Hepatitis C infected hemodialysis and renal transplant patients with elevated α-glutathione S-transferase have increased risk for liver damage

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Abstract: Patients on hemodialysis (HD) and renal transplant recipients (RT) have a high prevalence of HCV infection. The *aim* of our study was to determine the prevalence of HCV-RNA in the anti-HCV positive patients and to compare the biochemical parameters of PCR(+) and PCR(–) subgroups. *Methods:* The 525 sera were screened for anti-HCV. HCV-RNA was detected by polymerase chain reaction (PCR) and liver enzymes [SGOT, SGPT, GGT, α-glutathione S-transferase (GST)] were measured. *Results:* Active viraemia was found only in 187 of 289 (65%) seropositive HD patients in contrast to 53 of 53 (100%) of seropositive RT patients. Significantly increased ($p < 0.05$) GST values (9.9 μg/l) were found in the PCR(+) subgroups compared to GST levels (2.7 μg/l) of the PCR(–) subgroups. Elevated GST concentration was found in 80% (208/251) of PCR(+) patients. The measured enzymes were not elevated in HCV infected patients. Six percent of HD and 11% of RT patients were screened before seroconversion. Diagnostic sensitivity (80%) and specificity (79%) of GST were calculated as good for early liver damage caused by HCV. In contrast, the sensitivity of the measurement of other liver enzymes were very weak (SGOT: 8%; SGPT: 10%; GGT: 42%). *Conclusion:* The significantly higher viraemia of the RT subgroup could be related to the immunosuppressive therapy. Increased GST level may be a useful indicator of tissue damage during HCV infection. If HCV infection is suspected, PCR and GST measurement should be performed, even if anti-HCV result is negative.

Keywords: HCV infection, anti-HCV, HCV-PCR, α-glutathione S-transferase, renal transplant, patients on hemodialysis, hepatocellular damage, HCV carriers

Introduction

Since the introduction of preventive measures to reduce the risk of transmission of hepatitis B virus, most cases of dialysis-associated hepatitis are related to hepatitis C (HCV) [1].

Therefore hepatitis C virus infection is a major complication among patients undergoing hemodialysis (HD) therapy [2, 3]. There is a high prevalence (10%–50%) and incidence of new cases (up to 4.6 new cases/100 patients/year in dialysis units) [1, 4, 5, 6, 7, 8]. The renal transplant (RT) population may also have a high prevalence of HCV infection [9]. This is due to various causes, including blood transfusion [10] and chronic hemodialysis [6]. Some of the RT patients received multiple blood transfusions. It has also been documented that HCV can be transmitted by donated organs, although the magnitude of risk remains controversial [11, 12]. The principal screening method for HCV infection is based on the detection of antiviral antibodies (a-HCV) by ELISA/EIA assays [1, 13, 14]. Confirmation of these screening results can be done by more specific, but less sensitive antibody assays, namely RIBA2 or RIBA3 [15]. The estimated risk of HCV in the renal population varies from 6% to 66% if measured by ELISA [16, 17, 18]. Unfortunately, antibody tests may give an inaccurate estimate of the actual HCV infection rate in the renal population due to a low a-HCV antibody titer caused by immunosuppression [17, 19] and due to the compromised immune system of HD patients [20]. It has also been reported that an increased number of HCV-infected hemodialysis patients have severe histologic liver disease, which may not be suspected on clinical or biochemical grounds [1, 3]. The presence of viral RNA can be assessed by polymerase chain reaction (PCR). Previously, this method has not been employed as a routine procedure due to its high

cost and technical complexity [21, 22], but nowadays PCR technique is the most widely used technique in clinical laboratories for routine diagnostic measurements. Immunoserological testing can measure current and/or prior exposure to HCV, but cannot discriminate between the two, because no immunological assay for direct detection of HCV antigen is available. Furthermore, in cases of acute HCV infection, individuals may fail to produce antibody to HCV [23, 24], making diagnosis of the current HCV infection impossible by using only immunoserological techniques. A better alternative is to detect HCV-RNA by PCR, because it provides evidence for current infection. By using PCR, it is possible to detect HCV viraemia prior to immunological seroconversion [25, 26, 27].

The physician should also know whether the patient is merely an HCV-carrier without liver damage, or whether a small degree of hepatocellular loss has already occurred without any pathological change in the liver function test. Chronic hepatitis C is characterized by a silent evolution, a wide fluctuation of aminotransferase activities and potential presence of significant histological lesions in patients with normal concentrations of aminotransferases [24, 28, 29]. Only a histopathological examination can determine the degree of hepatocellular damage. Only liver biopsy provides definitive diagnosis, but false results may occur in rare cases. This is an invasive method with a risk of complications (severely impaired coagulation, thrombocytopenia) [30]. Usually it is only the biochemical evidence of progressive deterioration of liver function in conjunction with clinical signs that would lead us to suspect liver damage [31]. The most widely used laboratory indicator of early liver damage caused by HCV is the conventional liver function test, which includes the measurement of aminotransferases, alkaline phosphatase, bilirubin, prothrombin time, although none of these parameters indicate an early liver injury related to HCV [32]. For this reason HCV infection in HD and RT patients is common and underdiagnosed by using only the conventional biochemical parameters [9]. Therefore, a new sensitive marker is needed for early diagnosis of slight liver tissue damage in both HD and RT populations.

The purpose of the present study was to determine the prevalence of HCV-RNA in the seropositive HD and RT patients, to compare biochemical parameters of the PCR(–) and PCR(+) patients, and to predict the severity of liver disease of the HCV-infected subgroup by performing GST measurements.

Material, Method and Patients

A total of 525 samples were simultaneously evaluated for HCV serology, active viraemia, conventional liver biochemistry and for the relatively new GST measurement. Patients were grouped based on both serology and PCR results (Table I).

Blood samples were collected in sterile, native tubes (Becton Dickinson). After separation (centrifuged: $2000 \times g$ for 15 min), sera were assayed for SGOT, SGPT, α-glutamil-transferase (GGT) and serum bilirubin (Sebi) by standardized biochemical procedures. GST concentrations were measured (in µg/l) with a quantitative enzyme immunoassay designed for serum analysis (Biotrin HEPHKIT-alpha Biotrin International, Dublin, Ireland) in microtitre wells coated with anti-alpha GST IgG, on the ELISA reader (Anthos Reader 2001, Anthos Labtec Instrument) at 450 nm (reference: 620 nm), and 25 C by monitoring the increase of absorbance resulting from the release of chromogenic substrates. The GST assay has been improved so that it can now be completed within 4 hours [32].

The sera were screened for anti-HCV antibodies by ELISA method using commercially available assay (ETI-AB-HCVK-4, DiaSorin) [33]. HBsAg screening was carried out by HBsAg ELISA test (Hepanostika HBsAg Uni-Form II – one-step "sandwich" method) [34]. The HBsAg-positive patients were excluded from the study. HCV-RNA was detected by the PCR method (RT-PCR Roche version 2.0) as others have described previously [35, 36]. This assay is based on the bifunctional thermostable DNA polymerase, isolated from *Thermus ther-*

Table I Summary of the investigated patient groups, including the numbers and results of HCV serology and HCV-PCR

Groups	Anti-HCV	HCV-PCR	N
HD	Negative	Negative	88
HD	**Positive**	**Negative**	102
HD	Negative	**Positive**	6
HD	Positive	Positive	187
RT	Negative	Negative	38
RT	Negative	**Positive**	5
RT	Positive	Positive	53
CONT	Negative	Negative	46

There was a discrepancy between the presence of HCV antibodies and HCV viruses in 22% of the patients.

Abbreviations: HD = patient on hemodialysis; RT = renal transplant recipient; CONT = healthy control; N = the number of patients; anti-HCV = HCV antibody; HCV-PCR = active hepatitis virus detection by polymerase chain reaction method

mophilus. This enzyme possesses both RNA-dependent and DNA-dependent DNA polymerase activities, thereby obviating the need for separate reactions and enzymes for the generation of target RNA (complementary DNA) and for the amplification process [37, 38, 45, 46]. The sensitivity of our PCR assay is 60 IU/ml. Furthermore, with the appropriate selection of primers and probe, this method is able to detect all HCV genotypes [38, 56].

Statistical analysis. Data from all our measurements were entered into a database and analyzed with STATISTICA for Windows [39]. Data collected on the initial visits were coded and entered into a database using Microsoft Excel (version 7.0; Microsoft Co.; Redmond, Wa, USA). Analyses were conducted with the SAS statistical software package (version 6.12; SAS Institute Inc.; Cary, North Carolina 27513, USA). In the cases in which a question was left unanswered or data were not available, we excluded these elements from the analysis. Continuous variables were expressed as mean (SD). The homogeneity of variances was tested by Levene's test and the normality was tested by Shapiro-Wilk's test. Comparisons between groups (HD, RT, CONT and PCR-positive, PCR-negative patients) were performed by using analysis of variance (ANOVA) techniques. Dunnett's test was conducted as a post hoc test. For all statistical tests, $p \pm 0.05$ was used as the level of significance.

Results

The presence of hepatitis virus was investigated in the seropositive and seronegative patients (Table I). Active viraemia was found only in 187 of 289 (65%) seropositive HD patients in contrast with 53 of 53 (100%) of seropositive RT patients. Of the seropositive HD patients, 35% were not infected by hepatitis C viruses, even if HCV antibodies could be detected. Prior to seroconversion we could predict acute HCV infection in 6% (6/94) of the seronegative HD patients and in 11% (5/43) of the seronegative RT patients using the PCR technique.

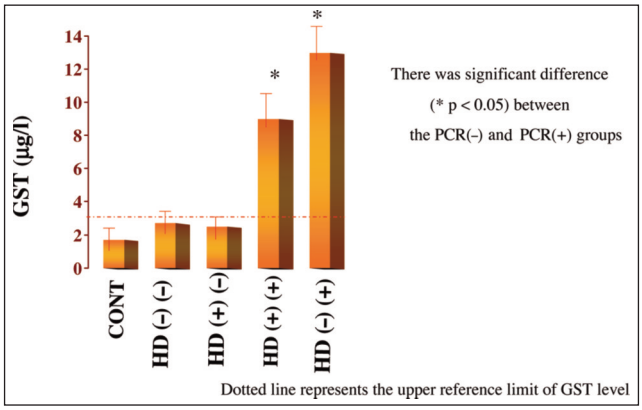

Fig. 1. Significantly increased ($p < 0.05$) GST value (9.8 ± 8.4 µg/l) were found in the PCR(+) subgroups ($n = 283$), as opposed to the average GST level (2.3 ± 1.7 µg/l) of the PCR(–) subgroups ($n = 242$)

Significantly increased ($p < 0.05$) GST values (9.8 ± 8.4 µg/l) were found in the PCR(+) subgroups ($n = 283$) compared to the average GST level (2.3 ± 1.7 µg/l) of the PCR(–) subgroups ($n = 242$) (Fig. 1). GGT enzyme levels were elevated compared to healthy controls in both PCR(+) and PCR(–) groups (Fig. 1). The SGOT and SGPT enzyme levels of HCV-infected patients were higher but still within the normal range, than those of the non-infected groups. We did not find significant difference in SGOT and SGPT enzyme levels between the PCR(+) and PCR(–) groups.

The upper reference limit of GST was determined. Based on the measurements of 46 healthy control patients, the average GST value (1.7 ± 1.0 µg/l) was deter-

Table II The percentage distribution of the sensitivities for early liver damage related to HCV at the different enzymes using the cut-off values. The calculated data represent in how many cases (as a percentage) we could measure the enzyme levels above the cut-off values in the different patient groups. These summarized data show that GST has strong sensitivity and specificity as well for early liver damage in contrast to the sensitivities of the other parameters (SGOT, SGPT, GGT and SEBI), which proved to be very weak

Groups Cut-off value:	GST 3.7 µg/l	SGOT 69 U/l	SGPT 60 U/l	GGT 75 U/l	SEBI 30 µmol/l
HD (–) (–)	25	2	6	30	0
HD (+) (–)	20	0	0	8	0
HD (–) (+)	**83**	0	0	60	0
HD (+) (+)	**79**	4	7	38	0.7
RT (–) (–)	12	5	0	21	0
RT (–) (+)	**100**	40	40	40	0
RT (+) (+)	**83**	13	13	54	2
CONT (–) (–)	0	0	0	0	0

The top row of the table indicates the respective cut-off value for each enzyme and for the serum bilirubin.

Abbreviations: HD (–) (–) = anti-HCV negative and PCR negative patients on hemodialysis; HD (+) (–) = anti-HCV positive and PCR negative patients on hemodialysis; HD (–) (+) = anti-HCV negative and PCR positive patients on hemodialysis; HD (+) (+) = anti-HCV positive and PCR positive patients on hemodialysis; RT (–) (–) = anti-HCV negative and PCR negative renal transplant patients; RT(–) (+) = anti-HCV negative and PCR positive renal transplant patients; RT(+) (+) = anti-HCV positive and PCR positive renal transplant patients

mined (Table II). Using two standard deviations, we calculated the cut-off value of GST as $1.7 + 2 \times 1.0 = 3.7$ µg/l (the average $+2\times$ standard deviations). With certain parameters (SGOT, SGPT, SGGT and SEBI), the generally accepted practice is to add fifty percent to the upper reference limit (see Table II).

Elevated GST concentration (higher than the cut-off value) was found in 80% (208/251) of the PCR(+) subgroups. The other enzymes were not elevated (SGOT: 19%; SGPT: 17%; GGT: 59%) in the PCR(+) patients. SEBI values above the upper reference limit were measured only in 3 patients. The sensitivity and specificity for the hepatocellular damage related to HCV were calculated. The sensitivity (80%) and specificity (79%) for GST can be considered a good result compared to the other investigated enzymes, where though the specificites were found to be very good (SGOT: 98%; SGPT: 100%; GGT: 91%), the sensitivities were very weak (SGOT: 8%; SGPT: 10%; GGT: 42%). The sensitivity of SEBI for the hepatocellular-damage-related HCV was also quite weak (1.2%). Data in Table II show that traditional liver function tests are not sensitive enough to predict early hepatocellular damage caused by hepatitis viruses at all.

Conclusion

The clinical utility of GST was evaluated in hepatitis C viraemic patients. Patients on hemodialysis (HD) and renal transplant recipients (RT) are at high risk for carrying HCV [40, 41, 42, 49]. According to our results standard liver test profiles (SGOT, SGPP, GGT, SEBI) and antibody screening tests gave incorrect results with respect to the actual HCV infection [20] and the degree of early hepatocellular damage in HCV viraemic HD and RT patients. The antibody detection method is limited in capacity, because there is a mean window period of 4 to 22 weeks between infection and seroconversion [43]. Furthermore, data on antibody status do not provide means to differentiate between current, active infection, chronic infection and prior, resolved infection [26]. The physician should never use serology results as a basis for diagnosing HCV infection, even if anti-HCV measurement is repeated with the same result. It should be noted that seronegativity is not a positive indication of the lack of HCV-RNA, just as seropositivity does not confirm the presence of HCV-RNA.

Many authors have questioned the measurement of conventional transaminases for monitoring liver function, as values may be normal in patients with chronic liver disease [44, 45]. It is also necessary to consider that normal activities of aminotransferases are lower in HD patients than in healthy population [6, 46]. Consequently some authors have found neither enough sensitivity nor enough specificity of these enzymes in the diagnosis of HCV [47]. Probably it is for the same reason that physicians have not found correlation between consistently elevated levels of SGOT and SGPT enzymes and the histological patterns of liver damage [6, 9]. This can be attributed to the different distribution of transaminases within the liver [48], which has the disadvantage that the periportal concentration of conventional transaminases is greater than those of found in the centrilobular region [5]. However, periportal hepatocytes are more susceptible to damage from hypoxia and toxin, whereas viruses cause hepatocellular damage in the centrilobular region in the very early stages [49].

This study aimed to evaluate very early liver damage caused by active hepatitis C infection by a non-invasive marker. Theoretically, there are numberous potential reasons why serum alpha GST measurements provided more sensitive indication of hepatocellular damage across the liver lobule [50]. GST spreads widely throughout the liver and is released equally by both centrilobular and periportal hepatocytes, as proven by immunohistochemical studies [51]. Hepatic alpha GST is a relatively small dimeric enzyme (MW~50,000) and is present in high concentration in the hepatocyte cytosol, constituting about 5% of the total cytosolic protein [32]. The cytologic, multifunctional, detoxifying liver enzyme alpha α-glutathione S-transferase (GST) may offer significant advantages over conventional transaminases for monitoring hepatocellular integrity [32]. A drawback with SGOT (glutamate-oxalacetate-transferase) and SGPT (glutamate-pyruvate-transferase) is that they are not distributed uniformly throughout the liver, because the periportal concentration is greater than the centrilobular [48]. In contrast, GST has been found to be equally distributed in both the centrilobular and periportal regions [51]. The centrilobular hepatocytes are susceptible to damage in a variety of clinical conditions including viral hepatitis [49, 52]. GST has been found to provide a more sensitive indication of hepatocellular injury than conventional transaminase activities [53, 54]. GST measurements also appear to correlate better with histological abnormalities [49], thus biopsy can be avoided if the GST level is normal at the time that the biopsy would have been performed [32]. Thus GST has been proposed as an alternative marker of slight hepatocellular damage and a more sensitive index than aminotransferases [32, 53, 55]. Since HCV appears not to cause aggressive liver disease in the early hemodialysis and post-transplant period [9], it is very useful to monitor and predict the HCV-related damage in the initial stage in order to avoid liver biopsy [56].

Thus the investigated GST is readily and rapidly released in a measureable quantity into the circulation following even a slight degree of hepatocellular damage.

We found that PCR-positive patients had significantly higher GST values than PCR-negative patients regardless of the presence or lack of HCV antibodies. It might be for this reason that GST is a new sensitive and specific alternative marker for early mild liver damage related to hepatitis C. This is a very useful tool in determining the severity of HCV infection, because HCV appears not to cause

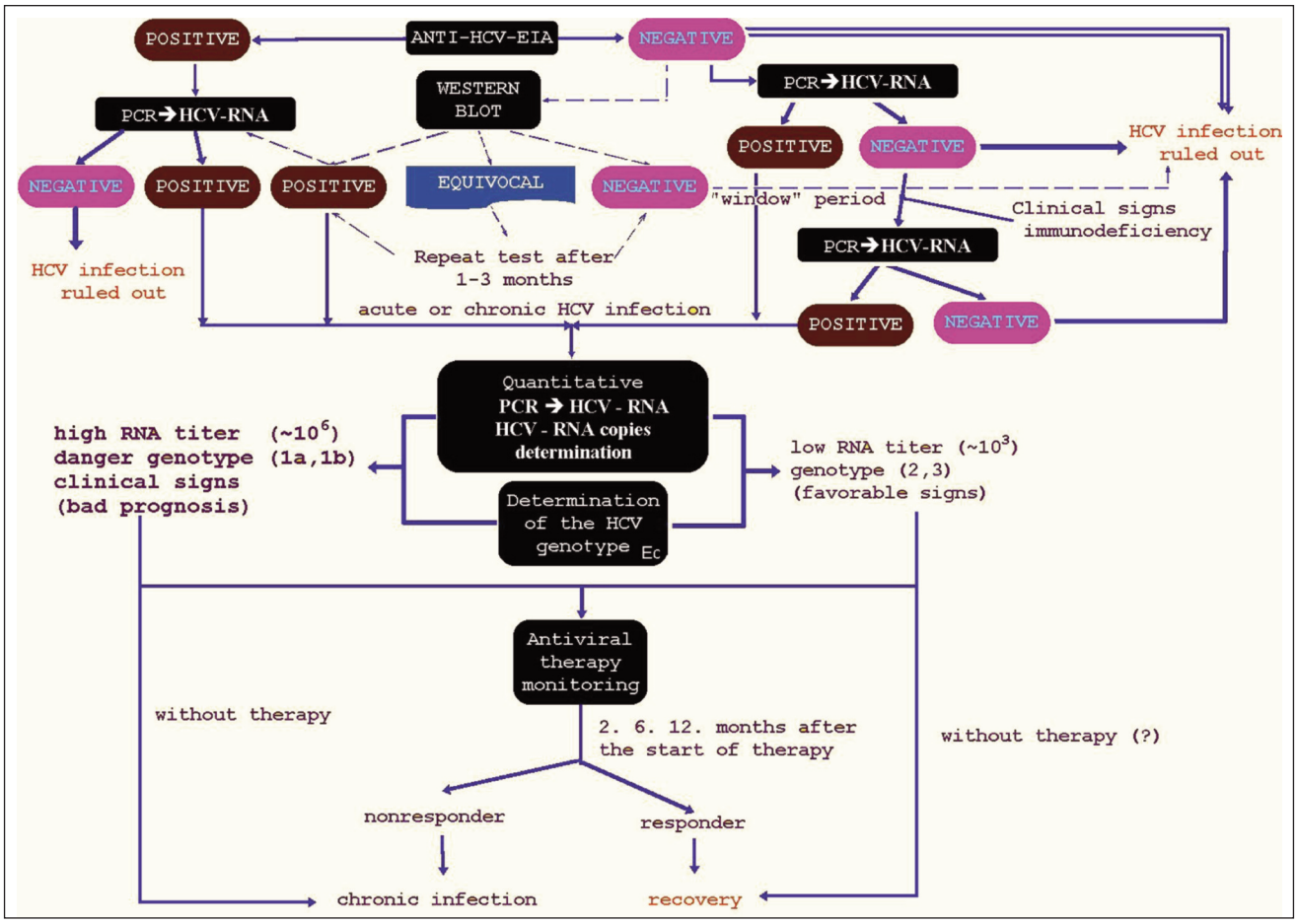

Fig. 2. Diagnostic evaluation and treatment of HCV infection. The diagram helps to select the adequate measurement(s) in logical order, when HCV infection is suspected. We emphasize the benefit of the PCR method. Monitoring of the HCV-RNA titer of the infected patients promotes to establish the fluctuation of the HCV-RNA copies and the efficacy of antiviral therapy. The test (Western Blot) indicated with dotted lines should not be assessed, if the PCR unambiguous

aggressive liver disease for the HD and RT patients in early hemodialysis treatment and in the early post-transplant period [9]. PCR monitoring of these patient groups is a conclusive indication of active viraemia, and GST determinations can predict the degree of liver damage, even if it is minimal. When GST value of a PCR(+) patient is normal, the liver seems to be intact. There is no reason to perform liver biopsy when the patient has no other clinical symptoms. Only PCR measurement gives reliable diagnosis for HCV infection, though it is more expensive than serology screening (Fig. 2). Seropositive but PCR-negative patients are not HCV-infected at the time of diagnosis, but have already recovered from the HCV infection. Thus they have antibodies to HCV without active viraemia (Fig. 2).

GST values of PCR(−) patients showed normal concentration (within reference range), indicating an intact liver [57]. When PCR method could not reveal HCV-RNAs, there were no viruses that could cause liver damage. Our data showed that GST was the most sensitive method, while the other investigated enzymes showed poor sensitivity (Table 2). According to our results, SGOT, SGPOT and GGT are specific for liver damage related to HCV, but much less sensitive than GST. For this reason the transaminases are not suitable for predicting early hepatocellular damage related to HCV. We suspect that 20% of PCR(+) patients are HCV carriers, without detectable hepatocellular damage (GST concentrations were similar to the healthy controls). These patients need longer follow-up, however, at the time of evaluation their liver is intact independently of the active viraemia [58].

Although HD patients do not receive immunosuppressive therapy, their immune systems are compromised; 35% of seropositive patients could eliminate HCV from their body. They had had prior HCV infections, but HCV was eliminated from their bodies by the time we performed PCR assessment.

Using PCR technique for detecting active viraemia is very important, not only for the accurate diagnosis of HCV infection [22] of the individual patient, but also for the epidemiological safety of the dialysis units [5, 57]. The seropositive but PCR-negative patients must not be hemodialyzed together (on the same instrument) with a PCR-positive patient, because this patient is indeed infected.

Significantly higher viraemia with the RT group could be related to the immunosuppressive therapy. Renal transplants recipients with weakened immune system are not able to defeat and eliminate hepatitis C infection. PCR-positive RT patients might be only carriers of the virus without any clinical symptoms and biochemical alterations; moreover most of them have no serious liver damage related to HCV in the early post-transplant period. The patients should be educated about their HCV infection, so they can avoid infecting others.

By using HCV-PCR measurements we can separate patients who are indeed infected from those whose serology test indicates a false positive result (anti-HCV-positive but PCR-negative), and we can predict active viraemia before seroconversion [59]. In many cases the seronegative results have to be confirmed by HCV-PCR (Fig. 2). The PCR verification is especially important in screening blood donors.

GST measurement is very useful in both HD and RT patients for predicting early hepatocellular lesion related to HCV. When a HCV-infected patient is undergoing antiviral therapy, GST measurements differentiate between responders and non-responders. GST concentration remains distinctly elevated in non-responders, while the SGOT and SGPT approaches normal levels on some occasions despite persistent viral RNA [5].

Long-term follow-up measurements of HCV-PCR together with alpha GST evaluations may have an accurate prognostic value for HCV-related early hepatocellular damage, and may reveal an increased incidence of late cirrhosis.

Acknowledgements

We are grateful to Mrs Marta Lakatos for a generous supply of the GST and PCR measurements. We are also thankful to Dr Gyorgy Tremko for completing Fig. 2 of this paper.

Abbreviations

a-HCV – anti-HCV antibody;
CONT – healthy control;
GST – α-glutathione S-transferase;
GGT – α-glutamil-transferase;
HCV – Hepatitis C virus;
HD – patient on hemodialysis;
PCR – Polymerase Chain Reaction;
PCR(–) – PCR negative;
PCR(+) – PCR positive;
RT – renal transplant recipients;
SD – standard deviation;
Sebi – serum bilirubin;
SGOT – glutamate-oxalacetate-transferase;
SGPT – glutamate-pyruvate-transferase

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