

# Autoimmune Neutropenia in Chronic Liver Disease Patients with Hepatitis C Virus at Suez Canal University Hospital

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## Abstract

**Background:** Chronic hepatitis C (CHC) continues to be a public health problem in Egypt with data suggesting that its incidence may be increasing. Hepatitis C virus (HCV) infection has been associated with a variety of extra-hepatic manifestations, including autoimmune disorders. **Aim:** To assess the prevalence of autoimmune neutropenia (AIN) in chronic liver disease (CLD) patients with HCV. **Patients and Methods:** A case-control design was used to fulfill the study objective. It was conducted on 60 subjects, divided into 2 groups: group 1 (30 HCV-infected patients with low total leucocytic count [TLC] as a study group) and group 2 (30 HCV-infected patients with normal TLC as a control group). AIN was evaluated by granulocyte immuno-fluorescence test (GIFT) to detect auto-antibodies against neutrophils in HCV-infected patients presented with neutropenia. **Results:** The mean age of the first group was 49.9±11.6 years, while the mean age of the second group was 52.7±11.3 years. Male gender was found in 53.3% of the low TLC group compared to 63.3% in normal TLC group; Positive GIFT was found in 13.3% of study group; while none of the control group has positive GIFT. The significant variables among patients with low TLC included low ANC (OR: 5.0, 95% CI: 1.5-16.5, low HB (OR: 7.0, 95% CI: 1.4-35.5), and low PLT count (OR: 15.5, 95% CI: 3.8-63.3). **Conclusion:** AIN is significantly higher among CHC patients with low TLC compared to normal TLC patients.

**Keywords:** low total leucocytic count, Granulocyte Immuno-fluorescence Test

## Introduction

HCV infection is a serious health problem affecting over 130 million people worldwide (Ref). Infection with the HCV affects 2-3% of the world's population<sup>(1)</sup>. Egypt has the highest recorded prevalence of HCV in the world, reaching 14.7% for HCV-antibody positivity

among 15- to 59-year-old population<sup>(2)</sup>. The viremic population of Egypt was estimated at over 6 million<sup>(3)</sup>. Approximately 85% of the HCV-infected population develops severe complications, such as CHC, cirrhosis, diabetes, and hepatocellular carcinoma (HCC). About 10-30% of CHC patients develop liver cirrhosis. <sup>(4,5)</sup> CHC is reported to be the

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major cause of 50-76% of all liver cancers globally and also the reason for two-third of all cases of liver transplantation. <sup>(6)</sup> Liver mortality in Egypt reaches 40,000 per year, making 10% of total mortality, and comes second after heart diseases<sup>(7)</sup>. Primarily, HCV infection appears to be confined to the liver, however, a wide variety of extra-hepatic disease manifestations have been reported to be associated with the infection. Extra-hepatic manifestations are mainly due to associated autoimmune disorders. A widely postulated, but controversial, hypothesis is that extra-hepatic disease manifestations are caused by extra-hepatic tropisms of HCV, particularly lymphotropism. The latter is thought to be involved in the production of auto-antibodies<sup>(8)</sup>. Persistent HCV infection is responsible for the production of a variety of auto-antibodies including non-organ-specific auto-antibodies and organ-specific auto-antibodies, as a virus-induced autoimmune phenomenon. The diversity of auto-antibodies in the sera of patients with HCV-related CLD has been shown<sup>(9,10)</sup>. Some auto-antibodies in CHC infection have biochemical, histological, or genetic characteristics, while other auto-antibodies may predict the response to anti-viral treatments, concomitant disorders, or prognosis in patients with HCV-related CLD<sup>(11)</sup>. AIN in adults are a heterogeneous group of diseases with clinical manifestations varying from being asymptomatic to having infectious complications with considerable morbidity and mortality. They are characterized by auto-antibodies directed against neutrophils, resulting in destruction of neutrophils. AIN can be

divided into two forms. In primary AIN, neutropenia is usually the sole hematologic abnormality and it is more common in children. Secondary AIN, which is more prevalent in adults, is associated with underlying autoimmune diseases, malignancies, infections, particularly viral, neurological diseases or drug exposure<sup>(12)</sup>. Diagnosis of AIN is based on the presence of neutropenia and demonstration of anti-neutrophil antibodies. The GIFT is a common method that can be used to test for antibodies against neutrophils in both the patient's serum and directly on autologous cells<sup>(13)</sup>.

## Patients and Methods

This case-control study was carried out at Internal Medicine and Clinical Pathology Departments, Suez Canal University Hospitals, Ismailia, Egypt. It included 60 CHC patients. They were classified into two groups; group 1 of 30 CHC patients with leucopenia which considered as the study group and group 2 of 30 CHC patients with normal TLC which considered as the control group. Data were collected from CHC patients using interview questionnaire. The recorded data included; patients' age, gender, Child-Pugh score, and laboratory parameters [hemoglobin (HB), platelets (PLT) count, TLC, absolute neutrophil count (ANC), total and direct bilirubin, Alanine transaminase (ALT), Aspartate transaminase (AST), albumin, total proteins, and prothrombin time (PT)]. GIFT is an indirect immunofluorescence assay for detection of granulocyte auto-antibodies in peripheral blood. Freshly air-dried peripheral

blood slides were individually wrapped in domestic aluminum foil or paraffin frozen within 2 hours of collection, transported on dry ice and stored at  $-25^{\circ}\text{C}$ . Frozen slides were reviewed within 2–3 weeks, but were stable for up to 3 months. A peripheral blood sample from normal person was frozen at the same time as each sample and used as a negative control. Two patient slides and the normal control slide were warmed to room temperature and fixed with 1% para-formaldehyde in phosphate buffered saline (PBS) for 5 min. Fixed neutrophils were incubated with the 1:2 dilution of patient serum at  $37^{\circ}\text{C}$  for 30 min. Known positive samples were analyzed with each sample batch. Slides were washed with PBS and incubated with fluorescein isothiocyanate (FITC) conjugated to human immunoglobulins IgG and incubated for further 30 min at room temperature in the dark. The dilution of the fluorescein conjugated was determined by titration for each batch of antibody (different dilutions of the fluorescein conjugated were applied to peripheral blood control slides incubated with known positive serum, the dilution that gave the best fluorescence was selected). The slides were subsequently rinsed and covered with a PBS–glycerol mountant. The slides were examined microscopically using epifluorescent ultraviolet illumination. A qualitative increase in cytoplasmic fluorescence of patient neutrophils over controls indicated the presence of auto-antibodies<sup>(14)</sup>. Positive and negative controls were run with each batch. The whole slide was reviewed microscopically with

the initial result validated by a second review. In cases where the results were not clear, a second individual performed independent analysis of the slides. If the quality of the slide was inadequate (because of sample preparation, storage or transport), these observations were recorded and reported as unsuitable for analysis.

## Results

The age ranged from 24–75 years. The mean age was  $49.9 \pm 11.5$  yrs versus  $52.7 \pm 11.3$ , in the study and the control groups respectively. About 53.3% were males in the study group compared to 63.3% in the control group. The groups were matched for age and gender without statistical significant differences between them ( $p > 0.05$ ). There were insignificant differences between the two groups regarding liver function tests and Child-Pugh scoring ( $p > 0.05$ ) (Table 1). Regarding hematological parameters, there was statistically significant higher mean ANC, HB and PLT count in the control group compared to the study group ( $p < 0.01$ ) (Table 1). According to GIFT results, the study group had significantly higher incidence of AIN compared to the control group (13.3% vs. 0.0%,  $p = 0.0002$ ) (Table 2). The significant variables among patients with low TLC included low ANC, low HB, and low PLT count.

## Discussion

HCV infection is a common cause of progressive liver disease. In addition, chronic HCV infection has been associ-

ated with a variety of extra-hepatic manifestations, including autoimmune disorders. HCV may skew the immune response toward the production of auto-antibodies. AIN is a relatively benign disorder characterized by neutrophil specific auto-antibodies, often se-

vere neutropenia and a paucity of severe bacterial infections. Anti-neutrophil antibodies are well recognized causes of neutropenia, producing both quantitative and qualitative defects in neutrophils and increased risk for infection<sup>(15)</sup>.

**Table 1:** Demographic, clinical and laboratory characteristics of the studied populations

Variables	Study group (n=30)	Control group (n=30)	p-value
Age (yrs)	49.90±11.56	52.70±11.30	0.347
Gender			0.432
Male	16 (53.3%)	19 (63.3%)	
Female	14 (46.7%)	11 (36.7%)	
Child-Pugh score			0.094
A	4 (13.3%)	0 (0.0%)	
B	17 (56.7%)	17 (56.7%)	
C	9 (30.0%)	13 (43.3%)	
T. bilirubin (mg/dL)	2.69±2.82	3.65±4.05	0.096
D. bilirubin (mg/dL)	1.25±0.64	1.68±0.623	0.589
ALT (U/L)	31.43±19.97	35.60±24.55	0.375
AST (U/L)	56.27±43.72	61.43±35.11	0.391
Albumin (mg/dL)	2.65±0.77	2.71±0.61	0.784
T. proteins (mg/dL)	6.18±1.23	6.65±0.66	0.135
PT (seconds)	19.72±4.99	19.63±6.41	0.882
TLC (cell/mm <sup>3</sup> )	2.44±0.64	7.11±2.36	0.001**
ANC (cell/mm <sup>3</sup> )	1.26±0.22	4.81±1.99	0.001**
HB (gm/dL)	8.64±1.71	9.83±1.73	0.017*
PLT count (cell/mm <sup>3</sup> )	64.04±38.68	128.87±90.43	0.001**

Data are presented as Mean ± SD ; \*Significant p-value ≤0.05, \*\*highly significant p-value ≤0.01, Alanine transaminase (ALT), Aspartate transaminase (AST), prothrombin time (PT), total leucocytic count (TLC), absolute neutrophil count (ANC), hemoglobin (HB), platelets (PLT).

Autoimmune neutropenia is believed to be triggered by a viral infection (as HCV) and, in some individuals, by immunization. When identified in chronic benign neutropenia, antibodies most commonly include immunoglobulin G (IgG) antibodies against neutrophil glycosylated isoforms. Bone marrow examination typically demonstrates increased cellularity of myeloid ele-

ments with an absent or decreased number of mature neutrophils. In some cases, mature forms of neutrophils are seen. This suggests that, in most cases, neutropenia is due to increased consumption of neutrophils in the peripheral circulation and in the tissues that result from phagocytosis of the antibody-coated neutrophils<sup>(16)</sup>. This case-control study was carried out aiming to detect presence of AIN in

chronic liver disease patients with HCV. When analyzing the results of the laboratory tests, our data showed a statistically significant difference between study and control group regarding ANC, HB and PLT count ( $p < 0.05$ ). In

patients with autoimmune neutropenia, hematological evaluation demonstrates a TLC count that is either decreased or within the reference range and a neutrophil count less than  $1000/\mu\text{L}$  (absolute neutropenia).

**Table 2:** Incidence of autoimmune neutropenia (AIN) according to granulocyte immuno-fluorescence test (GIFT)

Variables	Study group (n=30)	Control group (n=30)	p-value
AIN			0.0002**
Present	4 (13.3%)	0 (0.0%)	
Absent	26 (86.7%)	30 (100.0%)	

\*\*highly significant p-value  $\leq 0.01$ .

Performing sequential CBC counts with differential to document chronicity is important because most neutropenia in infants resolves with recovery from an acute infection. In individuals with autoimmune neutropenia, the ANC often remains less than  $500^{(17, 18)}$ . Our data revealed that the study group had

significantly lower levels of PLT count compared to the control group ( $64.04 \pm 38.68$  versus  $128.87 \pm 90.43$ , respectively,  $p < 0.05$ ). Thrombocytopenia is a common hematological toxicity among patients with chronic liver disease, occurring in 15% up to 70% of patients with cirrhosis<sup>(19)</sup>

**Table 3:** Odds ratio and 95% confident interval of the studied variables among patients with low and normal TLC

Variables	Odds ratio	95% CI	p-value
Age (<40 years)	2.8	0.50-15.7	0.42
Gender (female)	1.5	0.54-4.2	0.43
Total bilirubin	1.0	0.12-8.3	0.70
Direct bilirubin	0.75	0.04-15.0	0.71
ALT	1.08	0.50-2.3	0.50
AST	0.73	0.41-1.3	0.28
Albumin	1.0	0.04-24.5	0.79
Total proteins	0.86	0.044-16.9	0.73
PT	1.2	0.31-4.6	0.79
ANC	5.0	1.5-16.5	0.006**
HB	7.0	1.4-35.5	0.0098**
PLT count	15.5	3.8-63.3	<0.0001**

\*Significant p-value  $\leq 0.05$ , \*\*highly significant p-value  $\leq 0.01$ , Alanine transaminase (ALT), Aspartate transaminase (AST), prothrombin time (PT), total leucocytic count (TLC), absolute neutrophil count (ANC), hemoglobin (HB), platelets (PLT).

Thrombocytopenia can increase the risk of bleeding associated with inva-

sive or surgical procedures, causing delays or cancellations and preventing

initiation of selected planned therapies<sup>(20)</sup>. The pathophysiology of thrombocytopenia in CLD is complex and multi-factorial. Hypersplenism caused by portal hypertension and subsequent PLT sequestration were thought to be the primary pathogenic mechanisms; however, splenic PLT pooling does not account for thrombocytopenia in all patients; hypersplenism is not always observed in patients with cirrhosis and portal hypertension<sup>(21)</sup>. Some studies in patients with CHC suggest that HCV may cause direct bone marrow suppression and decreased thrombopoiesis with reduced hepatic production of the thrombopoietin (thrombopoietic growth factor)<sup>(22)</sup>. Other pathogenic mechanisms contribute to the decreased numbers of circulating PLT include increased PLT destruction mediated by immune mechanisms involving anti-platelet auto-antibodies and PLT-associated immune complexes<sup>(23,24)</sup>. Our data reported that about 13.3% of study group are GIFT positive, while there were no patients in the control group had positive GIFT. There was statistically significance difference between the two groups regarding GIFT. There is no statistically significance between two GIFT results regarding gender and Child-Pugh classifications. So, GIFT results are independent of the degree of liver fibrosis, cirrhosis, and splenomegaly. In Lane et al.<sup>(25)</sup> study, bone marrow immunofluorescence test (BMIFT) used to demonstrate auto-antibodies to granulocytes and their precursors on fresh-frozen bone marrow slides. It used to differentiate AIN from other causes of childhood neutropenia. GIFT was used in this study

to detect positive anti-neutrophil antibodies from peripheral blood of HCV patients. Sensitivity for antibody detection varies depending on the test. Indirect GIFT is more sensitive than monoclonal antibody-specific immobilization of granulocyte antigens (MAIGA) in detection of autoimmune neutropenia<sup>(26)</sup>. Antibody specificity is assessed by testing a panel of neutrophils from donors of known human neutrophil antigen (HNA) genotypes. Up to now, the combination of GIFT and granulocyte agglutination (GAT) has been the best means of detection, and this has become the standard approach in most laboratories. GIFT is considered to be the most sensitive of the two methods<sup>(27,28)</sup>. In our study, study group were found to have significantly lower levels of ANC. Positive GIFT was 13.3% in study group versus 0.0% in the control group with statistically significant difference. The positive GIFT indicates presence of auto-antibodies against neutrophils in CLD patients with HCV. In Jeffery et al<sup>(29)</sup> study, patients with HCV infection can develop peripheral blood cell count abnormalities that are commonly attributed to decreased thrombopoietin levels and/or autoimmune mechanisms. This was emphasized in this study, where cytopenias were occurred due to autoimmune responses in the absence of splenomegaly and/or antiviral medications, which were excluded from the study. The results of neutrophil antibodies may or may not be positive. Lalezali and colleagues<sup>(30)</sup> demonstrated antibodies in 98% of patients with chronic neutropenia, whereas Jonsson and Buchanan<sup>(31)</sup> demonstrated a posi-

tive result in only 13 of 28 patients studied. In conclusion, AIN is significantly higher among CHC patients with low TLC compared to normal TLC patients independent of the degree of liver fibrosis, cirrhosis, and splenomegaly.

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