

HOW TO DISTINGUISH NORMAL LIVER FROM CHRONIC HEPATITIS IN ANTI-HCV POSITIVE INDIVIDUALS WITH NORMAL ALANINE AMINOTRANSFERASE LEVELS

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Abstract The aim of this study was to characterize the biochemical, virological and histological profile of patients with HCV infection and persistently normal alanine aminotransferase levels (PNALT) in order to discriminate between normal liver and chronic hepatitis. Twenty eight anti-HCV positive patients with PNALT were studied. Twelve (42.9%) patients showed normal liver while 16 (57.1%) had chronic hepatitis. In patients with normal liver, the mean of ALT level differed from patients with chronic hepatitis (16.3 IU/L versus 25.6 IU/L, p 0.000089). By considering different ALT values as upper normal limit, sensitivity, specificity, positive and negative predictive values were calculated and the value of 18 IU/l showed 88, 90, 94 and 82%, respectively, in predicting chronic hepatitis. In conclusion, patients with normal liver had lower ALT levels than those with chronic hepatitis and by establishing 18 IU/L as a new upper limit of normal ALT value, it was possible to differentiate normal liver from chronic hepatitis in HCV patients with PNALT.

Key words: hepatitis C, alanine aminotransferase, chronic hepatitis

Resumen *Cómo distinguir el hígado normal de la hepatitis crónica en individuos anti-HCV positivos con valores normales de alanino aminotransferasa.* El objetivo de esta investigación fue caracterizar el perfil bioquímico, virológico e histológico de pacientes portadores de hepatitis por virus C (HCV) y valores persistentemente normales de alanino aminotransferasa (ALT) con el fin de evaluar un método no invasivo que permita discriminar en este grupo de pacientes a aquellos que presentan hepatitis crónica de los que presentan hígado normal. Se estudiaron 28 pacientes portadores de anticuerpos anti-hepatitis C (anti-HCV) y transaminasas persistentemente normales a quienes se les practicó una biopsia hepática. Doce pacientes (42.9%) presentaron hígado normal y 16 pacientes (57.1%) hepatitis crónica. El promedio de los niveles de ALT en pacientes con hígado normal fue significativamente más bajo que el de los pacientes portadores de hepatitis crónica (16.3 IU/L versus 25.6 IU/L, p 0.000089). Calculando la sensibilidad, especificidad, valor predictivo positivo y negativo de distintos valores de ALT, el valor de 18 UI/L mostró un 88% de sensibilidad y 90% de especificidad. Por lo tanto, a través de una modificación en el límite superior del valor normal de ALT a 18 UI/L, es posible diferenciar dentro de la población de pacientes con anticuerpos positivos para anti-HCV y ALT persistentemente normal, a aquellos que presentan hígado normal de aquellos que portan hepatitis crónica.

Palabras clave: hepatitis C, transaminasas, hepatitis crónicas, alanino aminotransferasa

Hepatitis C virus infection is a major health problem worldwide¹. The persistent nature of HCV infection is unique among positive-strand RNA viruses and is associated with chronic hepatitis, which eventually increases the risk of hepatocellular carcinoma².

Most of the individuals chronically infected by the HCV present alterations in the alanine aminotransferase values (ALT) that can fluctuate thoroughly; however, a third of the chronic carriers show persistently normal ALT values.

In the latter are included those who have cleared the infection as well as those who evidence histological lesions of chronic hepatitis³.

Alanine aminotransferase is routinely used for assessing liver function in subjects infected by HCV. However, on the basis of conventional reference ranges universally used (0 to 40 IU/l), patients with persistently normal ALT levels represent a challenge. Biochemical parameters do not allow to predict presence or absence of chronic liver damage and by means of ALT determinations it is not possible to discriminate past from active infection⁴. Furthermore, the management of patients with persistently normal ALT remains questionable because the natural history of this group of patients is unknown.

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The aim of this study was to characterize the biochemical, virological and histological profile of patients with HCV infection and persistently normal ALT levels in order to optimize the biochemical parameters in predicting liver histology and to assess a noninvasive approach to discriminate between normal liver and chronic hepatitis.

Patients and Methods

Between January 1998 and January 2000, all anti-HCV positive patients attending the Liver Unit at our Hospital were prospectively enrolled in the study if they showed persistently normal ALT levels (defined as repeatedly normal serum ALT and AST levels on at least 3 consecutive occasions during the last 12 months).

A total of 30 consecutive patients met the inclusion criteria and agreed to undergo blood examinations at scheduled intervals. The subjects were 12 men and 18 women, aged 21 to 61 years, mean 42.2 years. None of the patients received previous treatment with interferon or immunosuppressive agents. No patient had a previous history of autoimmune disease, alcohol intake, current intravenous drug use or other chronic liver disease. All patients were negative for hepatitis B surface antigen and anti-human immunodeficiency virus.

Epidemiological data were obtained from all patients and included sex, age, duration of infection and potential risk factors for HCV transmission (blood transfusion, intravenous drug addiction, tattooing, acupuncture, needle stick, professional exposure, sexual or familiar exposure). Clinical characteristics are shown in Table 1.

Written informed consent was obtained from all patients and the study protocol was approved by the local ethical committee in accordance with the 1975 Declaration of Helsinki.

Routine evaluation of the patients included medical history, physical examination, routine liver tests and measurements of ALT. Aminotransferase levels were evaluated by automated analysis in the same laboratory and monitored monthly. The cut-off value for ALT level was 40 IU/L.

Markers of hepatitis B infection were tested by enzyme-linked immunoassay test (ELISA, Abbott Laboratories, Chicago, IL) and antibody to hepatitis C was assessed by an enzyme-linked immunoassay test of second generation (ELISA, Abbott Labo-

ratories, Chicago, IL) in accordance with the manufacturer's instructions. Anti HCV reactive samples were confirmed using a second-generation anti-HCV recombinant immunoblot assay (RIBA II, Chiron Corporation, Emeryville, California).

Serum HCV RNA detection

To determine HCV RNA, a serum sample was taken from each patient at baseline and every month during the follow-up period. Each sample was divided into three 0.5 aliquots and then frozen at -70 °C within 2 hours under conditions known to best preserve RNA.

Total RNA was extracted by the acid guanidinium-phenol-chloroform method as previously described⁶. Briefly 150 µl serum as mixed with 500 µl of denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 0.1M 2-mercaptoethanol, 50 µl of 2 M NaAc (pH 4.0), 500 µl of phenol and 100 µl of chloroform. After centrifugation aqueous phase was recovered and precipitated over night at -20 °C with 650 µl of isopropanol and 20 µg dextran T500. The resulting pellet was washed with 70% ethanol and resuspended in 9 µg of water. RNA obtained was denatured at 78 °C for 5 min and primed with 0.4 µg of random hexamers. Reverse transcription conditions were 50 mM TrisHCl (pH 8.3), 25 mM KCl, 3 mM MgCl₂, 0.1 mM DTT, 1 mM dNTPs, 18 U of ribonuclease inhibitor (Promega) and 100 U of M-MLV reverse transcriptase (Gibco), reaction w°C for 5 min and chilling on ice, the cDNA was amplified. The 50 µl PCR reaction contained: 20 mM TrisHCl, 50 mM KCl, 50 pmoles of each primer for the 5UT region of HCV genome, 5UT1 (5' CCTGTGAGGAACTACTGTCTTCACGC 3') and 5UT2 (5' AGGTCTCGTAGA CCGTGCACC 3') and 1.25 U of Taq. The PCR reaction consisted of 40 cycles each with denaturing at 94 °C for 30 sec, annealing at 55 °C 30 sec, and polymerization at 72 °C for 45 sec. Nested PCR was done with 2 µl of PCR product as template, using internal primers 5UT3 (5' TCT AGC CAT GGC GTT AGT GCG AGT GT 3') and 5UT4 (5' CAC TCG CAA GCA CCC TAT CAG GCA GT 3'), in the same conditions of the first round. PCR products were analyzed by ultraviolet fluorescence after ethidium bromide staining. The stated cut off is 200 genome copies/ml

RNA Quantification

Non-competitive quantitative PCR-based Amplicor HCV Monitor Assay (Roche Molecular Systems) was used for HCV-RNA quantification according to the manufacturer's instructions. The stated cut off is 600 genome copies/ml (3 log₁₀ genome copies/ml). Viral load was measured in two samples, at baseline and 10.2 ± 3.3 months after, during the follow-up.

HCV Genotyping

HVC genotypes were assessed by commercial line-probe assay (INNO-LiPa, Innogenetics S.A., Gent, Belgium) that allows differentiation of the 6 most common genotypes. The LiPa test is a reverse hybridization assay based on highly conserved variations in the 5'UTR.

Characterization of HCV serotypes was performed by a qualitative immunoenzymatic assay. This assay utilizes synthetic peptides derived from serotype-specific sequences of the non-structural NS4.

Liver histology

Ultrasound assisted liver biopsy was performed using modified Menghini needle of 1.4 mm diameter (Hepafix, Braun, Germany). Liver specimens, previously fixed in formalin, were stained with hematoxylin and eosin, Perls blue and Prussian

TABLE 1.— Demographic and clinical features of the studied patients

	Patients with normal ALT (n: 28)
Age mean (years) ± SD	42.2 ± 12.1
Gender (M/F)	12/16
Duration of infection mean (years) ± SD	13.3 ± 8
Detectable HCV-RNA	
n (%)	10 (35.7)
Chronic hepatitis	16
Cirrhosis	0
Grading score	4.1 ± 3.0
Staging score	0.3 ± 0.4

SD: standard deviation, M: male, F: female, ALT: alanine aminotransferase, IU/L: international units/liter.

blue and were examined by two pathologist who were blinded to the ALT levels and clinical data of the patients.

Histological findings were assessed as: normal liver (NL), chronic hepatitis (CH) and cirrhosis (Cirr) using a numerical scoring system according to Ishak et al⁶, and grading, staging and total score were reported.

Follow-up

Genotype, RT-PCR HCV-RNA and liver biopsy were determined at the time of entry in the study. Clinical and biochemical follow-up was performed at 1-month interval.

Patients who had an average in ALT value higher than 40 IU/L during the follow-up were discharged from the study. Mean of follow-up period was 19.5 months.

Statistical analysis

Results were expressed as mean \pm standard deviation. ANVA and Scheffé test (post hoc comparisons of means) were used for comparisons. For categorical values Chi square, Yates corrected or Fisher's Exact test were used. A p value < 0.05 was considered statistically significant. Sensibility, specificity

positive and negative predictive values were calculated for different values of ALT. Receiver operating characteristic⁷ (ROC) curves were performed, and under the curve areas were calculated for ALT values and PCR-HCV. The area under the ROC curve and its standard error was calculated using the nonparametric method⁷.

Results

Demographic and clinical features

Twenty eight patients completed the study showing an average of ALT value lower than 40 IU/L during the follow-up period. A known risk factor for HCV transmission was present in 19 of 28 (68 %). None of the patients reported symptoms.

Liver histology

Twelve (42.9%) patients showed normal liver while 16 (57.1%) showed chronic hepatitis. No patients showed

TABLE 2.— Biochemical, virological and histological features in patients with persistently normal ALT according to the average of ALT level

Patient	Gender	Age (years)	ALT IU/L (mean)	HCV RNA	LB	G	S	Duration of	Source of infection ¹	Genotype Infection
1	F	41	12.81	U	NL	-	-	10	parenteral	ND
2	M	57	13.73	U	NL	-	-	UK	UK	2
3	F	27	13.75	U	NL	-	-	8	parenteral	3
4	F	42	15.27	U	NL	-	-	4	parenteral	1
5	M	38	16.12	U	CH	1	0	15	parenteral	3
6	M	54	16.58	U	NL	-	-	UK	UK	NT
7	F	39	16.85	U	NL	-	-	UK	UK	1
8	M	41	17.56	U	NL	-	-	10	parenteral	3
9	F	38	18.10	U	NL	-	-	13	parenteral	NT
10	F	57	18.33	U	CH	2	0	15	parenteral	ND
11	F	46	18.41	U	NL	-	-	UK	UK	2
12	F	33	18.90	U	CH	2	0	7	parenteral	3
13	M	30	19.90	U	CH	2	0	15	sexual	1
14	F	68	20.54	D	CH	2	1	UK	UK	2
15	F	27	21.60	U	NL	-	-	9	parenteral	1
16	F	61	23.79	D	CH	11	1	20	parenteral	2
17	F	56	24.50	D	CH	8	1	UK	UK	2
18	M	33	25.84	D	CH	2	0	UK	UK	2
19	M	39	26.40	D	CH	7	0	12	parenteral	4
20	M	53	26.90	U	NL	-	-	UK	UK	1
21	F	23	26.91	U	CH	2	0	UK	UK	1
22	F	21	27.73	D	CH	7	0	4	parenteral	1
23	F	31	30	D	CH	1	0	16	parenteral	3
24	F	48	30.06	D	CH	6	1	10	parenteral	2
25	M	37	30.29	U	CH	2	0	18	parenteral	1
26	M	44	32.62	U	NL	-	-	13	parenteral	NT
27	M	43	35.37	D	CH	5	0	40	parenteral	1
28	M	55	34.83	D	CH	5	0	40	UK	2

M: male, F: female. ¹ Time in years from probably date of infection. LB: liver biopsy. NL: normal liver. CH: chronic hepatitis.

G: grading, S: staging. UK: unknown. HCV-RNA: D: detectable; U: undetectable. NT: Non-type specific. ND: not done. IU/L: international units/liter.

TABLE 3.– Sensitivity, specificity, positive and negative predictive values in (%) percentage of different cutoff values of ALT

Cutoff value ALT (UI/L)	17	18	19	20	21	22	23	24
Sensitivity	94	88	82	75	69	69	69	69
Specificity	60	90	90	90	90	90	100	100
PPV	79	94	93	93	91	91	100	100
NPV	86	82	75	69	64	64	67	67

PPV: Positive predictive value; NPV: negative predictive value. IU/L: international units/liter.

cirrhosis. Mean grading and staging score was 4.06 ± 3 and 0.3 ± 0.4 respectively.

Biochemical features

The biochemical profile is shown in Table 2. There were $15.5 (\pm 6.7)$ ALT determinations per patient. Most of the patients maintained normal enzyme levels during follow-up, whereas four showed sporadic fluctuations at monthly monitoring that not exceeded 1.5 fold normal value.

Since patients number 20 and 26 showed ultrasound evidence of liver steatosis as well as high levels of plasma cholesterol, were not included in further analysis of predictive values.

In patients with normal liver, mean of ALT level was significantly lower than in patients with chronic hepatitis (16.3 ± 2.9 IU/L versus 25.6 ± 5.8 IU/L respectively, $p 0.00089$).

By considering different ALT values as upper normal limit, sensitivity, specificity, positive and negative predictive were calculated (Table 3). The value of 18 IU/l showed a sensitivity of 88% and a specificity of 90% in predicting chronic hepatitis.

Receiver operating characteristic (ROC) curves demonstrate the relationship between true positive ratio (sensitivity) and false positive ratio (1- specificity) as one modifies the definition of a positive test. While there are several reasons for examining ROC curves, in this study we have used ROC curves to discriminate patients with normal liver from chronic hepatitis comparing ALT level and the detection of HCV RNA by PCR. No significant difference was found between the areas under the two curves (ALT 0.93, standard error 0.05 and PCR 0.81, standard error 0.08, $p 0.095$).

Serum HCV-RNA profile and viral load

There were 6.2 ± 2.3 determinations of HCV-RNA per patient. No changes in RNA pattern was observed; patients who have detectable or undetectable HCV-RNA remained in the same condition during the follow-up period.

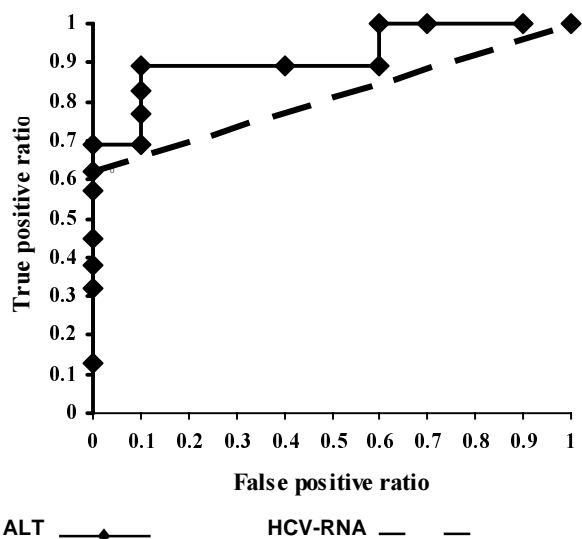


Fig. 1.– Receiver operating characteristic curve for ALT levels and HCV-RNA by PCR.

Serum HCV-RNA was not detectable in 18 patients (12 with normal liver and 6 with chronic hepatitis) and detectable in 10 patients (all with chronic hepatitis).

The diagnostic sensitivity and specificity for chronic hepatitis was 62.5% and 100%, positive predictive value 100% and negative predictive value 66.7%.

Mean of viral load was 3.9×10^5 gEq/ml, 6 patients showed a shift < 0.5 log between samples and 3 between 0.5-1 log.

No correlation was found between serum HCV RNA titers and grading and staging.

Discussion

It has been estimated that 20 to 33% of patients with chronic hepatitis C virus infection have persistently normal aminotransferase levels^{3, 4, 8}. Management of these patients is still a matter of controversy and several issues remain to be clarified. For instance, definition of the meaning of normal ALT level in patients with chronic

hepatitis C infection is required as well as an agreement about how long patients should be monitored to be classified in this category.

In the present study, we analyzed the biochemical, virological and histological characteristics of a cohort of patients with persistently normal ALT levels in order to improve the role of biochemical parameters in predicting liver histology.

The main findings of this study were: 1) ALT levels remained normal during the follow-up in the large majority of patients and showed a constant pattern, 2) patients with normal liver had lower ALT levels than those with chronic hepatitis (mean 16.3 IU/L vs. 25.6 IU/L respectively, $p: 0.000089$), 3) among patients with persistently normal ALT, 35.7% had detectable HCV viraemia; 4) 100% of patients with detectable HCV-RNA showed chronic hepatitis, 6) no changes in the RNA pattern were observed: patients who had detectable or undetectable HCV RNA remained in the same condition during the follow-up period.

Despite that the value of 40 IU/L is universally accepted as the cutoff for classifying patients having normal or elevated ALT, the normal value is arbitrarily defined by samples from healthy population and the cutoff is determined by calculation of standard deviation or predefined percentile⁹. Intrinsic factors related to the routine liver chemistry assays such as concentration of enzyme and substrates or temperature as well as extrinsic ones (control population gender and age, weight, race) may strongly influence the normal ALT limit making problematic the estimation of the normal range¹⁰. Additionally, cutoff level that define abnormality is rather arbitrary decreasing the specificity of the tests in apparently healthy patients¹¹.

We found that patients who showed chronic hepatitis had an average of ALT value significantly higher than patients with normal histology and it was possible to differentiate them using a biochemical parameter. This observations suggest that lowering the threshold for elevated ALT value to 18 IU/L may allow us to predict liver histology.

Normal liver was never observed in patients with detectable viremia and many patients showed significant liver damage despite persistently normal liver biochemistry. In our series, the specificity and positive predictive value of HCV-RNA testing in predicting chronic liver disease was 100%.

In HCV carriers with normal aminotransferase levels viremia did not predict the grade of HCV-related chronic liver disease and the amount of viral load was not different from the reported data in patients with elevated ALT levels¹²⁻¹⁴.

In conclusion, by establishing 18 IU/L as new upper limit of normal ALT value, it is possible to differentiate normal liver from chronic hepatitis in HCV patients with

persistently normal ALT levels with 88% of sensitivity and 90% of specificity.

Virological endpoint such as absence or presence of HCV viral RNA in serum have demonstrated efficacy in predicting liver histology; however, adding an inexpensive and equally efficacious biochemical endpoint seems to be quite reasonable.

Using a biochemical parameter has the advantage that its determination is done by a simple and reliable method. To date, no biochemical or virological tools to assess the presence of liver damage exist. Having a noninvasive means to discriminate between those patients with and without chronic hepatitis may allow the physicians to address rational decisions regarding their care.

References

1. World Health Organization: Global surveillance and control of hepatitis C. Report of a WHO consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. *J Viral Hepat* 1999; 6:35-47.
2. Tong MJ., El-Farra NS., Reikes AR, et al. Clinical outcomes after transfusion-associated hepatitis C. *N Engl J Med* 1995; 332: 1436-66.
3. Seeff LB. Natural history of hepatitis C. *Hepatology* 1997; 26 (Suppl 1): 21S-8.
4. Prieto M, Olaso V, Verdu C, et al. Does the healthy hepatitis C virus carrier state really exist? An analysis using polymerase chain reaction. *Hepatology* 1995; 22: 413-7.
5. Chomczynski SP, Sacchi N. Single step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* 1987; 162, 156-9.
6. Ishak K, Baptista L, Bianchi L, et al. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; 22: 696-9.
7. Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* 1982;143: 29-36.
8. Martinot-Peignoux M, Boyer N, Cazals-Hatem D, et al. Prospective study on anti-hepatitis C virus-positive patients with persistently normal serum alanine transaminase with or without detectable serum hepatitis C virus RNA. *Hepatology* 2001; 34: 1000-5.
9. Herrera L. The precision of percentiles in establishing normal limits in medicine. *J Lab Clin Med* 1995; 52: 34-42.
10. Nanji AA. Decreased activity of commonly measured serum enzymes: Causes and clinical significance. *Am J Medical Technology* 1983; 49: 241-5.
11. Leino A, Impivaara O, Irjala K, et al. Health-based reference intervals for ALAT, ASAT and GT in serum, measured according to the recommendations of the European Committee for Clinical Laboratory Standards (ECCLS). *Scand J Clin Lab Invest* 1995; 55: 243-50.
12. Lau JY, Davis GL, Kniffen J, et al. Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. *Lancet* 1993; 12; 341 (8859): 1501-4.
13. Persico M, Persico E, Suozzo R, et al. Natural history of hepatitis C virus carriers with persistently normal aminotransferase levels. *Gastroenterology* 2000; 118: 760-4.
14. Herve S, Savoye G, Riachi G, et al. Chronic hepatitis C with normal or abnormal aminotransferase levels: is it the same entity? *Eur J Gastroenterol Hepatol* 2001; 13: 495-500