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ORIGINAL ARTICLE

Lymphocyte subsets in alcoholic liver disease

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Abstract

AIM: To compare lymphocyte subsets between healthy controls and alcoholics with liver disease.

METHODS: The patient cohort for this study included individuals who were suspected to have alcoholic liver disease (ALD) and who had undergone liver biopsy (for disease grading and staging, doubts about diagnosis, or concurrent liver disease; n = 56). Normal controls included patients who were admitted for elective cholecystectomy due to non-complicated gallstones (n = 27). Formalin-fixed, paraffin-embedded liver biopsy specimens were sectioned and stained with hematoxylin and eosin and Perls' Prussian blue. The non-alcoholic steatohepatitis score was used to assess markers of ALD. Lymphocyte population subsets were determined by flow cytometry. T lymphocytes were identified ($CD3^+$), and then further subdivided into $CD4^+$ or $CD8^+$ populations. B lymphocytes ($CD19^+$) and natural killer (NK) cell numbers were also measured. In addition to assessing lymphocyte subpopulation differences between ALD patients and controls, we also compared subsets of alcoholic patients without cirrhosis or abstinent cirrhotic patients to normal controls.

RESULTS: The patient cohort primarily consisted of older men. Active alcoholism was present in 66.1%. Reported average daily alcohol intake was 164.9 g and the average lifetime cumulative intake was 2211.6 kg. Cirrhosis was present in 39.3% of the patients and 66.1% had significant fibrosis (perisinusoidal and portal/periportal fibrosis, bridging fibrosis, or cirrhosis) in their liver samples. The average Mayo end-stage liver disease score was 7.6. No hereditary hemochromatosis genotypes were found. ALD patients (n = 56) presented with significant lymphopenia (1.5×10^9/L ± 0.5×10^9/L *vs* 2.1×10^9/L ± 0.5×10^9/L, $P < 0.0001$), due to a decrease in all lymphocyte subpopulations, except for NK lymphocytes: $CD3^+$ (1013.0 ± 406.2/mm^3 *vs* 1523.0 ± 364.6/mm^3, $P < 0.0001$), $CD4^+$ (713.5 ± 284.7/mm^3 *vs* 992.4 ± 274.7/mm^3, $P < 0.0001$), $CD8^+$ (262.3 ± 140.4/mm^3 *vs* 478.9 ± 164.6/mm^3, $P < 0.0001$), and $CD19^+$ (120.6 ± 76.1/mm^3 *vs* 264.6 ± 88.0/mm^3, $P < 0.0001$). $CD8^+$ lymphocytes suffered the greatest reduction, as evidenced by an increase in the $CD4^+/CD8^+$ ratio (3.1 ± 1.3 *vs* 2.3 ± 0.9, $P = 0.013$). This ratio was associated with the stage of fibrosis on liver biopsy ($r_s = 0.342$, $P = 0.01$) and with Child-Pugh score ($r_s = 0.482$, $P = 0.02$). The number of $CD8^+$ lymphocytes also had a positive association with serum ferritin levels ($r_s = 0.345$, $P = 0.009$). Considering only patients with active alcoholism but not cirrhosis (n = 27), we found similar reductions in total lymphocyte counts (1.8×10^9/L ± 0.3×10^9/L *vs* 2.1×10^9/L ± 0.5×10^9/L, $P = 0.018$), and in populations of $CD3^+$ (1164.7 ± 376.6/mm^3 *vs* 1523.0 ± 364.6/mm^3, $P = 0.001$), $CD4^+$ (759.8 ± 265.0/mm^3 *vs* 992.4 ± 274.7/mm^3, $P = 0.003$), $CD8^+$ (330.9 ± 156.3/mm^3 *vs* 478.9 ± 164.6/mm^3, $P = 0.002$), and $CD19^+$ (108.8 ± 64.2/mm^3 *vs* 264.6 ± 88.0/mm^3, P

< 0.0001). In these patients, the $CD4^+/CD8^+$ ratio and the number of NK lymphocytes was not significantly different, compared to controls. Comparing patients with liver cirrhosis but without active alcohol consumption (n = 11), we also found significant lymphopenia (1.3 × 10^9/L ± 0.6 × 10^9/L *vs* 2.1 × 10^9/L ± 0.5 × 10^9/L, P < 0.0001) and decreases in populations of $CD3^+$ (945.5 ± 547.4/mm^3 *vs* 1523.0 ± 364.6/mm^3, P = 0.003), $CD4^+$ (745.2 ± 389.0/mm^3 *vs* 992.4 ± 274.7/mm^3, P = 0.032), $CD8^+$ (233.9 ± 120.0/mm^3 *vs* 478.9 ± 164.6/mm^3, P < 0.0001), and $CD19^+$ (150.8 ± 76.1/mm^3 *vs* 264.6 ± 88.0/mm^3, P = 0.001). The NK lymphocyte count was not significantly different, but, in this group, there was a significant increase in the $CD4^+/CD8^+$ ratio (3.5 ± 1.3 *vs* 2.3 ± 0.9, P = 0.01).

CONCLUSION: All patient subsets presented with decreased lymphocyte counts, but only patients with advanced fibrosis presented with a significant increase in the $CD4^+/CD8^+$ ratio.

Key words: Lymphocyte subsets; Flow cytometry; Alcoholism; Alcoholic liver disease; Liver fibrosis; Liver biopsy; *HFE* gene

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INTRODUCTION

Alcohol is the third highest cause of disease burden, worldwide, and the second highest cause in Europe. Alcohol abuse is inseparable from several social problems (*e.g.*, violence, abuse, children negligence, unemployment, and lost workdays)[1,2]. The World Health Organization estimates that worldwide alcohol ingestion is responsible for approximately 2.5 million deaths per year. Of those, 320 000 deaths are among young people aged 15 to 29 years old[1].

Despite alcoholism being frequently associated with cirrhosis and liver failure, only 30%-35% of chronic alcoholics develop liver disease (steatohepatitis and/or cirrhosis)[3,4] and only 10%-15% have documented cirrhosis on *post mortem* examination[5]. It is still not clear why this is, but certainly alcohol is a necessary factor for alcoholic liver cirrhosis[6].

It is usual to consider classical alcoholic liver disease (ALD) using various histological sub-types: steatosis, steatohepatitis, cirrhosis, and possibly hepatocellular carcinoma. Normally, two or more such sub-types coexist, representing the spectrum of liver response to alcohol injury. This division is useful for understanding ALD as a continuous evolution and, above all, to understand its spectrum of reversibility, as the disease is highly treatable at the point of pure steatosis but almost impossible at the point of cirrhosis[7-9].

As a disease involving liver inflammation, the role of lymphocytes in ALD has been the subject for different lines of research. Lymphocytes (together with neutrophils, macrophages, and plasma cells) are present in alcoholic steatohepatitis lobular inflammatory infiltrate[10]. It is widely accepted that immune system activation is relevant for alcoholic steatohepatitis pathogenesis and ALD progression. The causative endotoxins are likely lipopolysaccharides (LPS) secreted from Gram-negative bacteria, as LPS blood levels are increased in ALD. The increase has been attributed to jejunal bacterial overgrowth and to increased intestinal wall permeability caused by alcohol[11].

Portal blood LPS has been shown to stimulate liver Kupffer cells, by the direct activation of two cellular receptors, TLR-4 and CD14[12,13]. This activation leads to a downstream cascade of intracellular events, namely the activation of nuclear factor kappa B, activator protein 1, and interferon regulatory factors, and the alteration of pro-caspases 3 and 8 and c-Jun N-terminal kinase. Those events lead to a local immune response and the production of tumor necrosis factor alpha, interleukins 1β, 6, 8, 12 and 18, prostaglandins E_2 and D, transforming growth factor β, and leukotrienes. All of these changes cause polymorphonuclear neutrophil recruitment, increased production of cellular adhesion molecules, increased oxidative stress, and activation of hepatic stellate cells (HSC), leading to increased collagen deposition[13-16]. Humoral immunity may also play a role in ALD pathogenesis. Several autoantibodies, mostly antiphospholipid but also anti-nuclear, anti- double or -single stranded DNA (anti-dsDNA/ssDNA), and anti-lymphocytes, can be detected in 25%-60% of ALD patients[17-21].

The oxidative stress caused by alcohol metabolism can damage several cellular structures and trigger a process similar to idiosyncratic toxic hepatitis. In this process, a toxic metabolite forms haptens, which are presented by antigen-presenting cells to lymphocytes. These modified protein fragments can induce T cell clones, which then recognize self and non-self modified proteins and activate B cells. The newly activated B cells are then also able to produce immunoglobulins directed both to haptens and to native proteins[22].

Despite this immune deregulation, T lymphocyte activation and antigen-presenting cells are impaired in chronic alcoholism, leading to a subdued response to bacterial and viral infections. There is often an increased incidence of tuberculosis, pneumonia, and an increased susceptibility to human immunodeficiency virus and hepatitis c virus (HCV) infections in alcoholic patients. Alcohol also alters serum immunoglobulin levels, quantitative relations between the several lymphocyte populations, endocytic activity, and cytokine production by phagocytic polymorphonuclear cells[23,24].

The human hemochromatosis, or HFE, protein

and its encoding gene were uncovered in 1996, after researchers noticed that the mutation 845G→A in *HFE* (then named *HLA-H*) was homozygous in 80% of patients with hereditary hemochromatosis. This mutation caused the replacement of a cysteine by a tyrosine in position 282 of the HFE protein, then named HFE-C282Y[25]. Other common HFE mutations are H63D and S65C. Interpreting the presence of these mutations requires some caution. For instance, H63D homozygotes are not at risk for iron overload and the S65C allele only seems to have relevance when it is inherited with a heterozygous C282Y mutation. In these instances, it appears to worsen iron overload[26]. Consequently, the relationship between *HFE* mutations and iron overload/ALD progression has been controversial[27,28].

The aim of this study was to compare the lymphocyte subsets between ALD patients and healthy controls. We also compared lymphocyte populations between ALD patient subgroups, namely those with or without active alcoholism and with or without significant fibrosis.

MATERIALS AND METHODS

Both Tondela-Viseu Hospital Centre and the Ethics Committee of the Faculty of Medicine of University of Coimbra approved the study design, which conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Study population

Patients were recruited from the Liver Diseases Outpatient Consultation at Tondela-Viseu Hospital Centre. The cohort consisted of those patients with suspected ALD who had undergone liver biopsy (for disease grading and staging, doubts about diagnosis, or the presence of concurrent liver disease). They were selected with the following inclusion criteria: aged 18- to 80-year-old; male or female; liver biopsy compatible with alcoholic liver disease; with or without active alcohol consumption; no upper gastrointestinal bleeding in the previous three months; serum ferritin above normal inferior limits; without other relevant liver or systemic disease; and willing to give informed written consent. Fifty-six patients were enrolled in total.

As normal controls, we approached patients who had been admitted for elective cholecystectomy due to non-complicated gallstones. A small liver sample was collected during surgery for another study. The inclusion criteria were: male or female; alcohol consumption lower than 20 g/d in women and 40 g/d in men; aged between 18- and 80-year-old; without anemia and with serum ferritin above inferior limits of normality; with alanine aminotransferase (ALT), aspartate transaminase (AST), γ-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) lower than three times the upper normal limit; without other liver or systemic relevant diseases; without evidence of inflammation, as measured by C-reactive protein (CRP < 2.5 mg/dL); and willing to give informed written consent. Twenty-seven controls were enrolled in total.

Histopathologic evaluation

Liver tissue samples were collected, fixed in 10% formalin buffer, and embedded in paraffin. Tissue sections were cut and stained with hematoxylin and eosin and Perls' Prussian blue. Since a validated histological score for ALD does not exist, we used an adaption from the non-alcoholic steatohepatitis score[29]. A semi-quantitative evaluation was performed to measure several parameters, including steatosis (absent, light, moderate, or severe), necroinflammatory activity (absent, light, moderate, or severe), fibrosis (absent, periportal or perisinusoidal fibrosis, periportal and perisinusoidal fibrosis, bridging fibrosis, or cirrhosis) and iron stores grade (with Perls' coloration and evaluation using Scheuer's scale).

Laboratory and other evaluations

Patients and controls underwent routine laboratory evaluations for liver diseases, including complete blood counts, estimations of coagulation [by the international normalized ratio (INR)], glucose, kidney function, aminotransferases (ALT, AST), GGT, ALP, CRP, serological markers for HBV and HCV infections, ceruloplasmin, α1-antitrypsin, serum iron, ferritin, and transferrin saturation measurements. Also, the model for end-stage liver disease (MELD) and Child-Pugh scores were calculated. All patients also had a previous liver ultrasound evaluation.

HFE genotypes were evaluated with Hemochromatosis Strip Assay A (Vienna Lab, Vienna, Austria). This test also detects some very rare *HFE* mutations (including E168Q, E168X, V59M, H63H, P160delC, Q127H, Q283P, V53M, and W164X), as well as ferroportin 1 and transferrin receptor type 2 mutations.

Lymphocyte subsets were determined by flow cytometry, using an EPICS XL-MCL (Beckman Coulter) cytometer. T lymphocytes were identified ($CD3^+$), and then subdivided into $CD4^+$ or $CD8^+$ populations. B lymphocytes ($CD19^+$) and natural killer (NK) cells numbers were also measured.

Statistical analysis

Statistical analysis were performed using SPSS Statistics Data Editor 20 software (IBM, Armonk, NY). Groups were characterized using descriptive statistics, means, standard deviations, and percentages. Categorical variables were analyzed by the χ^2 test (with Yates continuity correction or with Fisher's exact test, when appropriate). Paired parametric numerical variables were analyzed for normal distribution, using the Shapiro-Wilk test, and then the differences between two sampled means was assessed for statistical significance using the Student's *t* test or the non-parametrical Mann-Whitney *U* test for non-normally distributed variables. Correlations between variables were evaluated with Spearman correlation coefficient. All *P* values were two-sided and a $P < 0.05$ was considered to be statistically significant.

Table 1 The clinical and laboratory comparison between alcoholic liver disease cases and controls

	Cases (n = 56)	Controls (n = 27)	P value
Age (yr)	53.7 ± 10.9	47.4 ± 14.4	0.036
Men (%)	83.9	14.8	<0.0001
Active alcoholism (%)	66.1	-	-
Daily consumption (g)	164.9 ± 58.3	-	-
Lifetime total consumption (kg)	2211.6 ± 992.4	-	-
Significant fibrosis[1] (%)	66.1	-	-
Cirrhosis (%)	39.3	-	-
Child–Pugh score	6.2 ± 1.7		
MELD score	7.6 ± 4.7	-	-
⩾ 1 *HFE* mutation (%)	37.5	22.2	NS
WBC (× 10^9/L)	6.2 ± 2.1	6.1 ± 1.4	NS
Neutrophils (× 10^9/L)	3.9 ± 1.7	3.4 ± 1.2	NS
RBC (× 10^{12}/L)	4.0 ± 0.7	4.5 ± 0.4	0.006
Hemoglobin (g/L)	13.4 ± 2.0	13.9 ± 1.2	NS
MCV (fL)	97.9 ± 6.5	90.8 ± 4.0	<0.0001
Platelets (× 10^9/L)	149.0 ± 82.0	231.0 ± 36.0	<0.0001
INR	1.2 ± 0.2	1.0 ± 0.1	<0.0001
Creatinin (mg/dL)	0.9 ± 0.3	0.8 ± 0.1	NS
Albumin (g/dL)	3.7 ± 0.8	3.9 ± 0.6	NS
Albumin/globulin ratio	1.0 ± 0.4	1.4 ± 0.3	<0.0001
Alkaline phosphatase (UI/dL)	119.2 ± 64.5	68.3 ± 14.0	<0.0001
GGT (UI/dL)	221.6 ± 218.8	37.9 ± 33.1	<0.0001
ALT (UI/dL)	43.2 ± 30.7	29.4 ± 15.3	<0.0001
AST (UI/dL)	60.9 ± 29.4	25.1 ± 7.9	<0.0001
Total bilirubin (mg/dL)	1.8 ± 2.7	0.7 ± 0.3	<0.0001
IgA (mg/dL)	583.4 ± 288.2	259.0 ± 146.7	<0.0001
IgG (mg/dL)	1484.3 ± 514.4	1061.1 ± 210.0	<0.0001
IgM (mg/dL)	167.8 ± 87.8	117.6 ± 43.3	0.008
Total Ig (mg/dL)	2035.4 ± 790.5	1437.7 ± 312.0	<0.0001
Serum iron (μg/dL)	115.7 ± 59.7	93.8 ± 34.1	NS
Serum ferritin (ng/mL)	474.6 ± 416.0	126.9 ± 109.8	<0.0001
Transferrin saturation (%)	39.6 ± 19.9	29.6 ± 10.8	0.052
CRP (mg/dL)	1.1 ± 1.6	0.7 ± 0.7	NS
Lymphocyte subsets			
Lymphocytes (× 10^9/L)	1.5 ± 0.5	2.1 ± 0.5	<0.0001
$CD3^+$ lymphocytes (/mm^3)	1013.0 ± 406.2	1523.0 ± 364.6	<0.0001
$CD4^+$ lymphocytes (/mm^3)	713.5 ± 284.7	992.4 ± 274.7	<0.0001
$CD8^+$ lymphocytes (/mm^3)	262.3 ± 140.4	478.9 ± 164.6	<0.0001
$CD4^+/CD8^+$ ratio	3.1 ± 1.3	2.3 ± 0.9	0.013
$CD19^+$ lymphocytes (/mm^3)	120.6 ± 76.1	264.6 ± 88.0	<0.0001
NK lymphocytes (/mm^3)	151.5 ± 134.9	178.7 ± 210.0	NS

[1]Perisinusoidal and portal/periportal fibrosis, bridging fibrosis or cirrhosis. MELD: Model for end-stage liver disease; WBC: White blood cell; RBC: Right blood cell; MCV: Mean cell volume; INR: International normalized ratio; GGT: γ-glutamyl transpeptidase; ALT: Alanine aminotransferase; AST: Aspartate transaminase; CRP: C-reactive protein; NK: Natural killer; NS: Not significant.

RESULTS

The clinical and laboratory comparison between ALD patients and healthy controls is shown in Table 1. Patients within the ALD cohort were slightly older and were predominantly men. Active alcoholism (defined as active ingestion of alcoholic beverages) was documented for 66.1% of the patients, who reported an average daily alcohol intake of 164.9 g and an average lifetime cumulative intake of 2211.6 kg. Cirrhosis was present in 39.3% of the patients and 66.1% of them had significant fibrosis (as evidenced by perisinusoidal and portal/periportal fibrosis, bridging fibrosis, or cirrhosis) on liver samples. The histological findings for patients' liver biopsies are shown in Table 2. The average MELD score was 7.6 and no hereditary hemochromatosis genotypes were found. The prevalence of *HFE* mutations was not significantly different between cases and controls.

Despite the difference in sex distribution, hemoglobin values were slightly higher in the control group, albeit not statistically significant. The number of red blood cells was lower in ALD patients, which was related to a significantly higher mean corpuscular volume. The number of platelets was lower in the ALD group. No significant differences were found concerning kidney function and CRP values.

While the total leukocyte and neutrophil numbers were not significantly different between cases and controls, the total lymphocyte count was significantly lower in the ALD group (1.5 × 10^9/L ± 0.5 × 10^9/L *vs* 2.1

Table 2 Histological findings on liver biopsy *n* (%)

Fibrosis	
Absent	8 (14.3)
Periportal or perisinusoidal	4 (7.1)
Periportal and perisinusoidal	7 (12.5)
Bridging fibrosis	15 (26.8)
Cirrhosis	22 (39.3)
Steatosis	
Absent	14 (25)
Mild (< 25%)	33 (58.9)
Moderate (25%-50%)	7 (12.5)
Severe (> 50%)	2 (3.6)
Necroinflammatory activity	
Absent	21 (37.5)
Mild	19 (33.9)
Moderate	11 (19.6)
Severe	5 (8.9)
Iron liver stores (Scheuer's Scale on Perls's stain)	
Absent	32 (57.1)
Grade 1	11 (19.6)
Grade 2	9 (16.1)
Grade 3	3 (5.4)
Grade 4	1 (1.8)

$\times 10^9$/L ± 0.5 $\times 10^9$/L, $P < 0.0001$). Similarly, all lymphocyte subsets were significantly lower, except for NK cells (Figure 1): $CD3^+$ (1013.0 ± 406.2/mm^3 *vs* 1523.0 ± 364.6/mm^3, $P < 0.0001$), $CD4^+$ (713.5 ± 284.7/mm^3 *vs* 992.4 ± 274.7/mm^3, $P < 0.0001$), $CD8^+$ (262.3 ± 140.4/mm^3 *vs* 478.9 ± 164.6/mm^3, $P < 0.0001$), $CD19^+$ (120.6 ± 76.1/mm^3 *vs* 264.6 ± 88.0/mm^3, $P < 0.0001$). This reduction was proportionally higher in $CD8^+$ lymphocytes, as evidenced by an increased $CD4^+/CD8^+$ ratio (3.1 ± 1.3 *vs* 2.3 ± 0.9, $P = 0.013$).

In other laboratory parameters, significant differences were found for levels of INR, alkaline phosphatase, GGT, aminotransferases, bilirubin, all classes of immunoglobulins, and ferritin. There was a trend for lower serum albumin levels and higher transferrin saturation in most cases of ALD. All of these laboratory abnormalities have been previously associated with ALD.

Considering ALD patients only, we noted a few additional associations. First, in terms of associations to fibrosis, that the $CD4^+/CD8^+$ ratio ($r_s = 0.342$, $P = 0.01$) was positively associated and that total lymphocyte numbers were negatively associated ($r_s = -0.398$, $P = 0.002$) with the grade of fibrosis. Second, that an increased $CD4^+/CD8^+$ ratio was correlated with the Child-Pugh score ($r_s = 0.482$, $P = 0.02$). Third, that the $CD8^+$ lymphocyte count had a positive association with serum ferritin levels ($r_s = 0.345$, $P = 0.009$).

We went on to divide ALD patients into subsets, in order to get more homogeneous groups that we could compare to normal controls. If we considered only patients with active ingestion of alcoholic beverages, but without cirrhosis (Table 3), we continued to observe a significant lymphopenia (1.8 $\times 10^9$/L ± 0.3 $\times 10^9$/L *vs* 2.1 $\times 10^9$/L ± 0.5 $\times 10^9$/L, $P = 0.018$), which was caused by lower numbers in all lymphocyte subsets except for NK cells (Figure 2A): $CD3^+$ (1164.7 ± 376.6/mm^3 *vs* 1523.0 ± 364.6/mm^3, $P = 0.001$), $CD4^+$ (759.8 ± 265.0/mm^3 *vs*

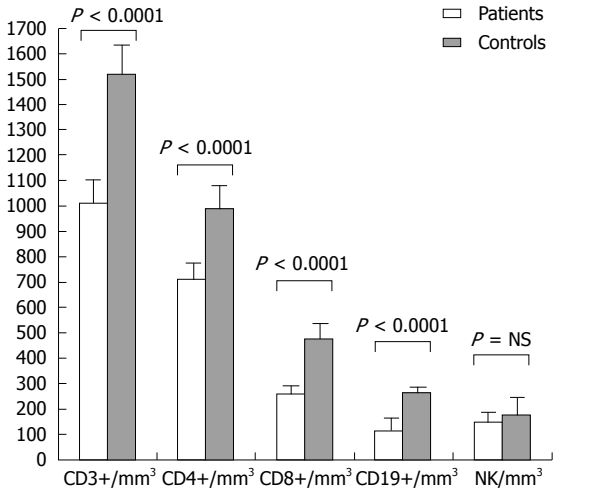

Figure 1 Lymphocyte subsets: All patients *vs* normal controls. NK: Natural killer; NS: Not significant.

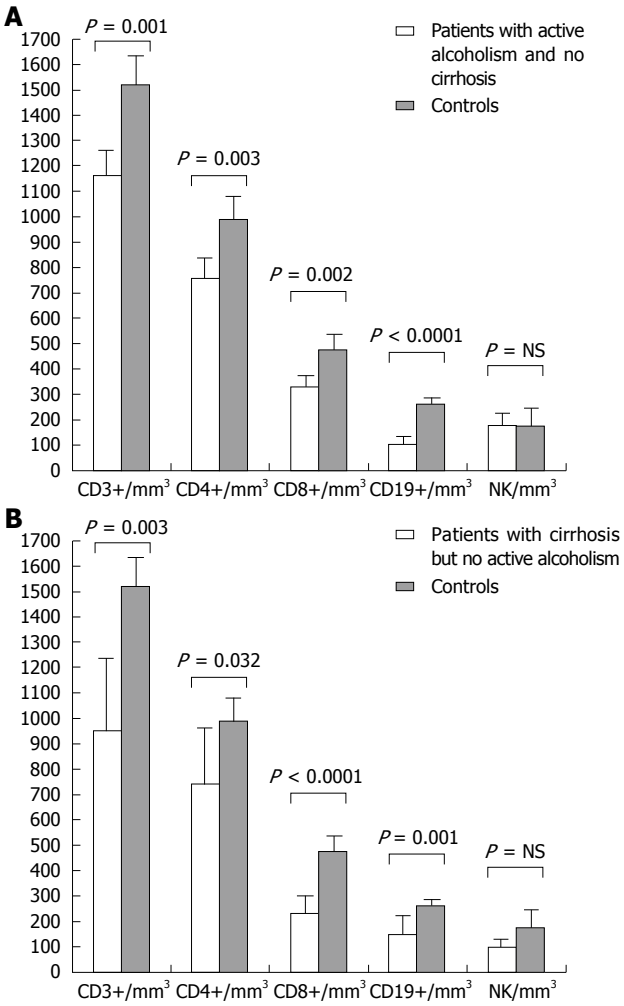

Figure 2 Lymphocyte subsets. A: Patients with active alcoholism/without cirrhosis *vs* normal controls; B: Patients with cirrhosis but no active alcoholism *vs* normal controls. NK: Natural killer; NS: Not significant.

992.4 ± 274.7/mm^3, $P = 0.003$), $CD8^+$ (330.9 ± 156.3/mm^3 *vs* 478.9 ± 164.6/mm^3, $P = 0.002$), and $CD19^+$ (108.8 ± 64.2/mm^3 *vs* 264.6 ± 88.0/mm^3, $P < 0.0001$). How-

Table 3 Clinical and laboratory comparison between alcoholic liver disease cases with active alcoholism *vs* controls with no cirrhosis

	Patients (n = 26)	Controls (n = 27)	P value
Age (yr)	44.4 ± 14.4	47.4 ± 14.4	
Men (%)	88.5	14.8	<0.0001
WBC (× 10^9/L)	6.9 ± 1.8	6.1 ± 1.4	NS
Neutrophils (× 10^9/L)	4.3 ± 1.5	3.4 ± 1.2	0.011
RBC (× 10^{12}/L)	4.1 ± 0.7	4.5 ± 0.4	NS
Hemoglobin (g/L)	14.0 ± 4.0	13.9 ± 1.2	NS
MCV (fL)	99.9 ± 5.9	90.8 ± 4.0	<0.0001
Platelets (× 10^9/L)	204.7 ± 84.6	231.0 ± 36.0	NS
INR	1.1 ± 0.2	1.0 ± 0.1	NS
Creatinin (mg/dL)	0.9 ± 0.2	0.8 ± 0.1	NS
Albumin (g/dL)	4.0 ± 0.5	3.9 ± 0.6	NS
Albumin/globulin ratio	1.4 ± 0.3	1.4 ± 0.3	NS
Alkaline phosphatase (UI/dL)	102.3 ± 74.1	68.3 ± 14.0	NS
GGT (UI/dL)	297.8 ± 33.1	37.9 ± 33.1	<0.0001
ALT (UI/dL)	55.8 ± 41.6	29.4 ± 15.3	<0.0001
AST (UI/dL)	72.2 ± 52.8	25.1 ± 7.9	<0.0001
Total bilirubin (mg/dL)	1.1 ± 0.6	0.7 ± 0.3	0.027
CRP (mg/dL)	1.1 ± 2.0	0.7 ± 0.7	NS
Lymphocyte Subsets			
Lymphocytes (× 10^9/L)	1.8 ± 0.3	2.1 ± 0.5	0.018
$CD3^+$ lymphocytes (/mm^3)	1164.7 ± 376.6	1523.0 ± 364.6	0.001
$CD4^+$ lymphocytes (/mm^3)	759.8 ± 265.0	992.4 ± 274.7	0.003
$CD8^+$ lymphocytes (/mm^3)	330.9 ± 156.3	478.9 ± 164.6	0.002
$CD4^+/CD8^+$ ratio	2.5 ± 1.1	2.3 ± 0.9	NS
$CD19^+$ lymphocytes (/mm^3)	107.8 ± 64.2	264.6 ± 88.0	<0.0001
NK lymphocytes (/mm^3)	180.2 ± 169.0	178.7 ± 210.0	NS

WBC: White blood cell; RBC: Right blood cell; MCV: Mean cell volume; INR: International normalized ratio; GGT: γ-glutamyl transpeptidase; ALT: Alanine aminotransferase; AST: Aspartate transaminase; CRP: C-reactive protein; NK: Natural killer; NS: Not significant.

ever, in this group, the $CD4^+/CD8^+$ ratio was not significantly different when compared to normal controls.

We performed one more analysis and considered only cases with documented cirrhosis but without active alcoholism (Table 4). Compared to normal controls, lymphopenia was again evident (1.3 × 10^9/L ± 0.6 × 10^9/L *vs* 2.1 × 10^9/L ± 0.5 × 10^9/L, $P < 0.0001$; Figure 2B), at the expense of all lymphocyte subsets except for NK cells: $CD3^+$ (945.5 ± 547.4/mm^3 *vs* 1523.0 ± 364.6/mm^3, $P = 0.003$), $CD4^+$ (745.2 ± 389.0/mm^3 *vs* 992.4 ± 274.7/mm^3, $P = 0.032$), $CD8^+$ (233.9 ± 120.0/mm^3 *vs* 478.9 ± 164.6/mm^3, $P < 0.0001$), and $CD19^+$ (150.8 ± 76.1/mm^3 *vs* 264.6 ± 88.0/mm^3, $P = 0.001$). A significant increase in the $CD4^+/CD8^+$ ratio was also evident in this group of patients with advanced fibrosis (3.5 ± 1.3 *vs* 2.3 ± 0.9, $P = 0.01$).

DISCUSSION

While the role of lymphocytes in ALD pathogenesis has been the subject of different lines of research, the results and ultimate interpretations are discrepant. Lymphopenia and an increased $CD4^+/CD8^+$ ratio in patients with liver failure due to alcoholic cirrhosis was first described by Couzigou *et al*[30]. A later paper reported no alterations in the total lymphocyte count, but did find an increased $CD4^+/CD8^+$ ratio in ALD patients[31]. Other studies have identified significant lymphopenia with decreased numbers of $CD4^+$ and $CD8^+$ populations, in both alcohol abusers without liver disease[32] and in patients with alcoholic cirrhosis[33,34]. A reduction in $CD3^+$ and $CD4^+$ T lymphocytes was also found to correlate with the severity of liver cirrhosis severity[35]. Despite these studies, Cook *et al*[36] found an increase in the number of T lymphocytes in alcoholic patients without liver disease, mainly due to an increased number of activated $CD8^+$ cells. Similarly, Arosa *et al*[37] reported a relative increase of some $CD8^+$ lymphocyte subtypes in alcoholic patients compared to healthy controls, however no significant differences were described for total $CD4^+$ or $CD8^+$ T lymphocyte counts.

Apart from alterations in lymphocyte count, alcohol appears to cause functional changes to lymphocyte subsets[38,39]. Th17 lymphocytes, a new $CD4^+$ effector subpopulation that has been recently described, are more populous in ALD and their absolute numbers are associated with fibrosis stage[40]. Several lymphocyte T integrins (namely CD29, VLA-3, VLA-4 and VLA-5) are also increased in ALD patients[41]. B lymphocytes were found to be significantly decreased in ALD patients, while a relative predominance of B cells characteristic of IgM antibody production was found. There is speculation on the possible contribution of these changes in autoimmunity and immunodeficiency to ALD pathology[42].

A decreased NK cell activity was also reported in ALD[43]. In recent years, the concept of resolution or

Table 4 Clinical and laboratory comparison between alcoholic liver disease cases with cirrhosis but no active alcoholism and controls

	Patients (n = 11)	Controls (n = 27)	P value
Age (yr)	56.8 ± 9.0	47.4 ± 14.4	0.03
Men (%)	100	14.8	<0.0001
MELD score	10.5 ± 4.6	-	
WBC (× 10^9/L)	5.4 ± 2.5	6.1 ± 1.4	NS
Neutrophils (× 10^9/L)	3.4 ± 2.0	3.4 ± 1.2	NS
RBC (× 10^{12}/L)	4.5 ± 0.4	4.5 ± 0.4	0.005
Hemoglobin (g/L)	12.9 ± 2.1	13.9 ± 1.2	NS
MCV (fL)	97.0 ± 6.0	90.8 ± 4.0	0.001
Platelets (× 10^9/L)	103.5 ± 39.4	231.0 ± 36.0	<0.0001
INR	1.4 ± 0.3	1.0 ± 0.1	<0.0001
Creatinin (mg/dL)	0.9 ± 0.3	0.8 ± 0.1	NS
Albumin (g/dL)	3.4 ± 0.7	3.9 ± 0.6	0.003
Albumin/globulin ratio	0.9 ± 0.3	1.4 ± 0.3	<0.0001
Alkaline phosphatase (UI/dL)	124.9 ± 32.6	68.3 ± 14.0	NS
GGT (UI/dL)	84.2 ± 55.5	37.9 ± 33.1	0.005
ALT (UI/dL)	32.6 ± 7.6	29.4 ± 15.3	NS
AST (UI/dL)	46.9 ± 10.9	25.1 ± 7.9	<0.0001
Total bilirubin (mg/dL)	2.2 ± 2.0	0.7 ± 0.3	<0.0001
CRP (mg/dL)	1.1 ± 1.5	0.7 ± 0.7	NS
Lymphocyte Subsets			
Lymphocytes (×10^9/L)	1.3 ± 0.6	2.1 ± 0.5	<0.0001
$CD3^+$ lymphocytes (/mm^3)	954.5 ± 547.4	1523.0 ± 364.6	0.003
$CD4^+$ lymphocytes (/mm^3)	745.2 ± 389.0	992.4 ± 274.7	0.032
$CD8^+$ lymphocytes (/mm^3)	233.9 ± 120.0	478.9 ± 164.6	<0.0001
$CD4^+/CD8^+$ ratio	3.5 ± 1.3	2.3 ± 0.9	0.01
$CD19^+$ lymphocytes (/mm^3)	150.8 ± 108.4	264.6 ± 88.0	0.001
NK lymphocytes (/mm^3)	99.5 ± 54.5	178.7 ± 210.0	NS

MELD: Model for end-stage liver disease; WBC: White blood cell; RBC: Right blood cell; MCV: Mean cell volume; INR: International normalized ratio; GGT: γ-glutamyl transpeptidase; ALT: Alanine aminotransferase; AST: Aspartate transaminase; CRP: C-reactive protein; NK: Natural killer; NS: Not significant.

regression of liver fibrosis has been described in various situations. The most important step in this process appears to be the apoptosis of HSCs[44]. This event can be induced by different mechanisms, with NK cell activation by interferon-α, interferon-β, and interferon-γ being one of the most important[45,46]. It is noticeable that 30%-50% of liver lymphocytes are NK cells, so further contributing to the link between NK cells and HSC activity regulation[46,47]. Furthermore, the experimental administration of alcohol greatly increases the resistance of HSCs to apoptosis, through NK cells and γ-interferon stimulation[48,49]. Other HSC interactions with immune system cells are also the subject of ongoing research. For instance, the phagocytosis of $CD8^+$ lymphocytes by activated HSCs was reported, but the relevance of this action is unknown[50]. There was also a report linking $CD8^+$ lymphocytes to HSC fibrogenic activity[51].

A decreased $CD8^+$ cell count, mainly $CD8^+/CD28^+$, was also described in hereditary hemochromatosis, as well as the presence of functional abnormalities in these cells. This may be due to an HFE polymorphism, linked to and located near the *HFE* gene, which is related to the regulation of lymphocyte subsets and $CD8^+$ maturation[52-56]. Furthermore, the number of peripheral blood $CD8^+$ lymphocytes inversely correlate with iron overload in hemochromatosis, which is more severe in cases of low $CD8^+$ counts[57,58].

The present study shows the impact of alcoholism in our country, as the majority of ALD patients were young or middle aged (the average age was 53.7 years old), mostly male, and with a high daily alcohol intake often since childhood. Also, most of the patients already displayed significant liver fibrosis.

The control group, chosen among healthy individuals admitted for elective cholecystectomy due to non-complicated gallstones, consisted mostly of women. This was expected, as gallstones are more common in women of childbearing age. Additionally, many potential male controls had to be excluded because they reported alcohol consumption higher than 40 g/d. This may have induced a bias when comparing serum iron, ferritin, and transferrin saturation, which we tried to keep to a minimum by excluding all individuals with hemoglobin and serum ferritin below normal or with analytical evidence of inflammation. Nevertheless, we found papers with control groups consisting mostly of women and gender differences do not seem to affect the type and number of circulating lymphocytes in peripheral blood[37].

As expected, ALD patients showed characteristic analytical changes, including macrocytosis, abnormal clotting, decreased platelets, elevated liver function tests, immunoglobulins, and serum ferritin. After analyzing the mean white cell counts, we found significant lymphopenia in ALD patients, compared to healthy controls. This

lymphopenia reflected a reduction of all lymphocyte subsets, except for NK lymphocytes, which did not show a statistically significant difference. The $CD4^+/CD8^+$ ratio was significantly increased in most cases, which was a reflection of the relative reduction in the number of $CD8^+$ cells.

However, after analyzing several subgroups in our study, it was evident that all ALD patients consistently presented with lymphopenia compared to healthy controls, but only patients with advanced fibrosis presented with an increased $CD4^+/CD8^+$ ratio, even accounting for active alcohol consumption. In fact, for ALD patients, the total lymphocyte count inversely correlated with the stage of liver fibrosis and the $CD4^+/CD8^+$ ratio directly associated with liver fibrosis.

Our work adds to the findings of significant lymphopenia in alcoholics (concerning B and T cells), with or without significant liver disease, as previously reported[30,32-34,42]. We found that an increased $CD4^+/CD8^+$ ratio was present only in the case of significant fibrosis, but this finding was also suggested by some previous studies which studied patients with advanced liver disease[30,31]. Our work does not support other papers that reported an increase in some lymphocyte subsets[36,37]. While we found a trend to lower NK cell counts, as previously reported[43], we could not demonstrate statistical significance for this phenomenon.

It should be noted that the association between the $CD4^+/CD8^+$ ratio and fibrosis stage was the opposite of what has been reported for viral hepatitis (*i.e.*, a low $CD4^+/CD8^+$ ratio being associated with an increased fibrosis stage)[50]. If this association is found to be specific for ALD, the $CD4^+/CD8^+$ ratio may eventually contribute to a panel of non-invasive markers of staging for ALD fibrosis. The $CD8^+$ lymphocyte count correlated directly with serum ferritin levels, unlike findings with hereditary hemochromatosis where lower $CD8^+$ counts were associated with increased iron overload. This may indicate that the lymphopenia etiopathogenic mechanism is different in ALD, when compared to other liver diseases, such as viral hepatitis or hemochromatosis.

COMMENTS

Background

Alcohol is the third highest cause of disease burden, worldwide, and the second highest cause in Europe. However, only 30%-35% of chronic alcoholics develop liver disease (steatohepatitis and/or cirrhosis) and only 10%-15% have documented cirrhosis. While the role of lymphocytes in alcoholic liver disease (ALD) pathogenesis has been the subject of different lines of research, the results and ultimate interpretations are discrepant.

Research frontiers

Apart from alterations in lymphocyte count, alcohol appears to cause functional changes to lymphocyte subsets.

Innovations and breakthroughs

Th17 lymphocytes, a new $CD4^+$ effector subpopulation that has been recently described, are more populous in ALD and their absolute numbers are associated with fibrosis stage. Several lymphocyte T integrins (namely CD29, VLA-3, VLA-4 and VLA-5) are also increased in ALD patients. B lymphocytes were found to be significantly decreased in ALD patients, while a relative predominance of B cells characteristic of IgM antibody production was found.

Applications

Our work adds to the findings of significant lymphopenia in alcoholics, with or without significant liver disease, ALD patients consistently presented with lymphopenia compared to healthy controls, but only patients with advanced fibrosis presented with an increased $CD4^+/CD8^+$ ratio. The total lymphocyte count inversely correlated with the stage of liver fibrosis and the $CD4^+/CD8^+$ ratio directly associated with liver fibrosis. If this association is found to be specific for ALD, the $CD4^+/CD8^+$ ratio may eventually contribute to a panel of non-invasive markers of staging for ALD fibrosis.

Terminology

$CD3^+$ is a marker of all T lymphocytes. Those can be subdivided into T helper cells ($CD4^+$), which help the activity of other immune cells by releasing T cell cytokines; and cytotoxic T cells ($CD8^+$), which are involved in direct cell killing after recognizing a specific antigen. B-lymphocytes, involved in antibodies synthesis, express the surface protein CD19.

Peer review

Stimulation of innate immunity is increasingly recognized to play an important role in the pathogenesis of alcoholic liver disease. Various studies have come up to speculate immune response in alcoholic liver disease (T Cell and B cell). Authors in this manuscript evaluated lymphocytic population subsets in alcoholic liver disease. It is an interesting manuscript.

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BRIEF ARTICLE

Dyspnea and respiratory muscle strength in end-stage liver disease

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Abstract

AIM: To investigate the prevalence of chronic dyspnea and its relationship to respiratory muscle function in end-stage liver disease.

METHODS: Sixty-eight consecutive, ambulatory, Caucasian patients with end-stage liver disease, candidates for liver transplantation, were referred for preoperative respiratory function assessment. Forty of these (29 men) were included in this preliminary study after applying strict inclusion and exclusion criteria. Seventeen of 40 patients (42%) had ascites, but none of them was cachectic. Fifteen of 40 patients (38%) had a history of hepatic encephalopathy, though none of them was symptomatic at study time. All patients with a known history and/or presence of co-morbidities were excluded. Chronic dyspnea was rated according to the modified medical research council (mMRC) 6-point scale. Liver disease severity was assessed according to the Model for end-stage liver disease (MELD). Routine lung function tests, maximum static expiratory (Pemax) and inspiratory (Pimax) mouth pressures were measured. Respiratory muscle strength (RMS) was calculated from Pimax and Pemax values. In addition, arterial blood gases and pattern of breathing (V_E: minute ventilation; V_T: tidal volume; V_T/T_I: mean inspiratory flow; T_I: duration of inspiration) were measured.

RESULTS: Thirty-five (88%) of 40 patients aged (mean ± SD) 52 ± 10 years reported various degrees of chronic dyspnea (mMRC), ranging from 0 to 4, with a mean value of 2.0 ± 1.2. MELD score was 14 ± 6. Pemax, percent of predicted (%pred) was 105 ± 35, Pimax, %pred was 90 ± 29, and RMS, %pred was 97 ± 30. These pressures were below the normal limits in 12 (30%), 15 (38%), and 14 (35%) patients, respectively. Furthermore, comparing the subgroups of ascites to non-ascites patients, all respiratory muscle indices measured were found significantly decreased in ascites patients. Patients with ascites also had a significantly worse MELD score compared to non-ascites ones (P = 0.006). Significant correlations were found between chronic dyspnea and respiratory muscle function indices in all patients. Specifically, mMRC score was significantly correlated with Pemax, Pimax, and RMS (r = -0.53, P < 0.001; r = -0.42, P < 0.01; r = -0.51, P < 0.001, respectively). These correlations were substantially closer in the non-ascites subgroup (r = -0.82, P < 0.0001; r = -0.61, P < 0.01; r = -0.79, P < 0.0001, respectively) compared to all patients. Similar results were found for the relationship between mMRC *vs* MELD score, and MELD score *vs* respiratory muscle strength indices. In all patients the sole predic-

tor of mMRC score was RMS (r = -0.51, P < 0.001). In the subgroup of patients without ascites this relationship becomes closer (r = -0.79, P < 0.001), whilst this relationship breaks down in the subgroup of patients with ascites. The disappearance of such a correlation may be due to the fact that ascites acts as a "confounding" factor. $PaCO_2$ (4.4 ± 0.5 kPa) was increased, whereas pH (7.49 ± 0.04) was decreased in 26 (65%) and 34 (85%) patients, respectively. PaO_2 (12.3 ± 0.04 kPa) was within normal limits. V_E (11.5 ± 3.5 L/min), V_T (0.735 ± 0.287 L), and V_T/T_I (0.449±0.129 L/s) were increased signifying hyperventilation in both subgroups of patients. V_T/T_I was significantly higher in patients with ascites than without ascites. Significant correlations, albeit weak, were found for $PaCO_2$ with V_E and V_T/T_I (r = -0.44, P < 0.01; r = -0.41, P < 0.01, respectively).

CONCLUSION: The prevalence of chronic dyspnea is 88% in end-stage liver disease. The mMRC score closely correlates with respiratory muscle strength.

Key words: Liver transplantation; Lung function testing; Maximum static mouth pressures; Pattern of breathing; Respiratory muscle strength

Kaltsakas G, Antoniou E, Palamidas AF, Gennimata SA, Paraskeva P, Smyrnis A, Koutsoukou A, Milic-Emili J, Koulouris NG. Dyspnea and respiratory muscle strength in end-stage liver disease. *World J Hepatol* 2013; 5(2): 56-63 Available from: URL: http://www.wjgnet.com/1948-5182/full/v5/i2/56.htm DOI: http://dx.doi.org/10.4254/wjh.v5.i2.56

INTRODUCTION

Liver transplantation has become the therapeutic option for patients with end-stage liver disease[1]. These patients are usually characterized by tiring easily, chronic fatigue, protein wasting and muscle mass loss[2]. The loss of muscle mass may affect both peripheral and respiratory muscles[3]. However, there are only a few reports on respiratory muscle function[4-8] or pattern of breathing in patients awaiting liver transplantation. These patients frequently report chronic dyspnea, however, its exact prevalence and severity remains unknown. To the best of our knowledge, no report can be found in the literature relating chronic dyspnea to respiratory muscle function in these patients. Therefore, we wondered whether chronic dyspnea is related to respiratory muscle strength and/or any other lung function index. We conducted this preliminary study to determine the prevalence of chronic dyspnea and the interrelationships among chronic dyspnea, measured with the modified Medical Research Council (mMRC) scale, respiratory muscle strength, and lung function in patients with end-stage liver disease[9-13].

MATERIAL AND METHODS

Subjects

Sixty-eight consecutive, ambulatory, Caucasian patients with end-stage liver disease, candidates for liver transplantation, were referred for preoperative respiratory function assessment. Twenty-eight patients were excluded applying the imposed (see below) strict inclusion and exclusion criteria [*i.e.*, chronic obstructive pulmonary disease (COPD) n = 11; bronchiectasis n = 2; previous abdominal and thoracic surgical operation n = 3; pleural effusion n = 5; unsatisfactory lung function testing performed n = 7]. Forty of them (29 men) were included in this preliminary study. The causes of end-stage liver disease among the patients finally included in the study were cirrhosis from hepatitis B or C (n = 23; 58%; 4 of them additionally had hepatocellular carcinoma), alcoholic liver disease (n = 9; 22%), cryptogenic cirrhosis (n = 2; 5%), primary biliary cirrhosis (n = 2; 5%), and other causes (n = 4; 10%; 1 of them additionally had hepatocellular carcinoma). Their anthropometric characteristics and lung function data are shown in Table 1.

Inclusion criteria were: (1) age 18 years and older; (2) the ability to perform a full lung function testing satisfactorily; and (3) stable clinical and functional state for at least four weeks before testing. Exclusion criteria were presence of any kind of: (1) cardiovascular disorders diagnosed by a cardiologist; (2) known lung disease caused by conditions other than liver, such as asthma or COPD requiring regular bronchodilator treatment; (3) pleural effusion; (4) previous abdominal and thoracic surgical operation; and (5) neuromuscular disorders. None of the patients studied was cachectic (body mass index < 18). Nineteen out of 40 patients had never been smokers. Fifteen of 40 patients had a history of hepatic encephalopathy, but none of them was symptomatic at study time. Seventeen of 40 patients presented with ascites, and 21 out of 40 patients were on b-blockers at study time.

The study was approved by the local ethics committee. All subjects gave informed consent.

Liver disease severity, dyspnea, and respiratory function

Liver disease severity was assessed according to the model for end-stage liver disease (MELD, United Network for Organ Sharing modification)[14]. The United Network for Organ Sharing and Euro Transplant currently use MELD score for prioritizing patients' allocation for liver transplantation instead of the older Child-Pugh score[15]. Serum laboratory data (used for MELD score calculation) were measured maximum ± 7 d the day of respiratory testing. The MELD score used was not corrected for the patients with hepatocellular carcinoma.

Chronic dyspnea was rated according to the modified mMRC 6-point scale[9]. The mMRC breathlessness scale comprises six statements that describe almost the entire range of respiratory disability from none (Grade 0) to almost complete incapacity (Grade 5). The score

Table 1 Anthropometric characteristics and routine respiratory function data of 40 end-stage liver disease patients, stratified in two groups according to the presence or absence of ascites

	All patients (n = 40)	Ascites (n = 17)	Non-ascites (n = 23)	P value
Gender, M/F	29/11	15/2	14/9	0.079
Age, yr	52 ± 10	51 ± 10	52 ± 9	0.752
Ht, m	1.69 ± 0.90	1.73 ± 0.90	1.66 ± 0.90	0.015
BMI, kg · m^{-2}	28 ± 4	26 ± 4	29 ± 5	0.144
Pack- years, yr	19 ± 22	27 ± 25	13 ± 18	0.087[1]
FVC	104 ± 20	91 ± 17	114 ± 16	< 0.001
FEV_1	100 ± 18	89 ± 15	108 ± 15	< 0.001
FEV_1/FVC, %	78 ± 3	79 ± 3	78 ± 3	0.083
PEF	95 ± 19	84 ± 18	103 ± 17	0.002
TLC	95 ± 20	86 ± 16	102 ± 21	0.011
FRC	97 ± 28	87 ± 24	101 ± 29	0.136
RV	92 ± 28	91 ± 25	93 ± 30	0.840
DL_{CO}	78 ± 18	70 ± 16	84 ± 18	0.013

[1]Mann-Whitney rank sum test was used. Values are mean ± SD obtained in a sitting position. Unless otherwise specified, values are expressed as % of predicted. Statistical significance tested with Student's t test between ascites and non-ascites groups. M: Male; F: Female; Ht: Height; BMI: Body mass index; FVC: Forced vital capacity; FEV_1: Forced expiratory volume in one second; PEF: Peak expiratory flow; TLC: Total lung capacity; FRC: Functional residual capacity; RV: Residual volume; DL_{CO}: Diffusing capacity for carbon monoxide.

is the number that best fits the patient's level of activity. Simple spirometry was measured with the Vmax apparatus (Vmax Encore 22: Sensor Medics, Yorba Linda, CA, United States) using the "fast inspiratory maneuver"[16]. Static Lung Volumes were determined by the multiple nitrogen washout technique[17] (Vmax Encore 22 apparatus). The diffusing capacity for carbon monoxide (DL_{CO}) single breath technique was also determined (Vmax Encore 22 apparatus)[18]. Predicted values for spirometry, static lung volumes, and DL_{CO} were from the European Community for Coal and Steel[19]. The arterial pH, PaO_2, and $PaCO_2$ were measured with the Stat Profile Critical Care Xpress apparatus (Nova Biomedical, Waltham, MA, United States).

Maximum static expiratory (Pemax) and inspiratory (Pimax) mouth pressures were measured with a plastic semi-rigid flanged mouthpiece fitted to a metallic stem incorporating a 3-way tap, manufactured according to the design of Ringqvist[10,11]. The dimensions of the metallic stem were length 27 cm and internal diameter 2.6 cm. A leak tube of length 3.7 cm and 2 mm internal diameter was incorporated into the stem 3 cm from the point of attachment to the plastic mouthpiece. A differential pressure transducer (range ± 340 cm H_2O, Validyne MP45-36-871, Validyne Co, Northridge, CA) connected to the 3-way tap with a 70 cm fine polyethylene catheter. The pressure transducer was calibrated before each study using a U-tube water manometer. Pressures were displayed on a computer screen. All studies were performed with a nose-clip on and with the subjects seated comfortably in a high-backed chair at 90° where they could view a computer screen. The flanged mouthpiece was held in the mouth behind the lips and gripped firmly by the teeth, the operator holding the stem. The subjects used their hands to hold the lips firmly onto the mouthpiece if a leak was noticed. Prior to a Pemax or Pimax effort, the operator closed the 3-way tap with the subject at total lung capacity or residual volume, respectively. All subjects were given verbal encouragement and received visual feedback from the computer screen. A period of learning the procedure preceded the definitive measurements[10]. All measurements followed the criteria of Ringqvist[11] such that: (1) no extra leakage occurred; (2) the three highest pressures were similar (within 5%) and later attempts did not yield higher results; and (3) the subjects felt that they had given a maximum effort. At least 1 min rest was allowed between efforts. Pressures maintained for less than 1 s were disregarded. The highest pressure generated by an individual was used for analysis. Predicted values for Pemax and Pimax standardized for age, height, and gender were obtained from Wilson *et al*[12]. Respiratory muscle strength (RMS) was also calculated as the arithmetic mean of Pemax and Pimax[13].

Pattern of breathing (V_E: minute ventilation; V_T: tidal volume; T_E: duration of expiration; T_I: duration of inspiration; T_{TOT}: total cycle duration; RR: respiratory rate; V_T/T_I: mean inspiratory flow; T_I/T_{TOT}: duty cycle) was also assessed during resting breathing. Subjects with a noseclip on breathed through a heated pneumotachograph (Screenmate-Box; Erich Jaeger GmbH and Co, Germany) while they were seated comfortably in a high-backed chair at 90° in a quiet room. In order to minimize the effects of anxiety, all indices were measured after the patient had become familiar with the procedure and actual measurements were made only when ventilation had remained constant for at least 10 min[20]. Normal values for the pattern of breathing were obtained from Tobin *et al*[21].

Statistical analysis

Data are expressed as mean ± SD, unless otherwise stated. For comparisons between groups Student's t test, Mann-Whitney rank sum test, and Fisher's exact test

Table 2 Serum laboratory data of the 40 patients with end-stage liver disease stratified according to the presence or absence of ascites

	All patients (n = 40)	Ascites (n = 17)	Non-ascites (n = 23)	P value
INR	1.40 ± 0.31	1.53 ± 0.34	1.30 ± 0.25	0.017[1]
Haemoglobin, g/L	121.00 ± 23.80	108.60 ± 24.00	130.10 ± 19.20	0.003
Creatinine, mg/dL	1.19 ± 1.02	1.43 ± 1.49	1.01 ± 0.39	0.328[1]
Total protein, g/L	72.20 ± 8.70	68.80 ± 8.80	74.70 ± 7.90	0.032
Albumin, g/L	34.80 ± 6.70	32.00 ± 6.90	37.00 ± 5.90	0.019
Total bilirubin, mg/dL	4.67 ± 9.61	6.68 ± 11.41	3.19 ± 7.97	0.004[1]
Sodium, mmol/L	137.00 ± 5.00	134.00 ± 5.00	139.00 ± 4.00	< 0.001

Values obtained maximum ± 7 d of respiratory tests day. [1]Mann-Whitney rank sum test was used. Statistical significance tested with Student's t test between ascites and non-ascites groups. INR: International normalised ratio for prothrombin time.

Table 3 Comparison of model for end-stage liver disease score, modified medical research council dyspnea score, and respiratory muscle strength indices between ascites and non-ascites liver disease patients

	All patients (n = 40)	Ascites (n = 17)	Non-ascites (n = 23)	P value
MELD	14 ± 6	17 ± 7	12 ± 5	0.006[1]
mMRC	2.0 ± 1.2	2.2 ± 1.1	1.9 ± 1.2	0.444[1]
Pemax, %pred	105 ± 35	87 ± 30	118 ± 33	0.004
Pimax, %pred	90 ± 29	72 ± 20	103 ± 28	< 0.001
RMS, %pred	97 ± 30	79 ± 23	110 ± 28	< 0.001

Values are mean ± SD obtained in a sitting position. Statistical significance tested with Student's t test between ascites and non-ascites groups. [1]For MELD and mMRC Mann-Whitney rank sum test was used. MELD: Model for end-stage liver disease; mMRC: Modified medical research council; Pemax: Maximum static expiratory mouth pressure; %pred: Percent of predicted; Pimax: Maximum static inspiratory mouth pressure; RMS: Respiratory muscle strength.

were used. Pearson and Spearman correlation coefficients, linear and backward stepwise regressions analyses were used where appropriate. A $P \leqslant 0.05$ value was considered as significant. Statistical analysis was performed using SigmaStat V3.5 and SigmaPlot V10.0 statistical software (Jandel Scientific, CA, United States).

RESULTS

Table 1 provides anthropometric and routine respiratory function data from the 40 end-stage liver disease patients stratified according to the presence or absence of ascites (17 with ascites; 42%). Non-ascites patients had better routine lung function compared to ascites ones. DL_{CO} was abnormal in 24 out of 40 patients, 13 of whom had ascites.

Serum laboratory data are shown in Table 2. Patients with ascites had abnormal values in the majority of the serum laboratory tests, whereas non-ascites patients had values almost within normal range.

Thirty-five (88%) of 40 patients reported various degrees of chronic dyspnea, ranging from 0 to 4 (Table 3). Pemax, Pimax, and RMS were below the normal limits[12,13] in 12 (30%), 15 (38%), and 14 (35%) patients, respectively. Furthermore, comparing the subgroups of ascites to non-ascites patients, all respiratory muscle indices measured were significantly decreased in ascites patients. Patients with ascites had significantly worse MELD score compared to non-ascites ones.

Arterial blood gases and pattern of breathing data are shown in Table 4. $PaCO_2$ was increased (< 4.7 kPa) and pH was decreased (> 7.45) in 26 (65%) and 34 (85%) patients, respectively. PaO_2 was within normal limits. V_E, V_T, and V_T/T_I were increased indicating hyperventilation in both subgroups of patients. V_T/T_I was significantly higher in patients with ascites than without ascites. Significant correlations, albeit weak, were found for $PaCO_2$ with V_E and V_T/T_I (r = -0.44, P < 0.01; r = -0.41, P < 0.01, respectively).

Significant correlations were found between chronic dyspnea and respiratory muscle strength indices. Specifically, mMRC score was significantly correlated with Pemax, Pimax, and RMS (r = -0.53, P < 0.001; r = -0.42, P < 0.01; r = -0.51, P < 0.001, respectively). These correlations were substantially closer in the non-ascites subgroup (r = -0.82, P < 0.0001; r = -0.61, P < 0.01; r = -0.79, P < 0.0001, respectively). Backward Stepwise Regression analysis showed that the sole predictor of mMRC score was RMS (r = -0.51, P < 0.001) (all parameters in Table 2 being eliminated) in all patients. In the subgroup of patients without ascites the predictive power of RMS was higher (r = -0.79, P < 0.001) (Figure 1). This prediction breaks down in the ascites subgroup. The correlation between mMRC score and MELD score was r = 0.43 (P < 0.01), whilst it was higher in the subgroup of end-stage liver disease without ascites (r = 0.51,

Table 4 Arterial blood gases and pattern of breathing of 40 end-stage liver disease patients, according to presence or absence of ascites

	All patients (n = 40)	Ascites (n = 17)	Non-ascites (n = 23)	P value
pH	7.49 ± 0.04	7.50 ± 0.04	7.48 ± 0.04	0.216
PaO_2, kPa	12.3 ± 1.3	12.0 ± 1.5	12.3 ± 1.3	0.508
$PaCO_2$, kPa	4.4 ± 0.5	4.3 ± 0.5	4.5 ± 0.4	0.261
V_E, L/min	11.5 ± 3.5	12.7 ± 3.2	10.6 ± 3.5	0.065
V_T, L	0.735 ± 0.287	0.800 ± 0.255	0.686 ± 0.305	0.218
T_E, s	2.26 ± 0.70	2.25 ± 0.68	2.26 ± 0.74	0.953
T_I, s	1.64 ± 0.40	1.65 ± 0.40	1.64 ± 0.42	0.941
RR, bpm	16 ± 4	17 ± 5	16 ± 4	0.938
V_T/T_I, L/s	0.449 ± 0.129	0.496 ± 0.130	0.414 ± 0.119	0.044
T_I/T_{TOT}	0.43 ± 0.05	0.43 ± 0.06	0.42 ± 0.05	0.928

Values are obtained in a sitting position. Statistical significance tested with Student's t test between ascites and non-ascites groups. PaO_2: Arterial oxygen tension; $PaCO_2$: Arterial carbon dioxide tension; V_E: Minute ventilation; V_T: Tidal volume; T_E: Duration of expiration; T_I: Duration of inspiration; RR: Respiratory rate; bpm: Breaths per minute; T_{TOT}: Total cycle duration; V_T/T_I: Mean inspiratory flow; T_I/T_{TOT}: Duty cycle.

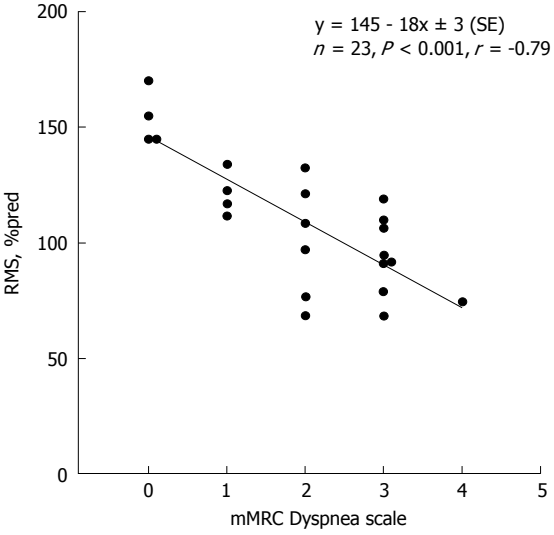

Figure 1 Relationship of respiratory muscle strength (percent of predicted) and modified medical research council dyspnea score in end-stage liver patients without ascites. Solid line: Linear regression. Linear regression equation and corresponding Pearson's correlation coefficient are shown. The slope of the line indicates that the dyspnea score increases, on average, by one modified medical research council score unit per approximately 20% decrease in respiratory muscle strength, percent of predicted (%pred). RMS: respiratory muscle strength; mMRC: modified medical research council.

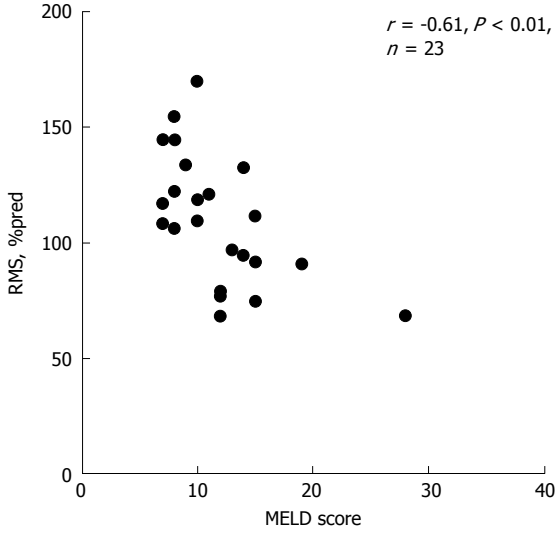

Figure 2 Relationship of respiratory muscle strength (percent of predicted) and end-stage liver disease score in end-stage liver patients without ascites. RMS: respiratory muscle strength; MELD: model for end-stage liver disease; %pred: percent of predicted.

P = 0.01). No correlation was found between mMRC score and MELD score in the ascites subgroup.

MELD score was correlated with Pemax, Pimax, and RMS (r = -0.48, P < 0.01; r = -0.48, P < 0.01; r = -0.48, P < 0.01, respectively). The latter correlations were higher in the subgroup of non-ascites patients (r = -0.64, P < 0.01; r = -0.47, P = 0.02; r = -0.61, P < 0.01, respectively) (Figure 2). MELD score was not correlated with respiratory muscle strength indices in the ascites subgroup.

DISCUSSION

The main findings of the present preliminary investigation in patients awaiting liver transplantation are: (1) most patients (88%) report a mild to severe degree of chronic dyspnea; (2) the degree of chronic dyspnea correlates well with respiratory muscle strength; and (3) respiratory muscle strength indices are weakly related to MELD score.

Most liver disease patients studied reported mild to severe chronic dyspnea (range: 0-4) (Table 3). Although chronic dyspnea is the predominant respiratory symptom in patients with liver disease[22], there are scarce reports on its prevalence and measurement. These reports show contradictory data regarding the prevalence of chronic dyspnea, ranging from 0% to 95%[3,23-26]. Nevertheless, none had assessed the severity of chronic dyspnea using such a widely accepted tool as the mMRC chronic dyspnea scale (Table 3). Thus, the prevalence and severity of chronic dyspnea may have been underestimated in previous reports.

There are also scarce data on respiratory muscle

strength. Pemax was abnormally low in 30%, Pimax in 38%, and RMS in 35% of our patients. These findings are in agreement with Hourani *et al*[3]. We have also found that the mean value for Pemax is higher than the mean value of Pimax, consistent with the findings of previous reports[3-5,7]. We also compared Pemax and Pimax in our patients, stratified according to the presence or absence of ascites (Table 3). Our findings are consistent with a few studies comparing these respiratory muscle indices before and after the removal of the ascitic fluid[8,27]. A possible explanation for the lower values of respiratory muscle strength indices in ascites compared to non-ascites group is that the former group of patients had more severe liver disease and a variable degree of mechanical compromise due to ascites *per se*. In fact, in patients undergoing continuous ambulatory peritoneal dialysis (a situation similar to ascites), Siafakas *et al*[28] and Prezant *et al*[29] reported that Pimax, measured in a sitting position, was low during this procedure and increased after the drainage of the fluid. In contrast, Duranti *et al*[8] found that large volume drainage in patients with tense cirrhotic ascites showed a lack of effect on Pimax, indicating that the cause is not solely mechanical.

Respiratory alkalosis, due to hyperventilation, is the commonest acid-base disorder in patients with chronic liver disease[30,31]. Indeed, $PaCO_2$ was decreased (< 4.7 kPa) in 85% of patients, whilst pH was increased (> 7.45) in 65% of patients in our study (Table 4). This respiratory alkalosis was due to increased V_E, V_T, and V_T/T_I (whilst T_I/T_{TOT} was normal) indicating increased inspiratory drive intensity (Table 4). Significantly, correlations were found for $PaCO_2$ with V_E and V_T/T_I, providing corroborative evidence. Furthermore, V_T/T_I was significantly higher in patients with ascites compared to those without ascites (Table 4). This result indicates that the presence of ascites and/or the progression of disease could lead to a further increase in central respiratory drive intensity. What triggers hyperventilation is yet unknown[32]. However, hyperventilation can explain in part the close correlation between chronic dyspnea and RMS in end-stage liver disease patients without ascites. Hyperventilation causes excessive use of the respiratory muscles, which may be mildly impaired. This results in neuromechanical dissociation between increased respiratory drive and respiratory muscle strength probably leading to an increased dyspnea sensation. Therefore, the combination of mild impairment of respiratory muscles and their increased use can cause dyspnea in patients with liver disease.

Correlations were found between chronic dyspnea severity and respiratory muscle strength indices (Pemax, Pimax, and RMS). In the subgroup of patients without ascites, Pemax was abnormally low in 13%, Pimax in 22%, and RMS in 22%. In this subgroup the correlations between respiratory muscle indices and mMRC score were very close, as a result of the absence of ascites (Figure 1). Our data show that patients studied could experience chronic dyspnea, which depends on RMS and not vice versa. Because it is difficult to suggest that chronic dyspnea, known to occur in liver disease patients, causes a decrease of RMS due to disuse atrophy, as increased intensity of respiratory drive tends to exercise, and hence strengthen, the respiratory muscles. Treatment with b-blockers in a subgroup of patients (n = 21; 52%) did not have any significant impact on dyspnea, respiratory muscle strength, and spirometric indices. In contrast, the presence of ascites acts as a strong confounding factor, hence the correlation between chronic dyspnea and respiratory muscle strength breaks down.

A weak correlation was found between mMRC score and MELD score. This correlation was relatively higher in the subgroup of end-stage liver disease without ascites compared to the subgroup of patients with ascites, possibly due to the above-mentioned confounding factor.

A good correlation was detected between MELD score and respiratory muscle indices only in the non-ascites subgroup (Figure 2). Only one study, by Galant *et al*[4], has shown a correlation between Pimax and MELD score. Few papers discuss the relationship between severity of liver disease as measured by Child-Pugh score and respiratory muscles strength indices[5,6].

In our study, statistical analysis showed that chronic dyspnea does not correlate with hypoxemia, ascites, anemia, and fluid retention.

Low DL_{CO} is the commonest lung function abnormality in end-stage liver disease. In our study, DL_{CO} was abnormally low in 60% of patients. This is in agreement with previous reports showing low DL_{CO} in 55% of liver transplantation candidates[3,33].

As Pimax is a predictor of weaning success from mechanical ventilation and Pemax is a predictor of effective cough[34], further investigations are needed to show whether preoperative measurement of the maximum static respiratory pressures (Pimax and Pemax) could be helpful in liver transplantation candidates.

In conclusion, a mild to severe degree of chronic dyspnea is quite common (88%) in patients with end-stage liver disease. Chronic dyspnea correlates with respiratory muscle strength in all patients. This correlation is close in the subgroup of patients without ascites. This correlation breaks down in the ascites subgroup despite the fact that these patients had weaker respiratory muscle strength. This indicates that the presence of ascites acts as a confounding factor. Respiratory muscle strength indices are weakly related to MELD score. Further studies with increased number of patients are needed to elucidate these findings.

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COMMENTS

Background

End-stage liver disease may cause mild reduction of respiratory muscle

strength and increase ventilation. The combination of both can induce various degrees of dyspnea. The cause of this increased ventilation still remains unknown.

Research frontiers

The exact prevalence and severity of chronic dyspnea in end-stage liver disease remains unknown. Additionally, no report can be found in the literature relating chronic dyspnea to respiratory muscle function in end-stage liver disease patients.

Innovations and breakthroughs

Although chronic dyspnea is the predominant respiratory symptom in patients with liver disease, there are few reports on its prevalence and measurement. These reports show contradictory data regarding the prevalence of chronic dyspnea, ranging from 0% to 95%. Nevertheless, none had assessed the severity of chronic dyspnea using such a widely accepted tool as the modified medical research council chronic dyspnea scale. Thus, the prevalence and severity of chronic dyspnea may have been underestimated in previous reports. There are also scarce data on respiratory muscles strength in end-stage liver disease patients. Measurement of Pemax and Pimax are not fully standardized. No reports in the literature describe in detail the assessment of Pemax and Pimax in liver disease patients. Furthermore, the correlation between chronic dyspnea and respiratory muscle function has not been previously reported in end-stage liver disease patients.

Applications

Further investigations are needed to show whether preoperative measurement of the maximum static respiratory pressures (Pimax and Pemax) could be helpful in the management of liver transplantation candidates.

Terminology

Maximum static expiratory (Pemax) and inspiratory (Pimax) mouth pressures are tests in which patients generate as low inspiratory and as high expiratory pressure as possible against a closed airway. These are tests of respiratory muscle function. Respiratory muscle strength is the arithmetic mean of Pemax and Pimax.

Peer review

The authors studied pulmonary functions of 40 patients with end -stage liver disease referred to a pretransplant clinic. They found that dyspnea was frequently reported by patients and was associated with respiratory muscle strength only in the patients without ascites. The paper is overall well written and provides useful information.

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BRIEF ARTICLE

Occult hepatitis B virus infection among Egyptian blood donors

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Abstract

AIM: To identify blood donors with occult hepatitis B virus (HBV) infection (OBI) to promote safe blood donation.

METHODS: Descriptive cross sectional study was conducted on 3167 blood donors negative for hepatitis B surface antigen (HBsAg), hepatitis C antibody (HCV Ab) and human immunodeficiency virus Ab. They were subjected to the detection of alanine aminotransferase (ALT) and aspartate transaminase (AST) and screening for anti-HBV core antibodies (total) by two different techniques; [Monoliza antibodies to hepatitis B core (Anti-HBc) Plus-Bio-Rad] and (ARC-HBc total-ABBOT). Positive samples were subjected to quantitative detection of antibodies to hepatitis B surface (anti-HBs) (ETI-AB-AUK-3, Dia Sorin-Italy). Serum anti-HBs titers > 10 IU/L was considered positive. Quantitative HBV DNA by real time polymerase chain reaction (PCR) (QIAGEN-Germany) with 3.8 IU/mL detection limit was estimated for blood units with negative serum anti-HBs and also for 32 whose anti-HBs serum titers were > 1000 IU/L. Also, 265 recipients were included, 34 of whom were followed up for 3-6 mo. Recipients were investigated for ALT and AST, HBV serological markers: HBsAg (ETI-MAK-4, Dia Sorin-Italy), anti-HBc, quantitative detection of anti-HBs and HBV-DNA.

RESULTS: 525/3167 (16.6%) of blood units were positive for total anti-HBc, 64% of those were anti-HBs positive. Confirmation by ARCHITECT anti-HBc assay were carried out for 498/525 anti-HBc positive samples, where 451 (90.6%) confirmed positive. Reactivity for anti-HBc was considered confirmed only if two positive results were obtained for each sample, giving an overall prevalence of 451/3167 (14.2%) for total anti-HBc. HBV DNA was quantified by real time PCR in 52/303 (17.2%) of anti-HBc positive blood donors (viral load range: 5 to 3.5 x 10^5 IU/mL) with a median of 200 IU/mL (mean: 1.8 x 10^4 ± 5.1 x 10^4 IU/mL). Anti-HBc was the only marker in 68.6% of donors. Univariate and multivariate logistic analysis for identifying risk factors associated with anti-HBc and HBV-DNA positiv-

ity among blood donors showed that age above thirty and marriage were the most significant risk factors for prediction of anti-HBc positivity with AOR 1.8 (1.4-2.4) and 1.4 (1.0-1.9) respectively. Other risk factors as gender, history of blood transfusion, diabetes mellitus, frequent injections, tattooing, previous surgery, hospitalization, Bilharziasis or positive family history of HBV or HCV infections were not found to be associated with positive anti-HBc antibodies. Among anti-HBc positive blood donors, age below thirty was the most significant risk factor for prediction of HBV-DNA positivity with AOR 3.8 (1.8-7.9). According to HBV-DNA concentration, positive samples were divided in two groups; group one with HBV-DNA ⩾ 200 IU/mL (n = 27) and group two with HBV-DNA < 200 IU/mL (n = 26). No significant difference was detected between both groups as regards mean age, gender, liver enzymes or HBV markers. Serological profiles of all followed up blood recipients showed that, all were negative for the studied HBV markers. Also, HBV DNA was not detected among studied recipients, none developed post-transfusion hepatitis (PTH) and the clinical outcome was good.

CONCLUSION: OBI is prevalent among blood donors. Nucleic acid amplification/HBV anti core screening should be considered for high risk recipients to eliminate risk of unsafe blood donation.

Key words: Hepatitis B virus; Total anti-HBc; Occult hepatitis B virus infection; Hepatitis B surface antigen; Hepatitis B virus-DNA

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INTRODUCTION

Hepatitis B virus (HBV) remains a major public health problem[1]. It is estimated that approximately 400 million people worldwide are chronically infected with HBV, where Egypt is considered as an area of intermediate endemicity[2].

Although, the incidence of transfusion-transmitted hepatitis B has been steadily reduced over the last four decades[3], HBV still remains the most frequent transfusion-transmitted viral infection[4-6]. Being the first-line of blood screening for HBV[7], different hepatitis B surface antigen (HBsAg) assays showed a range of sensitivity between < 0.1 and 0.62 ng of HBsAg per mL; 1 ng/mL corresponds to approximately 2 IU/mL[8,9], However, there is clear evidence that transmission by HBsAg-negative components occurs, in part, during the serologically negative window period, but more so during the late stages of infection[1], the later is referred to as occult HBV infection (OBI).

Occult HBV infection initially described in the late 1970 by Tabor *et al*[10] is characterized by the presence of HBV DNA in blood or tissues with undetectable HBsAg, with or without antibodies to hepatitis B core (anti-HBc) or hepatitis B surface (anti-HBs), outside the pre seroconversion window period[11,12].

Detection of OBI requires assays of the highest sensitivity and specificity with a lower limit of detection of less than 10 IU/mL for HBV DNA and < 0.1 ng/mL for HBsAg[13]. Most OBI are asymptomatic and would only be detected by systematic screening of large populations[14].No published guidelines are provided for categorizing those who should be screened for OBI[15]. Continuous progress in molecular biology techniques has led to greater recognition and diagnosis of OBI. It was reported in healthy blood donors, patients with chronic liver disease, and patients with hepatocellular carcinoma[13], in viral reactivation following immunosuppression, accidental transmission through transplantation, transfusion or experimental transmission to chimpanzees[16].

Published data reporting the infectivity of OBIs by transfusion are rare[17]. Pre-seroconversion window period (WP) infections are most likely transmit HBV but transmission from occult HBV infection remains debated[18]. In immunocompetent recipients, there is no evidence that anti-HBs-containing components (even at low titer) are infectious. Anti-HBc only, with HBV DNA, can be associated with infectivity, as can rare cases of HBV DNA without any serological HBV marker[11]. Addition of anti-HBc testing for donor screening, although will lead to rejection of a large number of donor units, will definitely eliminate HBV infected donations and help in reducing HBV transmission with its potential consequences, especially among the immunocompromised population[19].

The aim of this work is to determine the prevalence of occult HBV among Egyptian healthy blood donors and highlight the residual risks of transmitting HBV in blood banks through blood transfusion.

MATERIALS AND METHODS

A cross sectional study was undertaken including two main blood transfusion centers. Only donors negative for anti- hepatitis C antibody (HCV), anti-human immunodeficiency virus (HIV), and HBsAg were included in this study. All donors did not receive HBV vaccine. They were medically assessed and any known risk factors for viral infection listed in the questionnaire were recorded. Recipients' samples were collected before transfusion and a follow up sample was recollected whenever possible from those whose donor was found to be positive for total anti-HBc.

Hepatitis virus markers

Serum HBV total anti-HBc was performed by ELIZA technique [Monoliza Anti-hepatitis B core Plus-Bio-Rad] according to manufacturer's instructions. Sera were also investigated for alanine aminotransferase (ALT) and aspartate transaminase (AST) (Spectrum, Egypt). Anti-HBc positivity was confirmed with (ARC-Hepatitis B core total-ABBOT). Positive samples were subjected to quantitative detection of anti-HBs with commercially available kits (ETI-AB-AUK-3, Dia Sorin-Italy). Serum anti-HBs titer > 10 IU/L was considered positive. HBV DNA level was estimated mainly for blood units with baseline low or undetectable serum anti-HBs levels and also for 32 whose anti-HBs serum titers were > 1000 IU/L. All available recipients' samples as well as follow-up samples were investigated for ALT and AST HBV serological markers: HBsAg (ETI-MAK-4, Dia Sorin-Italy), anti-HBc, quantitative detection of anti-HBs as well as HBV-DNA.

Molecular assays

Real-time polymerase chain reaction quantification of HBV genome: HBV-DNA quantification by real-time polymerase chain reaction (PCR) was performed using automated system. Viral DNA was extracted from serum samples using QIAxtractor®, and VX kit as recommended by the manufacturer. QIAGEN- Germany. PCR setup was automated via QIAgility (QIAGEN, Germany). HBV real-time assays were performed in combination of Artus HBV RG PCR Kit (Artus™ GmbH, Hamburg Germany) and the Real time PCR instrument, Rotor-Gene Q (QIAGEN, Germany). Thermal profile was set according to manufacturer's guideline. Detection limit of HBV DNA in the current study assay is 3.8 IU/mL assessed by the World Health Organization (WHO) international standard (97/750)[20]. At least two negative controls, one non template control, and four standards (provided by the manufacturer) were added per run. Strict precautions were taken to avoid possible contamination. Only data that revealed no false-positive results in the negative controls and that were reproducible were used.

From the available archival sera of the recipients, AST, ALT, HBV markers as well as the HBV DNA was examined as mentioned above.

Ethical considerations

The study has been approved by the Al-Azhar University Ethical Committee and the procedures have been performed according to the World Medical Association Declaration of Helsinki, 1979; http://www.wma.net/e/policy/b3.htm. Informed, written consent at the time of sampling has been obtained from both donors and recipients.

Statistical analysis

Data entry and statistical analysis was done using SPSS under windows, version 13. χ^2 test was used in order to detect the difference in frequency for categorical variables. Fisher's exact test was used, instead of χ^2, when there was a cell in the 2 × 2 table with an expected frequency below 5. *t* test was used to assess the difference between two means of continuous variables. All tests were 2-sided and a *P* value < 0.05 was considered statistically significant. Multiple stepwise logistic analyses were done to predict the most important risk factors associated anti-HBc and HBV-DNA positivity.

RESULT

A descriptive cross sectional study was conducted on 3167 blood donors negative for HBsAg, HCV Ab and HIV Ab. The study included 491 blood donors from The National Blood Transfusion Center and 2676 blood donors as well as 265 blood recipients from the blood bank of Ain-Shams Maternity and Women's University hospital.

Anti-HBc detection in HBsAg-negative blood units

Total anti-HBc antibodies was positive in 525/3167 (16.6%) blood donors; 64% of those were positive for anti-HBs antibodies. Confirmation by ARCHITECT anti-HBc assay was carried out for 498/525 anti-HBc positive samples, where 451 (90.6%) were found positive. Reactivity for anti-HBc was considered confirmed only if two positive results were obtained, giving an overall prevalence of 451/3167 (14.2%) for total anti-HBc. The sensitivity of the assay was evaluated and 100 total anti-HBc ELIZA negative samples were retested by ARCHITECT for confirmation, three were positive, and only one showed HBV-DNA positivity by real time PCR. The prevalence of positive anti-HBc was significantly increased with increasing age (Figure 1). Other risk factors as gender, blood transfusion, diabetes mellitus, frequent injections, tattooing, previous surgery, hospitalization, Bilharziasis or positive family history of HBV or HCV infections were not found to be associated with positive anti-HBc antibodies ($P > 0.05$). Moreover, mean level of ALT and AST were found to be significantly higher among anti-HBc negative blood donors (42.7 ± 25.0 IU/L and 29.1 ± 17.1 IU/L) compared to anti-HBc positive blood donors (38.7 ± 20.0 IU/L and 26.0 ± 13.9 IU/L), respectively.

Five hundred and seventeen anti-HBc-positive samples were tested for anti-HBs and in eight cases samples were not enough to perform the assay. Anti-HBs was negative in 186 (36%) and positive in 331 (64%), where 92 (27.8%), 127 (38.4%) and 112 (33.8%) of them had anti-HBs levels of 10-99 IU/L, 100.0-999.9 IU/L and ⩾ 1000 IU/L respectively.

HBV DNA quantitation in HBsAg-negative, anti-HBc positive blood donor

HBV DNA was quantified in 303 samples with real-time PCR assay. It was detected in 52/303 (17.2 %) of anti-HBc positive blood donors. They were 88.2% males and 11.8% females with a mean age of 29.2 years (range, 20-47). Viral load range: 5 to 3.5 × 10^5 IU/mL with a

Table 1 Hepatitis B virus markers among 280 antibodies to hepatitis B core positive blood donors

Anti-HBc positive donors *n* (%)	Anti-HBc Architec	Anti-HBs	HBV-DNA
102 (36.4)	+ve	-ve	-ve
29 (10.4)	+ve	-ve	+ve
97 (34.6)	+ve	+ve	-ve
16 (5.7)	+ve	+ve	+ve
24 (8.6)	-ve	-ve	-ve
10 (3.6)	-ve	+ve	-ve
2 (0.7)	-ve	-ve	+ve

Anti-HBc: Antibodies to hepatitis B core; Anti-HBs: Antibodies to hepatitis B surface; HBV: Hepatitis B virus.

median of 200 IU/mL and a mean of $1.8 \times 10^4 \pm 5.1 \times 10^4$ IU/mL; 49% had a viral load < 200 IU/mL (low level of viremia that correlates with chronic resolving infections). According to the anti-HBs marker, the 51 occult cases could be divided into two groups: 35 subjects (68.6%) were only anti-HBc positive without detectable antibodies to the surface antigen, whereas 16 (30.8%) were positive for both anti-HBc and anti-HBs. Figure 2 shows the prevalence of HBV-DNA among blood donors with different anti-HBs levels ($P > 0.05$). All results are summarized in Figure 3.

Complete laboratory investigations were available for only 280 blood donors. Their results are summarized in Table 1.

Multivariate logistic analysis revealed that age above thirty and marriage were the most significant risk factors for prediction anti-HBc positivity among blood donors with AOR 1.8 (1.4-2.4) and 1.4 (1.0-1.9) respectively. Among anti-HBc positive blood donors, age below thirty was the most significant risk factor for prediction of HBV-DNA positivity with AOR 3.8 (1.8-7.9) (Table 2).

According to HBV-DNA concentration, positive samples were divided in two groups; group one is with HBV-DNA ⩾ 200 IU/mL (n = 27) and group two is with HBV-DNA < 200 IU/mL (n = 26). No significant difference was detected between both groups as regards to mean age, gender, liver enzymes or HBV markers (Table 3).

HBV status among recipients at baseline and follow-up

At baseline, total anti-HBc was positive in the sera of 49/265 (18.5%) recipients. Confirmation for anti-HBc by ARCHITECT revealed that 47 (95.9%) were positive. HBsAg were found positive in the sera of 6 blood recipients, 4 had also positive anti-HBc antibodies and 2 were negative. None of the recipients had HBV DNA.

It is noteworthy that getting follow-up samples from the recipients was not an easy task, mostly because they often refused to come back to give a new sample and also due to communication problems. This was reflected in lack of full characterization of the studied OBI cases. However, follow-up samples were acquired from 34 out of 216 recipients. Serological profiles of all followed up blood recipients showed that, all of them were negative for the studied HBV markers. No HBV DNA was detected among these recipients. No one developed post-transfusion hepatitis and the clinical outcome was good. It has to be mentioned that only 11/34 recipients received blood from anti-HBc positive blood donors. These 11 blood donors had total anti-HBc positive by both assays, 9 were negative for HBV-DNA (81.8%), and two were-HBV-DNA positive (18.2%); one was with HBV DNA concentration of 8 IU/mL and the other was of 3.3×10^4 IU/mL.

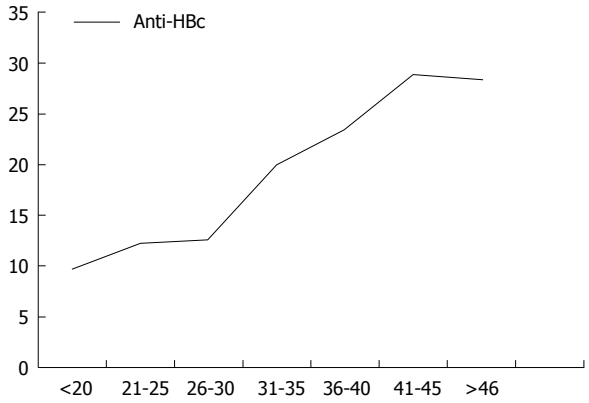

Figure 1 Prevalence of antibodies to hepatitis B core among hepatitis B surface antigen negative blood donors in different age groups. Anti-HBc: Antibodies to hepatitis B core.

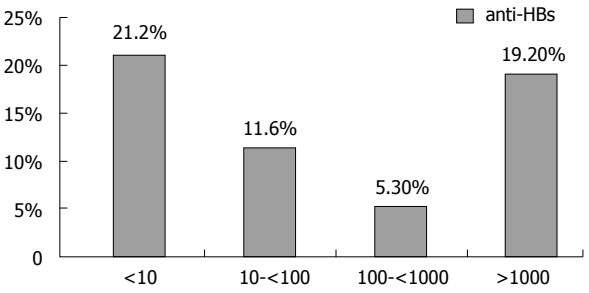

Figure 2 Prevalence of hepatitis B virus-DNA among blood donors with different antibodies to hepatitis B surface levels. Anti-HBs: Antibodies to hepatitis B surface.

DISCUSSION

Occult hepatitis B infection is one of the most challenging topics in the field of viral hepatitis. The frequency of detection of OBI is directly dependent on the sensitivity of assays of either or both HBV markers[14]; however, detection of virus specific nucleic acid does not always translate into infectivity[13]. In humans, HBV transmission was reported from donors in the window period and OBI donors showing HBV DNA load ⩽ 200 IU/mL[7, 21].

In Egypt, HBV screening in blood banks relies only on detection of HBsAg; however, introducing NAT in some major blood banks is under implementation. Discrepancies in analytical sensitivity and specificity have been recorded among commonly used EIAs for the detection of HBsAg from viruses of different genotypes[22,23]. Failure to detect HBsAg antigen and differences in signal

Table 2 Age as a risk factor for antibodies to hepatitis B core and hepatitis B virus DNA positivity among blood donors

Risk factor	*n* (%)	Crude odds ratio (95%CI)	Adjusted odds ratio Ω AOR (95%CI)
For anti-HBc +ve			
Age > 30 yr	196/853 (23.0)	2.1 (1.7-2.7)[b]	1.8 (1.4-2.4)[b]
Age ⩽ 30 yr	151/1233 (12.2)	®	®
Marital status			
Married	287/1530 (18.8)	2 (1.5-2.5)[b]	1.4 (1.0-1.9)
Single	94/898 (10.5)	®	®
For HBV-DNA+ve			
Age ⩽ 30 yr	29/95 (30.5)	3.6 (1.7-7.4)[b]	3.8 (1.8-7.9)[b]
Age > 30 yr	13/119 (10.9)	®	

[b]*P* value < 0.001, 95%CI: 95% confidence interval. ®: reference age group. Ω: Variables enter in the model of logistic analysis including age, donor site, gender, marital status, previous blood transfusion, tattooing, diabetic mellitus, previous surgery and hospitalization. Anti-HBc: Antibodies to hepatitis B core; HBV: Hepatitis B virus.

Table 3 Hepatitis B virus markers among antibodies to hepatitis B core positive blood donors in relation to hepatitis B virus DNA concentration *n* (%)

Variable	HBV-DNA ⩾ 200 (*n* = 27)	HBV-DNA < 200 (*n* = 26)	*P* value
Age (mean ± SD)	29.1 ± 6.7	29.2 ± 7.1	0.972
Gender			
Males	26 (57.8)	19 (42.2)	0.088
Females	1 (16.7)	5 (83.3)	
Marital status			
Single	7 (50.0)	7 (50.0)	0.75
Married	16 (55.2)	13 (44.8)	
Donation site			
Ain Shams Hospital	27 (54.0)	23 (46.0)	0.11
Central blood bank	0 (0.0)	3 (100.0)	
Previous blood donation	16 (64.0)	9 (36.0)	0.12
ALT (mean ± SD)	33.2 ± 14.7	34.4 ± 17.6	0.868
AST (mean ± SD)	25.5 ± 13.5	26.1 ± 10.8	0.671
Anti-HBs (mean ± SD)	156.1 ± 359.3	59.9 ± 203.8	0.634
Architect HBV			
+ve	23 (51.1)	22 (48.9)	0.99
-ve	2 (66.7)	1 (33.3)	
Anti-HBs level			
< 10	18 (51.4)	17 (48.6)	0.447
10-	5 (50.0)	5 (50.0)	
100-	0 (0.0)	1 (100)	
⩾ 1000	4 (80.0)	1 (20.0)	

ALT: Alanine aminotransferase; AST: Aspartate transaminase; Anti-HBs: Antibodies to hepatitis B surface; HBV: Hepatitis B virus.

intensity were mainly associated with mutations in the preS/S gene outside the "a" determinant[23].

In this study, the overall prevalence of total anti-HBc antibodies among HBsAg negative blood donors was 14.2% (451/3167). This is comparable to the older anti-HBc prevalence rates reported among HBsAg-negative blood donors in the Mediterranean region and Arab Peninsula; 15.03% in Greece[24] and 16.4% in Saudi Arabia[25], respectively. A previous Egyptian study, however, reported a prevalence of 10.9% in volunteer HBsAg-negative blood donors, where, HBV-DNA was detected in 11.54% of the anti-HBc positive units[26]. A more recent study among Egyptian healthy male HBsAg-negative donors showed that 80/1026 (7.8%) were reactive to anti-HBc. Of those, 5 (6.25%) had HBV-DNA as detected by real-time polymerase chain[27].

It was shown that low levels of viremia are detectable in 1.6% to 38% of HBsAg-negative/anti-HBc-positive donors when highly sensitive techniques for the detection of HBV-DNA were used[28-30]. Using a very sensitive real time PCR assay, 52/302 (17.2%) (95%CI: 14.7%-19.7%) anti-HBc positive blood donors were positive for HBV-DNA. This relatively high prevalence could be attributed to the high sensitivity of the assay. Furthermore, among 165 anti-HBs negative donors (all anti-HBs negative donors except 23) who were assayed for HBV-DNA by real time PCR, 35 (21.2%) tested positive.

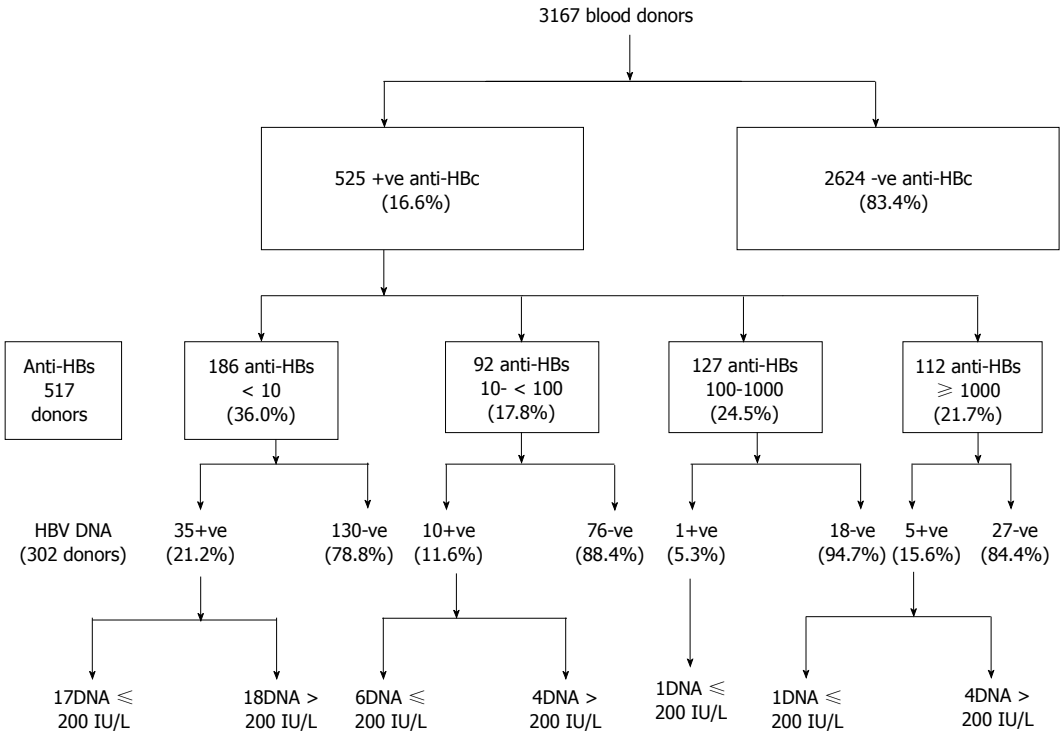

Figure 3 Results of antibodies to hepatitis B core, antibodies to hepatitis B surface and hepatitis B virus DNA among the studied blood donors. Anti-HBc: Antibodies to hepatitis B core; Anti-HBs: Antibodies to hepatitis B surface; HBV: Hepatitis B virus.

Thus it could be estimated that 2.8% of blood donor units have been viremic and otherwise transferable (525 anti-HBc-positives out of 3167 tested donors times 451 confirmed anti-HBc-positive out of 498 anti-HBc-initially reactive times 45 HBV-DNA-positive out of 244 confirmed anti-HBs positive tested for viremia). This equals to the release of one unit containing HBV-DNA in 36 donations or 27 in one thousand units eligible for transfusion.

It was previously shown that approximately 90% of blood donors carrying anti-HBc also carried anti-HBs signaling recovered HBV infection[31]. The remaining 10% were termed anti-HBc alone, the significance of which was extensively studied by many authors. It may be in part false positive anti-HBc due to poor assay specificity or true anti-HBc[32] that may originate either from recovered infections having lost detectable anti-HBs or from late stage chronic infections having lost detectable HBsAg[7].

In the current study, 17% of the HBsAg negative, anti-HBc positive blood donors were HBV-DNA positive with 6% also anti-HBs positive (Table 1). On the other hand, only 38% of anti-HBc positive donors who were HBV-DNA negative had anti-HBs denoting past infection. An earlier study similarly showed that up to 16% of anti-HBc/anti-HBs-positive donors have circulating HBV-DNA unbound to anti-HBs in their sera and thus in a potentially infective form[28]. Also, these results are in accordance with studies of Brojer *et al*[1], Candotti *et al*[33] and Katsoulidou *et al*[12] where, it was found that nearly 50% of OBI are asymptomatic, apparently healthy, blood donors carries anti-HBs. Satake *et al*[34] in Japan emphasized that no evidence of HBV infections was found when donations contained both anti-HBc and anti-HBs. Experiments in chimpanzees showed no HBV infection in animals transfused with blood from three anti-HBs-positive human plasmas, despite exposure to an HBV DNA dose known to be infectious in the absence of anti-HBs[35]. However, Levicnik-Stezinar *et al*[17] identified a case report of an OBI carrier who transmitted HBV to two immunocompetent transfusion recipients despite anti-HBs and concluded that the neutralizing capacity of low-level anti-HBs is limited and reinforced the validity of considering anti-HBs below 100 IU/L to be poorly protective from infectivity when HBV DNA is present.

A recent study conducted by Launay *et al*[36] showed that a small proportion of patients with "anti-HBc alone" have high viral loads, revealing the occurrence of infection with HBV mutants that escape detection even by multivalent HBsAg assays. Only 24 (8.6 %) donors' samples were false positive in our study, while 102 (36%) were "anti-HBc alone" positive by both evaluated assays (Table 1). A Lebanese study demonstrated that 56 out of 2505 (22%) blood donors screened for HBV markers were "anti-HBc alone" positive[37].

HBV nucleic acid amplification testing (NAT) is effective in reducing the risk of HBV transmission if performed on individual donations; nevertheless, the costs of such a strategy could be prohibitive until multiplex NAT testing for blood-borne viruses is available everywhere[38]. In addition, it was previously emphasized that the marginal yield of HBV NAT does not justify its implementation in routine screening of blood donors.

Thus, anti-HBc assays represent a second safeguard that may further reduces the need for HBV NAT implementation[39].

Different risk factors are known to be associated with OBI including detectable HBV DNA, anti-HBc, antibodies to HBsAg < 100 IU/mL, young age and male gender. On the other hand, a study by Minuk *et al*[40], demonstrated that age, gender and liver biochemistry findings do not identify those with OBI. The current study does not show an association between any of the former risk factors and OBI except for age below thirty which was the most significant risk factor for prediction of HBV-DNA positivity among anti-HBc positive blood donors (AOR 3.8; 1.8-7.9). This is recently reported by Allain *et al*[41] who found that OBI donors are generally older than 45 years except in Africa, carry very low viral load (median 11-25 IU/mL) and have normal alanine transaminase levels.

A recent study conducted in Egypt showed that HBV transmission is community rather than iatrogenic-acquired[42]. In several studies, low educational attainment had been associated with higher prevalence of hepatitis B in both developed and developing countries[43,44].

It is noteworthy, in this study, that only one subject was HBV-DNA positive when 100 HBV markers' negative blood donors were evaluated. Minuk *et al*[40] studied the prevalence of occult HBV in 487 HBsAg negative sera with/without serologic evidence of previous HBV infection. They concluded that the prevalence of OBI was 18% in those with serologic evidence of previous HBV infection and 8.1% in HBV seronegative individuals. HBsAg diagnostic failure and low viral replication in OBI carriers could possibly be attributed to multiple changes in envelope and polymerase regions, respectively[12]. Inhibition of HBsAg mRNA production and export are potential mechanisms of OBI occurrence[41].

The use of HBV anti core testing to eliminate the residual transfusion risk of transmission of HBV has not been evaluated in Egypt. The results of this study proved that anti-HBc testing would cause the exclusion of a consistent number of donors, more than 80% of whom are HBV-DNA negative. It was also shown that nearly half of anti core positive blood donors were also positive for HBs antibodies. A study conducted in Italy by Manzini *et al*[38] detected HBV-DNA among donors with an anti-HBs titer > 100 IU/L. They raise doubt about whether high titer anti-HBs blood may guarantee against HBV transmission, where countries such as Germany, Austria and Japan allow transfusion of units with anti-HBs titers higher than 100 IU/L[38].

Allain *et al*[11] reported that average HBV-DNA detection rates of 7% and 13% were observed in anti-HBc positive subjects with or without anti-HBs respectively, and in blood donors the rates ranged from 0% to 17%. Cases carrying anti-HBc alone are more infectious than those with low level of anti-HBs[41].

As for other viral infections, HBV infectivity depends on three main factors: the infectious dose, the level of immune complexing by neutralizing anti-HBs, and the immunocompetence of the host[45]. It is of note that proper donor selection, using a highly sensitive assay to rule out suspicious blood units together with application of quality control measures will aid in blood safety. In addition, it is important to measure the incidence rate of HBV infections in donor groups and to determine the major risk factors that cause an HBV infection in each country[46].

In 2008, an expert report meeting introduced a cutoff value for serum HBV DNA < 200 IU/mL for OBI[47]. In the current study, nearly half of HBV DNA viremic blood donors (27/53) were having DNA level ⩾ 200 IU/mL. This could be possibly attributed to escape mutations which could have altered the target epitope(s) of the HBsAg assay[48]. A recent study of OBI in Asian blood donors showed a viral load range between unquantifiable and 3670 IU/mL with a median 11 IU/mL[49]. In humans, HBV transmission was reported from donors in the window period and OBI donors showing HBV DNA load < 20 IU/mL[7,34,50].

There is preliminary evidence that immunocompromised patients are not only more susceptible to lower infectious dose in the presence of anti-HBs but also at higher risk of developing chronic infection[51]. The prevalence of occult HBV in children and adolescents with haematological disorders and malignancies was 21%, with a significantly increased frequency of HBV-DNA in the HBsAg negative (HCV-RNA positive-63.2%) compared with patients negative for HCV-RNA (25%)[52].

In the current study, none of the followed up recipients showed HBV infection when their sera were examined for HBV markers/HBV DNA within a period of 3-6 mo and none seroconverted to HBsAg positivity. These results are comparable to a recent Taiwanese study[53]. Tani *et al*[54] identified one case of post-transfusion HBV infection (rate, 0·0004675; 95%CI for the risk of transmission, 1 in 451-41 841). The background rates of HBV, infections in patients prior to transfusion was 3·4% (72/2139), Sixty-four anti-HBc- and/or anti-HBs-reactive blood components were transfused to 52 patients non-reactive for anti-HBc or anti-HBs before and after transfusion (rate, 0; 95%CI for the risk of transmission, < 1 in 22).

As confirmed by Prati *et al*[55], nosocomial sources should be carefully excluded before speculating that blood donors with OBI were involved in viral transmission. A decrease of HBV infection incidence was observed following HBV vaccine implementation in many countries with moderate to high HBV endemicity[56,57].

In conclusion, on the basis of available data, our findings proved that OBI exists among Egyptian blood donors. Thorough understanding of the infectivity of OBI in immunocompetent and immunosuppressed recipients and molecular characterization by sequencing is strongly indicated. New screening policy to further increase the safety of blood transfusion and exclude all

HBV DNA-positive donations has to be thoroughly evaluated. The cost effectiveness of eliminating potentially infectious donors with OBI by further assays is a major concern and has to be investigated.

The study, however, has some limitations. The post-transfusion follow-up schedule was not adequately fulfilled, as the sample size of recipients was small. It is of note that iatrogenic sources of infection have to be excluded by adequate donor follow-up together with pre- and post-transfusion testing of recipients. Molecular analysis by sequencing of virus infecting both donor and recipient is required for confirmation of transmission and consequence infectivity. HBV-anti core screening would possibly eliminate potentially infectious blood donations. Nucleic acid amplification should be considered as the primary screening method for high risk recipients. The provided data encourage further studies aimed at preventing HBV spread where specific management strategy for OBI should be implemented.

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COMMENTS

Background

Among the important clinical impacts of occult hepatitis B virus (HBV) infection (OBI) is transmission of HBV from OBI donors to recipients. Proper diagnosis will promote safety blood donation especially for high risk recipients.

Research frontiers

Hepatitis is a major worldwide public health problem. World Health Organization reports considered Egypt as an area of high hepatitis C virus (HCV) prevalence but of intermediate prevalence for HBV. The question is how a single environment like Egyptian environment promotes infection with HCV but not HBV, both have the same route of transmission, even though, the concentration of HBV in serum is much higher than that of HCV. Occult HBV infection has been reported in several clinical entities. Therefore, we conducted a series of researches to study the situation of occult HBV infection in Egypt (Chronic liver disease patients, polytransfused children and renal dialysis patients), and we come to conclusion that occult HBV do exist in our community. Blood donors with OBI have the risk of HBV transmission to their corresponding recipients. HBV transmission was previously reported from OBI donors who had circulating HBV DNA at a low level. Thus this study was carried out to identify apparently healthy blood donors with OBI in order to promote safe blood donation. The possibility of transmission of such infection through blood donation was also evaluated.

Innovations and breakthroughs

Among those who were positive for anti-HBs. Detection of HBV DNA does not always translated into infectivity.

Applications

The cost effectiveness of eliminating potentially infectious donors with OBI by further assays is a major concern and has to be investigated. New screening policy to further increase the safety of blood transfusion and exclude all HBV DNA-positive donations has to be thoroughly evaluated. Thorough understanding of the infectivity of OBI in immunocompetent and immunosuppressed recipients and molecular characterization by sequencing is strongly indicated.

Peer review

The authors addressed an interesting topic "Occult hepatitis B virus infection among egyptian blood donors". The manuscript is well written, and the manuscript is acceptable for publication.

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BRIEF ARTICLE

Significance of serum leptin and adiponectin levels in Egyptian patients with chronic hepatitis C virus associated hepatic steatosis and fibrosis

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Abstract

AIM: To study serum levels of leptin and adiponectin in patients with chronic hepatitis C virus infection genotype-4 (HCV-4) related steatosis and fibrosis.

METHODS: We prospectively studied 45 untreated men with chronic HCV-4, with proven steatosis (group Ⅰ, 30 patients), and fibrosis (group Ⅱ, 15 patients), on liver biopsy. In addition, 15 healthy men (group Ⅲ), matched for age, and body mass index were included. However, we excluded another five patients with steatohepatitis, and six patients with cirrhosis. We measured total serum leptin and adiponectin levels, as potential predictors for liver steatosis and fibrosis. Also, a correlation between these adipokines and various clinical and laboratory data were evaluated. All subjects were selected from Tropical and Internal medicine departments, Menoufiya University Hospital, Menoufiya, Egypt, during the period from February 2010 to August 2011.

RESULTS: In group Ⅰ, severity of hepatic steatosis was mild, moderate, and severe, in 19 patients (63.5%), 8 patients (26.5%), and 3 patients (10%), respectively. In contrast, in group Ⅱ, hepatic fibrosis was found to be in stage 1, 2, and 3, in 6 patients (40%), in 6 patients (40%), and in 3 patients (20%), respectively. On comparing group Ⅰ with group Ⅱ, there was a significant decrease in serum adiponectin levels (131.4 ± 7.91 pg/mL *vs* 436 ± 9.75 pg/mL, $P < 0.001$), while there was no significant difference between both groups regarding serum leptin levels (34.69 ± 7.69 ng/mL *vs* 35.17 ± 1.06 ng/mL, $P > 0.05$). However, in the same group, when compared with group Ⅲ, there was a significant increase in serum leptin levels (34.69 ± 7.69 ng/mL *vs* 10.69 ± 0.84 ng/mL, $P < 0.001$), while there was a significant decrease in serum adiponectin levels (131.4 ± 7.91 pg/mL *vs* 342.4 ± 44.48 pg/mL, $P < 0.001$). In contrast, in group Ⅱ, when compared with group Ⅲ, there was a significant increase in serum leptin and adiponectin levels (35.17 ± 1.06 ng/mL *vs* 10.69 ± 0.84 ng/mL, $P < 0.001$, and 436 ± 9.75 pg /mL *vs* 342.4 ± 44.48 pg/mL, $P < 0.05$, respectively), while there was no significant difference between both groups regarding serum creatinine (0.83 ± 0.34 *vs* 0.89 ± 0.24, $P > 0.05$). On the other hand, serum leptin was not correlated with serum adiponectin in group Ⅰ and in group Ⅱ ($r = 0.09$, $P > 0.05$, and $r = -0.1$, $P > 0.05$, respectively). However, serum adiponectin was significantly negatively correlated with serum aspartate transaminase in group Ⅰ, but no correlation detected in group Ⅱ ($r = -0.39$, $P > 0.05$, and $r = -0.03$, $P > 0.05$).

CONCLUSION: In male patients with chronic HCV-4, serum adiponectin levels are elevated in hepatic fibro-

sis, but decreased in steatosis. Therefore, in contrast to leptin, adiponectin may be used as a non-invasive marker.

Key words: Leptin; Adiponectin; Hepatitis C virus; Hepatic steatosis; Hepatic fibrosis

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INTRODUCTION

Leptin and adiponectin are the main metabolic products of adipose tissue. The former is expressed also in the stomach, placenta and mammary gland, while the latter is also secreted by hepatocytes[1,2].

Currently, there is an increasing interest in the role of these adipokines in the development of hepatic steatosis, and fibrosis, particularly in patients with nonalcoholic fatty liver disease (NAFLD) and chronic hepatitis C virus (HCV) infection[3].

Chronic HCV genotype-4 (HCV-4) is known to be endemic in Egypt, Central Africa and in the Middle East[4]. However, several recent studies carried out in Europe have indicated changes in genotype distribution and have underlined the increasing prevalence of HCV-4[5,6]. There are controversial data about the relationship between serum leptin levels and HCV-related steatosis[7,8].

The role of leptin in hepatic fibrosis is also less clear[9]. Moreover, the levels of adiponectin in patients with different stages of liver diseases, particularly in those with NAFLD and chronic HCV (especially genotype 4) infection, have been partly unraveled[10,11].

Therefore, the aim of this study was to measure serum leptin and adiponectin levels, as potential predictors of liver steatosis and fibrosis, for use in clinical practice, in patients with chronic HCV-4 infection, associated steatosis and fibrosis. Moreover, a correlation between these adipokines and different clinical and laboratory data were evaluated.

MATERIALS AND METHODS

Patients and study design

A total of 45 (30 with hepatic steatosis, and 15 with hepatic fibrosis) untreated Egyptian male patients with chronic HCV-4, who had undergone liver biopsy, were prospectively included in this study. We excluded another five patients with steatohepatitis, and six patients with cirrhosis, on liver biopsy. Patients were selected from Tropical and Internal medicine departments, Menoufiya University Hospital, Egypt, during the period from February 2010 to August 2011.

In addition, a control group comprised 15 healthy males matched for age and body mass index (BMI), from the same hospital. They were considered healthy on the basis of history, physical examination and laboratory tests. None received any medication and had normal liver enzymes and no clinical, laboratory or imaging evidence of liver disease.

We excluded all female patients, those with chronic HCV non-genotype 4, steatohepatitis or cirrhosis on liver biopsy, other causes of chronic liver disease (hepatitis B virus infection, alcoholism, Wilson's disease, haemochromatosis, and autoimmune hepatitis), seropositivity for anti-human immunodeficiency virus, evidence of cirrhosis or hepatocellular carcinoma, decompensated liver disease (evidence of ascites, variceal bleeding, or hepatic encephalopathy), history of heart failure, diabetes mellitus, thyroid diseases, abnormal renal function, obesity (*i.e.*, BMI $\geqslant$ 30), previous treatment with metformin, a thiazolidinedione, or interferon-based antiviral therapy, and use of drugs known to induce liver steatosis (corticosteroids, amiodarone, tamoxifen, valproic acid) within the last 6 mo.

Chronic HCV was defined as a positive serological test for HCV by at least a fourth-generation enzyme-linked immunosorbent assay (ELISA), positive HCV RNA results by polymerase chain reaction assay and compatible liver biopsy[9].

Clinical and laboratory data

A complete medical history was taken and physical examination carried out in all patients and controls. BMI was calculated according to the following equation: BMI = weight (in kilograms)/height^2 (in meters). Overweight was defined as a BMI of 25-29.9 kg/m^2, and obesity was defined as a BMI $\geqslant$ 30 kg/m^2[12].

Laboratory investigations included: liver function tests [serum aspartate transaminase (AST), alanine transaminase (ALT), prothrombin activity, serum proteins and albumin, total serum bilirubin and alkaline phosphatase (ALP)] kidney function tests (serum urea and creatinine), total serum leptin and adiponectin levels.

Sample collection and assay

After gaining the consent of all subjects studied, 10 mL of venous blood was withdrawn from all subjects after fasting for at least 10 h. 1.8 mL whole blood was added to 0.2 mL sodium citrate, then centrifuged at 4000 *g* for 5 min, then plasma was used for measuring prothrombin concentration using Fibrintimer Ⅱ instrument of Behring, Germany Using Sysmex K-21, Japan.

7 mL of venous blood was transferred slowly into a plain tube, allowed to clot, and then centrifuged for ten minutes. The clear supernatant was separated in several aliquots, kept frozen at -20 ℃, until analysis of the fol-

lowing: kinetic determination of ALT and AST[13], serum total bilirubin by a timed endpoint Diazo method[14]; serum albumin, using a method of enhanced specificity of bromcresol purple for albumin[15]; serum ALP activity by a kinetic method using a 2-amino-2-methyl-1- propanol buffer[16]; serum total protein by using the modified method of Biuret reaction[17]; colorimetric kinetic determination of serum creatinine, and colorimetric determination of serum urea[18].

Leptin and adiponectin assay

Serum leptin levels were determined by a solid phase ELISA based on the sandwich principle. The microlitre wells were coated with a monoclonal antibody directed towards a unique antigenic site on the leptin molecule. An aliquot of patient sample containing endogenous leptin was incubated in the coated well with a specific rabbit anti-leptin antibody. A sandwich complex was formed. After incubation the unbound material was washed off and anti-rabbit peroxidase conjugate was added for detection of bound leptin, the substrate solution was added and the intensity of color obtained was proportional to the concentration of leptin in the patient sample. (BioSource Europe S.A. 8 B-1400 Nivelles Belgium)[19]. For leptin, the intra-assay coefficient of variation (CV) was 6.91%, while inter-assay CV was 8.66%.

Regarding serum adiponectin levels, they were estimated by Human Adiponectin ELISA kits. This assay employed an antibody specific for human adiponectin coated on a 96-well plate. Standards, samples and biotinylated anti-human adiponectin were pipetted into the wells. Then, adiponectin was captured by the antibody immobilized to the wells and by the biotinylated adiponectin-specific detection antibody. After washing away unbound biotinylated antibody, horse radish peroxidase-conjugated streptavidin was pipetted into the wells. The wells were washed again. Following this second wash step, tetramethylbenzidine substrate solution was added to the wells, resulting in color development proportional to the amount of adiponectin bound. The Stop Solution changed the color from blue to yellow, and the intensity of the color was measured at 450 nm (FIN-00790 Helsinki, Finland)[20]. For adiponectin, the intra-assay CV was > 10%, while inter-assay CV was < 12%.

The study was approved by the Ethical Committee of our hospital. All patients and control subjects gave their verbal informed consent, and consented to the use of clinical data and serum for research purposes.

Liver histological assessment

The degree of hepatic fibrosis was assessed according to the modified Knodell scoring system[21]. Steatosis was identified and graded according to the histopathological criteria described by Burnt *et al*[22]. Based on the percentage of hepatocytes containing fat droplets, steatosis was graded as mild (< 33% of hepatocytes affected), moderate (33%-66% of hepatocytes affected) and severe (> 66% of hepatocytes affected)[22].

Statistical analysis

Results were collected, tabulated and statistically analyzed using an IBM personal computer and statistical package SPSS version 16 (SPSS Inc. Chicago, Illinois, United States). Student's *t* test was used for comparison between two groups having quantitative variables. analysis of variance (*F*) test was used for comparison among three groups having quantitative variables. Pearson correlation (*r*) was used to detect association between quantitative variables. χ^2 test to compare the qualitative data between different groups. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

In group Ⅰ, severity of hepatic steatosis was mild, moderate, and severe, in 19 patients (63.5%), 8 patients (26.5%), and 3 patients (10%), respectively. In contrast, in group Ⅱ, hepatic fibrosis was found to be in stage 1, 2, and 3, in 6 patients (40%), in 6 patients (40%), and in 3 patients (20%), respectively.

Table 1 shows the clinical and laboratory data of patients and controls studied. There was no significant difference between all groups studied regarding age and BMI ($P > 0.05$). In group Ⅰ, when compared with group Ⅱ, there was a significant increase in serum AST, prothrombin activity (PT), and albumin ($P < 0.001$), while, there was a significant decrease in serum total bilirubin, and serum adiponectin ($P < 0.001$, for each). There was no significant difference, between both groups regarding serum ALT, proteins, ALP, blood urea, serum creatinine, and leptin levels ($P > 0.05$). In contrast, for group Ⅰ, when compared with group Ⅲ, there was a significant increase in serum AST, ALT, total bilirubin, ALP, blood urea, and serum leptin levels ($P < 0.001$, 0.001, 0.001, 0.001, 0.05, 0.001, respectively), while, there was a significant decrease in serum PT, proteins, and serum adiponectin ($P < 0.001$, for each). There was no significant difference, between both groups regarding serum albumin, and serum creatinine levels ($P > 0.05$). Finally, in group Ⅱ, when compared with group Ⅲ, there was a significant increase in serum AST, ALT, total bilirubin, ALP, blood urea, serum leptin and adiponectin levels ($P < 0.001$, 0.001, 0.001, 0.001, 0.05, 0.001, 0.05, respectively), while, there was a significant decrease in serum PT, proteins, and albumin ($P < 0.001$, for each). There was no significant difference, between both groups regarding serum creatinine ($P > 0.05$).

Table 2 shows the correlation between serum leptin and other parameters in the patients studied. In group Ⅰ, there was a significant negative correlation between serum leptin levels, and serum AST, ALT, albumin, ALP, and creatinine ($r = -0.78, -0.39, -0.37, -0.70, -0.38$, and $P < 0.001$, 0.05, 0.05, 0.001, 0.05, respectively). However, there was a significant positive correlation with BMI, as well as serum total bilirubin ($r = 0.43$, 0.64, and $P < 0.05$, $P < 0.001$, respectively). There was no significant correlation detected with age, PT, serum

Table 1 Clinical and laboratory data of patients studied and controls (mean ± SD)

Parameter	Group Ⅰ (*n* = 30)	Group Ⅱ (*n* = 15)	Group Ⅲ (*n* = 15)	*F* test	*P* value
Age (yr)	39.87 ± 6.1	38.20 ± 6.09	39.40 ± 4.56	0.52	*P* > 0.05 NS
BMI (kg/m^2)	29.33 ± 1.92	28.20 ± 3.05	27.73 ± 1.62	1.71	*P* > 0.05 NS
AST (U/L)	62.40 ± 23.03	39.00 ± 8.7	27.00 ± 5.35	32.63	*P*1 < 0.001[1]
					*P*2 < 0.001[1]
					*P*3 < 0.001[1]
ALT (U/L)	42.60 ± 16.31	35.33 ± 7.67	25.67 ± 5.99	16.42	*P*1 > 0.05 NS
					*P*2 < 0.001[1]
					*P*3 < 0.001[1]
PT (%)	71.87 ± 8.92	61.33 ± 19.22	89.53 ± 5.85	22.04	*P*1 < 0.001[1]
					*P*2 < 0.001[1]
					*P*3 < 0.001[1]
Protein (gm/dL)	5.40 ± 1.03	5.00 ± 0.85	7.13 ± 0.88	5.59	*P*1 > 0.05 NS
					*P*2 < 0.001[1]
					*P*3 < 0.001[1]
Albumin (gm/dL)	2.46 ± 0.47	2.37 ± 0.24	4.35 ± 0.28	33.14	*P*1 < 0.001[1]
					*P*2 > 0.05 NS
					*P*3 < 0.001[1]
Total bilirubin (mg/dL)	1.30 ± 0.92	1.90 ± 0.97	0.53 ± 0.35	20.85	*P*1 < 0.001
					*P*2 < 0.001
					*P*3 < 0.001
ALP (U/L)	7.21 ± 3.99	4.30 ± 1.08	2.4 ± 0.82	24.97	*P*1 > 0.05 NS
					*P*2 < 0.001[1]
					*P*3 < 0.001
Urea (mg/dL)	28.2 0± 8.49	28.33 ± 6.45	23.27 ± 4.86	2.65	*P*1 > 0.05 NS
					*P*2 < 0.05[1]
					*P*3 < 0.05[1]
Creatinine (mg/dL)	1.10 ± 0.36	0.83 ± 0.34	0.89 ± 0.24	3.77	*P*1 > 0.05 NS
					*P*2 > 0.05 NS
					*P*3 > 0.05 NS
Leptin (ng/mL)	34.69 ± 7.69	35.17 ± 1.06	10.69 ± 0.84	107.28	*P*1 > 0.05 NS
					*P*2 < 0.001[1]
					*P*3 < 0.001[1]
Adiponectin (pg/mL)	131.40 ± 7.91	436.00 ± 9.75	342.40 ± 44.48	374.77	*P*1 < 0.001[1]
					*P*2 < 0.001[1]
					*P*3 < 0.05[1]

[1]Significant. Group Ⅰ: Patients with hepatic steatosis; Group Ⅱ: Patients with hepatic fibrosis; Group Ⅲ: Controls. *P*1: Comparison between group Ⅰ and group Ⅱ; *P*2: Comparison between group I and group Ⅲ; *P*3: Comparison between group Ⅱ and group Ⅲ. NS: Non significant; BMI: Body mass index; AST: Aspartate transaminase; ALT: Alanine transaminase; PT: Prothrombin activity; ALP: Alkaline phosphatase.

proteins, blood urea, and serum adiponectin (r = -0.24, 0.11, -0.28, -0.25, 0.09, and $P > 0.05$ for each). In group Ⅱ, there was a significant negative correlation between serum leptin levels, and age, serum AST, albumin, and ALP (r = -0.77, -0.99, -0.80, and -0.95, and $P < 0.001$ for each, respectively). However, there was a significant positive correlation with BMI, PT, serum total bilirubin, blood urea, and serum creatinine (r = 0.94, 0.93, 0.98, 0.95, 0.97, and $P < 0.001$ for each). There was no significant correlation detected with ALT, serum proteins, and adiponectin (r = -0.51, -0.93, -0.1 and $P > 0.05$ for each).

Table 3 shows the correlation between serum adiponectin and other parameters in the patients studied. In group Ⅰ, there was a significant negative correlation between serum adiponectin levels, and AST (r = -0.39, and $P < 0.05$). However, there was no significant correlation with all other parameters studied ($P > 0.05$, for each). In group Ⅱ, there was a significant negative correlation between serum adiponectin levels, and age (r = -0.55, and $P < 0.05$). However, there was a significant positive correlation with serum ALT, proteins, and albumin (r = 0.91, 0.95, 0.68 and $P < 0.001$ for each). There was no significant correlation detected with BMI, serum AST, PT, total bilirubin, ALP, blood urea, and serum creatinine (r = -0.45, 0.03, -0.46, 0.09, 0.41, -0.39, -0.35 and $P > 0.05$ for each).

DISCUSSION

The behavior of leptin concentrations in the course of liver disease due to HCV infection is still under investigation[23].

The relationship between leptin and hepatic fibrosis is controversial. Studies *in vitro* have clearly demonstrated a role in profibrogenic responses within the liver[24,25]. However, human studies describing the role of leptin in fibrosis are less convincing[26-28].

We found that serum leptin levels were significantly associated with fibrosis in patients with chronic HCV-4 infection. This is in agreement with many previous studies[23,29,30]. In contrast, several other studies have shown

Table 2 Correlation between serum leptin and other parameters in patients studied

Parameter	Group Ⅰ (n = 30)		Group Ⅱ (n = 15)	
	r	P value	r	P value
Age (yr)	-0.24	> 0.05 NS	-0.77	< 0.001[1]
BMI (kg/m^2)	0.43	< 0.05[1]	0.94	< 0.001[1]
AST (U/L)	-0.78	< 0.001[1]	-0.99	< 0.001[1]
ALT (U/L)	-0.39	< 0.05[1]	-0.51	> 0.05 NS
PT (%)	0.11	> 0.05 NS	0.93	< 0.001[1]
Protein (gm/dL)	-0.28	> 0.05 NS	-0.39	> 0.05 NS
Albumin (gm/dL)	-0.37	< 0.05[1]	-0.80	< 0.001[1]
Total bilirubin (mg/dL)	0.64	< 0.001[1]	0.98	< 0.001[1]
ALP (U/L)	-0.70	< 0.001[1]	-0.95	< 0.001[1]
Urea (mg/dL)	-0.25	> 0.05 NS	0.95	< 0.001[1]
Creatinine (mg/dL)	-0.38	< 0.05[1]	0.97	< 0.001[1]
Adiponectin (pg/mL)	0.09	> 0.05 NS	-0.10	> 0.05 NS

[1]Significant. Group Ⅰ: Patients with hepatic steatosis; Group Ⅱ: Patients with hepatic fibrosis. NS: Non significant; BMI: Body mass index; AST: Aspartate transaminase; ALT: Alanine aminotransferase; PT: Prothrombin activity; ALP: Alkaline phosphatase.

Table 3 Correlation between serum adiponectin and other parameters in patients studied

Parameter	Group Ⅰ (n = 30)		Group Ⅱ (n = 15)	
	r	P value	r	P value
Age (yr)	-0.09	> 0.05 NS	-0.55	< 0.05[1]
BMI (kg/m^2)	-0.24	> 0.05 NS	-0.45	> 0.05 NS
AST (U/L)	-0.39	< 0.05[1]	0.03	> 0.05 NS
ALT (U/L)	-0.15	> 0.05 NS	0.91	< 0.001[1]
PT (%)	-0.35	> 0.05 NS	-0.46	> 0.05 NS
Protein (gm/dL)	-0.09	> 0.05 NS	0.95	< 0.001[1]
Albumin (gm/dL)	-0.17	> 0.05 NS	0.68	< 0.001[1]
T.Bilirubin (mg/dL)	-0.16	> 0.05 NS	0.09	> 0.05 NS
ALP (U/L)	0.04	> 0.05 NS	0.41	> 0.05 NS
Urea (mg/dL)	-0.08	> 0.05 NS	-0.39	> 0.05 NS
Creatinine (mg/dL)	-0.10	> 0.05 NS	-0.35	> 0.05 NS

[1]Significant. Group Ⅰ: Patients with hepatic steatosis; Group Ⅱ: Patients with hepatic fibrosis. NS: Non significant; BMI: Body mass index; AST: Aspartate transaminase; ALT: Alanine aminotransferase; PT: Prothrombin activity; ALP: Alkaline phosphatase.

no association between serum leptin and fibrosis in HCV infection[31-33]. The reason for this discrepancy is not clear. It is possible that leptin levels intrahepatically, rather than in the serum, are more important determinants of hepatic fibrosis[9].

Meanwhile, we found that serum leptin was increased in patients with steatosis. In line with our work, patients with NAFLD[8,26], and alcoholic liver disease have increased levels of circulating leptin in their bodies[34].

In the present study, we observed that serum leptin levels correlated with BMI in our overweight HCV-4 patients. This is in accordance with Myers RP, study[9].

Hepatic steatosis is defined as excessive lipid accumulation within the hepatocyte cytoplasm. The prevalence of steatosis among different HCV genotypes is quite variable[35].

In the current study, hepatic steatosis was detected in about half of our patients with chronic HCV-4 associated steatosis, with predominance of mild steatosis in 63.5% of them. These findings are in accordance with other, similar, studies conducted by El-Zayadi *et al*[36], and Tsochatzis *et al*[37]. The mechanisms underlying the development of parenchymal steatosis in HCV infection are not precisely known[38].

Regarding serum adiponectin, we found that it was significantly elevated in patients with HCV-4 associated hepatic fibrosis versus controls. This coincides with other studies[39,40].

In contrast, the results of our study indicate that serum adiponectin was decreased with the presence of steatosis in patients with chronic HCV-4 infection. This is in accordance with other studies conducted in patients with HCV-4[41,42] and HCV of different genotypes[43]. However, Tiftikci *et al*[40], found an increase in serum adiponectin levels in patients (about 57% were females) with HCV (mostly with genotype 1). Also, Kara *et al*[44], found that serum adiponectin levels in HCV genotype 1 were similar to healthy control subjects. This difference might be due to the effect of gender and different HCV genotypes. In addition, circulating adiponectin concentrations may also be affected by renal clearance, as adiponectin levels are elevated in states characterized

by impaired renal function, such as macroalbuminuria[45].

We include only male patients in our study for two reasons: firstly, it is known that serum leptin and adiponectin levels are higher in females than males[27,46]; secondly, to avoid the confirmed negative role of menopause on steatosis, and the potential benefit of hormone replacement therapy on hepatic fibrosis in HCV patients[47].

In conclusion, serum leptin levels were elevated in male patients with both HCV-4 related hepatic steatosis and fibrosis, so it has a poor predictive value for either alone. In contrast, serum adiponectin levels were elevated in those patients with hepatic fibrosis, but decreased in hepatic steatosis, therefore, hypoadiponectinemia is a good predictor of hepatic steatosis in those patients.

Identification of individuals with hepatic fibrosis in chronic HCV-4 may be important for a number of reasons. Firstly, it can decrease the need for liver biopsy in those patients. Secondly, pharmacological treatments are currently being evaluated in NAFLD, but if successful agents are found it will be important to have identified a target population that can potentially be treated. Finally, once fibrosis is identified it may increase the imperative for patients to implement major lifestyle changes and clinicians to monitor the response to intervention[48].

On the other hand, serum adiponectin can be considered as a non-invasive marker for hepatic steatosis, and might decrease the need for liver biopsy in patients with chronic HCV-4 infection. Also, therapy to increase circulating adiponectin concentration, such as overweight reduction or thiazolidinediones, might represent a novel strategy to improve steatosis in those patients.

Limitations of the present study include the small sample size studied, which consisted of Egyptian male patients with chronic HCV-4, and thus, applicability to other populations requires further work and, the lack of data related to serum HCV load, which may not significantly impact the results. Indeed, HCV quantity is not an independent predictor of pathology[47]. Finally, we measured total serum leptin levels (which is composed of free and protein-bound components) and total serum adiponectin levels (which is composed of three forms); so, we cannot exclude the beneficial role(s) for measurement of these specific components for each one[1,49].

COMMENTS

Background

Leptin and adiponectin are the main metabolic products of adipose tissue. Recently, there is an increasing interest in the role of these adipokines in the development of hepatic steatosis and fibrosis, particularly in patients with nonalcoholic fatty liver disease (NAFLD) and chronic hepatitis C virus (HCV) infection. Chronic HCV genotype-4 (HCV-4) is known to be endemic in Egypt, Central Africa, Middle East, and recently, increasing in Europe. The role of leptin in hepatic steatosis and fibrosis is not clear. Moreover, the levels of adiponectin in patients with different stages of liver diseases, particularly in those with NAFLD and chronic HCV (especially genotype-4) infection, has been partly unraveled. Hence, the aim of this study was to measure serum leptin and adiponectin levels, as potential predictors of liver steatosis and fibrosis, for use in clinical practice.

Research frontiers

Liver biopsy is still the standard method for evaluation of liver steatosis and fibrosis. However, it is an invasive procedure and may carry some complications. So, authors tried to discover a simple blood test to measure a substance/substances which can address liver steatosis and fibrosis.

Innovations and breakthroughs

Many noninvasive methods for the evaluation of liver fibrosis and steatosis, in patients with HCV-4, have been proposed, but none are easy nor simple methods. The present study shows that estimation of serum adiponectin , in patients may potentially be used for identification of hepatic steatosis and fibrosis.

Applications

Diagnosis of hepatic fibrosis in chronic HCV-4 is important because it can decrease the need for liver biopsy. Also, pharmacological treatments are currently being evaluated in NAFLD. Furthermore, once fibrosis is identified it may increase the imperative for patients to implement major lifestyle changes. In contrast, serum adiponectin can be considered as a non-invasive marker for hepatic steatosis, and might decrease the need for liver biopsy in patients with chronic HCV-4 infection. Also, therapy to increase circulating adiponectin concentration, such as overweight reduction or thiazolidinediones, might represent a novel strategy to improve steatosis in those patients.

Terminology

Serum leptin and adiponectin are produced mainly by adipose tissue. However, recently, it was found that they may have an important role in NAFLD and HCV.

Peer review

This study focuses on the potential use of serum adiponectin in identification of liver steatosis and fibrosis in people with HCV-4. Although this study is primary, but avoiding liver biopsy through estimation of these substances in the serum is promising. So, this study may be interesting for the readers, particularly those with HCV.

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CASE REPORT

Acute renal failure associated with acute non-fulminant hepatitis B

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Abstract

A 38-year-old female presenting with a high fever of 39 ℃ developed severe liver dysfunction and acute renal failure (ARF). In tests for a hepatitis associated virus, an Immunoglobulin M-anti-hepatitis B virus core antibody was the only positive finding. Moreover, the progression of ARF coincided with the pole period of liver damage and all the other assumed causes for the ARF were unlikely. Therefore, this case was diagnosed as ARF caused by acute hepatitis B. ARF associated with non-fulminant hepatitis has been infrequently reported, usually in association with acute hepatitis A. This case is considered to be an extremely rare and interesting case.

Key words: Acute hepatitis B; Acute renal failure; Non-fulminant hepatitis; Acute tubular necrosis; Hyperimmune response

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INTRODUCTION

Acute renal failure (ARF) with fulminant hepatitis is a common complication and the functional kidney failure induced by hepatocellular failure is known as hepatorenal syndrome[1]. On the other hand, ARF with non-fulminant acute hepatitis has also been reported, but this represents a different condition from hepatorenal syndrome and most cases are due to hepatitis A virus (HAV)[2-5]. Recently, a case of ARF accompanied with non-fulminant acute hepatitis presented to our clinic, and this hepatitis case was attributed to a hepatitis B virus (HBV) infection. We report this case here because it is extremely rare.

CASE REPORT

The patient was a 38-year-old female who presented with a fever of 39 ℃ without respiratory symptoms or gastrointestinal symptoms in early November. The following day she received 1 g intravenous Ceftriaxone and 200 mg levofloxacin orally for 2 d. Diclofenac sodium of 25 mg was also administered only once. A few days later, general fatigue and nausea developed. On a return clinic visit, she had jaundice and had developed severe liver disorder [aspartate aminotransferase (AST) 2751 IU/L,

alanine aminotransferase (ALT) 5754 IU/L, total bilirubin (T-Bil) 4.7 mg/dL]. Therefore, the next day, she was referred to a nearby general hospital and was admitted. However, she was transferred to our hospital because of the development of ARF [blood urea nitrogen (BUN) 173.4 mg/dL, creatinine (Cr) 10.8 mg/dL].

There was nothing remarkable in her medical history and family history, and there was no record of transfusion. No weight gain or loss was recognized. The body temperature was 36.6 ℃, blood pressure was 120/70 mmHg without orthostatic change, and the pulse rate was 70/min and regular. Her consciousness was alert and no involuntary movement, such as a flapping tremor, was recognized. Her skin had normal moisturization and did not have petechia or other eruptions. Her jugular venous pressure was normal. Her lungs were clear in auscultation. Her cardiac examination was normal, without a murmur or rub. The liver was palpable two finger-breadths in the right hypochondrium, but it was smooth and non-tender. There was no splenomegaly, and no fluctuation was recognized. She had slight pitting pretibial edema bilaterally.

Urine protein and occult blood were both strongly positive, and an erythrocyte, leukocyte, and a hyaline cast were found in the urinary sediment, but no granular or cellular casts were found. She was in an oliguric state and the fraction sodium excretion rate (FENa) rose by 15.5%. A complete blood count revealed anemia (Hb 9.5 g/dL) and slight thrombocytopenia. Prothrombin (PT) activity was slightly decreased (63%).

Biochemical findings revealed severe azotemia (BUN 201.5 mg/dL, Cr 14.05 mg/dL), remarkable hyperuricemia (UA 22.2 mg/dL), slightly elevated serum C-reactive protein CRP was 1.11 mg/dL, increased hepatic enzyme levels (AST 252 IU/L, ALT 2000 IU/L), and moderate hyperbilirubinemia, mainly direct bilirubin (T-Bil 4.5 mg/dL), whereas no increase in ammonia was observed, creatinine phosphokinase levels were normal, myoglobin was slightly increased, and endotoxin was negative.

The patient did not exhibit autoantibodies, including antinuclear antibodies and antineutrophilic cytoplasmic antibodies. Slight hypocomplementemia was found, but immune complex (C1q) was negative.

The findings of serological tests for herpes simplex virus, Epstein-Barr virus, cytomegalovirus, HAV, and E viruses were negative. As for HBV, the HB surface (HBs) antigen was negative, but the IgM-HB core (HBc) antibody was positive. In additional tests, the HB envelope (HBe) antigen was negative, the HBe antibody was positive, and IgG-HBc antibodies were weakly positive. The HBs antibody of a low titer showed a gradual rising trend.

On admission, 7 d after the onset of illness, she demonstrated extensive renal insufficiency and oliguria. Ultrasound revealed findings compatible with ARF, including increased bilateral kidney size, enlarged medullary pyramids and a distinct corticomedullary boundary. The ARF was thought to be strongly associated with acute hepatitis because it developed in parallel to the progression of the hepatic disorder. Hemodialysis was initiated since she was in an oliguric state. The PT activity, platelet count and bilirubin value did not aggravate after admission, but improved immediately. Moreover, no encephalopathy developed through the course. On the 7th day, the urine volume exceeded 2000 mL and hemodialysis was withdrawn (Figure 1).

Acute tubular necrosis was suspected as the cause of ARF because of the oliguria and increased FENa. However, percutaneous kidney biopsy was performed on the 16th day because of nephritis-like urine findings on admission.

The light microscopic findings revealed no glomerulus change. Edema, degeneration and regeneration of the renal tubule epithelium, a slight cast in renal tubules, and slight cellular infiltration were recognized in the interstitium (Figure 2). Fluorescent antibody staining showed that IgG, IgA, IgM, C3, C4, C1q, κ, and λ were all negative. An electron dense deposit was not revealed in the electron microscopic findings. On the basis of the above findings, she was diagnosed with acute tubular necrosis.

On the 35th day of hospitalization, she recovered and left the hospital. Her liver function was normalized before discharge, and her renal function including urinary findings normalized 1 mo after discharge.

DISCUSSION

Initially, hypovolemia due to acute hepatitis, hyperuricemia and administered medicines were suspected to be the cause of the ARF of this case. However, there was no sign of weight loss, hypotension or tachycardia, so hypovolemia was ruled out as a possible cause of this ARF. Remarkable hyperuricemia was not thought to be a possible cause of the ARF because the formation of the cast was found only in a small part of the kidney biopsy specimen. It was necessary to rule out interstitial nephritis caused by an antimicrobial agent or non-steroidal anti-inflammatory drugs (NSAIDs). However, there was no clinical manifestation such as eosinophilia or pyrexia, and no findings that suggested interstitial nephritis in the kidney biopsy specimens. Therefore, it was unlikely to have been the cause of the ARF. Moreover, ARF due to NSAIDs through the inhibition of prostaglandin synthesis was highly unlikely because it was used only one time. Therefore, acute hepatitis was diagnosed as the cause of this ARF because the ARF developed in association with the progression of hepatitis. The findings of IgM-anti-HBc antibodies of high titer, HBs antigen being negative in spite of the acme phase of hepatitis, and the occurrence of seroconversion suggested that this hepatitis was not generated from an HBV carrier[6], but was generated from a primary infection instead. An assay for hepatitis E infection that has been reported in a superinfection to HB[7] was negative.

There have been almost no reports of ARF associated with acute non-fulminant HB until now. Wilkinson

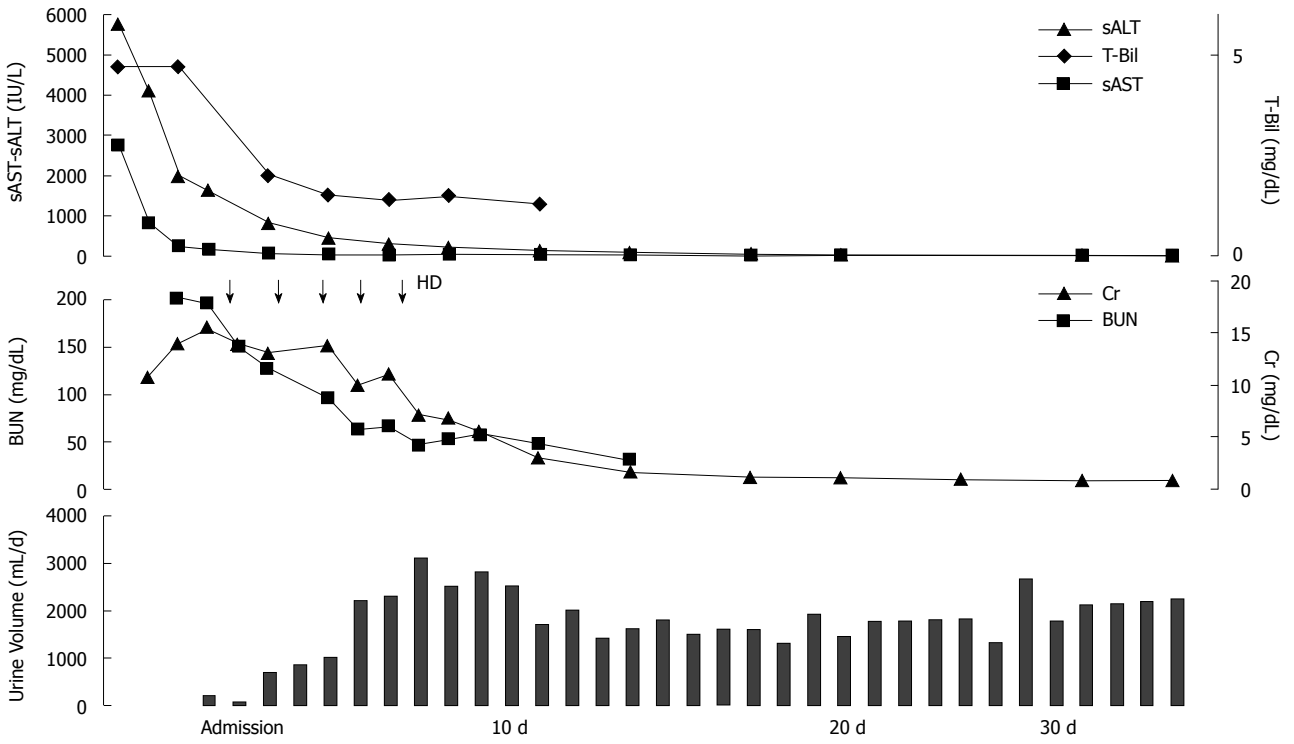

Figure 1 Clinical course of the present case. T-Bil: Total bilirubin; Cr: Creatinine; BUN: Blood urea nitrogen.

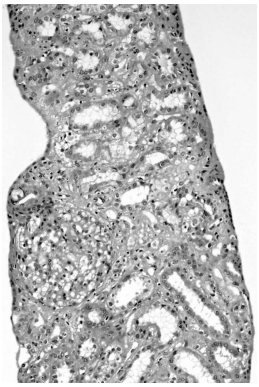

Figure 2 Light microscopic findings of the renal tissue showing dilated renal tubules, edema and inflammatory infiltrate in the interstitium (hematoxylin and eosin stain, ×100).

et al[2] reported that only 2 cases of positive HB antigen were found in 12 cases of acute hepatitis with ARF. One of these cases was considered pre-renal ARF due to frequent emesis; another case was considered to be acute tubular necrosis to the point of oliguric ARF without proteinuria based on the findings of urine chemistry. The latter case died despite receiving peritoneal dialysis, and no renal biopsy was performed. Obana *et al*[8] also reported a similar case that was acute aggravation of hepatitis from an HBV carrier, and the findings of a renal biopsy 40 d after onset showed interstitial nephritis that was thought to have been due to concomitantly used drugs. However, in this case, a direct correlation between hepatitis and ARF was also suspected because of deposition of HBe antigen and IgG in the glomerulus.

Not only pre-renal factors, such as hypovolemia due to digestive symptoms, but also endotoxinemia[2], hyperbilirubinemia[3], vasoconstriction induced by the renin-angiotensin system[5], and glomerulonephritis induced by the immune complex[4] have been reported as factors associated with ARF associated with acute HA. Although such causes were considered, none were seen as a reasonable cause in the present case. There is a report[9] pointing out that a case of HA with ARF has a greater tendency to become more severe than one without ARF. It is noted that HAV induces a host immune response more powerfully than HB or HC viruses. Therefore, HA rarely becomes fulminant and common to heal as acute hepatitis.

The considerable rise of aminotransferase in the current patient was different from common acute HB. The HB antigen was already negative and the HB antibody was developing during the phase when the level of aminotransferase increased. This was thought to be due to a strong immune reaction, known as the hyperimmune response[6] in severe acute HB. The mechanism of the hyperimmune response has not been unexplained. The specific mechanism in ARF associated with acute HA is also unclear, but a common mechanism in cases of such severe acute hepatitis has been suggested as a cause of ARF. Moreover, since the seriousness of hepatitis correlates with the cause of co-morbid ARF, it is possible that there may be some pathological condition overlapping with ARF in fulminant hepatitis. The current case was thought to have been caused by sudden hepatic cell annihilation due to a non-typical strong immune reaction as in acute serum hepatitis, although the details were indistinct. This abnormal state was thought to induce ARF as can be seen in an acute HA case.

Acute hepatitis was suspected because of the preced-

ing pyrexia and remarkable liver injury. Generally, HB antigens are only measured when screening for a HB virus infection, but the measurement of IgM-HBc established the diagnosis for this case. The phenomenon of the hyperimmune response thus brings to mind past cases that may have not reached a diagnosis of acute HB. The current case suggested that not only acute HA but also HB may be associated with ARF. Therefore, in the future, it is necessary to check for HB in addition to HA when a patient presents with ARF associated with acute hepatitis.

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CASE REPORT

Myxedema ascites with high CA-125: Case and a review of literature

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Abstract

Ascites appearing in a previously healthy female patient is usually ascribed to a variety of causes, among which, is a cancerous process, especially if it comes with a raised CA-125 level. Although the CA-125 antigen is present on more than 80% of malignant epithelial ovarian tissue of non-mucinous type, it is also found on both healthy and malignant cells of mesothelial and non-mesothelial origin. Myxedema ascites which is caused by hypothyroidism is a rare entity, but on the other hand is easy to treat. It is one of the differential diagnoses when the ascites is refractory to treatment and no other obvious cause can be identified. If the diagnosis is delayed, patients will frequently receive unnecessary procedures, while treatment has very good response rates and ascites resolve with serum CA-125 normalization after adequate hormonal treatment.

Key words: Hypothyroidism; Refractory ascites; High CA-125; Hormonal therapy; Abdominal paracentesis

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INTRODUCTION

Of the many nonspecific clinical manifestations of hypothyroidism, ascites is one of the least frequently reported and it accounts for less than 4% of cases[1]. Isolated cases of myxedema ascites have been reported in the absence of cardiac, hepatic and renal failure or peritoneal inflammation.

Frequently, other obvious signs of hypothyroidism are lacking and an inflammatory or malignant disease is suspected. Usually such patients are subjected to diuretic therapy, diagnostic laparotomies and paracentesis for long periods before hypothyroidism is diagnosed[2]. In such cases, the use of thyroid hormone replacement usually leads to a progressive decrease in ascites, which will ultimately disappear[3]. Therefore, when any patient presents with ascites of uncertain etiology, hypothyroidism should be considered as a differential diagnosis.

In some cases of ascites, CA-125 levels can be as high as those seen in patients with cancer, suggesting that any patient with ascites and a raised CA-125 concentration should have thyroid function measured as part of their initial evaluation. Here we describe a case of a patient with refractory ascites of unknown etiology, who was found to have severe hypothyroidism with high CA-125, that was diagnosed as myxedema ascites and

that responded to thyroid hormone replacement therapy.

CASE REPORT

A 78-year-old female was referred to our hospital because of a hip fracture after a fall. In the emergency room she was found to have severe abdominal distention due to ascites which she mentioned had been present for eight months; for this she had multiple abdominal paracentesis with recurrence of the ascites that the medical treatment prescribed failed to resolve.

Her past medical history was positive for acute right lower limb ischemia 4 years previously, for which she was treated with a vascular shunt. She did not smoke or consume alcoholic beverages.

On physical exam, her blood pressure was 130/80, her pulse 110 beats/min, her weight was 55 kg and her height was 163 cm. she was conscious, cooperative and well oriented. She was afebrile. Neck examination was normal. Cardio-pulmonary exam was normal. The abdomen was soft, distended, with a circumference of 101 cm. There was shifting dullness and fluid waves evident on palpation indicating the presence of ascites but without any stigmata of chronic liver disease. The liver and spleen were not palpable. No pretibial edema.

Complete blood count was normal. C-reactive protein was 28 mg/L. The prothrombin time and the activated partial thromboplastin time were normal. The liver function tests were normal. Amylase, lipase, total cholesterol, triglycerides, urea, creatinine and electrolytes were normal. The total protein level was 6 g/dL and albumin 2.9 g/dL. The urinalysis result showed numerous red blood cells, white blood cells and epithelial cells. Urine culture was negative. Hepatitis B and C serologies were negative. Cortisol 18.41 mg/dL (normal 5-25); Tumor markers (CEA, CA 15-3, CA 19-9) were negative except for an elevated CA-125 of 1255 U/mL (normal 0-35); negative Protein Purified Derivative (3 mm).

Chest radiology, electrocardiogram and Doppler ultrasound of the abdomen were normal. Abdominal ultrasound and computed tomography scan of the abdomen and pelvis (Figure 1) were performed showing marked ascites.

A diagnostic abdominal paracentesis yielded turbid yellowish, transparent ascitic fluid with a protein concentration of 4.1 g/dL, sugar 110 mg/dL and albumin 1.9 g/dL. The white blood cell count in the fluid was 100/mm^3 and 90% of the cells were lymphocytes. Serum-to-ascites albumin gradient (SAAG) was 1 g/dL. On histological exam, no malignant cells were found. Gram staining and cytology were negative. Bacterial, fungal and mycobacterial cultures were also negative.

Gynecological evaluation showed no abnormalities. Echocardiography showed normal sized cardiac chambers, and an ejection fraction of 65%. With the high protein component of the ascites fluid, we performed thyroid function tests. These showed the following values: free T3 0.56 pg/mL (normal 2.2-4.4 pg/mL), free T4 < 0.08 ng/dL (normal 0.7-1.6 ng/dL) and thyroid-stimulating hormone level (T_{SH}) > 100 miU/mL (normal 0.2-5.0 miU/mL). These findings necessitated further thyroid tests. The anti- thyroxin peroxidase was negative.

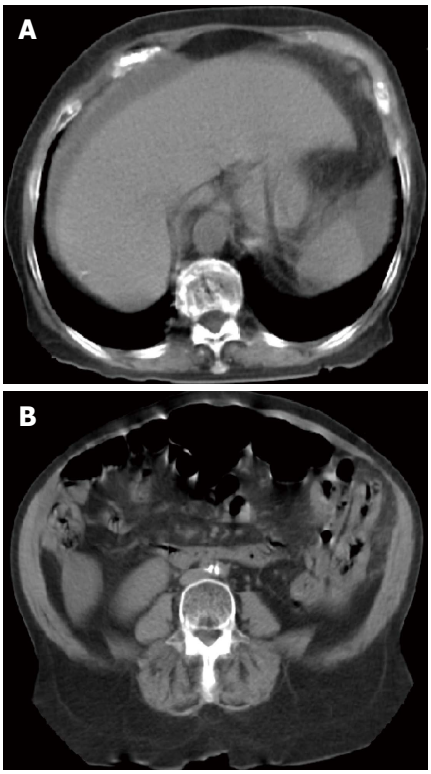

Figure 1 Computed tomography of the abdomen and pelvis showing massive ascites and normal-sized liver and spleen.

Ultrasonography of the thyroid gland showed that both lobes were enlarged in size. Thyroid hormone replacement therapy was started with gradually increasing doses of levothyroxine, from 0.05 to 0.15 mg daily. Over the following three months, she became euthyroid with complete resolution of her ascites and CA-125 returned to normal. Her body weight decreased by 8 kg and the abdominal circumference was reduced by 12 cm. One year later, there had been no recurrence of ascites and her euthyroid condition was maintained.

DISCUSSION

Hypothyroidism is a relatively rare cause of ascites. However, the importance of its diagnosis is that use of thyroid hormone replacement results in complete resolution. If there is new onset ascites, diagnostic workup should begin with the analysis of ascitic fluid. Usually total protein in the ascitic fluid and the SAAG value give a useful framework for analysis. Of the various causes, peritoneal malignancies, tuberculous peritonitis, pyogenic peritonitis and pancreatic ascites can all lead to high-protein ascites. Patients with liver cirrhosis and congestive heart failure show low protein ascites. The SAAG corre-

lates directly with portal pressure. Ascitic fluid associated with portal hypertension shows a low total albumin level, and the SAAG is greater than 1.1 g/dL (high gradient). SAAG is usually high in patients with liver cirrhosis and congestive heart failure. A gradient of <1.1 g/dL (low) usually suggests that the ascites is not caused by portal hypertension. The SAAG is low in patients with peritoneal malignancies, tuberculous peritonitis, pyogenic peritonitis and pancreatic ascites.

Portal hypertension secondary to liver cirrhosis is the leading cause of ascites (more than 80% of cases) and peritoneal involvement in patients with malignant diseases is the second at about 10%[4]. Therefore, if the composition of ascitic fluid and ultrasonography are not consistent with portal hypertension or other specific diseases, the physician should consider peritoneal malignancy. If the ascitic fluid shows a high protein content, then hypothyroidism should be considered as a differential diagnosis. In this patient, the ascitic fluid analysis revealed a high protein content (SAAG was < 1.1 g/dL) and there was a lack of esophageal varices or gastropathy on esophagogastroduodenoscopy without portal hypertension on abdominal ultrasound, we performed thyroid function testing, which proved decisive.

Prompt recognition of myxedema ascites prevents the inappropriate use of diuretics and unnecessary procedures, including repeated paracentesis, liver biopsies and exploratory laparotomies[5]. A constant feature was the positive response to thyroid hormone replacement therapy, which led to elimination of the ascites in every instance.

There has been a suggestion that the SAAG may exceed 1.1 in patients with myxedema ascites, based on a review of eight patients[6]. Because so few cases have been studied and portal hypertension or heart failure do not seem to be the mechanisms causing ascites in patients with myxedema, we cannot conclude that a high SAAG is a typical feature in this disease[7]. Moreover, the patient reported here showed a low SAAG.

The mechanism of ascites fluid formation in patients with myxedema is unclear. There are two main hypotheses. The first is that low levels of circulating thyroid hormones cause increased extravasation of plasma proteins because of abnormal capillary permeability and the lack of a compensatory increase in lymph flow and protein return rate[8]. The second hypothesis is that hyaluronic acid accumulates in the skin and produces edema by a direct hygroscopic effect. However, hyaluronic acid has only been found in minute quantities in patients with myxedema ascites; not large enough to exert a direct hygroscopic effect. It could, however, interact with albumin to form complexes that prevent the lymphatic drainage of extravasated albumin[9].

Although the CA-125 antigen is present on more than 80% of malignant epithelial ovarian tissue of non-mucinous type, it is also found on both healthy and malignant cells of mesothelial (pleural, pericardial, peritoneal, and endometrial) and non-mesothelial (amniotic membrane, tracheobronchial and cervical epithelium) origin. Raised serum CA-125 levels have therefore been reported in various conditions involving these cells, including pleural and pericardial effusions and ascites[10].

The mechanism of elevated CA-125 is not yet understood. Several theories have been proposed. One theory is that stretching of the peritoneum with ascites is a cause since paracentesis alone leads to a decrease in the serum value of CA-125. The other theory is that peritoneal cells shed a lot of CA-125 antigen and that it enters the blood *via* lymphatic absorption of ascites[11].

In conclusion, myxedema ascites is rare but easy to treat. Treatment with thyroid hormone replacement therapy leads to complete regression of the ascites. Once routine evaluation of ascites excludes common causes such as liver cirrhosis, peritoneal malignancies, infections, congestive heart failure and pancreatic ascites, thyroid function tests should be performed on patients with high protein levels in the ascitic fluid. A high CA-125 makes the diagnosis more difficult, but with extensive workup to rule out malignancies, it can be attributed to ascites. Early diagnosis is important to prevent inappropriate use of diuretics or even unnecessary laparotomies to determine the cause.

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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