SCANDINAVIAN JOURNAL OF GASTROENTEROLOGY
2021, VOL. 56, NO. 4, 443–452
https://doi.org/10.1080/00365521.2021.1880627

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ORIGINAL ARTICLE

Associations of neopterin and kynurenine–tryptophan ratio with survival in primary sclerosing cholangitis

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ABSTRACT

Background and aims: Biomarkers of inflammation may be of clinical utility in primary sclerosing cholangitis (PSC). We aimed to investigate the interferon gamma-related biomarkers neopterin and kynurenine–tryptophanratio (KT-ratio) in PSC.

Methods: Circulating neopterin, tryptophan and kynurenine were measured with LC-MS/MS in multiple cross-sectional cohorts comprising in total of 524 PSC patients and 100 healthy controls from Norway, Germany and Sweden.

Results: Neopterin and KT-ratio were significantly increased in PSC patients compared with controls in both a discovery and a validation cohort from Norway. Furthermore, high neopterin and KT-ratio levels were associated with a shorter transplantation-free survival in the PSC patients in the Norwegian discovery cohort and the German validation cohort. However, in the validation PSC cohort from Sweden, no relationship between neopterin and KT-ratio and liver transplantation-free survival was observed. The correlations between neopterin and KT-ratio were moderate to strong and similar in all cohorts (rho 0.50–0.67). Neopterin and KT-ratio also correlated with C-reactive protein (rho 0.17-0.63) and revised Mayo risk score (rho 0.23–0.42) in all cohorts.

Conclusions: Neopterin and KT-ratio were elevated in PSC and associated with liver transplantation-free survival in two independent PSC cohorts, highlighting a possible role of interferon gamma-driven inflammation in the pathogenesis. However, the lack of association with survival in one of the cohorts reduces the potential clinical value of neopterin and KT-ratioas biomarkers and highlights the need to validate new biomarkers in PSC in multiple cohorts.

ARTICLE HISTORY
Received 2 December 2020
Revised 11 January 2021
Accepted 19 January 2021

KEYWORDS
Biliary disease; sclerosis cholangitis; inflammation; tryptophan; neopterin; kynurenine

Introduction

Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease characterized by multifocal intra- and extrahepatic bile duct strictures [1]. The nature of the disease is progressive leading to liver cirrhosis and often a need for liver transplantation. The disease causes are unknown, and there are no medical interventions available to slow the disease progression [2]. Biomarkers of disease activity, severity and prognosis are important tools to improve clinical practice and facilitate development of new therapies. Circulating biomarkers correlating with key features of the disease such as fibrosis and inflammation may therefore be of clinical utility in PSC [3,4]. The levels and predictive features of biomarkers may also shed light on disease pathogenesis.

Multiple inflammatory pathways and immune cells are likely involved in PSC. Macrophage activation has recently been linked to disease severity in experimental PSC models [5]. Circulating soluble CD14, often regarded as a macrophage activation marker, is increased in PSC and predict liver transplantation-free survival. Furthermore, one of the strongest genetic risk factors in PSC is a single-nucleotide

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Supplemental data for this article can be accessed here.

polymorphism in the Macrophage stimulating 1 (*MST1*) gene [6]. Taken together, macrophages may be relevant for disease activity and progression in PSC. Another marker of macrophage activity is neopterin. Neopterin is produced from guanosine triphosphate by monocytes, monocyte dendritic cells and macrophages after stimulation with interferon ɣ (IFNɣ), which is released from activated T-cells during the cellular immune response [7]. Its production is increased in many infectious, autoimmune, cardiovascular and malignant diseases, [8,9] but data in PSC are scarce. One early study reports higher levels of neopterin in patients with liver disease compared to healthy controls, and also in patients with liver cirrhosis compared to non-cirrhotic patients [8]. Increased IFNɣ activity has also recently been reported in PSC and relevant mouse models [10]. Notably, another event occurring during IFNɣ stimulation is activation of indoleamine 2,3-dioxygenase, which is a first-step of the kynurenine pathway of tryptophan catabolism. The degradation of tryptophan to kynurenine, assessed as the kynurenine/tryptophan ratio (KT-ratio) is therefore another marker of IFNɣ-related inflammatory activity which could be of relevance in PSC.

Based on the above, the aim of this study was primarily to analyze neopterin and KT-ratio in several cohorts of PSC patients to investigate its possible clinical impact as biomarker for prognosis.

Patients and methods

Study design and patient populations

As shown in Figure 1, step I used a cross-sectional design, comparing biomarkers in PSC patients in a Norwegian discovery cohort (Norway I, $n = 191$ recruited at Oslo University Hospital Rikshospitalet, Oslo between 2008–2015) compared with a cohort of healthy controls recruited from the Norwegian Bone Marrow Donor registry (Controls I, $n = 48$, collected in Oslo between 2009–2010). For validation, a Norwegian validation cohort (Norway II, $n = 42$ sampled at Haukeland University Hospital, Bergen in 2017–2018) was compared with a second cohort of healthy controls (Controls II, $n = 52$, collected in Oslo between 2009 and 2010). In step II, the aim was to investigate the association of biomarkers with liver transplantation-free survival and other clinical characteristics (Figure 1). The Norway I patients were used as discovery cohort and validation was attempted in a German cohort (Germany, $n = 150$ recruited at the University Hospital in Heidelberg, Heidelberg between 2008 and 2017) and a Swedish cohort (Sweden, $n = 141$ recruited at Karolinska University Hospital, Stockholm between 2008 and 2012). The population characteristics are shown in Table 1. The cohort sizes were determined by sample availability.

Diagnosis of PSC was based on typical findings on magnetic resonance cholangiography or endoscopic retrograde cholangiography according to established criteria [11]. The first pathological radiologic finding defined the time of PSC diagnosis. Clinical and demographic information including laboratory data were acquired from patient records and research databases. Inflammatory bowel disease was diagnosed based on endoscopy and histological findings, according to accepted criteria [12]. Revised Mayo risk score was calculated according to the established algorithm [13]. Liver stiffness was measured at the time of blood sampling in the Norway II cohort as well as in a subset of the Swedish patients, using transient elastography (Fibroscan, Echosens, Paris, France) in both cohorts.

Plasma was collected from the Norwegian patient and control cohorts (no previous freeze-thaw cycles) and serum from the German and Swedish patient cohorts (at least one previous freeze-thaw cycle per sample), according to a standard protocol in each country.

Biochemical analyses

Plasma and serum concentrations of neopterin, kynurenine and tryptophan were analyzed in the same assay using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described [14]. The inter-assay variation

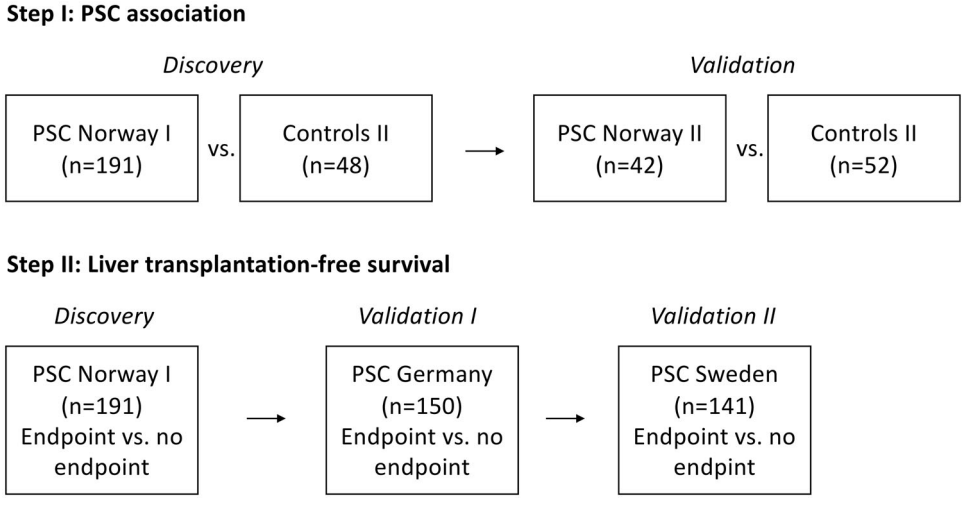

Figure 1. Overall study design. The figure shows the study design and the use of the four included PSC cohorts and two cohorts of healthy controls. In step I, associations with PSC are analyzed by comparing PSC patients with healthy controls in two independent cohorts. In step II, the association between liver transplantation-free survival and the biomarkers are investigated in three independent cohorts.

Table 1. Participant characteristics.

	Norway I (PSC, $n = 191$)	Norway II (PSC, $n = 42$)	Germany (PSC, $n = 150$)	Sweden (PSC, $n = 141$)	Controls I (Norway, $n = 48$)	Controls II (Norway, $n = 52$)
Males, n (%)	151 (79)	33 (79)	94 (63)	95 (67)	28 (58)	31 (60)
Age at sampling, years, median (range)	41 (16–72)	50 (19–84)	43 (18–74)	42 (21–77)	40 (29–55)	40 (28–56)
Age at PSC diagnosis, years, median (range)	35 (13–72)	38 (12–73)	–	34 (8–72)	–	–
IBD, n (%)	139 (74)	35 (83)	96 (64)	105 (74)	–	–
Cholangiocarcinoma, n (%)	24 (13)	2 (2)	10 (7)	5 (4)	–	–
Follow-up, years, median (range)	4 (0–8)	NA	4 (0–10)	8 (0–11)	–	–
Liver transplantation, n (%)	67 (35)	NA	9 (6)	37 (26)	–	–
Deaths, n (%)	20 (10)	NA	7 (5)	10 (7)	–	–
Mayo risk score, median (range)	0.27 (-2.37–4.13)	−0.45 (-1.88–2.95)	−0.10 (-2.2–5.1)	0.04 (-1.8–3.3)	–	–
ALP, U/L, median (range)	237 (51–1459)	168 (59–863)	182 (47–1622)	126 (30 − 1350)	–	–
ALT, U/L, median (range)	97 (14–1008)	58 (11–657)	50 (6–489)	49 (9–436)	–	–
AST, U/L, median (range)	77 (16–1683)	50 (18–299)	46 (5–1178)	40 (14–311)	–	–
Albumin g/L, median (range)	40 (23–51)	45 (31–51)	43 (20–49)	38 (22–48)	–	–
Total bilirubin, μmol/L, median (range)	24 (3–591)	11.50 (5–99)	15 (3–460)	12 (3–160)	–	–
Creatinine, mmol/L, median (range)	65 (5–137)	74 (54–98)	68 (33–187)	70 (38–105)	–	–
Platelets x10^9, median (range)	276 (22–903)	236 (80–430)	250 (47–1127)	235 (36–555)	–	–

ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; IBD: inflammatory bowel disease; NA: not applicable.

coefficients of the assays are for tryptophan 6%, kynurenine 6–9% and neopterin 6–10%, while the intra-assay variation coefficients are 3–4% for tryptophan and 3–5% for kynurenine and neopterin. Samples were analyzed 82 samples in each batch, with eight additional calibrators/quality control samples in each batch. Standard biochemical analyses were performed as part of clinical routine, including liver function tests, CRP and international normalized ratio (INR).

Statistical analyses

Unless stated otherwise, data are presented as median (range), except survival (in years) which is presented as mean (95% CI).The Mann–Whitney U-test was applied for continuous variables. Bivariate correlations were assessed by the Spearman's rank correlation test (rho). Primary endpoints were liver transplantation or death. Liver transplantation-free survival was analyzed using Kaplan-Meier plots and log-rank tests, and univariate and multivariate Cox regression. In multivariable regression, patients lacking Mayo risk score data were excluded. The KT-ratio was calculated as 100*kynurenine:tryptophan (μmol:μmol). Cut-offs for high versus low levels of neopterin and KT-ratio were defined by Youden's index when performing analysis of receiver operating characteristics/area under the curve. Statistical analyses were performed using SPSS (version 25; SPSS, Inc., Chicago, IL), GraphpadPrism (version 8, GraphPad Software, San Diego, CA) and MedCalc (MedCalc Software bvba, Ostend, Belgium). P-values <.05 were considered statistically significant.

Ethics

Written informed consent was obtained from each study participant. The study was carried out in accordance with the Declaration of Helsinki. The Regional Committee for Medical and Health Research Ethics of South-Eastern Norway approved the study (reference 2015/2140).

Results

Step I discovery and validation. Increased neopterin and KT-ratio in Norwegian PSC patients

Neopterin levels were higher in the plasma of PSC patients in Norway I, median (range) 11.9 (5.8–74.4), than in Controls I, 10.2 (4.7–42.0), $p = .003$ (Figure 2(A)). This finding was validated when comparing Norway II PSC patients with Controls II, 11.9 (5.7–27.9), vs. 11.4 (4.9–20.2), $p = .05$ (Figure 2(B)). Likewise, KT-ratio was increased in PSC in both Norway I and Norway II compared with controls (Figure 2(C–D)). Neopterin and KT-ratio correlated strongly in both the Norway I and Norway II cohorts, rho: 0.61, $p < .001$ and rho: 0.50, $p = .001$, respectively (Figure 2(E–F)).

Step II discovery. Neopterin and KT-ratio associated with survival in Norwegian PSC patients

Next, the associations between neopterin and KT-ratio and liver transplantation-free survival were investigated in the discovery PSC cohort Norway I. PSC patients who died or were liver-transplanted (reached an endpoint) during follow-up ($n = 87$, 45%) had higher neopterin levels than those who did not, 13.6 (6.6–74.4) compared with 11.2 (5.8–43.3) nmol/L, respectively ($p < .001$, Figure 3(A)). When stratifying neopterin into quartiles, a Kaplan–Meier survival analysis showed an inverse association between higher levels of neopterin and liver transplantation-free survival, log rank $p < .001$ (Figure 3(B)).

Similar results were observed in Norway I for KT-ratio, which was higher in PSC patients who reached an endpoint during follow-up compared with those who did not (Figure 3(C)). Accordingly, KT-ratio stratified into quartiles showed an inverse association with liver transplantation-free survival in the Norway I cohort ($p < .001$, Figure 3(D)).

Both neopterin and KT-ratio were able to differentiate PSC patients who reached an endpoint during follow-up from those who did not, with AUCs of 0.68 (95% CI 0.61–0.75) and

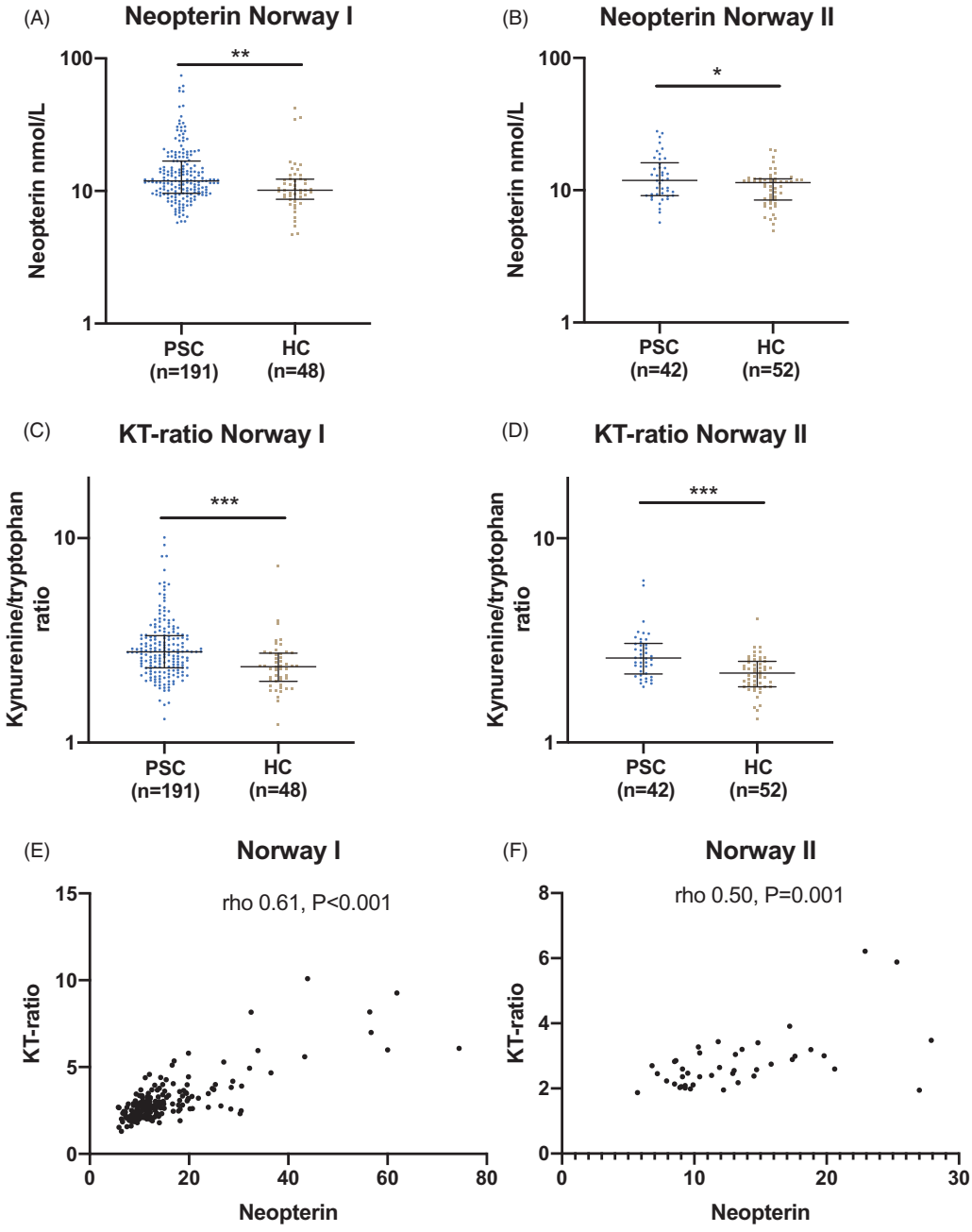

Figure 2. Neopterin and KT-ratio in PSC patients compared with healthy controls. (A-B) Increased neopterin concentration in two cohorts of Norwegian PSC patients compared with controls. (D-E) Increased kynurenine–tryptophan-ratio (KT-ratio) in two cohorts of Norwegian PSC patients compared with controls. (C and F) Moderate-to-strong correlation between neopterin and KT-ratio in both the Norway I ($n = 191$) and Norway II ($n = 42$) cohorts of PSC patients. *$p \leq .05$, **$p < .01$, ***$p < 0.001$. Results in (A), (B), (D), (E) are shown as median, interquartile range on a logarithmic scale.

0.69 (95% CI 0.62–0.76), respectively. The optimal cut-offs were 11.8 nmol/L for neopterin (Figure 3(E)) and 2.89 for KT-ratio (Figure 3(F)).

Step II validation. Neopterin and KT-ratio associated with survival in German PSC patients

In line with the findings in Norway I, patients reaching an endpoint in the German PSC cohort ($n = 16$, 11%) had higher neopterin concentration than patients not reaching an endpoint, 18.3 (7.0–105.0) compared with 10.0 (3.0–145.0) nmol/L, $p = .006$ (Figure 4(A)). When stratifying neopterin into quartiles, a Kaplan–Meier survival analysis validated the inverse relationship between higher levels of neopterin and liver transplantation-free survival ($p = .012$, Figure 4(B)). High neopterin as defined by the 11.8 nmol/L cut-off was strongly associated with reduced liver transplantation-free survival also in the German cohort (Figure 4(C)).

In line with the results for neopterin, KT-ratio was also higher in the German patients reaching an endpoint than in those without endpoint (Figure 5(A)). Accordingly, KT-ratio stratified into quartiles associated with liver transplantation-free survival in Kaplan–Meier analysis ($p = .002$, Figure 5(B)).

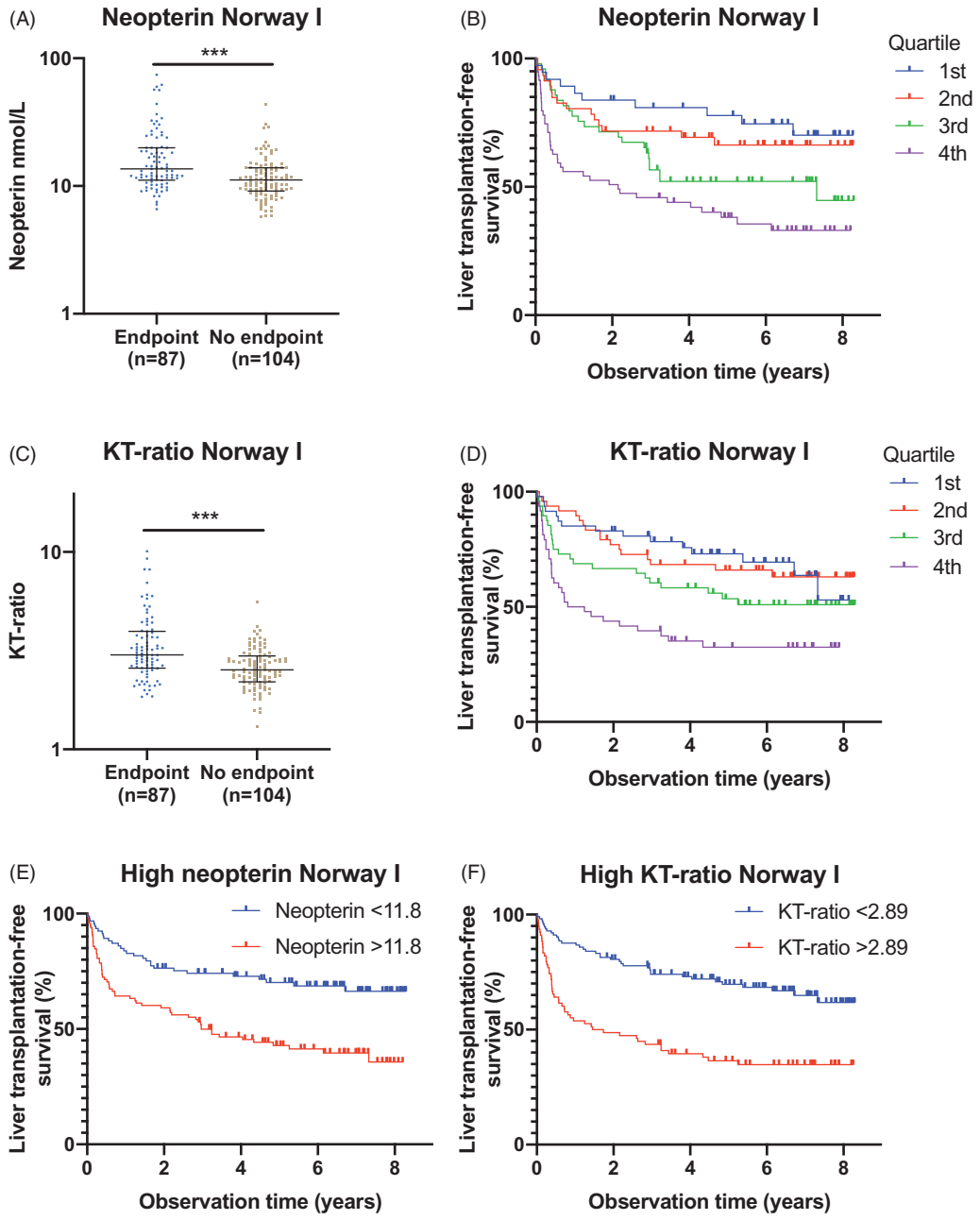

Figure 3. Neopterin, KT-ratio and liver transplantation-free survival in the Norwegian discovery cohort. (A) Increased neopterin concentration in PSC patients in the Norway I cohort, who were liver transplanted or died during follow-up. (B) Liver transplantion-free survival in the PSC patients in the Norway Icohort according to quartiles of neopterin, showing shorter liver transplant-free survival with higher values of neopterin ($p < 0.001$). (C) Higher KT-ratio level in PSC patients from the Norway I cohort, who were liver transplanted or died during follow-up. (D) Liver transplantation-free survival in the PSC patients in the Norway I cohort according to quartiles of KT-ratio, showing shorter liver transplant-free survival with higher values of KT-ratio ($P < 0.001$). (E) PSC patients in the Norway I cohort with neopterin $>$ 11.8 nmol/L had significantly shorter liver transplant-free survival (log rank $p < 0.001$). (F) PSC patients in the Norway I cohort with KT-ratio $>$ 2.89 had significantly shorter liver transplantation-free survival (log rank $p < 0.001$). ***: $p < 0.001$; KT-ratio: Kynurenine–tryptophan-ratio. Results in (A), (C) are shown as median, interquartile range on a logarithmic scale.

High KT-ratio (>2.89) was strongly associated with reduced liver-transplantation-free survival (Figure 5(C)).

Step II validation. Neopterin and KT-rationot associated with survival in Swedish PSC patients

In the Swedish validation cohort, neopterin was distinctly higher than seen in the three other PSC cohorts (Figure 4(D)). $N = 134$ (96%) of the Swedish patients had neopterin >11.8 nmol/L compared with 98 (51%) of the PSC patients in Norway I, 21 (50%) in Norway II and 63 (42%) in the German cohort. In the Swedish cohort, patients who reached an endpoint during follow-up had neopterin levels similar to the patients without an endpoint (Figure 4(E)). Correspondingly, a Kaplan-Meier analysis after stratifying neopterin into quartiles showed no association between neopterin levels and liver transplantation-free survival (Figure 4(F)). The predictive effect of high neopterin (>11.8 nmol/L) was not analyzed since only 6 (4%) were defined as low.

KT-ratio was lower in the Swedish PSC patients than in the Norway I cohort, while there were no statistically significant

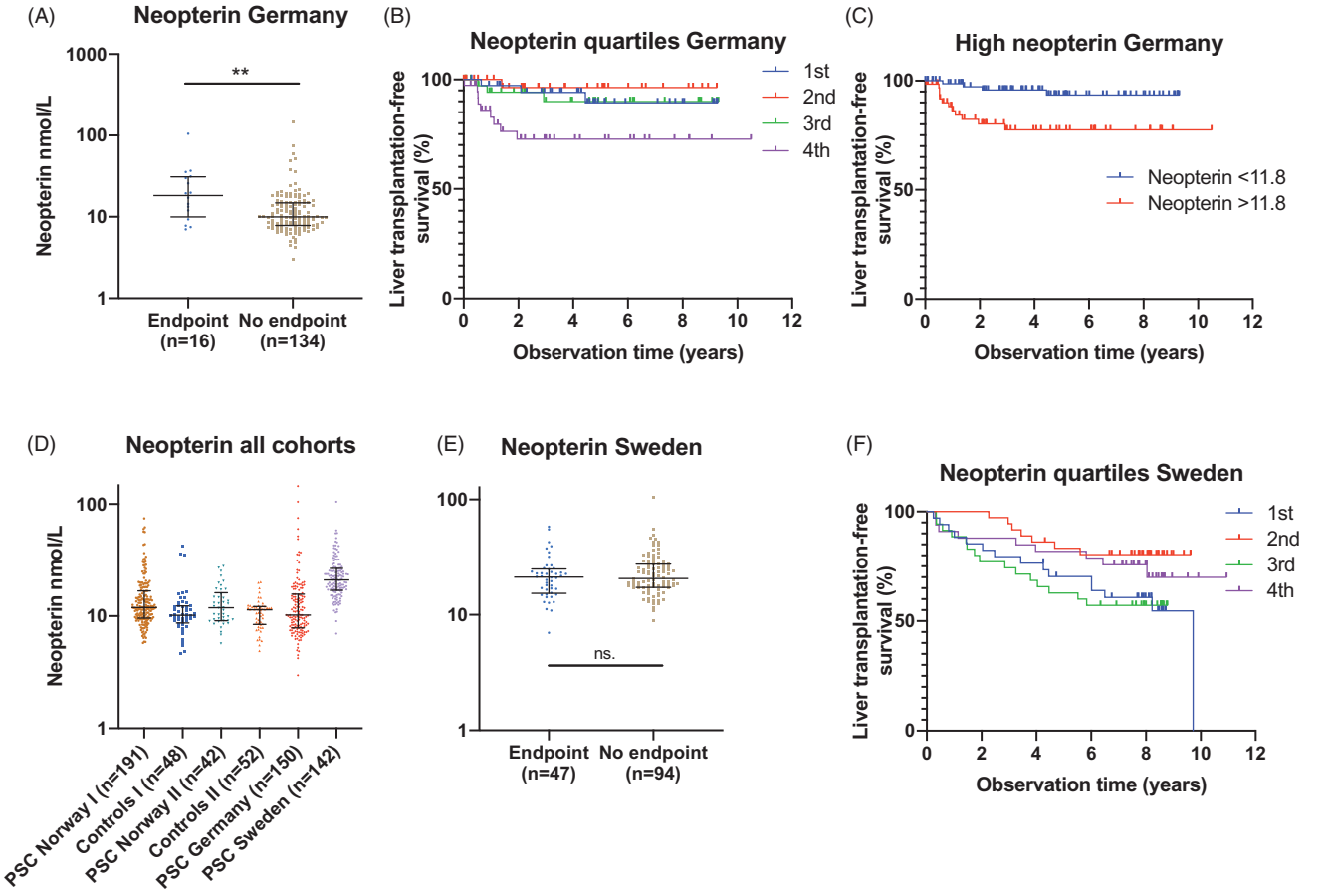

Figure 4. Neopterin in German and Swedish PSC patients. (A) Increased neopterin concentration in PSC patients from Germany, who were liver transplanted or died during follow-up. (B) Liver transplantation-free survival in the German PSC according to quartiles of neopterin, showing shorter liver transplant-free survival with higher values of neopterin (log rank $p = 0.012$). (C) German PSC patients with neopterin > 11.8 nmol/L had significantly shorter liver transplant-free survival (log rank p=.003). (D) Neopterin concentrations in all investigated cohorts, showing higher levels in Swedish PSC compared with Norway I (p<.001), Norway II (p<.001) and Germany (p<.001). E. No difference in neopterin concentration in PSC patients from Sweden who reached an endpoint (liver transplantation or death) or not during follow-up. F. Liver transplantation-free survival in the PSC patients from Sweden according to quartiles of KT-ratio, showing no significant association (log rank p=.07). **p<.01. Results in (A), (D), (E) are shown as median, interquartile range on a logarithmic scale.

differences between Sweden and Germany or Norway II (Figure 5(D)). KT-ratio was similar in Swedish PSC patients with and without endpoint (Figure 5(E)), and accordingly, KT-ratio stratified into quartiles (Figure 5(F)) or above the cut-off of 2.89 (Supplementary Figure 1) was not associated liver transplantation-free in the cohort of Swedish patients.

The correlations between KT-ratio and neopterin were similar in the German and Swedish cohorts (rho: 0.67, $p < .001$ and rho: 0.62, $p < .001$, respectively, Supplementary Figure 2 A-B), which was within the range of the observations in the two Norwegian cohorts (Figure 2(C–D)).

Correlations between neopterin and KT-ratio and disease severity

Neopterin and Mayo risk score showed a positive correlation in all PSC cohorts (Figure 6), with Spearman's rho ranging from 0.23 in the German cohort to 0.42 in the Norway II cohort (Supplementary Figure 3). Similar correlations were observed between KT-ratio and Mayo risk score, ranging from 0.26 in the German cohort to 0.40 in the Norway I cohort (Supplementary Figure 4). In a Cox regression model including neopterin and Mayo risk score, high neopterin (>11.8 nmol/L) predicted survival independent of Mayo risk score in the Norway I cohort, $HR_{high\ neopterin}$ of 1.62 (95% CI 1.0–2.6), $p = .044$, but not significantly in the German cohort, $HR_{high\ neopterin}$ 2.8 (95% CI 0.8–9.0), $p = .093$. In a similar analysis in the Swedish cohort, high neopterin (as defined by above median, 21.1 nmol/L, because of higher values) did not, HR 0.7 (95% CI 0.4–1.3). The results were similar for high KT-ratio (>2.89) corrected for Mayo risk score (data not shown).

Liver stiffness as measured by transient elastography correlated with neopterin and KT-ratio in the Norway II cohort, rho 0.31, $p = .045$ and rho 0.34, $p = .027$, respectively. In the Swedish patients, neither neopterin nor KT-ratio correlated with transient elastography (rho −0.03 for both). Median transient elastography was 7.9 (range 4–61) kPa in the Norway II cohort and 7.1 (3–26) in the subset of the Swedish patients, with 6/42 and 5/57, respectively, above the established cut-off of 14.4 kPa for cirrhosis [15]. There was a significant correlation between transient elastography and Mayo risk score in both the Norway II and the Swedish cohort (rho 0.59, $p < .001$ and 0.41, $p = .002$, respectively).

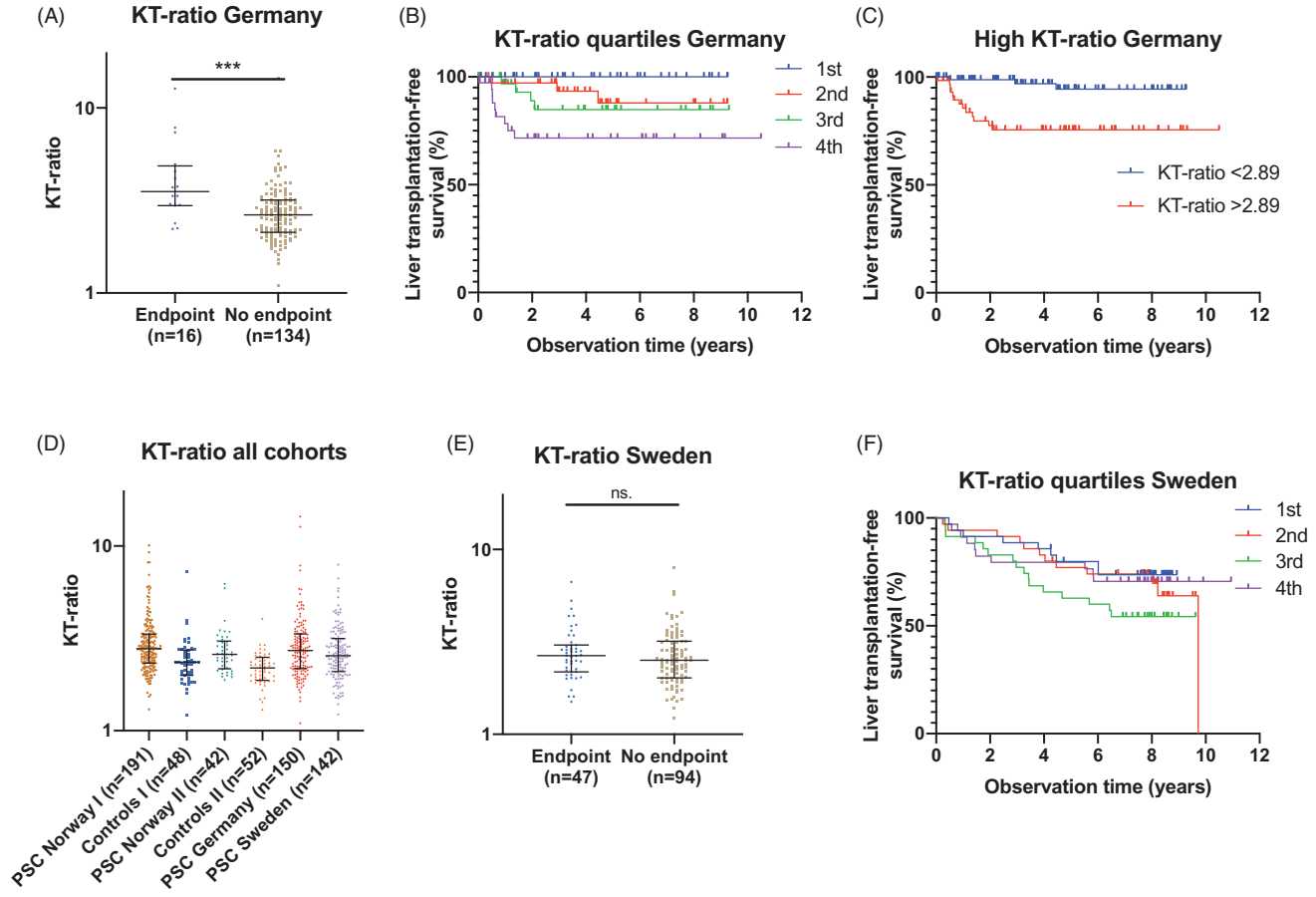

Figure 5. KT-ratio in German and Swedish PSC patients. (A) Increased KT-ratio in PSC patients from Germany, who were liver transplanted or died during follow-up. (B) Liver transplantation-free survival in the German PSC according to quartiles of KT-ratio, showing shorter liver transplant-free survival with higher values of KT-ratio (log rank p=.002). (C) German PSC patients with KT-ratio > 2.89 had significantly shorter liver transplant-free survival (log rank p=.001). (D) KT-ratio compared between all investigated cohorts, showing higher levels in the Norway I PSC patients compared with patients from Sweden (p=.018), otherwise no differences between the patient cohorts. (E) No difference in KT-ratio in PSC patients from Sweden who reached an endpoint (liver transplantation or death) or not during follow-up. (F) Liver transplantation-free survival in the PSC patients from Sweden according to quartiles of KT-ratio, showing no significant association (log rank p=.28) ***p<.001. KT-ratio: Kynurenine–tryptophan-ratio. Results in (A), (D), (E) are shown as median, interquartile range on a logarithmic scale.

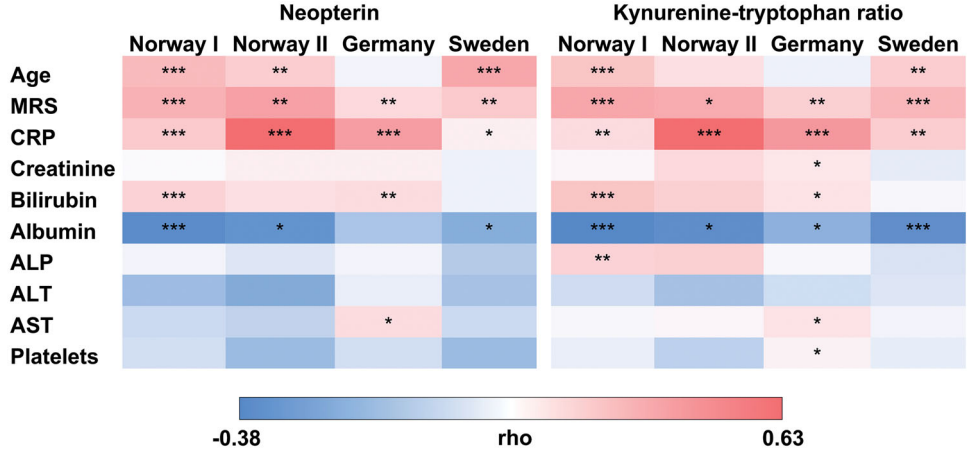

Figure 6. Neopterin and KT-ratio correlations with biochemistry and clinical characteristics. Heat map showing the correlation (Spearman rank) of clinical and biochemical characteristics of the PSC patient cohorts and neopterin and kynurenine-tryptophan ratio, respectively.The exact rho values are shown in Supplementary Figure 5. *: $p \leq .05$, **: $p < .01$, ***: $p < .001$. MRS: Revised Mayo risk score; CRP: C reactive protein; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

Correlations with clinical and biochemical characteristics in all cohorts

Given the deviating results in the Swedish validation cohort, we explored the cohort characteristics to identify possible clinical differences between the patients which could explain the observations. The Swedish PSC cohort was demographically relatively similar to the Norway I cohort (Table 1). Correlations between clinical and biochemical parameters

and neopterin and KT-ratio are shown in Figure 6 and Supplementary Figure 5. Notably, neopterin and KT-ratio correlated with CRP in all investigated cohorts (rho 0.17–0.63). Considering the Swedish cohort specifically, the perhaps most noteworthy difference was a lack of association of neopterin and KT-ratio with bilirubin, which was present in the other cohorts. Data on medication was available from the German cohort, where all patients were on ursodeoxycholic acid, 35 (23%) on immunosuppression 44 (29%) on 5-ASA therapy. Neopterin and KT-ratio were not different between patients with and without immunosuppression or 5-ASA (data not shown). In the German cohort, patients with concomitant IBD had higher neopterin and KT-ratiothan patients without IBD ($p < .05$), while neopterin or KT-ratio did not vary with IBD status in the three other cohorts (Supplementary Figures 6–7).

Discussion

In this study of in total 524 PSC patients from three countries, circulating neopterin and KT-ratio were higher in PSC patients compared with healthy controls in two independent Norwegian cohorts. In addition, increasing levels of neopterin and KT-ratio associated with reduced liver transplantation-free survival in independent cohorts from Norway and Germany, while in a PSC cohort from Sweden, neither neopterin nor KT-ratio predicted endpoints, despite similar patient demographics and correlations with biochemistry and Mayo risk score. Overall, the results suggest that IFNɣ-driven inflammation occurs in PSC patients, but the non-consistent association with survival suggests either that neopterin or tryptophan degradation to kynurenine at a given time point do not determine survival per se, or that circulating levels of neopterin or kynurenine do not always accurately reflect the process in the liver. It also highlights the need for validating biomarkers in multiple PSC cohorts before implementation in clinical practice.

Neopterin and KT-ratio were elevated in PSC compared with healthy controls. In the published literature, one small study has shown increased kynurenine and unchanged tryptophan in PSC, which would lead to increased KT-ratio [16]. Also, neopterin has been observed to be increased in multiple chronic liver diseases, including autoimmune etiologies, but no PSC patients were investigated [8,17]. Both neopterin and KT-ratio increase with IFNγ signaling, and the data are therefore supported by the observations of increased IFNγand the IFNγ-driven chemokines CXCL10 and CXCL11 found in serum in small cohorts of PSC patients [10,18]. IFNγ concentration was also increased in PSC bile, [19] increased IFNγ mRNA expression has been found in PSC explants, [20] and more IFNγ-producing T cells were found in colons of ulcerative colitis patients with concomitant PSC compared with ulcerative colitis patients without PSC [21]. Furthermore, the main source of neopterin is macrophages, and the results would fit with the observation of increased numbers of macrophages in PSC livers and accumulation of macrophages in the peribiliary areas of the liver [5,22–24]. Overall, there is therefore strong reason to believe that IFNγ-driven inflammation with subsequent increased neopterin and KT-ratio occurs in PSC.

Since biomarkers of inflammation in PSC could reflect disease activity in the biliary tree and subsequently progression of disease, we investigated the relationship with liver transplantation-free survival. There was a strong association between high neopterin and KT-ratio and reduced survival in both the Norwegian discovery cohort and the German cohort. Previous studies have shown a predictive value in PSC of biomarkers of inflammation like soluble CD14 and interleukin-8 [25,26]. Furthermore, IFNɣ signaling has been shown to be an important mediator in the progression of the PSC-relevant mouse *Mdr2* knockout model, [10] and inhibition of macrophage recruitment in an acute model of sclerosing cholangitis attenuated liver injury, [5] both supporting the potential relevance of neopterin and KT-ratio as biomarkers of disease severity in PSC. An important question is whether increased neopterin and KT-ratio reflect increased disease activity or later disease stage. There were only moderate correlations between neopterin and Mayo risk score in the included cohorts, and only a moderate correlation between neopterin and KT-ratio and liver stiffness in the Norway II cohort. Furthermore, both predicted liver transplantation-free survival independent from Mayo risk score in the Norway I cohort, while in the German cohort neopterin and KT-ratio were no longer significantly associated with survival after correction for Mayo risk score, although the risk estimates were higher in the German than the Norway I cohort. Overall, these observations may suggest the predictive values of neopterin and KT-ratio reflect disease activity in PSC and not only disease stage, but it cannot be concluded with certainty.

In contrast to the findings in the Norwegian and German PSC patients, there were no signs of association of neopterin or KT-ratio with survival in the Swedish cohort. Notably, the neopterin concentrationswere much higher in the Swedish patient serum than in the German serum or Norwegian plasma, potentially reflecting differences in critical preanalytical factors. Speaking against that being the case, KT-ratio were similar in the cohorts and the well-known strong correlation between KT-ratio and neopterin, [27] was observed in all cohorts, despite neopterin, kynurenine and tryptophan having different biochemical characteristics including more stable concentrations during storage for kynurenine and tryptophan than neopterin [28]. Taken together, this suggests that the pre-analytical factors do not explain the heterogeneity observed.

The deviating results in the Swedish cohorts could reflect true biological and clinical differences in the Swedish patients. Notably, there was no correlation of liver stiffness with neopterin or KT-ratio in the subset investigated in the Swedish cohort, in contrast to the observations in the Norway II cohort. However, the overall patient characteristics and biochemistry were not conspicuously different. Also, Mayo risk score predicted liver transplantation-free survival in all three cohorts and both neopterin and KT-ratio correlated with Mayo risk score in all three cohorts, although the correlation with CRP was weaker and with bilirubin absent in

the Swedish patients. Importantly, while the proportion of patients with IBD was comparable in the patient cohorts, it cannot be excluded that IBD activity may differ. Intestinal indoleamine 2,3-dioxygenase activity may influence plasma KT-ratio, [29] and it has previously been shown that KT-ratio may be elevated in patients with IBD, particularly pronounced in those with severe disease activity [30]. However, the differences in neopterin and KT-ratio between PSC patients with and without IBD were minor and only statistically significant in the German cohort, speaking against a major effect of IBD in this study.

The strengths of the present study are in particular the large number of patients from multiple cohorts from three countries, long follow-up of the patients, in addition to the use of well-validated and precise LC-MS/MS-based analysis methodology. Regarding limitations, the use of plasma in the Norwegian samples and serum in the German and Swedish samples as well as differences in storage time may add some heterogeneity, although only similar samples were compared. Furthermore, all serum samples were thawed and frozen at least once, with could add some additional variation to the subsequent biochemical analyses. The large number of samples and hence multiple batches analyzed and the inter- and intra-assay variation of the analyses could also influence the results, although this was minimized by the use of internal standards and controls. Regarding clinical data, there is a lack of detailed information for many of the patients on disease stage besides core biochemistry and complications of cirrhosis, and there is a lack of details on the distribution and activity of the concomitant IBD. The heterogeneity of the results makes a clear interpretation difficult and reduces the possible clinical value of neopterin or KT-ratio as candid biomarkers in PSC. Notably, among other investigated biomarkers in PSC, fibrosis markers like enhanced liver fibrosis test and pro-C3 appear to be perhaps stronger than inflammation markers, as exemplified by interleukin-8, which was no longer and independent predictor of survival after correction for enhanced liver fibrosis test [4,26,31,32]. Still, neopterin and KT-ratio are clearly elevated in PSC compared with controls, and the survival effects observed in two of the cohorts were strong. It is therefore still possible that therapies targeting inflammatory pathways are of relevance in PSC. However, the study highlights the general need for validating biomarkers in multiple PSC cohorts before clinical implementation.

Conclusions

Overall, neopterin and KT-ratio were elevated in PSC and associated with liver transplantation-free survival in two independent PSC cohorts, highlighting a possible role of IFNγ-driven inflammation in the pathogenesis and these markers as measures of prognosis and disease activity in PSC. However, the lack of association with survival in one of the investigated cohorts questionsthe potential clinical value of neopterin and KT-ratio and highlights the need to validate new biomarkers in PSC in multiple cohorts, preferably with detailed characterization of stage and IBD activity taken into account.

Acknowledgements

Liv Wenche Thorbjørnsen at the Norwegian PSC Research Center is acknowledged for help with biobank material. The Norwegian Bone Marrow Donor Registry is acknowledged for providing healthy controls.

Ethics approval

The study was carried out in accordance with the Declaration of Helsinki. The Regional Committee for Medical and Health Research Ethics of South-Eastern Norway approved the study (reference 2015/2140). Written informed consent was obtained from allstudy participants.

Author contributions

(i) Guarantor of the article: Johannes R. Hov is acting as the submission's guarantor.

(ii) Specific author contributions: AKD, MT and JRH conceived and planned the study. AKD, CR, AB, RV, TF, THK, MV, MK, JRH collected samples and clinical data. ØM and PMU performed biochemical analyses. AKD, MK and JRH performed statistical analyses. AKD, CR, AB, MT, ØM, PMU, MV, MK and JRH contributed to the interpretation of the results. AKD and JRH wrote the first draft.

(iii) All authors critically reviewed and revised the manuscript and approved the final version.

Disclosure statement

There was no conflict of interest.

Funding

AKD and JRH were funded by the Research Council of Norway (no. 240787/F20). MK was funded by the Regional Health Authority of South-Eastern Norway (no. 2016067).

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