of the assay could allow more than two mutations to be analyzed, thereby leading to a cost savings. We believe this technique can be used to easily develop multiplex assays for additional CF mutations and for other genes.

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**Effects of Glucocorticoid Immunosuppression on Serum Cystatin C Concentrations in Renal Transplant Patients, Lorenz Risch,<sup>1</sup> Roberto Herklotz,<sup>1</sup> Alfred Blumberg,<sup>2</sup> and Andreas R. Huber<sup>1\*</sup>** (Departments of <sup>1</sup>Laboratory Medicine and <sup>2</sup>Nephrology, Kantonsspital Aarau, 5001 Aarau, Switzerland; \* author for correspondence: fax 41-62-838-5399, e-mail andreas.huber@ksa.ch)

Cystatin C is a nonglycosylated basic protein (13.36 kDa) and can be found in a variety of biologic fluids (1). Cystatin C serum concentration is not influenced by gender, inflammation, or lean tissue mass and is regarded to be mainly determined by glomerular filtration rate (GFR) (2–5). Cystatin C has been described as meeting many of the characteristics of an ideal GFR marker (e.g., endogenously produced at a constant rate, freely filtered in the glomerulus, neither reabsorbed nor secreted in the renal tubule, not extrarenally eliminated) and has been reported to be at least as accurate as the commonly used serum creatinine to detect impaired renal function in various patient groups, including renal transplant patients (6–24).

Despite this, Bokenkamp et al. (25) reported that cystatin C is higher in children with renal transplants than in children with other renal pathologies but comparable GFRs, suggesting an underestimation of GFRs by cystatin C in renal transplant patients. This finding suggested immunosuppression as a major influencing factor because all patients had received prednisolone and cyclosporin A medication. Interestingly, neither prednisolone nor cyclo-

sporin A was believed to change cystatin C concentrations because no dose-dependent increase of cystatin C was found. In contrast, an *in vitro* study by Bjarnadottir et al. (26) described a dose-dependent increase of cystatin C production in HeLa cells exposed to dexamethasone. To further clarify these findings, we conducted a nested case-control study in a cohort of renal transplant patients who were prospectively monitored during a 1-year period (20). The present study aimed to elucidate the influence of glucocorticoid immunosuppression on cystatin C concentrations in serum from renal transplant patients.

The patients were seen for routine follow-up, which included assessment of clinical data and laboratory results. To evaluate the influence of immunosuppressive regimens, especially glucocorticoids, on serum cystatin C, 20 clinically stable patients on immunosuppression therapy with low-dose glucocorticoids were matched with 20 clinically stable patients receiving cyclosporin A alone and 20 clinically stable patients receiving cyclosporin A together with azathioprine (Table 1).

Clinical stability was defined as the absence of acute rejection, febrile infection, and cyclosporin A toxicity, as well as stability of creatinine clearance as estimated by the formula of Cockcroft and Gault (27). The three groups were matched for estimated creatinine clearance (CrCl) and had comparable gender, age, and time since transplantation. To reduce the influence of the known biologic variation of cystatin C (20, 28), all patients had six measurements during subsequent visits that demonstrated stable clinical condition. Means from cystatin C reciprocals, as well as from CrCl estimates, were calculated and used for data analysis.

Furthermore, 13 patients receiving a short course of high-dose methylprednisolone (500 mg intravenously per day for 3 days) for deteriorating renal function were analyzed to observe a possible dose-dependent effect of glucocorticoid administration. None of the 73 patients was receiving additional glucocorticoid or mineralocorticoid medication.

The group receiving short-course, high-dose methyl-

prednisolone had results from four time points available: (a) the visit before methylprednisolone commencement (median, 17 days; range, 2–67 days; 2 patients with missing values); (b) the day methylprednisolone was started (before medication); (c) after 3 days of methylprednisolone therapy; and (d) on a follow-up visit (median, 8 days after last dose; range, 6–11 days).

Serum cystatin C was measured by a particle-enhanced turbidimetric immunoassay (PETIA; Dako) on a Cobas Mira (Roche) (9). Serum creatinine was measured with a modified kinetic Jaffe method (Dimension RXL; Dade-Behring) (29). Creatinine clearance was estimated by the formula of Cockcroft and Gault (27), which in renal transplant patients has been validated against Iohexol clearance with creatinine measurements by a kinetic Jaffe method (30, 31).

Data are presented as mean  $\pm$  SD or as median and interquartile range (IQR) where appropriate after testing for gaussian distribution. For linearization of the hyperbolic relationship between cystatin C and GFR, reciprocals of cystatin C concentrations were calculated. Multiple comparisons were done by Kruskal–Wallis one-way ANOVA. Linear regression lines were compared by analysis of covariance. For comparison of serial measurements in the same group, the Friedman repeated-measure ANOVA on ranks followed by the Dunn test was used. The study was approved by the Institutional Review Board and is in accordance with the Helsinki Declaration of 1975, as revised in 1983. All patients gave informed consent.

Reciprocals of cystatin C and CrCl estimates showed a linear relationship in all four groups. Equations of the linear regression lines between cystatin C reciprocals and CrCl estimates were as follows:  $1/\text{cystatin C} = 0.3413 + [0.00685 \times \text{CrCl}]$  ( $r = 0.65$ ) in the cyclosporin A alone group;  $1/\text{cystatin C} = 0.173 + [0.00850 \times \text{CrCl}]$  ( $r = 0.76$ ) in the cyclosporin A-azathioprine group;  $1/\text{cystatin C} = 0.0860 + [0.00838 \times \text{CrCl}]$  ( $r = 0.86$ ) in the low-dose glucocorticoid group; and  $1/\text{cystatin C} = 0.1318 + [0.00534 \times \text{CrCl}]$  ( $r = 0.69$ ) in the high-dose glucocorticoid

**Table 1. Characteristics of renal transplant patients with different immunosuppression.**

Characteristic <sup>a</sup>	Immunosuppression			
	Cyclosporin A	Cyclosporin A/ azathioprine	Low-dose glucocorticoid <sup>b</sup>	High-dose glucocorticoid <sup>c</sup>
Number	20	20	20	13
Age, years	55 $\pm$ 15	51 $\pm$ 12	49 $\pm$ 14	44 $\pm$ 12
Time since transplantation, years	4.5 $\pm$ 3.5	6.1 $\pm$ 3.7	4.3 $\pm$ 2.7	6.3 $\pm$ 5.7
Gender, M/F	11/9	13/7	15/5	8/5
Body weight, kg	66 $\pm$ 11	71 $\pm$ 10	77 $\pm$ 14	71 $\pm$ 10
Creatinine clearance estimate, mL/min <sup>d</sup>	51 $\pm$ 14	52 $\pm$ 14	49 $\pm$ 14	43 $\pm$ 12
Cystatin C, mg/L, median (IQR) <sup>e</sup>	1.50 (1.19–1.83)	1.63 (1.42–2.06)	1.92 (1.73–2.44)	2.69 (2.34–3.5)

<sup>a</sup> Except for cystatin C concentration, data are presented as mean  $\pm$  SD.

<sup>b</sup> Prednisone dose per day: 5 mg, n = 6; 7.5 mg, n = 6; 10 mg, n = 8. Fourteen patients received cyclosporin A, prednisone, and azathioprine and 6 received cyclosporin A and prednisone.

<sup>c</sup> 500 mg of intravenous methylprednisolone daily for 3 days. All patients received cyclosporin A, methylprednisolone, and azathioprine.

<sup>d</sup> Not significant ( $P = 0.29$ ).

<sup>e</sup>  $P < 0.001$ .

group. Whereas correlation coefficients and slopes among the different groups did not differ significantly, significant differences of elevations in the regression lines could be observed: cystatin C reciprocals in the low-dose glucocorticoid group were significantly lower than in the cyclosporin A-azathioprine group ( $P = 0.016$ ) and the cyclosporin A alone group ( $P < 0.001$ ), but significantly higher than in the high-dose glucocorticoid group ( $P = 0.008$ ). No significant difference of increase was seen between the groups with cyclosporin A alone and cyclosporin A-azathioprine ( $P = 0.15$ ). Together, glucocorticoid administration was associated in a dose-dependent fashion with increased cystatin C values, leading to systematic underestimation of GFR in renal transplant patients.

To estimate the magnitude of cystatin C increases among the different groups, hyperbolic relationships between cystatin C and CrCl estimates, as derived from linear regression equations, are shown in Fig. 1A. In comparison with the group receiving cyclosporin A alone, the group receiving low-dose glucocorticoids had cystatin C concentrations that differed by the following: 0.20 mg/L at  $80 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ ; 0.27 mg/L at  $70 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ ; by 0.37 mg/L at  $60 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ ; by 0.52 mg/L at  $50 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ ; by 0.75 mg/L at  $40 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ ; by 1.13 mg/L at  $30 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ ; and by 1.85 mg/L at  $20 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ .

Because patients receiving long-term, low-dose glucocorticoid therapy demonstrated higher cystatin C concentrations than controls, cystatin C kinetics after glucocorticoid administration and withdrawal were investigated. High-dose methylprednisolone given intravenously led to significant differences in cystatin C values at different time points (before administration, after three doses, and 8 days after discontinuation;  $P < 0.001$ ). After three daily doses of 500 mg, cystatin C concentrations increased from 2.13 mg/L (IQR, 1.72–2.80) to 2.69 mg/L (IQR, 2.34–3.5;  $P < 0.05$ ). Eight days after discontinuation, cystatin C concentrations significantly decreased to 1.96 mg/L (IQR, 1.63–2.4;  $P < 0.05$ ; Fig. 1B) At these time points, neither the CrCl estimate ( $45 \pm 13 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ ,  $43 \pm 12 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ , and  $46 \pm 14 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ ;  $P = 0.08$ ) nor the serum creatinine concentrations ( $175 \mu\text{mol/L}$ , IQR, 156–202;  $178 \mu\text{mol/L}$ , IQR 161–203; and  $158 \mu\text{mol/L}$ , IQR 144–199;  $P = 0.17$ ) underwent significant changes.

By ROC analysis of all four groups, cystatin C had higher diagnostic accuracy than serum creatinine in detecting a creatinine clearance estimate  $\leq 60 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$  (data not shown). This finding was significant in the low-dose glucocorticoid group ( $P = 0.005$ ). Furthermore, in vitro interference of the administered immunosuppressive drugs (cyclosporin A, azathioprine, and methylprednisolone) on the cystatin C and creatinine assays used was tested and excluded as a cause of the false increase of cystatin C (data not shown).

This study demonstrates that renal transplant patients receiving glucocorticoid medication have higher cystatin C than two comparable groups with glucocorticoid-free

immunosuppression. Because patients receiving 500 mg of methylprednisolone had significantly higher cystatin C values than patients receiving  $\leq 10 \text{ mg}$  of prednisone, a dose-dependent influence of the administered glucocorticoid dose is suggested. Thus, glucocorticoid medication leads to systematic underestimation of GFR in renal transplant patients. The hyperbolic relationships deriving from linear regression lines allow for an estimate of cystatin C increase at specific levels of CrCl.

Similar to our findings, Bjarnadottir et al. (26) observed that dexamethasone caused a dose-dependent increase in the cystatin C secretion of cultivated HeLa cells. Further-

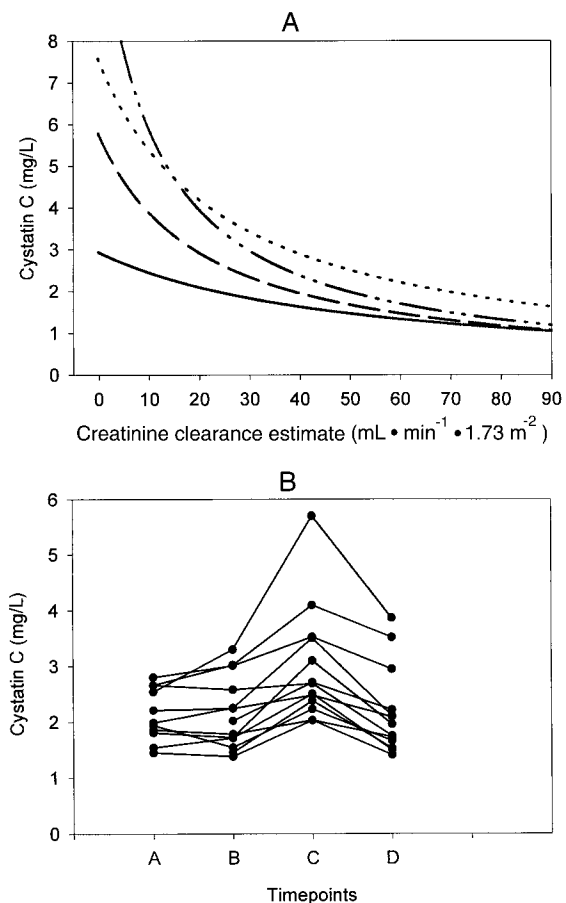


Fig. 1. Serum cystatin C concentrations in renal transplant patients receiving different immunosuppressive medication.

(A), glucocorticoid medication is associated in a dose-dependent fashion with significantly increased cystatin C. Hyperbolic relationships derived from linear regression equations are shown. The low-dose glucocorticoid group received  $\leq 10 \text{ mg}$  prednisone daily combined with cyclosporin A and azathioprine, and the high-dose glucocorticoid group received three doses of 0.5 g of methylprednisolone in combination with cyclosporin A and azathioprine. The groups without glucocorticoids had no significant difference in cystatin C concentrations. . . . ., high-dose glucocorticoid; — — —, low-dose glucocorticoid; — — —, cyclosporin A plus azathioprine; —, cyclosporin A alone. (B), time course of cystatin C concentrations after administration of a high-dose methylprednisolone therapy. Each line represents the course of a patient. Point A, determinations 17 days before methylprednisolone course was started (median range, 2–67 days; 2 patients with missing values); point B, before first dose; point C, after three daily doses of 500 mg of methylprednisolone; point D, follow-up 8 days after cessation of methylprednisolone (range, 6–11 days). Increases from points B to C and decreases from points C to D are both significant ( $P < 0.05$ ). Serum creatinine and CrCl estimates underwent no significant changes.



more, when an expression system was transfected in HeLa cells by chimeric plasmid constructs of the cystatin C promoter coupled to the structural gene coding for human growth hormone, a statistically significant increase of human growth hormone secretion after dexamethasone administration could be detected. These findings suggest that the glucocorticoid-induced increase of cystatin C production reflects a promoter-mediated increase in transcription of the cystatin C gene. Despite these *in vitro* results, cystatin C serum concentration *in vivo* is thought to be mainly determined by GFR, although some exceptions have been reported (25, 32).

Bokenkamp et al. (25) reported that serum cystatin C in pediatric renal transplant patients is higher than in non-renal transplant children with comparable GFR. It was not stated, however, how many participants in the control group had received a glucocorticoid-free medication. Furthermore, the control group was matched only for GFR, age, and gender, but not for kidney transplantation. With this, it was not possible to determine whether glucocorticoid medication or renal transplantation led to the difference in cystatin C concentrations. In contrast to our observations, a dose-dependent influence of glucocorticoid medication on cystatin C could not be demonstrated, probably because the differences in individual prednisolone doses were too small to be reflected in cystatin C concentrations.

A similar effect of glucocorticoid medication has been reported in asthmatics by Cimerman et al. (32). Comparable to our observations in renal transplant patients receiving a 3-day course of 500 mg of methylprednisolone per day, they found a highly significant increase of cystatin C after 1 week of 40 mg of methylprednisolone daily. The present study demonstrates that the increase is a transitory phenomenon, because after a median of 8 days after cessation of methylprednisolone, cystatin C concentrations decreased to values observed before high-dose glucocorticoid administration.

Our finding that glucocorticoid medication is associated with increased cystatin C could explain observations in another study by Newman et al. (7), who found three outliers exhibiting increased cystatin C, when comparing cystatin C concentrations and GFR measurements by <sup>51</sup>Cr-EDTA clearance in 209 patients. One of these outliers originated from a renal transplant patient after several rejection episodes that were treated with steroids and full immunosuppression therapy. Although not stated in the report, the other two patients may also have been on glucocorticoids because one suffered from systemic sclerosis and the other patient had severe rheumatoid arthritis.

In summary, glucocorticoid medication in adult renal transplant patients is associated in a dose-dependent manner with increased cystatin C, leading to systematic underestimation of GFR. This does not preclude the use of cystatin C in detecting impaired renal function in renal transplant patients with glucocorticoids, because this study and others (19–22) showed cystatin C to be significantly more accurate in detecting impaired renal function

in this patient group. Moreover, our data illustrate the need for specific reference intervals in patients on glucocorticoid therapy. Depending on steroid dose and CrCl impairment, the cystatin C increase can be estimated to be 0.20–1.85 mg/L in patients receiving low-dose glucocorticoids and is even higher in patients receiving high-dose glucocorticoids. However, a more detailed study evaluating dose effects is needed. In conclusion, in clinical routine settings, as well as in future clinical studies, it is important to take glucocorticoid medication into account when interpreting serum cystatin C concentrations in renal transplant patients and, presumably, in other patient groups.

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**Comparison of Bathophenanthroline Sulfonate and Ferene as Chromogens in Colorimetric Measurement of Low Hepatic Iron Concentration.** Laurence Pieroni,<sup>1</sup> Lina Khalil,<sup>1</sup> Frederic Charlotte,<sup>2</sup> Thierry Poynard,<sup>3</sup> Annie Piton,<sup>1</sup> Bernard Hainque,<sup>1</sup> and Françoise Imbert-Bismut<sup>1\*</sup> (<sup>1</sup> Laboratoire de Biochimie B, <sup>2</sup> Service d'Anatomopathologie, and <sup>3</sup> Service d'Hépatogastroentérologie, Groupe Hospitalier Pitié-Salpêtrière, 47 Boulevard de l'Hôpital, F 75651 Paris Cedex 13, France; \* author for correspondence: fax 33-01-42-16-20-33, e-mail francoise.bismut@psl.ap-hop-paris.fr)

Sensitive and accurate measurement of hepatic iron concentration (HIC) is required to investigate liver fibrogenesis (1) and its influence on the outcome of interferon therapy for chronic viral hepatitis C (2, 3). Hepatic iron content can be measured by a quantitative chemical method and/or evaluated by semiquantitative histologic scoring. Quantitative chemical methods assess all liver iron forms, whereas histologic scoring evaluates only the hemosiderin form. A colorimetric method using bathophenanthroline sulfonate as chromogen was recommended in 1978 by the International Committee for Standardization in Hematology (ICSH) for determination of serum iron (4, 5). It was adapted by Barry and Sherlock (6) to the determination of HIC, and we recently evaluated it for measurement of low HIC (7). In 1990, the ICSH replaced bathophenanthroline sulfonate with ferene, a more sensitive chromogen, in the determination of serum

iron (8). The aim of the present study was to evaluate the replacement of bathophenanthroline sulfonate with ferene to improve the sensitivity of the colorimetric determination of low HIC.

We used samples of a frozen Wistar rat liver for quality control and determination of reliability criteria of both assays. We compared the results obtained with the two chromogens on 66 liver biopsies from patients with chronic liver diseases hospitalized in the Department of Hepatogastroenterology of the Pitié-Salpêtrière Hospital. The clinical diagnoses of these patients are summarized in Table 1. We determined the CV for HIC measurements on two separate samples from the same liver specimen for each chromogen on 38 human liver biopsies. Histologic iron scoring was according to the method of Deugnier et al. (9); among the 66 biopsies, 20 had no stainable iron (score of 0), and the 46 others exhibited iron overload (score  $\geq 6$ ). Biopsies were fixed in 40 g/L formaldehyde and embedded in paraffin as part of routine histologic processing for better preservation and transport.

Paraffin-embedded tissues were heated at 60 °C to remove the paraffin and washed in three successive baths of xylene followed by three baths of ethanol. Liver samples were dried at 120 °C for 24 h and weighed on a microbalance with 0.01 mg precision. The mean dry weight (dw) of the liver samples was 1 mg (SD, 0.4 mg; range; 0.5–2.5 mg). Liver samples were digested with an equivolume mixture of sulfuric acid and nitric acid for 5–8 min over a low flame. The working solutions were prepared by adding 10 mL of H<sub>2</sub>O (10). Samples of thawed rat liver were dried, weighed, and treated as described above.

HIC was measured according to the colorimetric method described by Barry and Sherlock (6). The iron in 3 mL of each working solution was reduced with 50  $\mu$ L of thioglycolic acid (Sigma Aldrich) and 2.5 mL of an acetic acid–acetate buffer (Merck Eurolab) at pH 4.5, which is optimal for the reduction of ferric to ferrous iron and for absorbance measurements of iron complexed with bathophenanthroline sulfonate (4,7-diphenyl-1,10-phenanthroline disulfonic acid), disodium salt or ferene {3-(2-pyridyl)-5,6-bis(2[5-furyl sulfonic acid])-1,2,4-triazine} (11).

**Table 1. Clinical diagnoses of patients with chronic hepatic disorders.**

Pathology	Histologic iron score	
	0	$\geq 6$
Hepatitis C only	15	30
Hepatitis C + HIV	2	5
Hepatitis C + hepatitis B	0	2
Hepatitis C + alcoholism	1	0
Hepatitis B only	1	1
Alcoholism	0	1
Hemochromatosis	0	2
Dysmetabolic syndrome	1	1
Other	0	4
Total	20	46