**Research Paper**

*Running title: B-cell Subsets and Fibrosis Indices in Cirrhosis*

# Peripheral B-cell immunophenotypic alterations correlate with non-invasive fibrosis indices in viral and non-viral cirrhosis

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**ABSTRACT**

**Background**

Liver fibrosis is a key determinant of disease progression in cirrhosis. Non-invasive indices such as APRI, FIB-4 and FIB-6 are widely used to estimate fibrosis severity; however, their relationship with immune alterations, particularly B-cell subsets, remains incompletely understood.

**Objective**

To investigate the association between peripheral B-cell subset distribution and non-invasive fibrosis indices (APRI, FIB-4 and FIB-6) in cirrhotic patients.

**Methods**

This case–control study included 80 participants divided into four groups: HCV-related cirrhosis, HBV-related cirrhosis, non-HBV/non-HCV cirrhosis, and healthy controls. Peripheral blood B cell subsets were analyzed using multiparameter flow cytometry. APRI, FIB-4 and FIB-6 scores were calculated for fibrosis assessment and correlations were analyzed.

**Results**

Total $CD19^+$ B cells, $CD19^+CD24^+CD27^+$, and $CD19^+CD5^+$ subsets were significantly increased in cirrhosis, whereas $CD19^+CD24^+CD73^+$ and $CD19^+IgM^+$ subsets were significantly reduced ($p < 0.05$). APRI, FIB-4 and FIB-6 showed positive correlations with $CD19^+$, $CD19^+CD24^+CD27^+$, and $CD19^+CD5^+$ B cells, and negative correlations with $CD19^+CD24^+CD73^+$ and $CD19^+IgM^+$ subsets.

**Conclusion**

Peripheral B-cell subset alterations are significantly associated with fibrosis severity in cirrhosis. These findings suggest that B-cell immunophenotyping may serve as a complementary immunological marker for disease progression.

**Keywords:** B cells; Cirrhosis; APRI; FIB-4; FIB-6; Flow cytometry; Liver fibrosis.

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**Introduction:**

Chronic liver diseases caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) remain major global health challenges, accounting for a substantial proportion of liver cirrhosis, hepatocellular carcinoma, and liver-related mortality worldwide. Recent estimates indicate that hundreds of millions of individuals are chronically infected, with viral hepatitis contributing to more than one million deaths annually due to progressive liver disease and its complications (1,2). Despite advances in antiviral therapies, liver fibrosis remains a key determinant of clinical outcome and long-term prognosis.

Assessment of liver fibrosis has evolved significantly with the development of non-invasive indices such as the aspartate aminotransferase-to-platelet ratio index (APRI), fibrosis-4 (FIB-4) and fibrosis-6 (FIB-6) scores. These indices provide simple, cost-effective, and reliable tools for estimating fibrosis severity and have been widely validated in chronic viral hepatitis and cirrhosis (3,4,5). However, while these markers reflect

structural liver damage, they do not directly capture the underlying immunological mechanisms driving disease progression.

Chronic HBV and HCV infections are characterized by persistent antigenic stimulation and immune dysregulation, which play central roles in hepatic inflammation and fibrogenesis. While T-cell exhaustion has been extensively studied in chronic viral hepatitis, increasing evidence highlights the importance of B lymphocytes in modulating immune responses during chronic infection (6,7). B cells contribute not only to humoral immunity through antibody production but also function as antigen-presenting cells and cytokine-secreting cells, thereby influencing both innate and adaptive immune pathways (8).

B-cell populations are functionally heterogeneous and include distinct subsets with pro-inflammatory or regulatory properties. Effector B cells have been implicated in promoting inflammatory responses and may contribute to liver injury and fibrosis progression, whereas regulatory B cells (Bregs), particularly interleukin-10-producing B cells, exert immunosuppressive effects and help maintain immune homeostasis (9,10). In chronic viral hepatitis, expansion of regulatory B-cell populations has been associated with impaired antiviral T-cell responses and persistence of infection (11–13).

In addition, experimental and clinical studies suggest that B cells may directly influence fibrogenesis through interactions with hepatic stellate cells and modulation of cytokine networks (14). Alterations in B-cell phenotype and function have been reported in patients with chronic liver disease, indicating a potential link between immune dysregulation and fibrosis progression (15,16). However, the relationship between specific peripheral B-cell subsets and the severity of liver fibrosis remains incompletely defined.

In particular, limited data are available regarding the association between peripheral B-cell immunophenotypes and non-invasive fibrosis indices such as APRI, FIB-4 and FIB-6 in cirrhotic patients of different etiologies. Understanding this relationship may provide important insights into the immunopathogenesis of cirrhosis and identify potential immunological markers reflecting disease severity.

To the best of our knowledge, few studies have comprehensively evaluated peripheral B-cell subset distribution in relation to fibrosis indices in cirrhotic patients. Therefore, the present study aimed to investigate the association between peripheral blood B-cell subsets and non-invasive fibrosis indices, namely APRI, FIB-4 and FIB-6 in patients with cirrhosis of different etiologies.

**Materials and Methods:**

**Study Design and Population**

This was a case–control study conducted to evaluate the association between peripheral blood B-cell subsets and non-invasive fibrosis indices in cirrhotic patients. A total of 80 participants were enrolled and divided into four equal groups; patients with hepatitis C virus (HCV)-related cirrhosis (n = 20):, patients with hepatitis B virus (HBV)-related cirrhosis (n = 20):, patients with non-HBV/non-HCV (cryptogenic) cirrhosis (n = 20):, and healthy controls (n = 20).

Patients were recruited from the Egyptian Liver Research Institute and Hospital (ELRIAH), Mansoura, Egypt, in collaboration with the Clinical Pathology Department, Faculty of Medicine, Suez University.

**Ethical Considerations**

The study was conducted in accordance with the principles of the World Medical Association Declaration of Helsinki. Ethical approval was obtained from the Research Ethics Committee of the Faculty of Medicine, Suez University (Approval No. 42). Written informed consent was obtained from all participants prior to enrollment.

**Inclusion and Exclusion Criteria**

Adult patients aged ≥18 years with clinically and radiologically confirmed liver cirrhosis were included. Chronic HBV and HCV infections were confirmed by polymerase chain reaction (PCR) testing.

Patients were excluded if they had autoimmune diseases (including autoimmune hepatitis), diabetes mellitus (for the non-viral cirrhosis group), active malignancy, severe systemic comorbidities affecting immune status, or were pregnant or lactating.

**Assessment of Liver Fibrosis**

Non-invasive fibrosis assessment was performed using APRI, FIB-4 and FIB-6 indices.

- APRI was calculated according to the method described by Wai, et al. (3)
- FIB-4 was calculated as described by Sterling, et al. (4)
- FIB-6 was calculated as described by Shiha, et al., (5)

These indices were selected due to their validated utility in assessing fibrosis severity in chronic liver disease.

**Sample Collection and PBMC Isolation**

Peripheral venous blood samples were collected from all participants under aseptic conditions. Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation with BD Vacutainer CPT tubes (BD Biosciences, USA).

Isolated cells were resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated human AB serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin.

**Flow Cytometric Immunophenotyping**

Multiparameter flow cytometry was performed to assess peripheral B-cell subsets using fluorochrome-conjugated monoclonal antibodies targeting CD19,

CD24, CD38, CD27, CD73, CD5, IgM, and IgD (Miltenyi Biotec, USA). The monoclonal antibodies are combined in two tubes as following:
CD45 PerCP, CD19 APC, CD38 PE, CD27 AF700, CD24 FITC.
CD19 APC, CD73 PE, CD5 PE AlexaFluor, IgM FITC and IgD AF700.
The selected fluorochrome panel was designed to match the optical configuration of the instrument while minimizing spectral overlap.
Cells were incubated with fluorochrome-conjugated monoclonal antibodies in the dark at room temperature, washed, and resuspended in PBS prior to acquisition. At least 50,000 events were acquired per sample. Data acquisition was performed using the Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific), which utilizes acoustic focusing technology to align cells prior to laser interrogation, allowing improved resolution and high-throughput analysis.

**Gating Strategy**
Lymphocytes were initially identified based on forward and side scatter properties. CD19+ B cells were then gated, followed by identification of specific B-cell subsets based on surface marker expression:

- Transitional B cells: CD19+CD24+CD38+
- Memory B cells: CD19+CD24+CD27+
- Regulatory-associated subset: CD19+CD73+
- CD19+CD5+ B1 cells
- CD19+IgM+ B cells
- CD19+IgD+ B cells

Fluorescence minus one (FMO) controls were used to accurately define gating boundaries and minimize false-positive signals.
The selected panel was designed to capture key functional B-cell subsets relevant to immune regulation and chronic inflammation. CD24 and CD38 identify transitional B cells, CD27 marks memory B cells, while CD73 and CD5 have been associated with regulatory and immunomodulatory B-cell phenotypes. IgM and IgD expression were included to distinguish naïve and unswitched B-cell populations.
This combination allows comprehensive phenotypic characterization of B-cell subsets implicated in immune dysregulation in chronic liver disease.

**Statistical Analysis**
Statistical analysis was performed using SPSS software (version XX). Quantitative data were expressed as mean ± standard deviation (SD). Comparisons between multiple groups were performed using one-way ANOVA or Kruskal–Wallis test depending on data distribution. Post hoc analysis was conducted using Tukey's test. Correlations between fibrosis indices (APRI, FIB-4 and FIB-6) and B-cell subsets were assessed using Spearman's correlation coefficient

**Results**

**1. Baseline Characteristics of the Study Population**
The mean age was highest in the HCV cirrhosis group, followed by the HBV cirrhosis group, while patients with cryptogenic cirrhosis were comparatively younger. Male predominance was observed in both viral cirrhosis groups, whereas a relatively higher proportion of females was noted among the cryptogenic cirrhosis and control groups (Table 1).

**2. Distribution of Fibrosis Indices (APRI, FIB-4 and FIB-6)**
Among the cirrhotic groups, the HCV-related cirrhosis group demonstrated the highest mean APRI and FIB-4 values, followed by the HBV group, while the cryptogenic cirrhosis group showed the highest mean FIB-6 values, followed by the HCV group (Table 2,3).

**3. Distribution of Peripheral B-Cell Subsets**
Flow cytometric analysis revealed significant alterations in peripheral B-cell subset distribution across study groups (Table 4).
Total CD19+ B cells were significantly increased in cirrhotic patients compared with controls ($p < 0.05$). Similarly, CD19+CD24+CD27+ (memory B cells) and CD19+CD5+ B cells showed a significant increase in cirrhotic groups.
In contrast, CD19+CD73+ B cells and CD19+IgM+ B cells were significantly reduced in cirrhotic patients compared with controls. Different B cell subsets showed variable distribution across groups most of them with consistent statistically significant differences.
Overall, these findings demonstrate a shift in B-cell homeostasis in cirrhosis toward expansion of activated/memory-associated subsets and reduction of naïve or regulatory-associated subsets.

**4. Correlation Between APRI, FIB-4, FIB-6 and B-Cell Subsets**
Significant correlations were identified between APRI, FIB-4 and FIB-6 scores and several B-cell subsets.
The 3 scores showed positive correlations with total CD19+ B cells, CD19+CD24+CD27+ (memory B cells) and CD19+CD5+ B1 cells. While the 3 scores showed negative correlations with gated lymphocyte percentage, CD19+CD73+ B cells and CD19+IgM+ B cells.
These correlations indicate that increasing fibrosis severity, as estimated by APRI, is associated with expansion of activated B-cell subsets and reduction of naïve/regulatory-associated populations (Figure 1-3 & Table5-7)

**6. Summary of Key Findings**
Taken together, the results demonstrate that cirrhosis is associated with significant alterations in peripheral B-cell subset distribution. Importantly,

these alterations correlate with non-invasive fibrosis indices, suggesting that progressive liver fibrosis is accompanied by a shift toward activated and memory B-cell phenotypes with a concomitant reduction in naïve and regulatory-associated subsets

**Discussion**

The present study investigated the relationship between peripheral B-cell subset distribution and non-invasive fibrosis indices in cirrhotic patients. The findings demonstrate that cirrhosis is associated with significant alterations in B-cell homeostasis, characterized by expansion of activated and memory-associated B-cell subsets and reduction of naïve and regulatory-associated populations. Importantly, these alterations showed consistent correlations with fibrosis severity as assessed by APRI, FIB-4 and FIB-6, suggesting a potential link between B-cell dysregulation and progression of liver fibrosis.

One of the key findings of this study was the significant increase in total $CD19^+$ B cells and memory B cells ($CD19^+CD24^+CD27^+$) in cirrhotic patients. This observation is consistent with previous studies reporting expansion of activated and atypical B-cell populations in chronic viral hepatitis, reflecting persistent antigenic stimulation and ongoing immune activation (15). Chronic exposure to viral antigens in HBV and HCV infections drives continuous B-cell activation and differentiation toward memory phenotypes, contributing to sustained inflammatory responses and immune-mediated liver injury (6).

In addition, the observed increase in $CD19^+CD5^+$ B cells may reflect expansion of innate-like B-cell populations with immunomodulatory properties. $CD5^+$ B cells have been implicated in both regulatory and pathogenic roles, depending on the inflammatory context, and their expansion has been reported in chronic inflammatory and autoimmune conditions (16). In cirrhosis, this expansion may represent a compensatory but insufficient mechanism to counterbalance ongoing immune activation.

Conversely, the study demonstrated a significant reduction in $CD19^+CD73^+$ B cells and $CD19^+IgM^+$ B cells in cirrhotic patients. These subsets are generally associated with naïve or regulatory B-cell compartments, and their decline may indicate impaired immune regulation and loss of tolerance. Regulatory B cells, particularly those producing interleukin-10, play a crucial role in suppressing excessive immune responses and maintaining immune homeostasis (9,10). Their reduction may therefore contribute to persistent inflammation and progressive fibrogenesis.

A major strength of this study is the demonstration of significant correlations between B-cell subsets and fibrosis indices. Both APRI, FIB-4 and FIB-6 showed similar patterns, with positive correlations with total B cells, memory B cells, and $CD5^+$ B cells, and negative correlations with naïve/regulatory-associated subsets. The consistency between these three independent fibrosis indices strengthens the validity of the findings and supports the concept that B-cell alterations reflect fibrosis severity rather than merely disease etiology.

These findings are in line with experimental evidence suggesting that B cells actively contribute to liver fibrogenesis. B cells have been shown to promote fibrosis through cytokine production, modulation of T-cell responses, and interaction with hepatic stellate cells, which are key effectors of extracellular matrix deposition (14). Furthermore, intrahepatic B-cell accumulation has been correlated with fibrosis stage in patients with chronic hepatitis, supporting their role in disease progression (17).

From an immunological perspective, the observed shift toward activated B-cell subsets and away from regulatory-associated populations may represent a hallmark of cirrhosis-associated immune dysfunction. Cirrhosis is characterized by a paradoxical state of systemic inflammation and immune deficiency, in which chronic immune activation coexists with impaired immune regulation (18). The present findings suggest that B-cell dysregulation may be an important component of this immune imbalance.

Notably, patients with HCV-related cirrhosis exhibited higher fibrosis indices compared with other groups, which may reflect more pronounced immune activation and B-cell perturbation in this subgroup. HCV is known to directly interact with B cells and has been associated with B-cell activation and lymphoproliferative disorders, which may contribute to the more marked immunological alterations observed (19).

To the best of our knowledge, this study is among the few to comprehensively evaluate peripheral B-cell subsets in relation to non-invasive fibrosis indices using multiparameter flow cytometry in cirrhotic patients. The findings highlight the potential of B-cell immunophenotyping as a complementary approach for assessing disease severity and understanding the immunopathogenesis of liver fibrosis.

However, several limitations should be acknowledged. First, the cross-sectional design precludes establishing causal relationships between B-cell alterations and fibrosis progression. Second, regulatory B cells were identified based on phenotypic markers rather than functional assays such as intracellular interleukin-10 detection, which may limit precise characterization of their immunosuppressive capacity.

Despite these limitations, the present study provides important insights into the association between B-cell dysregulation and fibrosis severity in cirrhosis. Future studies incorporating functional immunological assays and longitudinal follow-up

are warranted to further elucidate the role of B-cell subsets in liver disease progression and their potential as therapeutic targets.

In conclusion, the present study demonstrates that cirrhosis is associated with significant alterations in peripheral B-cell subset distribution, characterized by expansion of activated and memory-associated B-cell populations and reduction of naïve and regulatory-associated subsets. These alterations showed consistent correlations with non-invasive fibrosis indices, namely APRI, FIB-4 and FIB-6 suggesting a close relationship between B-cell dysregulation and fibrosis severity.

These findings support the potential role of B-cell immunophenotyping as a complementary tool for assessing disease progression and provide further insight into the immunopathogenesis of liver fibrosis. Future studies incorporating functional assays and longitudinal follow-up are warranted to validate these observations and explore their clinical implications.

**Ethical Approval:**

Approved by the Research Ethics Committee, Faculty of Medicine, Suez University (Approval No. 42).

**Consent to Participate:**

Written informed consent was obtained from all participants.

**Conflict of Interest:**

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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| Variables | HCV Cirrhotic (No.=20) | HBV Cirrhotic (No.=20) | Non HCV / HBV Cirrhotic (No.=20) | Normal control (No.=20) | P value |
|---|---|---|---|---|---|
| Gated lymph % | 26.60±6.56 | 24.06±9.65 | 28.05±9.78 | 33.35 ±7.32 | P=0.01 |
| CD19+ | 20.85 ±7.74 | 17.90 ±3.02 | 20.25±7.31 | 12.40 ±1.94 | P<0.00 |
| CD19+CD24+CD38+ | 3.41 ±1.18 | 6.38 ±0.89 | 4.84 ±1.26 | 3.82± 0.26 | P<0.00 |
| CD19+CD24+CD27+ | 23.12±6.18 | 15.59 ±5.16 | 18.60±4.70 | 5.65± 1.12 | P<0.00 |
| CD19+CD73+ | 13.82±5.24 | 14.12±3.85 | 14.87±5.86 | 25.90 ±6.36 | P<0.00 |
| CD19+CD5+ | 11.50±4.87 | 26.81±8.65 | 11.25±3.05 | 5.30± 2.20 | P<0.00 |
| CD19+IgM | 22.95±6.05 | 24.46±3.79 | 17.15±4.80 | 40.90 ±10.25 | P<0.00 |
| CD19+IgD | 18.10±5.04 | 13.56±7.51 | 13.20±4.42 | 4.85± 1.81 | P<0.00 |

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**Table (1) Demographic Characteristics of the Study Population**

| Group | FIB-4 (median, IQR) | FIB-4 (mean ± SD) | FIB-6 (median, IQR) | FIB-6 (mean ± SD) |
|---|---|---|---|---|
| Control | 0.67 (0.45–0.80) | 0.68 ± 0.29 | 0.5 | 0.98 ± 0.34 |
| HBV Cirrhosis | 3.82 (2.62–5.21) | 4.19 ± 2.24 | 1.58 | 3.7 ± 1.09 |
| HCV Cirrhosis | 5.52 (3.58–8.80) | 6.55 ± 4.13 | 1.5 | 3.99 ± 1.1 |
| Cryptogenic | 3.77 (2.26–6.07) | 4.64 ± 3.52 | 1.7 | 4.14 ± 1.53 |

| Group | APRI (median, IQR) | APRI (mean ± SD) | % APRI <0.5 | % APRI 0.5–1.5 | % APRI 1.5–<2.0 | % APRI ≥2.0 |
|---|---|---|---|---|---|---|
| Control | 0.17 (0.14–0.19) | 0.17 ± 0.04 | 100 | 0 | 0 | 0 |
| HBV-Cirrhosis | 0.63 (0.60–1.44) | 1.09 ± 0.82 | 15 | 65 | 15 | 5 |
| HCV, Cirrhosis | 2.06 (0.84–2.69) | 2.17 ± 1.73 | 10 | 35 | 5 | 50 |
| Cryptogenic | 1.21 (0.58–2.03) | 1.82 ± 2.23 | 20 | 40 | 15 | 25 |

**Table (2). APRI score among study groups**

**Table (3). FIB-4 and FIB-6 scores among study groups**

| Variable | HCV Cirrhosis (n=20) | HBV Cirrhosis (n=20) | Non-HBV/Non-HCV Cirrhosis (n=20) |
|---|---|---|---|
| Age (years), mean ± SD | 60.85 ± 7.98 | 57.56 ± 10.21 | 48.15 ± 13.95 |
| Male sex, n (%) | 14 (70%) | 16 (80%) | 11 (55%) |

**Table (4):** B cell subsets in patients and normal controls.

**Table (5). Correlation between APRI score and B-cell subsets**

| B-cell subset | Spearman r | P value | Interpretation |
|---|---|---|---|
| Gated lymph % | -0.38 | 0.0006 | Moderate negative |
| CD19+ | +0.56 | <0.001 | Strong positive |
| CD19+CD24+CD38+ | +0.19 | 0.084 | Not significant |
| CD19+CD24+CD27+ | +0.58 | <0.001 | Strong positive |
| CD19+CD73+ | -0.40 | <0.001 | Moderate negative |
| CD19+CD5+ | +0.41 | <0.001 | Moderate positive |
| CD19+IgM+ | -0.51 | <0.001 | Strong negative |
| CD19+IgD+ | +0.51 | <0.001 | Strong positive |

Values are presented as Spearman correlation coefficients (r) with corresponding p-values.

**Table (6). Correlation between FIB-4 score and B-cell subsets**

| **B-cell subset** | **Spearman r** | **P value** | **Interpretation** |
|---|---|---|---|
| Gated lymph % | -0.31 | 0.00451 | Moderate Negative |
| CD19+ | 0.54 | 0.000 | Strong positive |
| CD19+CD24+CD38+ | 0.20 | 0.0687 | Not significant |
| CD19+CD24+CD27+ | 0.55 | 0.000 | Strong positive |
| CD19+CD73+ | -0.36 | 0.000 | Moderate Negative |
| CD19+CD5+ | 0.44 | 0.000 | Moderate positive |
| CD19+IgM+ | -0.45 | 0.000 | Moderate Negative |
| CD19+IgD+ | 0.50 | 0.000 | Moderate positive |

Values are presented as Spearman correlation coefficients (r) with corresponding p-values.

**Table (7). Correlation between FIB-6 score and B-cell subsets**

| **B-cell subset** | **Spearman r** | **P value** | **Interpretation** |
|---|---|---|---|
| Gated lymph % | -0.31 | 0.004 | Moderate Negative |
| CD19+ | 0.620 | 0.000 | Strong positive |
| CD19+CD24+CD38+ | 0.265 | 0.025 | Weak positive |
| CD19+CD24+CD27+ | 0.619 | 0.000 | Strong positive |
| CD19+CD73+ | -0.508 | 0.000 | Moderate Negative |
| CD19+CD5+ | 0.500 | 0.000 | Moderate positive |
| CD19+IgM+ | -0.554 | 0.000 | Moderate Negative |
| CD19+IgD+ | 0.517 | 0.000 | Moderate positive |

Values are presented as Spearman correlation coefficients (r) with corresponding p-values.

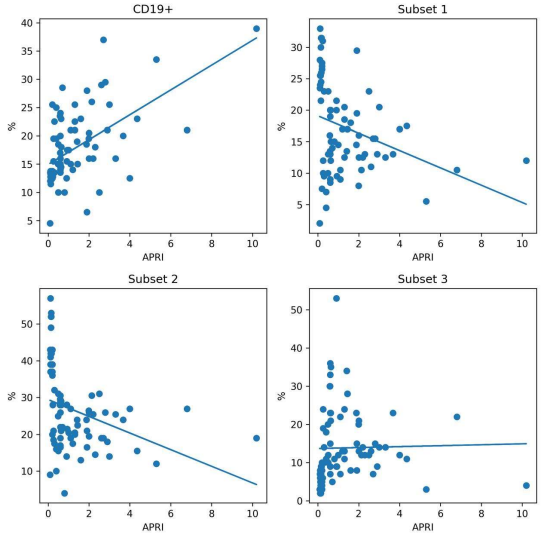


Figure 1. Correlation between APRI score and peripheral B-cell subsets.
(A) $CD19^+$ B cells, (B) $CD19^+CD24^+CD27^+$ B cells, (C) $CD19^+CD73^+$ B cells, and (D) $CD19^+IgM^+$ B cells.
Spearman correlation analysis was performed. Each dot represents an individual subject.

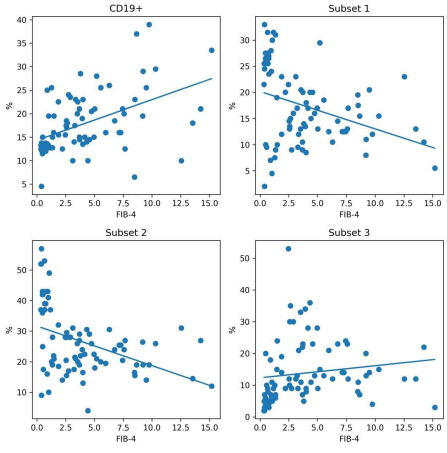


Figure 2. Correlation between FIB-4 score and peripheral B-cell subsets.

(A) $CD19^+$ B cells, (B) $CD19^+CD24^+CD27^+$ B cells, (C) $CD19^+CD73^+$ B cells, and (D) $CD19^+IgM^+$ B cells.
Spearman correlation analysis was performed. Each dot represents an individual subject.