Neutrophil to lymphocyte ratio as a reliable marker to predict insulin resistance and fibrosis stage in chronic hepatitis C virus infection

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Background : **Hepatitis C virus (HCV) is one of the most noxious infectious diseases. Chronic hepatitis C (CHC) had biochemical evidence of insulin resistance (IR). The neutrophil/lymphocyte ratio (NLR) integrates information on the inflammatory milieu and physiological stress.**

Aim : **We aimed to investigate the clinical utility of NLR to predict the presence of IR and fibrosis in CHCvirus infection.**

Methods : **The study included 234 CHC patients and 50 healthy controls. The CHC group was divided into two subgroups ; CHC with HOMA-IR > 3 and CHC with HOMA-IR ≤ 3. Liver biopsy, homeostasis model assessment-IR (HOMA-IR), neutrophil and lymphocyte counts were recorded ; and NLR was calculated. Pro-inflammatory cytokines [tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)] were measured by an enzyme-linked immunosorbent assay.**

Results : **Patients with HOMA-IR > 3 had a higher NLR compared with patients with HOMA-IR ≤ 3 [2.61 ± 0.95 and 1.92 ± 0.86, respectively, $P < 0.001$]. The NLR ratio was positively correlated with HOMA-IR, C-reactive protein, TNF-α and IL-6 cytokines ; $P < 0.001$). Patients with advanced fibrosis (F3-4) had an elevated N/L ratio [2.4 ± 0.99] compared with patients with fibrosis stage 1-2 [1.86 ± 0.66], $P < 0.001$.**

Conclusions : **The N/L ratio is higher in patients with CHC with HOMA-IR > 3 and advanced fibrosis. This ratio can be used as a novel noninvasive marker to predict IR and advanced disease.** (Acta gastroenterol. belg., **2015**, 78, **386-392**).

Key words : CHC, IR, HOMA, fibrosis, NLR.

Introduction

Worldwide, about 170 million individuals are chronically infected with hepatitis C virus (HCV) (1). In the western world, chronic HCV infection is the major etiology for the development of hepatocellular carcinoma (HCC), hepatic fibrosis, cirrhosis, and is the essential etiology for liver transplantation (2). Insulin resistance (IR) is one of the pathological characteristics in patients with HCV infection that subsequently leads to type IIdiabetes development. Different complications associated with HCV infection may be related to the presence of insulin resistance. Insulin resistance associated with HCV may lead to hepatic steatosis, fibrosis, resistance to anti-viral treatment and HCC. Therefore, insulin resistance associated with HCV is a therapeutic concern at any stage of infection (3).

Different explanations have been suggested that HCV induced insulin resistance, involving up-regulation of inflammatory cytokines, like tumor necrosis factor-α, phosphorylation of insulin-receptor substrate-1, up-regulation of gluconeogenic genes like glucose 6 phosphatase, phosphoenol pyruvate carboxykinase 2, and accumulation of lipid droplets (4).

In cardiovascular diseases ; white blood cells (WBCs) and their differential counts are known as conventional inflammatory indicators (5). The neutrophil-to-lymphocyte ratio (NLR) was outlined as a new prospective marker to estimate inflammation in noncardiac and cardiac disorders (6). This ratio merges data on two various immune courses – the lymphocytes that illustrate the regulatory pathway and the neutrophils that clarify the ongoing inflammatory processes (7-9). Therefore, the NLR is a marker of the inclusive inflammatory events of the body.

However, the relationship between insulin resistance and NLR, especially in HCV-infected patients has not been evaluated so far. Therefore, in the current study, we aimed to investigate the clinical utility of NLR to predict the presence of insulin resistance and hepatic fibrosis in the context of hepatitis C infection ; in relation to pro-inflammatory cytokines (TNF-α and IL-6).

Patients and Methods

This prospective work was carried out on consecutive 332 patients referred to the outpatient clinic of the Tropical Medicine Department (Mansoura University- Egypt) with hepatitis irrespective to their etiologies between May 2012 and June 2014. Only 234 patients with chronic hepatitis C (CHC) were selected to be enrolled in our study. In addition, the control group included 50 healthy age-matched and sex- matched participants.

Exclusion criteria included patients with chronic HBV, history of co-infection with either human immunodeficiency virus (HIV) or HBV, chronic HCV-infected patients who were negative for genotype IV, decompensated liver disease (ascites, esophageal varices and

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Submission date : 05/06/2015
Acceptance date : 09/06/2015

encephalopathy), liver transplant, HCC, patients who had received immunomodulatory therapy or antiviral therapy within the last 12 months, and patients who had inadequate biopsy results. Exclusion criteria also included patients with alcohol intake ; immunosuppressant drugs, patients with a fasting blood glucose level > 6.2 mmol/L or under anti-diabetics medications, with clinically overt hyper- or hypo-thyroidism, and patients who undergoing dialysis. Moreover ; patients with the peripheral vascular disease, heart failure, hyperlipidemia, hypertension, or autoimmune diseases were also excluded from this study, other possibilities, e.g., infections, inflammatory diseases that may affect the levels of cytokines were excluded. All studied patients were submitted to laboratory and radiological assessment, percutaneous ultrasound-guided liver biopsy, clinical examination, and complete history taking.

Clinical and Laboratory Assessments

Via an interview, data on sex, age, and smoking habits were evaluated. At the same time of liver biopsy, after fasting for at least 8-12 h, venous blood samples were collected between 8.30 and 10.30 am. The blood samples were stored at -20 °C. All patients were subjected to : liver function profile [alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltranspeptidase (γ-GT), alkaline phosphatase (ALP), bilirubin and albumin (ALB)] and fasting blood glucose were assessed on a Dimension Xpand plus chemistry analyzer (Roche Diagnostics, Basel, Switzerland) using commercially available reagents and an enzyme-based kit. A complete blood count (CBC) containing white blood cell (WBC), neutrophils, lymphocytes, (NLR) was calculated, platelet count and hemoglobin (HB) was performed with CELL-DYN Emerald cell counter (Abbott, Wiesbaden, Germany). Prothrombin time (PT) and INR was assessed using kits provided by Siemens Healthcare diagnostic Inc (Erlangen, Germany). Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) were assessed by an enzyme-linked immunosorbent assay using kits supplied by Dia Source (Rue du Bosquet, Louvain-la-Neuve, Belgium). C-reactive protein (CRP) was evaluated on COBAS c111 Chemistry Analyzer (Roche Diagnostics, Basel, Switzerland) using commercially obtainable reagents. Serum insulin was assessed using an immunoradiometric assay kit (Insulin Riabead II kit ; Dainabot, Tokyo, Japan). The inter- and intra-assay coefficients of variation of the assay were 2.1% and 2.0%, respectively.

The homeostasis model assessment (HOMA) method [10] was evaluated as follows : Insulin resistance (HOMA-IR) = Fasting insulin (μU/ mL) X fasting glucose (mmol/ L) / 22.5 ; normal insulin resistance ≤ 3, moderate insulin resistance (between 3 and 5), and severe insulin resistance ≥ 5 (10).

- *Assessment of HCV RNA viral load :*

Viral load of HCV in the plasma was assessed in all cases. Qiagen Viral RNA Kit (Hilden, Germany). A detection limit of the PCR is 12 IU/ml.

- *Detection of HCV genotype IV :*

Extracting parts of HCV-RNA from patient's sera was achieved by Qiagen Viral RNA mini Kit (Hilden, Germany). Firstly, using particular primers common to all genotypes (11) ; the core region was amplified by RT-PCR in the thermocycler (Biometra Analytik Jena Company, Germany). The output was then amplified in the second PCR with type IV genotype specific primers (11) and Taq DNA polymerase (Qiagen, Hilden, Germany).

Histopathology of percutaneous ultrasound-guided liver biopsy

Hepatic tissues were evaluated after paraffin embedding, 5 μm sections were obtained for Prussian Blue staining, Masson's trichrome staining, and Hematoxylin and Eosin staining. The hepatic tissue sample was diagnosed depending on the presence of at least 10 complete portal tracts has long been considered the 'gold standard' to estimate stages fibrosis, and degree of disease activity (12). In the present study, the stages of histologic hepatic fibrosis and the degree of inflammation was scored using the METAVIR scoring system (13). Indeed, hepatic steatosis was assessed as the percentage of hepatocytes containing fat droplets and it was graded as absent (< 5%), grade 1 (5-33%), grade 2 (33- 66%) and grade 3 (> 66%). Moderate/severe steatosis was considered as the presence of fat in > 33% of hepatocytes (14). While advanced fibrosis was defined with fibrotic score > 2 and mild fibrosis was defined as fibrotic score ≤ 2.

Liver biopsy was assessed by a pathologist (blinded to clinical and laboratory data).

Ethics

This work was approved by the Ethical Committee of Mansoura University, and before participating in any protocol specific procedure, all patients provided written informed consent. The study was achieved in accordance with the guidelines of the Helsinki Declaration.

Statistical analysis

All statistical analyses were carried out using the SPSS version 17.0 software (SPSS Inc., Chicago, Illinois, USA). Categorical variables were expressed as frequency (percentages) and continuous variables were presented as mean ± SD values. The Kolmogorov-Smirnov test was used to estimate the distribution of variables. Student's t test (independent-sample t-test) was used for continuous variables with normal distribution, and the

Mann-Whitney U test was used for continuous variables without normal distribution. In addition ; the χ^2 test was used for categorical variables. Spearman's correlation analyses were used to assess the relationships. Receiver operating characteristics (ROC) curve analysis was used to assess the role of NLR in distinguishing patients with high grades of IR and hepatic fibrosis. A value of $P < 0.05$ was accepted as the significance level.

Results

Demographic and laboratory data of the patients and the control group :

This work included 332 patients ; a total of 98 patients were excluded from this study (28 patients with CHB, 25 patients with nonalcoholic steatohepatitis (NASH),17 patients with combined HCV and HBV, 14 patients with autoimmune hepatitis, 5 patients with uncontrolled hyperthyroidism, 4 cases with SLE and 5 patients with renal failure on dialysis). Besides, the control group included 50 healthy sex- and age- matched individuals (male/female = 31/19). All of them had no autoimmune diseases, collagen diseases, hyperlipidemia, hypertension, diabetes mellitus, or any other comorbid diseases.

There was no significant statistical difference in body mass index (BMI), smoking, age, and gender (all $P > 0.05$) between patients compared with the control group. In chronic hepatitis C (CHC) patient group ; their stages of fibrosis were F0 (n = 16 ; 6.8%), F1 (n = 80 ; 34.2%), F2 (n = 70 ; 29.9%), F3 (n = 50 ; 21.4%), and F4 (n = 18 ; 7.7%), and the grades of hepatic steatosis were none (n = 132 ; 56.4%), grade 1 (n = 84 ; 35.9%) and grade 2 (n = 18 ; 7.7%). There was statistically increased γ-GT, ALP, AST, and ALT in CHC patients versus control group (all $P < 0.05$). Moreover ; fasting blood sugar, fasting serum insulin and HOMA-IR were significantly elevated in CHC group versus the control group (all $P < 0.001$). On the other hand ; there was no statistical changes in hemoglobin levels, serum albumin (ALB), bilirubin, and INR between two groups (all $P < 0.05$), as shown in Table 1.

Table 1. — **Demographic and laboratory data of the studied groups**

Variables	Patient group (n = 234)	Control group (n = 50)	P value
Age (years)	50.42 ± 6.31	49.49 ± 6.56	NS
Gender (M/F)	144/90	31/19	NS
Smoking	116 (49.6%)	26 (52%)	NS
BMI (kg/m²)	27.81 ± 4.11	26.95 ± 5.23	NS
Staging of liver biopsy			
F0	16 (6.8%)	–	–
F1	80 (34.2%)	–	–
F2	70 (29.9%)	–	–
F3	50 (21.4%)	–	–
F4	18 (7.7%)		–
Liver steatosis			
0	132 (56.4%)	–	–
Grade 1	84 (35.9%)	–	–
Grade 2	18 (7.7%)	–	
Grade 3	0	–	–
Plasma HCV RNA load (×10⁵ IU/ml)	17.69 ± 9.54	–	–
AST (U/L)	51.43 ± 19.46	32.43 ± 10.23	< 0.001
ALT (U/L)	49.67 ± 22.15	31.56 ± 11.69	< 0.001
ALB (g/dL)	3.99 ± 0.406	4.06 ± 0.112	NS
Bilirubin (mg/dL)	1.4 ± 0.12	1.1 ± 0.17	NS
ALP (IU/L)	146 ± 55.6	121 ± 22.5	0.002
γ-GT (IU/L)	56 ± 19.8	32 ± 17.5	< 0.001
INR	1.2 ± 0.7	1.1 ± 0.5	NS
Hemoglobin (g/dL)	12.33 ± 1.71	11.66 ± 1.23	NS
Fasting blood glucose (mmol/l)	4.9 ± 0.75	4.1 ± 0.42	< 0.001
Fasting insulin (μU/l)	12.85 ± 4.5	5.9 ± 1.75	< 0.001
HOMA	3.98 ± 1.41	1.92 ± 0.74	< 0.001

M, male ; F, female ; BMI, basal metabolic index ; HCV, hepatitis C virus ; AST, aspartate aminotransferase ; ALT, alanine aminotransferase ; ALB, albumin ; ALP, alkaline phosphatase ; γ-GT, γ-glutamyl transpeptidase ; INR, international normalized ratio ; HOMA, homeostasis model assessment ; NS, not significant.

Table 2. — **Laboratory Data of the Groups**

Variables	CHC with HOMA-IR		Control group (n = 50)	*P* value
	CHC with HOMA-IR > 3 (n = 98)	CHC with HOMA-IR ≤ 3 (n = 136)		
Platelets (10^3/L)	247.47 ± 72.17	256.43 ± 47.51	231.14 ± 53.75	NS
WBCs (10^3/L)	7.74 ± 1.25	7.65 ± 1.44	6.72 ± 1.18	NS
ESR, mm/h	23.55 ± 14.66	9.76 ± 7.68	6.33 ± 4.05	< 0.001*
CRP (mg/dL)	2.48 ± 2.27	0.95 ± 0.67	0.69 ± 0.55	< 0.001*
TNF-α (pg/ml)	13.64 ± 7.89	8.11 ± 6.16	7.31 ± 4.87	< 0.001*
IL-6 (pg/ml)	63.3 ± 27.65	44.4 ± 21.34	35.6 ± 18.65	< 0.001*
Neutrophils (10^3/L)	5.56 ± 1.75	4.34 ± 1.34	3.72 ± 1.22	< 0.001*
Lymphocytes (10^3/L)	1.92 ± 0.65	2.65 ± 0.66	2.14 ± 0.48	< 0.001*
N/L ratio	2.61 ± 0.95	1.92 ± 0.86	1.80 ± 0.76	< 0.001*

* Significant difference between the two groups.

WBC, white blood cell count ; ESR, *Erythrocyte sedimentation rate* ; CRP, C-reactive protein ; TNF-α, tumor necrosis factor alpha ; IL-6, interleukin-6 ; NLR, neutrophil-to-lymphocyte ratio ; NS, not significant.

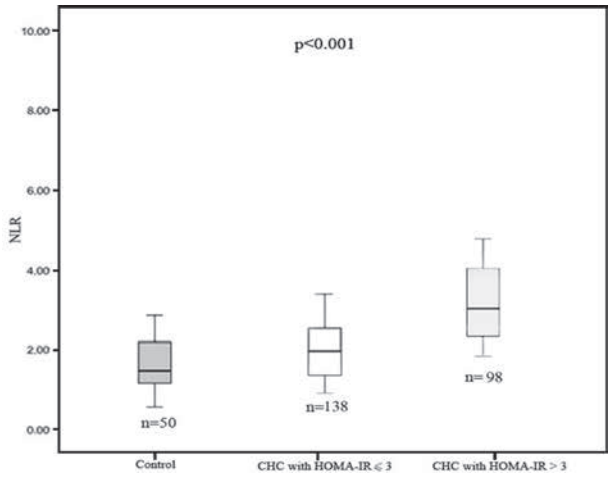


Fig. 1. — Mean neutrophil-to-lymphocyte ratio (NLR) values in the groups.

After the assessment of the biochemical data of the chronic hepatitis C (CHC) and control groups, CHC patients were divided into two sub-groups according to the degree of HOMA-IR ; CHC with HOMA-IR> 3 [n = 98 (42%)] and CHC with HOMA-IR ≤ 3 [n = 136 (58%)]. There were no statistically differences in terms of gender, age, BMI, and smoking habits in these sub-groups. The mean NLR levels of the CHC with HOMA-IR patients were significantly elevated than those of the healthy control group ($P < 0.001$) as shown in table 2, and NLR values of the patients in CHC with HOMA- IR > 3 were elevated than those of the patients with CHC with HOMA-IR ≤ 3 ($P < 0.001$) (Fig. 1). Moreover ; in terms of NLR, there was a significant statistical difference between stages of fibrosis in CHC group ($P < 0.05$) (Fig. 2 A).

In CHC patients, there was a positive correlation between NLR values and HOMA-IR, stages of liver fibrosis, hepatic steatosis, C-reactive protein, TNF-α, and IL-6 values ($r = 0.65$, $P < 0.001$; $r = 0.67$, $P < 0.001$; $r = 0.74$, $P < 0.001$; $r = 0.78$, $P < 0.001$, $r = 0.68$, $P < 0.001$ and $r = 0.66$, $P < 0.001$ respectively) (Fig. 2 B, and Fig. 3). In the same group, HOMA-IR was positively correlated with the stages of liver fibrosis, C-reactive protein, TNF-α, and IL-6 values ($r = 0.66$, $P < 0.001$; $r = 0.74$, $P < 0.001$; $r = 0.64$, $P < 0.001$; and $r = 0.72$, $P < 0.001$, respectively).

No significant differences were reported as regard platelet values and hemoglobin between patients with fibrosis score ≥ F2 and patients with fibrosis score (F0 + F1 + F2). While ; NLR, HOMA-IR, CRP, TNF-α, and IL-6 value was significantly higher in patients with fibrosis score ≥ F2 versus (F0 + F1 + F2) (all $P < 0.001$) (Tables 3 and Fig. 2).

The relationship between NLR and the presence of high degree of IR > 3 and fibrosis stages

The Receiver Operating Characteristic curve analysis mentioned that a cutoff value of 2.01, the NLR for evaluating cases with CHC with HOMA > 3, has the highest specificity (74.9%) and sensitivity (72.3%) ; with an area under the curve (AUC) of 0.821 (95%CI : 0.682-0.876), ($P < 0.001$), with NPV and PPV for NLR of 79.6 and 72.1%, respectively as shown in Fig. 4 (A).

Regarding, the hepatic histopathological characteristics ; a group with mild fibrosis composed of 166 patients and a group with advanced fibrosis consisted of 68 patients. We compared NLR values between the advanced and mild fibrosis subgroups of CHC and reported a statistically significant difference (1.86 ± 0.66 and 2.4 ± 0.99, respectively, $P < 0.001$), as outlined in table 3 and figure 2 (A). The ROC curve analysis of NLR for the estimation of advanced fibrosis in CHC was statistically significant ; at cut-off value 2.2, the NLR for identifying patients with advanced fibrosis, has the highest specificity (73.5%) and

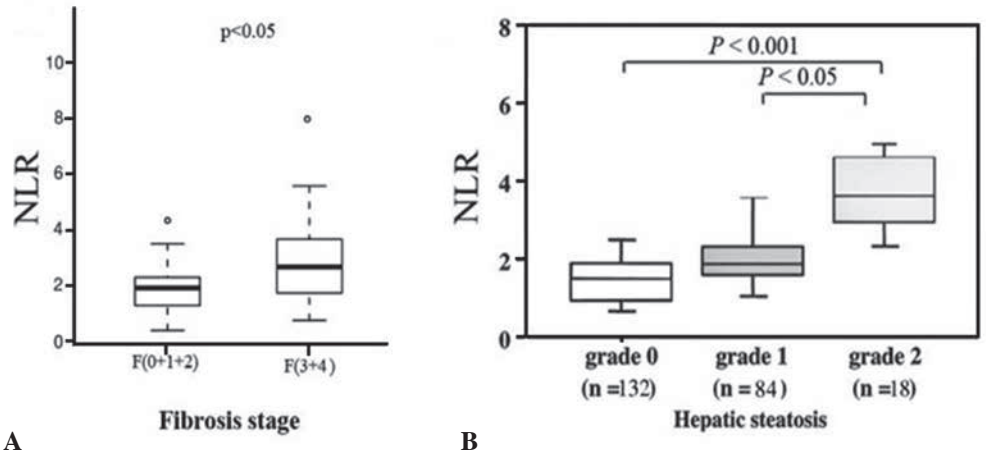


Fig. 2. — Mean neutrophil-to-lymphocyte ratio (NLR) values in chronic hepatitis C patients ; according to stage of liver fibrosis (A) and hepatic steatosis (B).

sensitivity (77.2%) with (AUC = 0.793, 95%CI : 0.669 -0.994, $P < 0.001$), with NPV and PPV for NLR of 79.4 and 69.3%, respectively as shown in Figure 4 (B).

Discussion

The world's highest prevalence of HCV infections was represented in Egypt (15). IR is mediated by HCV genotype-4 irrespective of the severity of liver disease. IR initiates early in infection of HCV and props the progression of HCC development and hepatic fibrosis (16).

NLR is an elementary biomarker of inflammation used to estimate systemic inflammation processes (17). It

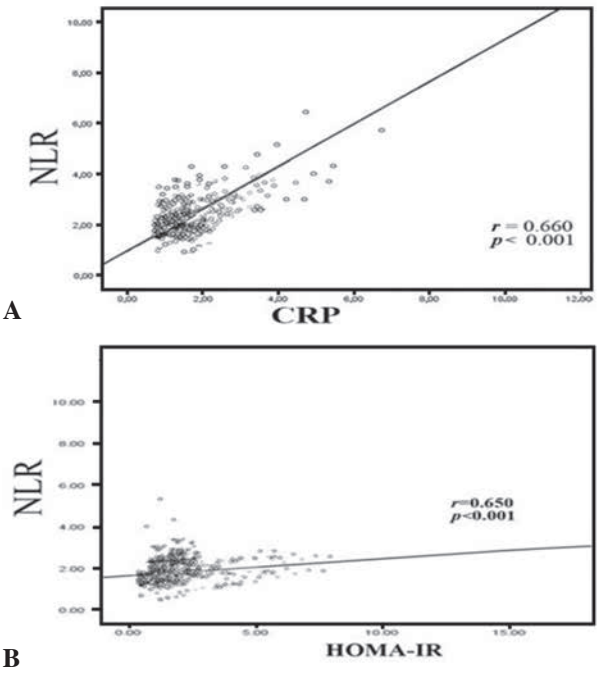


Fig. 3. — The association between neutrophil-to-lymphocyte ratio (NLR) and C-reactive protein (CRP) (A) and HOMA-IR (B).

was shown that diabetes, essential hypertension, valvular heart diseases, renal and/or hepatic failure, metabolic syndrome, acute coronary syndromes, thyroid function abnormalities, inflammatory diseases, local or systemic infections, many drugs and malignancy may probably influence the NLR (18-20).

This study of our best of knowledge is the first study to describe the use of NLR as a novel and reliable marker to diagnose IR in chronic hepatitis C virus infection.

Our findings explained that : (i) NLR is increased in patients with CHC with HOMA-IR > 3 compared with CHC with HOMA-IR ≤ 3 patients, and a cutoff value of 2.01 can be used to detect patients with higher degrees of IR > 3, (ii) NLR is positively correlated with the histopathological characteristics of chronic hepatitis, particularly the stages of fibrosis and steatosis ; and a cutoff value of 2.2 can be used to detect patients with advanced fibrosis in CHC.

Proinflammatory cytokines such as various interleukins and TNF-α can locally influence insulin sensitivity at their site of production as well as in distant adipose tissue (21). Besides ; interleukin-6, TNF-α, and platelet inhibitors and/or activators are the main proinflammatory adipokines ; that plays a vital function in the pathogenesis of inflammatory disorders (22). Therefore, systemic inflammation creates IR and IR immortalizes inflammation (23).

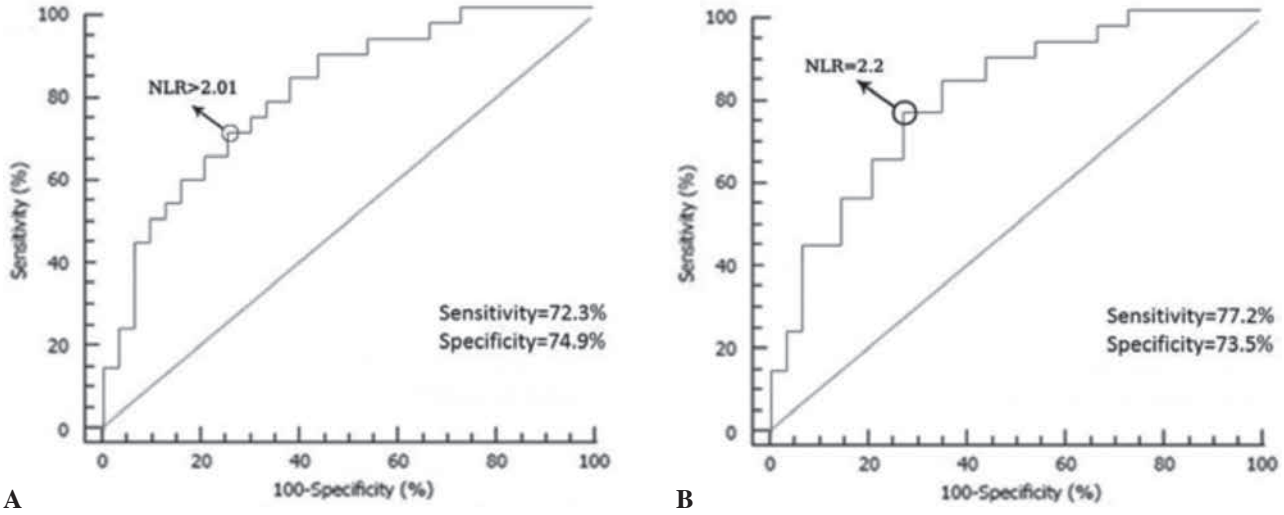
A previous study reported that patients with CHC had laboratory proof of IR (24). These data are further boosted by literature suggesting an increased risk of diabetes in patients with CHC (25). In HCV-infected patients ; obesity, family history of diabetes, the degree of liver fibrosis, and older age correlate with the development of diabetes (26).

In our work ; a positive correlation between TNF-α, IL-6, CRP, and NLR approved our elementary hypothesis that NLR could represent ceaseless systemic inflammatory responses in HCV infection and related fibrosis. This observation together with elevated NLR in patients

Table 3. — **Comparison of hematological values, HMOA-IR and proinflammatory cytokines according to METAVIR staging**

Variables	F0 + 1 + 2 (n = 166)	≥ F3 (n = 68)	P value
Haemoglobin (g/dL)	12.33 ± 1.71	11.93 ± 1.43	NS
Platelets (10^3/L)	211.33 ± 61.26	196.53 ± 51.32	NS
NLR	1.86 ± 0.66	2.4 ± 0.99	< 0.001
HOMA-IR	2.11 ± 0.95	3.87 ± 1.89	< 0.001
CRP (mg/dL)	1.75 ± 0.78	2.33 ± 1.27	< 0.001
TNF-α (pg/ml)	9.34 ± 4.67	12.88 ± 6.98	< 0.001
IL-6 (pg/ml)	42.5 ± 22.11	61.2 ± 26.21	< 0.001

NLR, neutrophil-to-lymphocyte ratio ; HOMA, homeostasis model assessment ; CRP, C-reactive protein ; TNF-α, tumor necrosis factor alpha ; IL-6, interleukin-6 ; NS, not significant.



A **B**

Fig. 4. — Receiver operating characteristic (ROC) curve analysis of NLR for the identification of CHC patients with HOMA-IR. A cutoff value of 2.01 had the highest sensitivity and specificity to identify patients with high HOMA-IR > 3 (A) ; and for the identification of CHC patients with fibrosis. A cutoff value of 2.2 had the highest sensitivity and specificity to identify patients with advanced stages (F3 + F4) of liver fibrosis (B).

with CHC with HOMA-IR > 3 compared with CHC with HOMA-IR ≤ 3 patients are propped by the findings of previous researches that demonstrated ; low grades (subclinical) inflammation is gradually leading to diabetes mellitus development and under the effect of proinflammatory cytokines, the endothelial dysfunction may have been progressed. In adipose tissue, especially visceral adiposity, these cytokines are secreted (27,28). Moreover Buyukkaya *et al*. demonstrated that, a significant positive correlation between inflammation and the criteria of metabolic syndrome on the basis of NLR. Thus, increasing the severity of metabolic syndrome leads to an elevation in NLR (18).

We strongly believe that through serious inflammation ; elevated counts of neutrophils may intercede IR. The increment in NLR denotes underlies the elevated levels of the pro-inflammatory process, as evidenced from the enhanced release of neutrophil proteases and the persistent neutrophil activation in type 2 diabetic patients (29). In adipose tissue, especially in mice fed with a high-fat diet, incremented neutrophil recruitment has been observed (maybe via secreted elastase) (30).

Surprisingly, the neutrophils can be added to the extensive repertoire of immune cells that collaborate on pro-inflammatory processes that underlie IR (31).

We strongly believe that the observations of this study will enable us to use NLR as a superior marker for identifying patients with higher grades of IR and histopathological patterns of HCV, especially hepatic fibrosis earlier in the course of disease progression.

The main power of this work is the inclusion of patients with biopsy-proven CHC with the full spectrum of disease and measurements of TNF-α, IL-6, and CRP that allowed us to identify the relationship between hepatic histopathological changes with NLR. However, there are several limitations to the current study that merit consideration and discussion. First, the cross-sectional design of our work. Second, our sample size was somewhat

small and larger studies are needed to estimate the authenticity of this marker in various clinical settings.

In conclusion, our work showed that in CHC patients, NLR is related to histological severity and could be used to detect patients with advanced hepatic fibrosis. It's well known that ; NLR is inexpensive, more applicable, widely available, and easily repeatable. Although the preciseness of NLR for estimating IR and significant fibrosis is sufficient ; NLR in combination with other markers may help to detect patients at increased risk of having advanced disease and higher grades of IR. NLR could be used to estimate the fibrotic process of different diseases. It may become one of the cornerstones of fibrosis and inflammation if assured in large scale population based studies in the future.

Acknowledgments

We would like to express our sincere gratitude to the patients, laboratory technicians, and staff of the Tropical Medicine Department, for their honorable efforts in this work.

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