Original Article

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# Relation of Lymphocyte Subsets and Cytokines in Different Grades of Alcoholic Cirrhosis

Pathology Section

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## ABSTRACT

**Introduction:** The pathogenesis of Alcoholic Liver Disease (ALD) shows immune dysregulation with decreasing lymphocyte subsets and increasing CD4/CD8 ratio. T lymphocyte activation leads to secretion of cytokines like Tumour Necrosis Factor-α (TNF-α) and interleukins causing inflammation and fibrosis.

**Aim:** To correlate lymphocyte subsets and TNF-α and Interleukin 6 (IL-6) in different grades of alcoholic cirrhosis.

**Materials and Methods:** This was a prospective, cross-sectional study carried out over a period of two years at a tertiary care hospital and research centre in Western Maharashtra. The study cohort consisted of 51 patients diagnosed clinically as alcoholic cirrhosis. They were classified into grades A, B and C by Child-Pugh's score. Lymphocyte subsets were determined by flow cytometry. T lymphocytes were identified (CD3+), and further subdivided into CD4+and CD8+cells. TNF-α and IL-6 were measured by ELISA. All parameters were measured using one-way Analysis of Variance (ANOVA test).

**Results:** There was insignificant change in the total number of lymphocytes with different grades of cirrhosis. T lymphocytes and CD4+cells showed increase in count (885.38±464.19/mm$^3$ to 1002.81±338.52/mm$^3$) and (489.63±248.23/mm$^3$ to 689.05±263.08/mm$^3$) respectively, with corresponding decrease in CD8 count (364±230.37 to 3440.38±165.91). There was also an increase in CD4/CD8 ratio (1.48 to 2.18) along with raised TNF-α (25.16±18.45 to 30.15±36.37) and IL-6 (58.27±50.52 to 175.38±241.85) with increasing grades of cirrhosis.

**Conclusion:** Alcoholic cirrhosis is a complex entity caused due to interaction between various components of immune systems and elaboration of cytokines. We found increase in T lymphocytes and CD4+cells along with increase in cytokines TNF-a and IL-6 with increase in grades of cirrhosis.

**Keywords:** Flow cytometry, Interleukin-6, Tumour necrosis factor-α

## INTRODUCTION

In recent times, there has been a rapid and significant increase in per capita consumption of alcohol especially in the developing countries of the Asian continent [1].

Of all those deaths, people aged 15 to 29 years account for about 320,000 deaths per annum. ALD is the most common medical consequence of excessive alcohol intake accounting for about 70% of recorded mortality [2].

ALD consists of three major categories as the disease progresses. The spectrum consists of fatty liver, alcoholic hepatitis and cirrhosis and finally Hepatic Cell Carcinoma (HCC). Cirrhosis can further be classified into grades A, B and C according to Child-Pugh classification based on total bilirubin, serum albumin, prothrombin time, ascites and hepatic encephalopathy. This classification measures severity of liver disease based on the above-mentioned clinical parameters [3].

Studies have suggested the role of chronic inflammation in alcoholic liver injury. Several studies have shown infiltration by CD4+ and CD8+T lymphocytes in alcoholic hepatitis and active cirrhosis [4]. It is well documented that there is activation of B-cells which leads to increased in-vivo synthesis of immunoglobulins in alcoholic liver damage [5]. Studies have also described a decrease in lymphocyte count as well as its subsets, and increase in CD4/CD8 ratio in ALD when compared to healthy controls [6]. Chronic alcohol treated mice show increased release of cytokines such as TNF-α, TNF-β, IL-6, IL-10 and IL-12 [7,8]. These cytokines cause morphological changes in the hepatic parenchyma.

With this background, this study was undertaken to determine the lymphocyte population and CD4/CD8 ratio and to see relation of TNF-α and IL-6 in different grades of alcoholic cirrhosis based on Child-Pugh classification.

## MATERIALS AND METHODS

This was a prospective, cross-sectional study carried out over a period of two years from July 2015 to June 2017, at a tertiary care hospital and research centre in Western Maharashtra, India. The study cohort consisted of 51 patients diagnosed clinically as alcoholic cirrhosis and admitted in our hospital. These patients had a history of average daily intake of 40-80 mL of alcohol over a period of 10 years or more, with clinical signs and symptoms ranging from nausea, anorexia, malaise, jaundice, upper abdominal pain, ascites, variceal bleeding or encephalopathy. The evaluation of stage of Liver disease was established with the help of Ultrasonography (USG). Abdominal USG revealed liver parenchymal disease with coarse echotexture along with ascites and/or splenomegaly. The liver function tests were done. Patients suffering from HIV, Diabetes Mellitus, Tuberculosis, malignancy, or on any Immunosuppressant drug were excluded from the study as lymphocyte subsets get altered due to these conditions. These cases were further classified into Child-Pugh's score which is further graded A, B and C as shown in [Table/Fig-1].

| Measure | 1 point | 2 points | 3 points |
|---|---|---|---|
| Total bilirubin, µmol/L (mg/dL) | <34 (<2) | 34-50 (2-3) | >50 (>3) |
| Serum albumin, g/dL | >3.5 | 2.8-3.5 | <2.8 |
| Prothrombin time, prolongation(s)<br>OR<br>International Normalized Ratio (INR) | <4.0<br><br><1.7 | 4.0-6.0<br><br>1.7-2.3 | >6.0<br><br>>2.3 |
| Ascites | None | Mild (or suppressed with medication) | Moderate to severe (or refractory) |
| Hepatic encephalopathy | None | Grade I-II | Grade III-IV |
| Grade A score: 5-6, Grade B score: 7-9, and Grade C score: 10-15. | | | |

**[Table/Fig-1]:** The Child-Pugh's score.

All procedures performed in the current study were approved by Ethics Committee, Dr. DY Patil Vidyapeeth, Pune (Ref: DYPV/EC/360/15 Dated: 07/03/2015) in accordance with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all individual participants included in the study.

Five millilitres of peripheral blood was obtained from each patient from which 3 mL was collected in EDTA vacutainer and 2 mL in plain vacutainer. EDTA vacutainer was used to obtain Total and differential WBC count along with absolute lymphocyte count using BenespheraTM 5-part Differential Haematology Analyser. Flow cytometric analysis was done to determine the T lymphocyte subset using FACS Jazz (Beckton Dickinson, San Jose, CA, USA). BD CalibriteTM 3 beads were used for the calibration (Beckton Dickinson, San Jose, CA, USA).

The subsets of lymphocytes measured were CD45+, CD3+, CD4+ and CD8+using Fluorescent tagged antibodies. Five units of Anti-CD45 V450, 10 units each of Anti-CD3 FITC (BD Multitest CD3 fluorescein isothiocyanate), Anti-CD4 PE (Phycoerythrin), and Anti-CD8 APC (Allophycocyanin) were added with appropriate dilution to the 100 µL sample of blood. It was mixed by tapping and then incubated at room temperature for 30 minutes. A two mL of FACS lysing solution was added to each tube, which was then vortexed for 10 seconds. This was followed by incubation in dark for 10-15 minutes and centrifugation at 2000 rpm for 2 minutes. This procedure was repeated twice for washing and then 500 µL of sheath fluid was added to the cell pellet. The resulting fluid was then vortexed and stored in a refrigerator at 4°C. This was run in the flow cytometer within 24 hrs. CD45+ cells were gated and CD3+ cells were identified. These were further separated into CD4+ and CD8+ cells. Ratio was also calculated. The blood collected in plain vacutainer was used for analysis of TNF-α and IL-6 which was done by DIA source TNF-α and IL-6 ELISA kit respectively. The DIA source ELISA kits are solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiterplate. The assay used monoclonal antibodies (MAbs) directed against distinct epitopes of TNF-α/IL-6. Calibrators and samples reacted with the capture monoclonal antibody (MAb 1) coated on microtiter well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase. After an incubation period allowing the formation of a sandwich: coated MAb 1-human TNF-α/IL-6-MAb 2-HRP, the microtiterplate was washed to remove unbound enzyme labelled antibody. Bound enzyme-labelled antibody was measured through a chromogenic reaction. Chromogenic solution was added and incubated. The reaction was stopped with the addition of Stop Solution and the microtiterplate is then read at the appropriate wavelength. The amount of substrate turnover was determined colourimetrically by measuring the absorbance, which was proportional to the TNF-α/IL-6 concentration. A calibration curve was plotted and TNF-α/IL-6 concentration in samples was determined by interpolation from the calibration curve.

Samples were run in three batches of 17 cases each. Since one of the batches revealed analytical error, their results were excluded. Hence the sample size of TNF-α and IL-6 with correct results (n=34) were considered for statistical analysis.

## STATISTICAL ANALYSIS

All parameters were measured using one-way Analysis of Variance (ANOVA test). The results were statistically analysed using SPSS Statistics data editor 20 software version 7.0 and Microsoft Office. Groups were characterised using descriptive statistics, means, standard deviations, and percentages. These tests were considered to be statistically significant when their p-values were <0.05 and extremely significant for values <0.01.

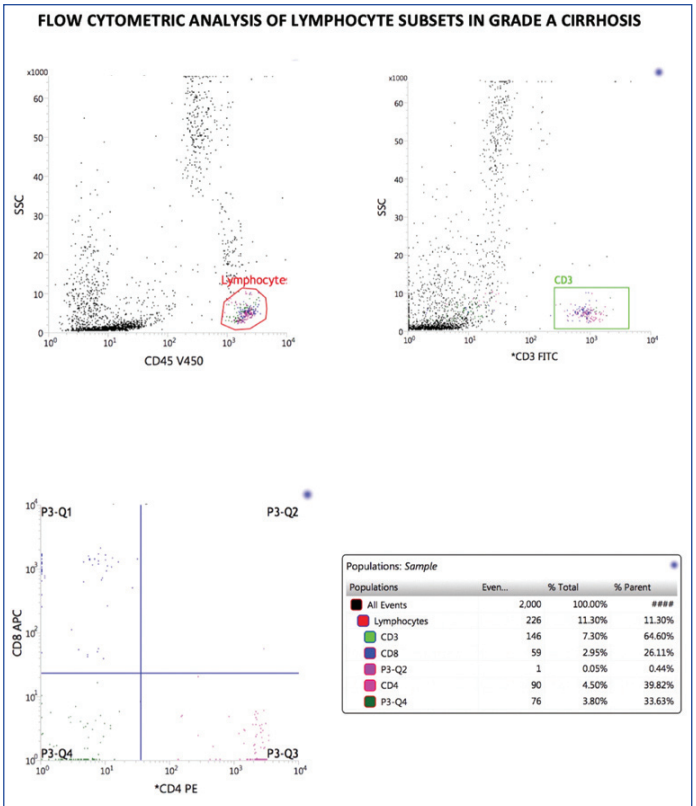
## RESULTS

The number of patients with grade A, B and C were 8, 22 and 21 respectively. The age ranged from 30-65 years. The mean age of grade A, B and C were 44.75, 46.73, and 44.57 years. Male predominance was observed.

Total CD45+lymphocyte count showed an increase in the counts as the severity of cirrhosis increased, however it is a statistically insignificant relationship (F Value: 0.23, p-value: 0.80). Likewise, an increase in T lymphocytes with CD4+ subset as the grade of cirrhosis increases; however the relationship is also not statistically significant. CD8+ counts were reduced resulting in increase in CD4/CD8 ratio with higher grades of cirrhosis [Table/Fig-2]. Flow cytometric analysis in different grades of cirrhosis is shown in [Table/Fig-3-5]. Results of TNF-α and IL-6 are shown in [Table/Fig-6,7] respectively. There was an increasing trend as the disease progressed, however it was not statistically significant.

| | Cirrhosis | | | | | | F value | p-value |
|---|---|---|---|---|---|---|---|---|
| | Grade A (n=8) | | Grade B (n=22) | | Grade C (n=21) | | | |
| | Mean | SD | Mean | SD | Mean | SD | | |
| CD45 | 1332 | 681.50 | 1501.45 | 815.89 | 1396.67 | 503.19 | 0.23 | 0.80 |
| CD3 | 885.38 | 464.19 | 1088.68 | 635.47 | 1002.81 | 338.52 | 0.50 | 0.61 |
| CD4 | 489.63 | 248.23 | 635 | 360.65 | 689.05 | 263.08 | 1.22 | 0.30 |
| CD8 | 364 | 230.37 | 452.05 | 329.92 | 344.38 | 165.91 | 0.99 | 0.38 |
| CD4/CD8 | 1.48 | 0.45 | 1.81 | 0.85 | 2.18 | 0.998 | 2.14 | 0.13 |

**[Table/Fig-2]:** Lymphocyte subsets in different grades of cirrhosis.

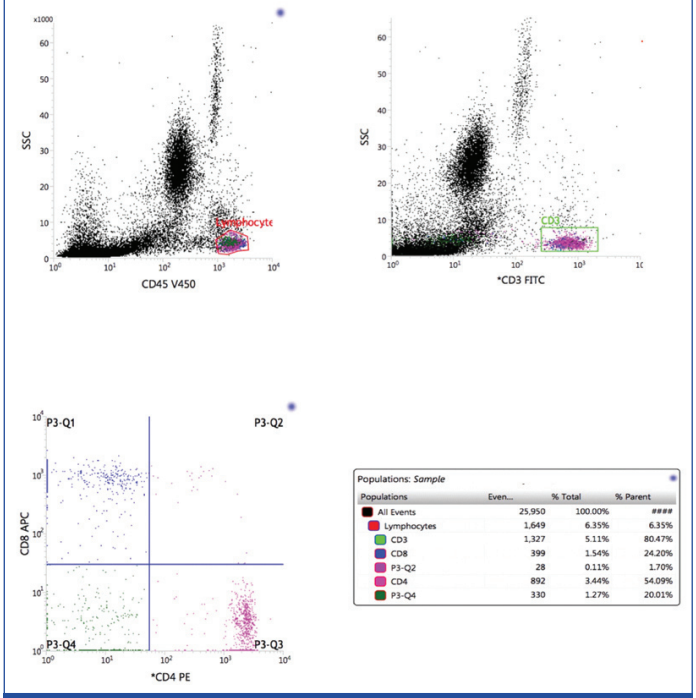



**[Table/Fig-3]:** Lymphocyte population in Grade A cirrhosis was gated in a CD45 side scatter plot, from which the CD3+ T cells were gated. Subsequently CD4+ and CD8+ cells populations were identified.

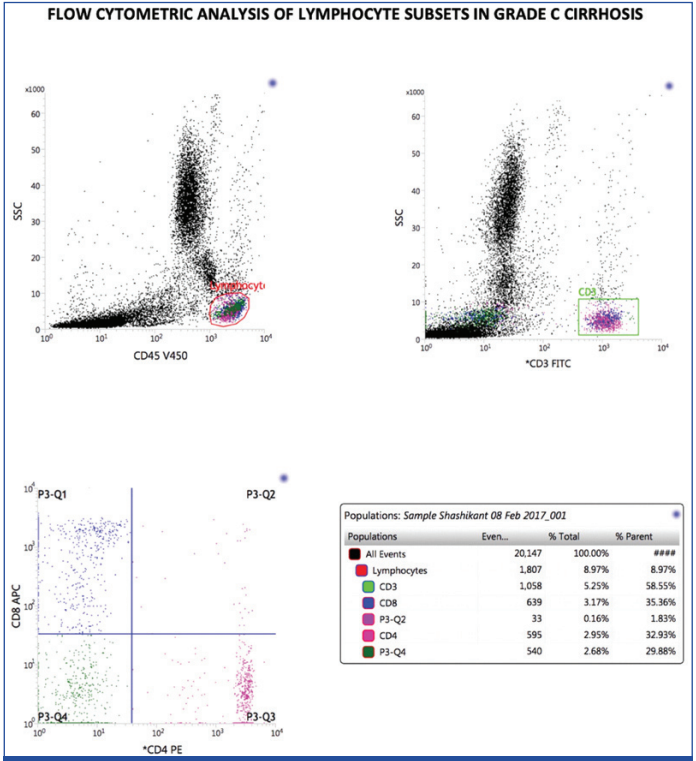
## DISCUSSION

Lymphocyte subsets play an important role in the pathogenesis of ALD [9]. It is an established fact, first observed by Couzigou P et al., that patients with liver failure due to alcoholic cirrhosis show lymphopenia and increased CD4/CD8 ratio with progression of the disease [6]. A reduction in T lymphocyte (CD3+) and its subset CD4+ T lymphocytes was also found to correlate with the severity of liver cirrhosis [10,11]. Several other studies reported relative increase in the number of T lymphocytes in alcoholic patients, mainly due to increased number of activated CD8+cells [12,13].

Alcohol alters the gut microbiota, increases the gut permeability and increases lipopolysaccharides in liver triggering liver damage




**[Table/Fig-4]:** Lymphocyte population in Grade B cirrhosis was gated in a CD45 side scatter plot, from which the CD3+ T cells were gated. Subsequently CD4+ and CD8+ cells populations were identified.




**[Table/Fig-5]:** Lymphocyte population in Grade C cirrhosis was gated in a CD45 side scatter plot, from which the CD3+ T cells were gated. Subsequently CD4+ and CD8+ cells populations were identified.

| Cirrhosis | n | TNF-α | | F value | p-value |
|---|---|---|---|---|---|
| | | Mean | SD | | |
| Grade A | 4 | 25.16 | 18.45 | 0.61 | 0.55 |
| Grade B | 14 | 19.25 | 10.75 | | |
| Grade C | 16 | 30.15 | 36.37 | | |

**[Table/Fig-6]:** Comparison of TNF-α according to cirrhosis grade in study group.

via TLR4 activation. This leads to increase proliferation of several proinflammatory cytokines such as TNF-α and IL-6. It increases the cholestasis and acute phase reactants. This in turn leads to release

| Cirrhosis | n | IL-6 | | F value | p-value |
|---|---|---|---|---|---|
| | | Mean | SD | | |
| Grade A | 3 | 58.27 | 50.52 | 1.13 | 0.33 |
| Grade B | 15 | 336.37 | 484.26 | | |
| Grade C | 16 | 175.38 | 241.85 | | |

**[Table/Fig-7]:** Comparison of IL-6 according to cirrhosis grade in study group.

of other cytokines by the lymphocytes which are responsible for fibrosis and consequently cirrhosis [14,15].

ALD ultimately leading to cirrhosis is accompanied by several secondary inflammations following release of toxic metabolites derived from degradation of alcohol by cytochrome CYP2E1 [16]. This leads to formation of reactive oxygen species along with production of TNF-α. Other interleukins are also released by Helper T cells which play an important role in pathogenesis of alcoholic cirrhosis. Liver damage is directly influenced by severity of inflammation [17]. The literature reveals that there is decrease in lymphocyte count and subsets from fatty liver to cirrhosis. This study revealed that amongst the cirrhotic patients there was mild lymphocytosis with increase in T lymphocytes with increase in grade of cirrhosis. There was an increase in CD4+ count and decrease in CD8+ count with an increase in CD4/CD8 ratio with increase in grade of cirrhosis. There was also an increase in TNF-α and IL-6 with increased grade of cirrhosis. Though all these results were statistically insignificant, we observed a relation between the values of cytokines and lymphocyte subsets. Grade B cirrhosis revealed higher range of cytokines which reflected with the corresponding increase in the lymphocyte subsets. This finding is in tune with the immune regulation and release of cytokines as a basis of pathogenesis of liver cirrhosis.

Costa Matos L et al., had found lymphopenia with reduction of all lymphocyte subsets and significantly increased CD4/CD8 ratio reflecting relative reduction in the number of CD8+ cells. They also concluded that patients with advanced fibrosis presented with an increased CD4/CD8 ratio accounting for active alcohol consumption [9]. There was also consistent increase in CD4/CD8 ratio which signifies fibrosis and hence severity of clinical parameters of Child-Pugh's score, leading to poor prognosis and decreased survival. It is believed that CD4/CD8 ratio and levels of cytokines like TNF-α and IL-6 may eventually contribute as a non-invasive marker of staging for alcoholic cirrhosis.

The reagents and the equipment used in the study are costly, require good infrastructure and trained staff which may not be feasible to utilise this test as a routine practice. However, in future, further studies with larger sample size with strict selection criteria and comparison with tissue diagnosis may strengthen the validity of this argument and reliability of these tests.

## LIMITATION

This sample size was small which could be the reason for insignificant p-value despite the correlating trends of lymphocyte subsets and cytokine levels. There are many variables which affects the CD4+ and CD8+ counts like age, sex, diurnal variations, history of smoking, BMI, and associated undiagnosed viral infections which may give rise to variabilities in the results.

Liver biopsy for tissue diagnosis was not done to quantify the amount of fibrosis and to rule out other contributing causes of cirrhosis which was another limitation of our study.

## CONCLUSION

Alcoholic cirrhosis is a complex entity caused due to interaction between various components of immune systems and elaboration of many cytokines. This study adds to the finding of significant lymphopenia in alcoholics with slight increase in lymphocyte count as the grade of cirrhosis increases. There was increased CD4/CD8

ratio along with raised TNF-α and IL-6 with increase in grade of cirrhosis, suggesting its direct association with liver cirrhosis, which can eventually contribute as a non-invasive marker for staging of alcoholic cirrhosis.

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