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Urinary Porphyrin Excretion in Hepatitis C Infection

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A high prevalence of hepatitis C virus infection in porphyria cutanea tarda in some populations suggests a close link between viral hepatitis and alteration of porphyrin metabolism. Moreover, there is evidence of a role of porphyrinopathies in hepatocarcinogenesis. The aim of our study was to obtain data on the prevalence and patterns of heme metabolism alterations in patients with chronic hepatitis C virus infection. Urinary porphyrin excretion was prospectively studied in 100 consecutive outpatients with chronic hepatitis C infection without signs of photosensitivity, using an ion-pair high-performance liquid chromatography method. Increased total porphyrin excretion was found in 41 patients, with predominant excretion of coproporphyrins (whole study group: mean 146 µg/g creatinine, interquartile range 76–186; normal < 150), in 10 patients excretion exceeded 300 µg/g creatinine. In the majority of all patients studied (75/100) an increased ratio of the relatively hydrophobic coproporphyrin isomer I to isomer III was found. In just one case, urinary porphyrin pattern characteristic for chronic hepatic porphyria was present (uroporphyrin > coproporphyrin, heptacarboxyporphyrin III increased) but the total porphyrin excretion was only slightly elevated in this case. In the whole group, total urinary porphyrin excretion correlated well with serum bilirubin and was inversely correlated with albumin and thrombin time. In conclusion, secondary coproporphyrinuria occurs frequently in heptatitis C infection, whereas in Germany, preclinical porphyria cutanea tarda seems to be rare in these patients.

Key words: Hepatitis C; Secondary porphyrinuria; Chronic hepatic porphyria; Coproporphyrin isomers.

Introduction

The identification of chronic viral hepatitis C as a frequent coexisting disease in porphyria cutanea tarda (PCT) with an increasing gradient from northern to southern Europe (1–4) suggests association between viral hepatitis and porphyrinopathies. Increased urinary porphyrin excretion has been recognised for many years in various hepatobiliary diseases (5, 6). Whereas PCT, in which uroporphyrin and heptacarboxyporphyrin excretion are largely elevated (7), causes characteristic dermatological symptoms, the mere excess of urinary coproporphyrins is generally considered to be a biological indicator not related to any symptoms.

There is growing evidence to suspect that chronic disturbance of heme metabolism may be involved in hepatocarcinogenesis (8, 9): a high risk for hepatocellular carcinoma has been recognised in patients with chronic hepatic porphyria and cirrhosis (10, 11). In acute intermittent porphyria an increased risk of cirrhosis and hepatocellular carcinoma (HCC) has been demonstrated (12–14); a case of HCC associated with the rare hereditary coproporphyria has been described (15). Massively elevated levels of urinary coproporphyrin have been found in patients with HCC compared to patients with liver diseases without HCC (16). Several cases of PCT related to benign or malignant hepatocellular tumors have been reported (17, 18). Besides these epidemiological observations, there are important experimental data indicating a role of altered porphyrin metabolism in hepatocarcinogenesis: toxic compounds that induce HCC (such as polyhalogenated biphenyls or ketoconazole in animal models) usually exhibit potent porphyrinogeneic properties (19–23).

Based on these findings we decided to reevaluate possible pathophysiological and prognostic significance of secondary porphyrinuria in liver diseases, and especially in chronic hepatitis C infection with its high rate of progression to cirrhosis and finally HCC (24). Only few systematic studies on urinary porphyrin excretion, with conflicting results, have been performed in well defined groups of patients with liver diseases (5, 6, 25–29); therefore, links between abnormal porphyrin metabolism, disease progression to cirrhosis and hepatocarcinogenesis in heptatitis C are so far unclear. In order to obtain basic epidemiological data on urinary porphyrin excretion in liver diseases for further study on prognostic implications we studied urine porphyrin excretion patterns in patients with chronic HCV infection. One hundred consecutive HCV patients were included in the study to answer three main questions:

(i) What is the prevalence of increased urinary porphyrin excretion in patients with HCV infection?

(ii) What patterns of the main porphyrin compounds (coproporphyrin isomers I and III, uroporphyrin I and III) are found; to what extent can the pattern typical for chronic hepatic porphyria (i.e. markedly increased uroporphyrin excretion) as "preclinical" PCT be found?

(iii) Is there any association of porphyrin excretion patterns with routine laboratory tests of liver cell integrity, function or biliary excretion, that characterise the stage of liver disease?

Patients and Methods

One hundred consecutive patients with serologically confirmed hepatitis C infection (HCV-RNA positive, elevated ALT) attending the hepatology outpatients' service were included in the study. In all cases, the reason for the patients' presentation was an evaluation for a potential therapy with interferon. Forty-eight patients were female, 52 male, the median age was 47 years (range 22–78). After informed consent, patients were interviewed for dermatological abnormalities and the sun exposed skin was examined. Patients with significant (> 20 g per day) alcohol consumption were not included. A random sample of urine was collected from each patient and immediately protected from light; the urine was stored at –20 °C until analysis. No additional blood sampling was performed for this study. The results of clinically indicated routine laboratory serum tests, and hematological and coagulation laboratory tests (determined on the day of urine collection) were recorded (bilirubin, AST, ALT, γ-glutamyltransferase (GGT), alkaline phosphatase (AP), albumin, ferritin, transferrin saturation calculated from serum transferrin and iron, and thrombin time); mechanised standard clinical chemistry methods run on autoanalyzers were used (Roche Diagnostics, Mannheim, Germany). Data on viral load (HCV-RNA quantification, by HCV-Amplicor monitor®, Roche Diagnostics) were recorded as well.

Patients were stratified into three groups according to the total porphyrin excretion: group I had porphyrin excretion within the normal range (< 150 µg/g creatinine); group II had porphyrin excretion up to twice the reference range (150–300 µg/g creatinine); group III had porphyrin excretion more than twice the reference range (> 300 µg/g creatinine).

In addition, urinary porphyrin excretion patterns were studied in 18 patients with cholestasis due to bile duct obstruction.

Urinary porphyrin analysis

An ion-pair high-performance liquid chromatographic (HPLC) method was used as described previously (30); this method is routinely performed in our institution.

Sample preparation

Six ml of urine was oxidised with 0.3 ml of a solution containing 5 g I_2 and 10 g KI in 100 ml water for 5 min, to convert quantitatively porphyrinogens into the corresponding porphyrins. Excess iodine was removed with $Na_2S_2O_3$, and subsequently 6 ml 1.2 mol/l H_3PO_4 were added. The porphyrins were adsorbed on SepPak C_{18} cartridges (Waters, Milford MA, USA) and eluted with 5 ml of methanol/acetone (1+1, by volume). The solvents were removed under vacuum, and the residue was dissolved in 1.9 ml of 50 mmol/l methanolic tetrabutylammonium phosphate; as internal standard, 100 µl of 4 µmol/l mesoporphyrin (Sigma, Deisenhofen, Germany) in 50 mmol/l methanolic tetrabutylammonium phosphate was added. After centrifugation, 50 µl of this solution was injected for HPLC analysis.

HPLC conditions

Precolumn: LiChrospher 4x4 RP_{18} 5 µm (Merck, Darmstadt, Germany). Analytical column: LiChrospher 125x4 RP_{18} 5 µm (Merck). Mobile phase: multilinear gradient starting with 62 % aqueous phosphate buffer (40 mmol/l, pH 5.4) and 38 % methanolic phase (12.5 mmol/l tetrabutylammonium phosphate, pH 7.2). Final composition after 40 min: 20 % aqueous phase and 80 % methanolic phase. Fluorescence detection: excitation wavelength 395 nm, emission wavelength 625 nm.

The interassay coefficient of variation (n = 11) of this method is 4 % to 7 % depending on the respective porphyrin compound. The recovery rate varies between 95 % and 97 %.

Uroporphyrin I and III, coproporphyrin I and III, and heptacarboxyporphyrin III concentrations were calculated in relation to the respective urinary creatinine concentration and the total urinary porphyrin excretion was determined by summing up the concentrations of these analytes. Normally, less than 150 µg porphyrin per g creatinine are excreted (< 25 µg uroporphyrin and < 125 µg coproporphyrin; n = 20); the normal ratio of coproporphyrin III to I isomers is approximately 75:25. With this method the intermediate types of porphyrins (hexacarboxy- and pentacarboxyporphyrins) are also completely separated and detected.

Median and interquartile ranges of the laboratory tests and of quantitative results related to the porphyrin excretion pattern (percentage of coproporphyrin I in total coproporphyrins, and percentage of uroporphyrins in the total porphyrin excretion) were calculated for the three groups. After an F-test, Student's T-test was applied to test for significant differences in the variables between the groups II and III compared to group I (significance was assumed for $p < 0.05$). Using the data of the entire group studied, linear regression was calculated between the total porphyrin excretion and albumin, bilirubin, ALT, AST, AP, GGT, ferritin, and thrombin time, and Pearson's coefficient of correlation was calculated.

Results

Normal total porphyrin excretion, including the respective porphyrinogens, was found in 59/100 of the patients (< 150 µg/g creatinine); in 31 % total porphyrin excretion was increased up to twice the normal range (150–300 µg/g creatinine). Ten out of 100 patients had more marked porphyrinuria of more than 300 µg/g creatinine. Mean total porphyrin concentration calculated for all patients was 146 µg/g creatinine and median was 123 µg/g creatinine (interquartile range 76–186 µg/g creatinine, range 24–483).

Coproporphyrin excretion clearly exceeded that of uroporphyrin in the majority of patients with porphyrinuria. Increased coproporphyrin excretion (> 125 µg/g creatinine) was found in 42 patients, the median excretion in all patients was 105 µg/g creatinine (interquartile range 60–160 µg/g creatinine, mean 125 µg/g creatinine). The ratio of the more hydrophobic coproporphy-

rin isomer I to isomer III was increased in the majority of patients studied (75/100).

Increased excretion of uroporphyrin was found in 19 patients; an excretion of more than 50 µg/g creatinine (i.e. twice the reference range) was found in only three patients. The median uroporphyrin excretion was 15 µg/g creatinine (mean 19 µg/g creatinine). The highest uroporphyrin excretion found was 133 µg/g creatinine; this was the only case where the excretion of uroporphyrin exceeded that of coproporphyrin (32 µg/g creatinine). This 51 year-old female patient also was the sole patient with a marked excretion of heptacarboxyporphyrin III (59 µg/g creatinine, normal < 5 µg/g) and slightly increased pentacarboxyporphyrin excretion (9 µg/g creatinine, normal < 5 µg/g) offering the typical patterns of chronic hepatic porphyria. The total porphyrin excretion, however, was only moderately elevated (224 µg/g creatinine). No patient had signs of abnormal photosensitivity of the skin. Significant amounts (> 5 µg/g creatinine) of hexacarboxy- or pentacarboxyporphyrin were not found in the other samples studied.

Median values (with interquartile ranges) of the laboratory tests and of characteristics of the urinary porphyrin pattern among the three groups according to total porphyrin excretion are given in Table 1. Among the ten patients of group III with marked porphyrinuria, five had normal serum bilirubin, albumin, and plasma thrombin time.

In the entire group a significant positive correlation between total porphyrin excretion and serum bilirubin was found ($r = 0.46$; $p < 0.001$), and a significant inverse correlation with thrombin time ($r = -0.53$; $p < 0.001$) and albumin ($r = -0.58$; $p < 0.001$); no significant correlations were found between the total porphyrin excretion and AST, ALT, AP, GGT, ferritin, and transferrin saturation in the entire group. However, in the group of patients with marked porphyrinuria, 60 % had elevated serum ferritin and 43 % had elevated transferrin saturation, in the group of patients with moderate porphyrinuria 29 % had increased ferritin and 21 % had increased transferrin saturation. Patients without porphyrinuria had increased ferritin in 17 % and increased transferrin saturation in 10 %. No correlation was found between HCV-RNA viral load (data not shown) and total urinary porphyrin excretion.

In the two groups of patients with increased total urinary porphyrin excretion the proportion of uroporphyrin to total porphyrin was lower than in normal subjects.

In the control group of patients with bile duct obstruction (n = 18; bilirubin 79 µmol/l, range 39–376) median urinary coproporphyrin excretion was 241 µg/g creatinine (interquartile range 144–355), with an increased relative proportion of isomer I (58 %; interquartile range 50–69, normal < 25 %).

Discussion

Epidemiological as well as experimental data encourage a reevaluation of porphyrinurias in chronic liver disease with special respect to disease progression to hepatocellular carcinoma. In this report we present basic epidemiological data on the porphyrin excretion in unselected patients infected with the HCV in Germany; these data are the prerequisite for further investigations on a possible prognostic value of porphyrin excretion in viral hepatitis.

A coproporphyrin-dominated porphyrinuria was found in nearly half of the HCV infected patients studied; in an even higher proportion (75 %) of the patients

Tab. 1 Liver function tests and porphyrin excretion patterns of 100 patients with chronic hepatitis C infection, categorised according to the total urinary porphyrin excretion.

	Normal	Group I	Group II	Group III
Total urinary porphyrin excretion (µg/g creatine)*	< 150	< 150	150–300	> 300
N		59	31	10
Age (median, years)		46	47	52
Sex		25 f, 34 m	20 f, 11 m	2 f, 8 m
Bilirubin (µmol/l)	< 17	10.3 (7.0–15.4)	12.0 (8.6–22.2)	20.5 (15.4–44.5)[a]
Albumin (g/l)	35–50	49 (47–50)	46 (42–48)[a]	39 (37–47)[a]
Thrombin time (%)	70–100	90 (85–95)	85 (75–90)[a]	70 (60–70)[a]
AST (U/l)	5–17	23 (16–35)	40 (24–58)[a]	44 (30–61)[a]
ALT (U/l)	5–24	38 (27–58)	58 (33–88)	42 (27–58)
GGT (U/l)	4–28	23 (12–41)	33 (21–49)	36 (25–46)
AP (U/l)	50–190	121 (100–151)	135 (110–152)	170 (156–188)[a]
Ferritin (ng/ml)	30–300	104 (62–209)	176 (100–367)	414 (164–531)[a]
% Coproporphyrin I[1)]	20–30	39 (28–50)	39 (21–50)	51 (40–58)
% Uroporphyrins[2)]	15–25	14 (10–24)	9 (7–13)[a]	9 (7–10)[a]

(median with interquartile range)

[a]: $p < 0.05$ with respect to group I

[1)] Percentage of total coproporphyrins

[2)] Percentage of total porphyrins

* Conversion factor for creatinine: 1 g/l = 8.85 mmol/l.

an increased ratio of the more hydrophobic coproporphyrin isomer I to isomer III was found, ranging from 30 % to 58 % (normal 20 %–30 %). In contrast, a porphyrin pattern typical of chronic hepatic porphyria was found in just one patient; in this case, total porphyrin excretion was only moderately elevated. A constellation that might be called "preclinical PCT" could be found by measuring porphyrin excretion in patients with chronic HCV infection. This condition, however, is obviously rare in Germany.

In the entire study group, there was a weak correlation of total porphyrin excretion with laboratory markers of impaired liver function; porphyrin excretion did not correlate with serum transaminases, and GGT or AP as markers of cholestasis. Likewise marked porphyrinuria (above twice the reference range) was also found in patients with normal or only slightly impaired liver function. The group of patients with moderate porphyrinuria did show significant differences in liver function tests in relation to the group without porphyrinuria; however, median values of serum albumin, bilirubin, and thrombin time in this group were within normal limits. We found altered porphyrin excretion in HCV infection with a great interindividual variability which was in part independent of global liver function or disease activity. Our data show that a stratification of HCV patients based on their porphyrin excretion is only partially in accordance with established liver function tests.

In a series of 56 HCV-infected patients, Cribier *et al.* (26) found slightly elevated median urinary porphyrin excretion, however, data on the distribution of the respective values were not given; in addition, porphyrin concentrations of random urine samples were not corrected for urinary creatinine in this study, and coproporphyrin isomer distribution was not investigated. In another cohort of 34 HCV-infected patients, O'Reilly *et al.* (27) were not able to demonstrate significant urinary porphyrin changes. Bonkovsky *et al.* (29) report a rather low prevalence (15 %) of porphyrinuria in HCV-infected patients in North America.

Several mechanisms may be responsible for secondary porphyrinuria in HCV infection: impaired biliary excretion of coproporphyrins, increased porphyrin formation or a combination of both. Coproporphyrins, especially the isomer I, are relatively hydrophobic and thus are predominantly excreted into bile; mild cholestasis may lead to a coproporphyrin accumulation in the plasma with spillover into the urine, as can be observed in bile duct obstruction. In our series, total porphyrin excretion did not correlate with AP and GGT as the typical laboratory markers of cholestasis, but there was a significant correlation to serum bilirubin. Thus urinary coproporphyrin seems indeed to be a sensitive marker of intrahepatic cholestasis; the increased proportion of the more hydrophobic isomer I to isomer III in urine found in the study group with normal total urinary porphyrin excretion suggests that this ratio is an even more sensitive indicator of early cholestasis (6). This view is in agreement with the urinary porphyrin patterns found in the control group of patients with bile duct obstruction who had both increased urinary coproporphyrin and a raised proportion of isomer I. Uroporphyrins are less affected by cholestasis as they are rather hydrophilic and are predominantly excreted in the urine.

It is still speculative to assume increased whole body total porphyrin formation (in the liver as well as in other organ systems) in patients with porphyrinuria because total porphyrin production can only be assessed by summation of urinary and fecal excretion rates. Quantification of overall fecal porphyrin excretion is cumbersome in a routine setting and is difficult to standardise. In a small series of patients (n = 8) with chronic liver diseases and porphyrinuria we did find a distinctly reduced fecal porphyrin excretion (median coproporphyrin 3 µg/g dried stool, interquartile range 2–7 µg/g, compared to controls 3–24 µg/g; median percentage of isomer I of total coproporphyrins 66 %, interquartile range 59 %–71 %, compared to controls 66 %–74 %). Mechanisms that might lead to increased porphyrin synthesis in liver diseases are (i) increased heme turnover to meet higher cytochrome requirements or by non-specific induction of aminolevulinic acid synthase or porphobilinogen deaminase (31, 32), (ii) ineffective heme synthesis, possibly by decreased ferrochelatase activity or uroporphyrinogen decarboxylase deficiency (33, 34) leading to precursor accumulation (heme precursor underutilisation), (iii) increased irreversible oxidation of porphyrinogens by oxidative stress, (iv) a decreased activity of heme oxigenase. Porphyrins, with the exception of protoporphyrin, are notably byproducts that have escaped from the biosynthetic pathway by irreversible oxidation of the corresponding porphyrinogens; oxidised porphyrins are thought to interfere with several enzymes in the metabolic pathway of heme (35). Increased concentrations of porphyrins in the liver tissue of HCV-infected patients without PCT have been shown (34). It has to be emphasised, that secondary porphyrinuria is not specific for liver disease but may also be found in neoplastic, blood and infectious diseases (6, 36, 37).

In our series, a high proportion of patients with marked porphyrinuria had elevated serum ferritin concentrations and transferrin saturation as a sign of liver iron overload. This observation suggests an association of iron status and secondary porphyrinopathies in HCV infection and merits further investigation. Iron seems to play an important pathophysiological role in HCV infection as, for example, effects of iron depletion on disease activity suggest (38, 39). Increased porphyrin excretion has been described in patients with hemochromatosis (40). Among PCT patients, a large proportion of 44 % was found to have mutations of the hemochromatosis gene, HFE (41). Iron has been found to be an important cofactor in experimental chemically induced porphyrias (21).

Whereas preclinical PCT is probably rare in HCV infection, as shown in this cross-sectional study, coproporphyrinuria is a frequent but not obligate finding in chronic HCV infection. This variable may be understood as a screening test of altered heme metabolism and porphyrin excretion. With respect to possible links

between altered heme metabolism and disease progression to cirrhosis and HCC, further investigations on the underlying pathophysiological mechanisms, including total body porphyrin synthesis rate, and the prognostic significance seem reasonable. In a follow-up investigation of our series we intend to study the course of porphyrinuria in individual patients. To clarify the prognostic significance of urinary porphyrin excretion with respect to disease progression and the occurrence of HCC, a larger prospective long-term study would be required.

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