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Soluble CD163 and mannose receptor as markers of liver disease severity and prognosis in patients with primary biliary cholangitis

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List of abbreviations

PBC: Primary biliary cholangitis; sCD163: soluble CD163; sMR: soluble mannose receptor; LT: liver transplantation; IQR: interquartile range; AMA: anti-mitochondrial antibody; HBV: hepatitis B virus; HCV: hepatitis C virus; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatehepatitis; AIH: autoimmune hepatitis; ACLF: acute-on-chronic liver failure; HCC: hepatocellular carcinoma; UDCA: Ursodeoxycholic acid; ELISA: enzyme-linked immunosorbent assays; ULN: upper limit of normal; LLN: lower limit of normal; ALP: alkaline phosphatase; ALT: alanine aminotransferase; HR: hazard ratio; C-index: the concordance index; CI: confidence interval; EASL: European Association for the Study of the Liver; MELD: model for end-stage liver disease.

Lay Summary

Primary biliary cholangitis (PBC) is a chronic cholestatic autoimmune liver disease with inflammation in the intra-hepatic bile ducts leading to fibrosis and end-stage liver disease. In this study, we explore markers of a specific immune cell, the macrophage, and their association with disease severity and prognostic abilities in patients with PBC. We found that the macrophage activation markers are elevated in patients with more severe disease and further identified them to be associated with prognosis in PBC patients.

Abstract

Introduction: In primary biliary cholangitis (PBC) macrophages are involved in liver inflammation and fibrosis. The macrophage activation markers, soluble (s)CD163 and mannose receptor (sMR) are associated with liver disease severity and prognosis in other chronic liver diseases. We aimed to investigate sCD163 and sMR in PBC patients.

Methods: We investigated PBC patients from the *Italian PBC Study Group* cohort and measured macrophage activation markers in serum at study enrolment. Patients were followed from enrolment until they experienced an event or were censored at their last visit. Events were defined as follows: (1) death from a liver-related cause; or (2) liver transplantation (LT) for PBC. We used Cox regression to investigate the association between sCD163 and sMR and long-term prognosis.

Results: Two-hundred-two PBC patients were included. Median age was 62 years (interquartile range (IQR), 53-71) at enrolment and 93% were women. Median sCD163 was 3.43 mg/L (IQR 2.48-5.35) and median sMR was 0.35 mg/L (IQR 0.28-0.45). There was an increase in sCD163 and sMR with increasing alkaline phosphatase. Two-hundred-one patients were followed for a median of 8.6 years, and sCD163 and sMR predicted long-term risk of liver related death or LT in univariate analyses, while sCD163 was also associated with outcome after confounder adjusting (adjusted HR=1.14, 95% CI 1.00-1.30). Finally, we showed an increase in the prediction accuracy of poor outcome by adding sCD163 to the UK-PBC risk score.

Conclusion: The macrophage activation markers sCD163 and sMR represent a non-invasive measure of PBC disease severity that provides useful long-term prognostic information.

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Introduction

Primary biliary cholangitis (PBC) is a chronic, cholestatic autoimmune liver disease characterized by biliary inflammation and destruction of intrahepatic bile ducts with subsequent ductopenia, and portal fibrosis[1]. The natural history of the disease is usually a slow progression towards biliary cirrhosis, with a substantial part of the patients developing end-stage liver disease and subsequent need of liver transplantation (LT)[2,3]. However, the disease presentation and progression is very heterogeneous and several at-risk phenotypes have been identified[4]. Thus, identification of non-invasive risk stratifiers to distinguish between patients with high and low risk of disease progression and need of LT is of paramount importance[5,6].

In PBC, inflammation is attributed to an immune response to mitochondrial autoantigens followed by a serologic response of anti-mitochondrial antibodies (AMAs) and accompanied by inflammation of small bile ducts. The pathogenesis includes both CD4 and CD8 cells, which, in the presence of biliary cells expressing the 2-oxo-dehydrogenase pathway (PDC-E2), activate macrophages via granulocyte macrophage colony–stimulating factor. The activated macrophages, together with AMAs, produce a pro-inflammatory response with subsequent liver inflammation and fibrosis[7-9]. Further, macrophages comprise around 30% of mononuclear cells found in the cellular infiltrate at biopsies in PBC patients[10]. Thus, macrophages seem to be involved in PBC pathogenesis; however, macrophage activation markers have not yet been investigated in PBC patients.

The macrophage activation marker soluble CD163 (sCD163[11,12]) has been investigated in a number of liver diseases over the last years. Increased levels are associated with liver fibrosis/cirrhosis in patients with chronic viral hepatitis (HBV and HCV)[13], non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH)[14,15], and alcoholic liver disease (alcoholic hepatitis and cirrhosis)[16], and liver disease severity including risk of portal hypertension and development of complications and mortality[17,18]. Moreover, in patients with autoimmune hepatitis (AIH) sCD163 levels were associated with liver disease severity and treatment response[19]. Similar data have been published for the soluble mannose receptor (sMR), which is expressed on macrophages but also on dendritic cells and endothelial cells[20,21]. Moreover, a recent study showed that mannose phosphate isomerase gene expression downregulates as fibrosis progress in patients with NAFLD and HBV[22]. In 2015, an association between sCD163 and sMR and liver disease severity and prognosis in patients with Acute-on-Chronic Liver Failure (ACLF) was shown[23].

The aim of this study was to investigate the association between the macrophage activation markers sCD163 and sMR and long-term prognosis in PBC patients. We hypothesized that higher sCD163 and sMR will be associated with a worse long-term prognosis.

Methods

Participants and study design

We performed a cross-sectional study in 202 PBC patients enrolled in the *Italian PBC Study Group* cohort, already described[24]. We identified patients with available serum samples and known survival status. Some patients were enrolled into the cohort at the time of PBC diagnosis,

while others were included at a later time. For the latter, the time since diagnosis was recorded. The date of diagnosis of PBC was defined as the date of the first positive test for AMA or, for seronegative patients, the date of the diagnostic liver biopsy.

Patients were followed from enrolment until a combined endpoint of either (1) death from a liver-related cause, meaning liver failure, variceal hemorrhage, hepatorenal syndrome, or hepatocellular carcinoma (HCC); or (2) LT for PBC. When no events occurred, the observation was censored at the time of last patient contact.

Data collection

At enrolment, data on patient age, sex, year of diagnosis, and Ursodeoxycholic acid (UDCA) therapy were collected, and blood samples were taken. Further, all patients had ultrasonography of the liver. After at least 12 months of UDCA treatment data on alkaline phosphatase (ALP) were obtained in those patients included at the time of PBC diagnosis to determine UDCA response; those with normal ALP were considered responders and those with abnormal ALP were considered non-responders. Serum samples were stored at -80^{0}C until analyses. No data on symptoms or histology were available.

Measurements of sCD163 and sMR

sCD163 and sMR measurements were performed using blood samples taken at enrolment into the *Italian PBC Study Group* cohort, i.e. at the same time as those used to measure liver biochemistry at baseline. Serum concentrations of sCD163 and sMR were measured in duplicate by in-house sandwich enzyme-linked immunosorbent assays (ELISA) using a BEP-2000 ELISA-analyser (Dade Behring)[20,25]. sCD163 has a reference interval of 0.69-3.86 mg/L[11], and the reference interval for sMR is 0.10-0.43 mg/L[20]. Soluble CD163 and sMR are resistant to repeated freezing and thawing[20,25].

Statistical analysis

To account for inter-laboratory variability of liver functions tests we used a ratio of upper limit of normal (ULN) or lower limit of normal (LLN) when analysing these.

Counts, percentages, quartiles and ranges were used to describe the patients' characteristics. sCD163 and sMR values were described by quartiles. We used the Kruskal-Wallis test to compare levels of sCD163 and sMR in groups of ALP, alanine aminotransferase (ALT) and bilirubin.

In order to evaluate sCD163 and sMR as risk factors for liver-related death or LT we applied a univariate Cox model after assessing the proportional hazard assumption. We also applied a Cox model adjusting for confounding by age at recruitment because sCD163 increases with age and age is a strong predictor of mortality[11]. Based on the values of sCD163 and sMR the hazard ratio (HR) was calculated for a 1.0 mg/L increase in sCD163 and a 0.1 mg/L increase in sMR.

The concordance index (C-index) for right-censored survival time data was used to evaluate the prognostication of sCD163 and sMR alone and in combination with the UK-PBC risk score at 5, 10 and 15 years in 139 patients with all necessary data available[26].

Results

Patient Characteristics

We identified 202 patients diagnosed with PBC between 1982 and 2015 from 16 Italian National Health Service Hospitals. Ninety-three percent were women, median age at diagnosis was 52 years (interquartile range (IQR) 45-62) and median age at study enrolment was 62 (IQR 53-71). Ninety-nine (49%) patients were enrolled at the time of PBC diagnosis. One-hundred-twenty-six patients (76%) had abnormal ALP at diagnosis. The remaining had been diagnosed through liver biopsy showing features compatible with PBC. Ninety-eight percent were treated with UDCA. Seventeen patients had liver cirrhosis on ultrasonography at study enrolment.

Basic demographic, clinical, and biochemical data for the PBC patients are presented in **Table 1.**

sCD163, sMR and patient characteristics

At enrolment the median ALP was 1.65 x ULN (IQR 1.03-2.87); ALT was 1.31 x ULN (IQR 0.90-1.94); serum bilirubin level was 0.55 x ULN (IQR 0.41-0.76); albumin was 1.13 x LLN (IQR 1.02-1.20); and platelet count was 1.54 x LLN (IQR 1.27-1.95).

Median sCD163 was 3.43 mg/L (IQR 2.48-5.35) and median sMR was 0.35 mg/L (IQR 0.28-0.45).

There was an increase in sCD163 and sMR with increasing ALP, but not with increasing bilirubin and ALT (**Figure 1**). Also, serum levels of sCD163 and sMR were higher in patients above 50 years at enrolment than in patients below 50 years.

Of the 99 newly diagnosed patients included, data on UDCA and ALP after 12 months follow up were available in 92 patients. Forty-eight (52%) were UDCA responders with normal ALP levels. At baseline these responders had lower sCD163 (3.21 mg/L [IQR 2.44-4.04] vs. 4.11 mg/L [IQR

3.10-5.86], p=0.004) and sMR (0.30 mg/L [IQR 0.25-0.38] vs 0.40 mg/L [IQR 0.31-0.51], p=0.025) levels than non-responders.

sCD163, sMR and long-term outcomes

We followed 201 patients from enrolment for a median of 8.6 years. At the end of follow-up 177 patients were still alive, 10 had undergone LT and 14 had died, of whom 8 had died from liver related causes. One patient was lost to follow-up and was excluded from the follow-up analysis.

The unadjusted Cox model showed a positive association between both sCD163 and sMR values and liver-related events. The HR for 1 mg/L increase of sCD163 was 1.15 (95% CI: 1.02-1.29, p=0.027), while the HR for 0.1 mg/L increase of sMR was 1.17 (95% CI: 1.02-1.35, p=0.022) (**Table 2)**. One patient had an outlier value of sCD163 of 17.80 mg/L. If excluding that one outlier from the unadjusted analysis, the HR for 1 mg/L increase of sCD163 was 1.28 (95% CI: 1.09-1.51, p=0.003). When adjusting for confounding by age, sCD163 was still associated with outcome (adjusted HR=1.14, 95% CI 1.00-1.30, p=0.04) (**Table 2**). This was also the case for sMR, although the confidence interval and p-value suggest that this may be by chance (adjusted HR=1.13, 95% 0.98-1.31, p=0.10) (**Table 2**).

Finally, in the subgroup of patients, for whom ALP, ALT, total bilirubin, albumin and platelet count were available (n=139) the addition of sCD163 to the UK-PBC risk score improved the prognostic abilities compared to UK-PBC risk score itself at 5 (C-index = 0.91), 10 (C-index = 0.74) and 15 years (C-index = 0.89) (**Table 3)**.

Discussion

This is the first study to systematically investigate markers of macrophage activation in PBC patients. The main finding of our study was that sCD163 and sMR increase with increasing ALP and that they were associated with long-term risk of liver related events in PBC patients. Furthermore, we showed an increase in the prediction accuracy of poor outcome gained by adding sCD163 to the UK-PBC risk score, a risk stratification tool recommended by the clinical practice guidelines from the European Association for the Study of the Liver (EASL).

The data presented here stems from an Italian multicentre cohort with PBC patients included at various time points during the course of the disease. Almost 25% of patients had normal ALP at

diagnosis, reflecting early stage disease with normal ALP, and hence diagnosed based on positive AMA and findings on liver biopsy. sCD163 and sMR were measured from blood samples taken at enrolment to the Italian PBC cohort, where liver biochemistry was also measured. Thus, we could successfully investigate the relation between the macrophage activation markers and liver biochemistry from different disease stages as some patients had early stage disease while others had late disease stage.

Hepatic macrophages play a central role in the pathogenesis of chronic liver injury, including inflammation and fibrosis[27,28], and this has increased the interest for systemic markers of macrophage activation. Further, the critical role of monocyte-derived macrophages in PBC to produce pro-inflammatory cytokines in response to biliary apotopes in the presence of AMAs is well described.

Levels of markers of macrophage activation in PBC reported here are comparable with those observed in early disease stages in other chronic liver diseases[13,21], and lower than those observed in patients with acute inflammation or decompensated cirrhosis[16-19,29,30]. In patients with alcoholic or viral cirrhosis, sCD163 is in the range of 5–10 mg/L (i.e. up to twice the ULN and higher than in our PBC cohort) and is related to both the severity of the cirrhosis (defined by MELD and Child–Pugh score) and to the degree of portal hypertension[18]. In our study, we found that sCD163 and sMR at enrolment were higher in those later defined as UDCA non-responders than in those defined as responders. This may be of potential interest suggesting both less severe liver inflammation but also a better response to UDCA treatment. However, this finding should be interpreted carefully, as we do not have data on sCD163 and sMR at the time point of UDCA-response determination, and thus, it should be confirmed in prospective studies with samples before and after UDCA treatment and preferably in newly diagnosed patients.

Both circulating monocytes, recruited hepatic macrophages and Kupffer cells shed the soluble macrophage activation markers, which cannot be separated when analysed in blood samples. In other liver diseases, CD163 expression examined on liver biopsies with immunohistochemistry was markedly elevated in patients with simultaneous increased levels of soluble macrophage activation markers[16,19], and in patients with NASH, hepatitis C, and hepatitis B it correlates with inflammation and fibrosis scores based on liver histology[13,15]. Further, in hepatitis C, hepatitis B,

AIH, and NASH previous studies have found that levels of soluble macrophage activation markers decrease after treatment initiation[14,15,19,31,32]. Hence, there is good reason to believe that the levels of sCD163 and sMR measured in our study stems from macrophages from the liver, which is also supported by a gradient across the liver as shown in alcohol cirrhosis and NASH patients[15,17]. Thus, our results suggest that macrophages play a pivotal role in disease development and progression in PBC, further supported by the known pathophysiological mechanism of PBC[7-9].

Current trends in PBC research focus on identifying non-invasive markers of disease progression and prognosis[5,6]. Here we present data to support the macrophage activation markers sCD163 and sMR as markers of disease progression and prognosis in PBC patients, and thus, as possible surrogate endpoints in future trials of PBC treatments. Moreover, the addition of sCD163 improved the prediction accuracy of the UK-PBC risk score. Unfortunately, we did not have a second cohort to validate our findings, hence we encourage future studies to validate our results in other PBC longitudinal cohorts, and further investigate the relation to other prognostic markers in PBC, e.g. histology and fibroscan.

Major strengths of our study are the inclusion of a large number of well-characterised patients with a rare condition like PBC and the longitudinal follow-up. A major limitation to the study is the lack of longitudinal measurements of sCD163 and sMR; we could not investigate the possible dynamic changes of the macrophage activation markers during disease progression.

In conclusion, the macrophage activation markers sCD163 and sMR increase with increasing ALP and are prognostic markers of liver related death or LT in PBC patients. These findings confirm that macrophages play a role in the PBC inflammatory process. Moreover, a UK-PBC+sCD163-based score was superior to the existing UK-PBC score alone for outcome prediction in PBC patients.

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Figure legends

Fig. 1. sCD163 and sMR distribution by bilirubin, ALP and ALT at baseline.

(A) sCD163 distribution by baseline bilirubin level. Levels of significance: $p = 0.711$ (Kruskal-Wallis test). (B) sMR distribution by baseline bilirubin level. Levels of significance: $p = 0.131$ (Kruskal-Wallis test). (C) sCD163 distribution by baseline ALP level. Levels of significance: $p = 0.006$ (Kruskal-Wallis test). (D) sMR distribution by baseline ALP level. Levels of significance: $p = 0.005$ (Kruskal-Wallis test). (E) sCD163 distribution by baseline ALT level. Levels of significance: $p = 0.971$ (Kruskal-Wallis test). (F) sMR distribution by baseline ALT level. Levels of significance: $p = 0.352$ (Kruskal-Wallis test). Box-whisker plots report minimum, maximum, and 3 quartiles values. Values that are far from the box by more than 1.5 times the interquartile range are reported by empty dots.

Footnotes: ALP: alkaline phosphatase; ALT: alanine transaminase; ULN: upper limit of normal; sMR: soluble mannose receptor; sCD163: soluble CD163.

Table 1: Characteristics of the PBC patient cohort

	N.of missing	N(%) / Median [IQR]	Range
Total		N=202	
Female sex (%)		188 (93)	
Age at diagnosis (yrs)	1	52 [45-62]	26-85
Age at enrolment, yrs	1	55 [48-64]	31-85
Time from diagnosis to enrolment, yrs	1	1 [0-3]	1-26
Year of diagnosis	1	2006 [2001-2009]	1982-2015
UDCA treated	1	197 (98)	
Bilirubin xULN	53	0.55 [0.41-0.76]	0.21-9.09
Bilirubin >ULN (%)		18 (12)	
ALPxULN	36	1.65 [1.03-2.87]	0.40-9.23
ALP >ULN (%)		126 (76)	
ALTxULN	39	1.31 [0.90-1.94]	0.30-8.46
ALT >ULN (%)		112 (69)	
Albumin xLLN	53	1.13 [1.02-1.20]	0.71-1.66
Platelet xLLN	30	1.54 [1.27-1.95]	0.38-3.36

ULN: upper limit of normal; IQR: interquartile range; LLN: lower limit of normal; ALP: alkaline phosphatase; ALT: alaninetransferase; UDCA: ursodeoxycholic acid.

Table 2: Hazard ratios from univariate and adjusted hazard ratios from Cox regressions

Model	Variable	HR (95% CI)	P-value
Univariate	sCD163, per mg/L increase	1.15 (1.02-1.29)	0.0265
	sMR, per 0.1 mg/L increase	1.17 (1.02-1.35)	0.0221

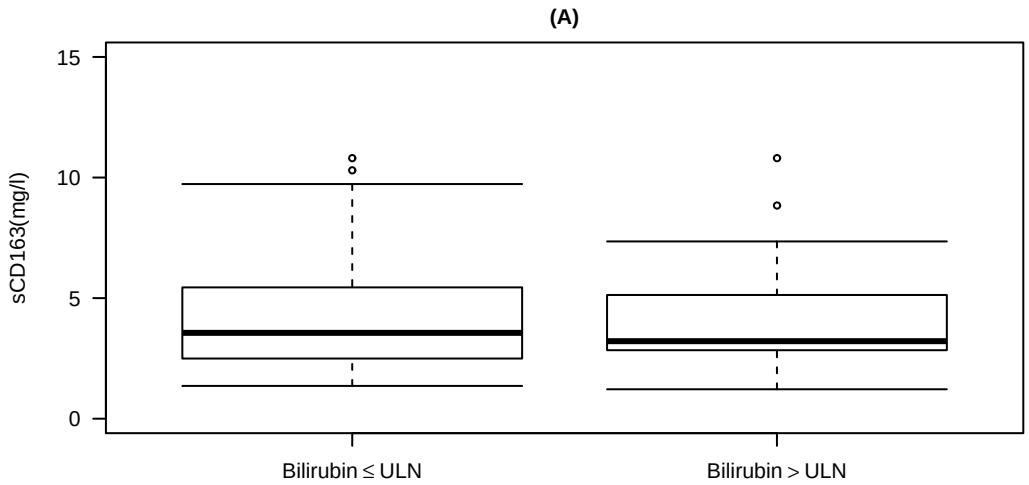
Multivariable, sCD163	sCD163, per mg/L increase	1.14(1.00-1.30)	0.0428
	Age, per year increase	1.04(1.00-1.09)	0.0741
Multivariable, sMR	sMR, per 0.1 mg/L increase	1.13(0.98-1.31)	0.1005
	Age, per year increase	1.04(0.99-1.09)	0.1577

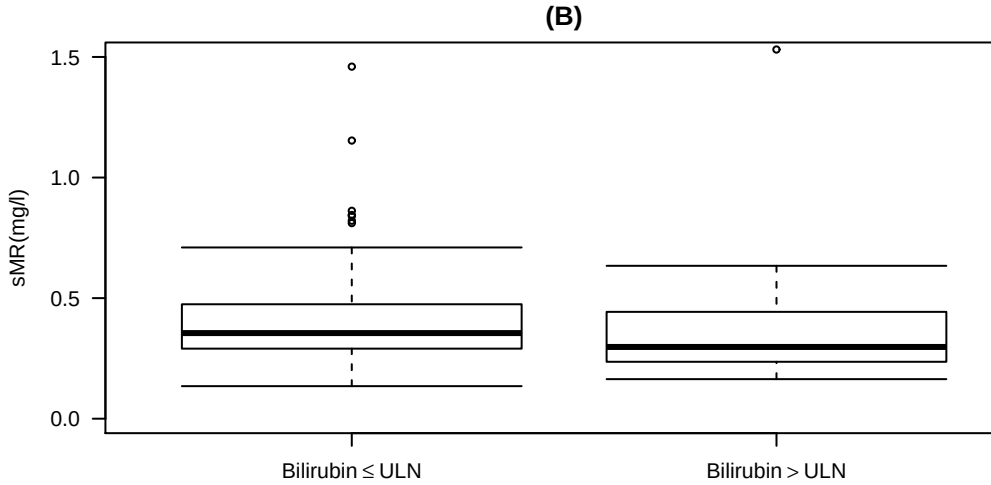
HR: hazard ratio; CI: confidence interval; sCD163: soluble CD163; sMR: soluble mannose receptor

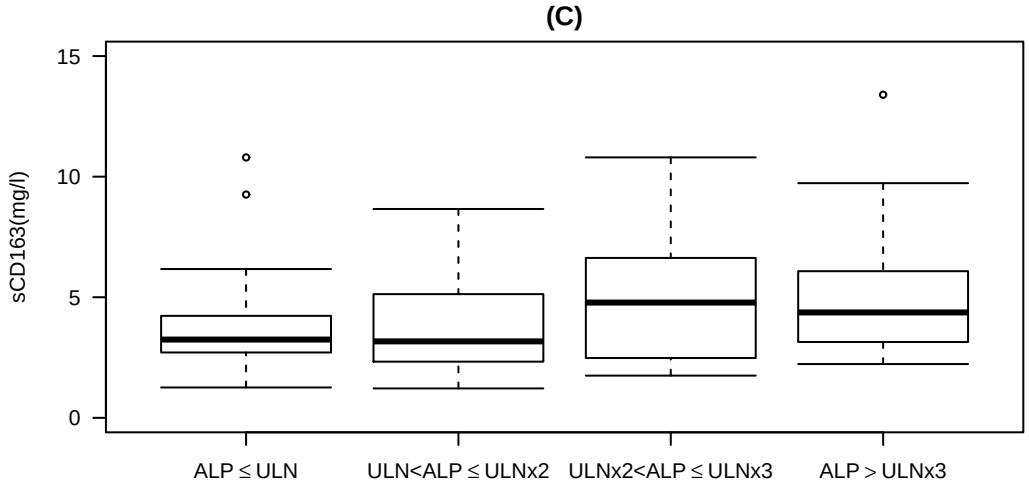
Table 3: C-index for sCD163, sMR and the UK-PBC risk score alone and in combination at 5, 10 and 15 years. Patients = 139, 8 events.

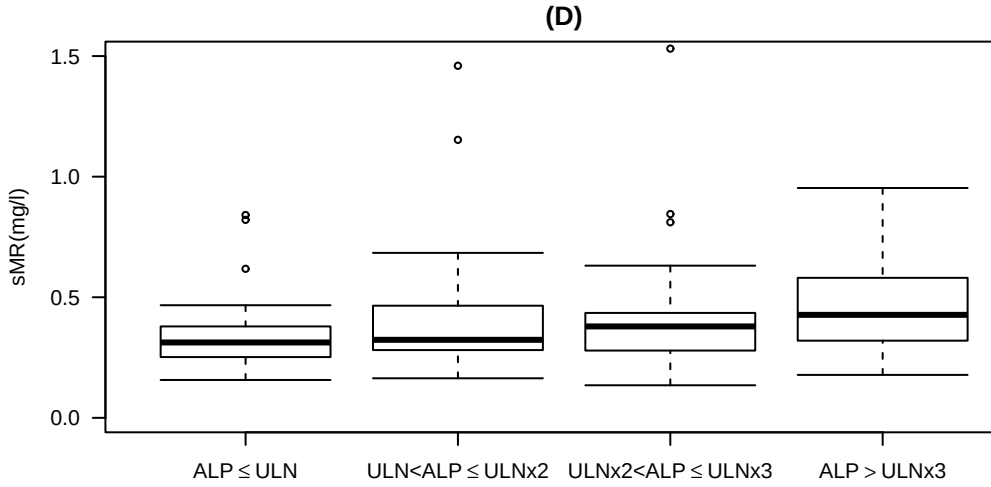
	5 years	**10 years**	**15 years**
sCD163	0.60	0.58	0.66
sMR	0.46	0.32	0.48
UK-PBC score	0.76	0.65	0.88
UK-PBC score + sCD163	0.91	0.74	0.89
UK-PBC score + sMR	0.54	0.66	0.81

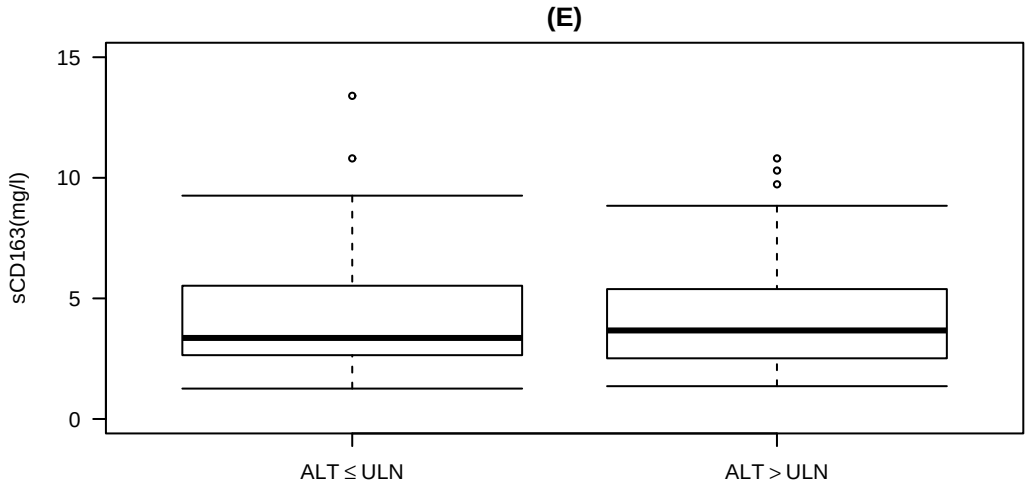
C-index: concordance index; sMR: soluble mannose receptor; sCD163: soluble CD163

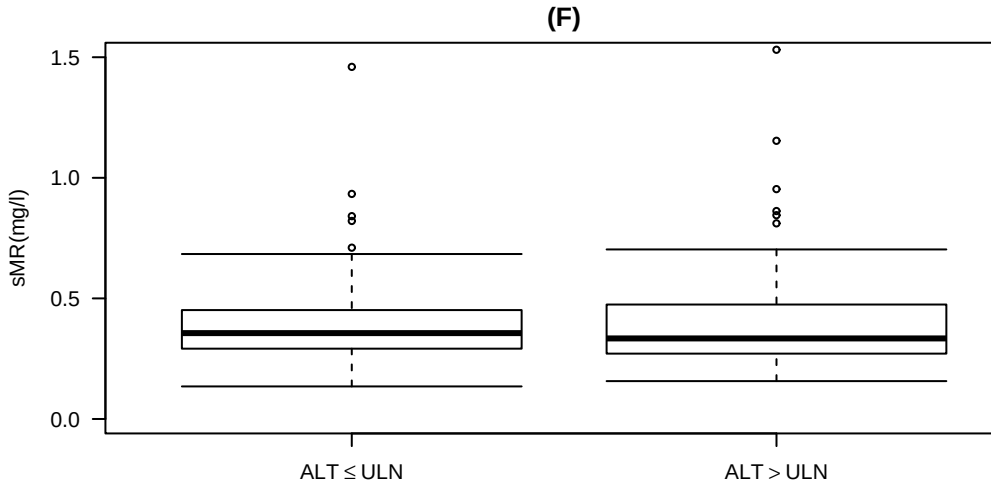

(A)
sCD163(mg/l)
15
10
5
0
Bilirubin ≤ ULN
Bilirubin > ULN


(B)
sMR(mg/l)
1.5
1.0
0.5
0.0
Bilirubin ≤ ULN
Bilirubin > ULN


(C)
sCD163(mg/l)
15
10
5
0
ALP ≤ ULN
ULN<ALP ≤ ULNx2
ULNx2<ALP ≤ ULNx3
ALP > ULNx3


(D)
sMR(mg/l)
1.5
1.0
0.5
0.0
ALP ≤ ULN
ULN<ALP ≤ ULNx2
ULNx2<ALP ≤ ULNx3
ALP > ULNx3


(E)
sCD163(mg/l)
15
10
5
0
ALT ≤ ULN
ALT > ULN


(F)
sMR(mg/l)
1.5
1.0
0.5
0.0
ALT ≤ ULN
ALT > ULN