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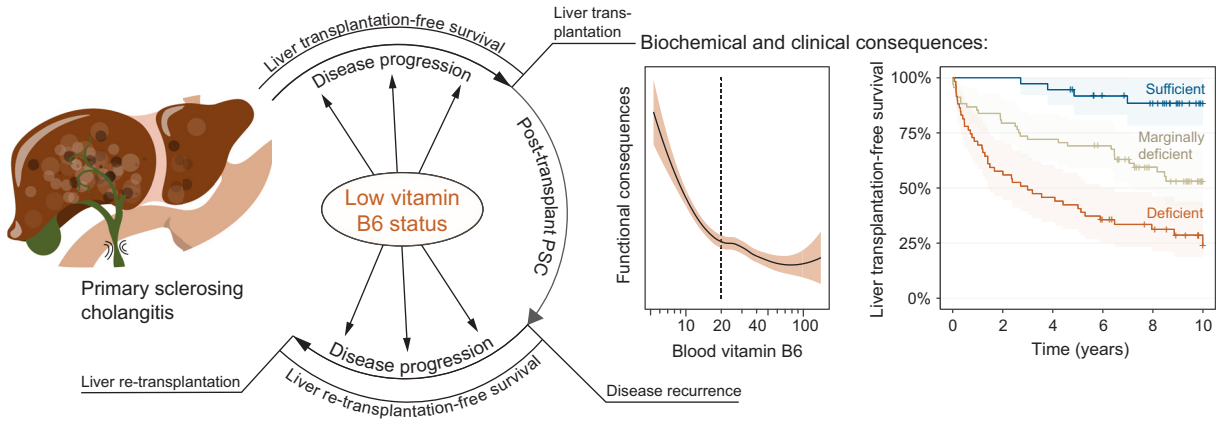
Graphical abstract



Highlights

- Vitamin B6 deficiency is common in people with primary sclerosing cholangitis.
- Low vitamin B6 impaired PLP-dependent pathways important for health.
- Low vitamin B6 was associated with short liver transplantation-free survival.
- The addition of vitamin B6 to current risk models improved survival prediction.
- After liver transplantation, low B6 persisted and was associated with poor outcomes.

Impact and implications

We previously found that people with PSC had reduced gut microbial potential to produce essential nutrients. Across several cohorts, we find that the majority of people with PSC are either vitamin B6 deficient or have a marginal deficiency, which remains prevalent even after liver transplantation. Low vitamin B6 levels strongly associate with reduced liver transplantation-free survival as well as deficits in biochemical pathways dependent on vitamin B6, suggesting that the deficiency has a clinical impact on the disease. The results provide a rationale for measuring vitamin B6 and to investigate whether vitamin B6 supplementation or modification of the gut microbial community can help improve outcomes for people with PSC.

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Clinical and biochemical impact of vitamin B6 deficiency in primary sclerosing cholangitis before and after liver transplantation

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Background and aims: We previously demonstrated that people with primary sclerosing cholangitis (PSC) had reduced gut microbial capacity to produce active vitamin B6 (pyridoxal 5'-phosphate [PLP]), which corresponded to lower circulating PLP levels and poor outcomes. Here, we define the extent and biochemical and clinical impact of vitamin B6 deficiency in people with PSC from several centers before and after liver transplantation (LT).

Methods: We used targeted liquid chromatography-tandem mass spectrometry to measure B6 vitamers and B6-related metabolic changes in blood from geographically distinct cross-sectional cohorts totaling 373 people with PSC and 100 healthy controls to expand on our earlier findings. Furthermore, we included a longitudinal PSC cohort (n = 158) sampled prior to and serially after LT, and cohorts of people with inflammatory bowel disease (IBD) without PSC (n = 51) or with primary biliary cholangitis (PBC) (n = 100), as disease controls. We used Cox regression to measure the added value of PLP to predict outcomes before and after LT.

Results: In different cohorts, 17-38% of people with PSC had PLP levels below the biochemical definition of a vitamin B6 deficiency. The deficiency was more pronounced in PSC than in IBD without PSC and PBC. Reduced PLP was associated with dysregulation of PLP-dependent pathways. The low B6 status largely persisted after LT. Low PLP independently predicted reduced LT-free survival in both non-transplanted people with PSC and in transplant recipients with recurrent disease.

Conclusions: Low vitamin B6 status with associated metabolic dysregulation is a persistent feature of PSC. PLP was a strong prognostic biomarker for LT-free survival both in PSC and recurrent disease. Our findings suggest that vitamin B6 deficiency modifies the disease and provides a rationale for assessing B6 status and testing supplementation.

Introduction

Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease of the bile ducts characterized by progressive fibrotic strictures and a strongly elevated risk of inflammation-driven biliary cancer.[1] The disease primarily affects young adults, and there are no available medical therapies proven to modify the disease course. PSC is a common indication for liver transplant(ation) (LT), which is an effective treatment, but there is a considerable risk of recurrent PSC (rPSC) in the new liver despite considerable immunosuppression.[2]

The liver is directly exposed to gut microbial factors via the portal circulation, which can either be increased or decreased depending on the context. PSC is well known to entail a markedly lowered gut microbial diversity and altered composition[3–5] which persists after LT.[6] Inflammatory bowel disease (IBD) is present in up to 80% of people with PSC and associates with a shorter LT-free survival[7] and a higher risk of recurrence after LT,[8] with the latter potentially being more pronounced in the case of ulcerative colitis.[9] In alignment with this, a colectomy appears to mitigate the risks of both disease progression prior to LT[10] and rPSC after LT,[8,11] despite some conflicting evidence.[9] Thus, the likely influence of gut pathology and/or modifications on PSC etiology suggests that gut microbial factors influence disease development and activity both before and after LT.

In a previous work aimed to define the contribution of the gut microbiome in PSC, we performed full metagenomic (shotgun) sequencing of microbial DNA from stool samples.[3] One of the main findings was that the genetic potential of the

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gut microbiota to synthesize the coenzyme form of vitamin B6, pyridoxal 5'-phosphate (PLP), was reduced in people with PSC compared to healthy controls.[3] Accordingly, people with PSC had lower circulating levels of PLP than healthy controls, and low PLP was associated with adverse outcomes. Plasma PLP is the most commonly used marker of B6 status, but the specificity is questioned, since it is affected by factors such as inflammation, alkaline phosphatase (ALP) activity, albumin concentration and alcohol consumption,[12] several of which are relevant in the setting of PSC. These drawbacks have motivated the development of functional markers of B6 status in blood, which reflect the metabolic activities of PLP in tissues. One example of such markers is the HKr index, which is defined as 3-hydroxykynurenine and divided by the sum of several downstream derivatives (kynurenic acid, anthranilic acid, xanthurenic acid, hydroxyanthranilic acid), all of which are generated in PLP-dependent enzymatic reactions. The HKr index represents a measure of the overall effect of PLP in the kynurenine pathway, and is characterized by high specificity, including a minor influence of inflammation.[13]

In the present study, we therefore analyzed an expanded set of metabolites reflecting vitamin B6 homeostasis in two cross-sectional cohorts to determine whether we could confirm low circulating PLP levels in PSC and whether low B6 status could be substantiated by the functional B6 marker HKr. We also measured PLP in relevant disease controls (*e.g.* people with IBD without PSC and primary biliary cholangitis [PBC]). Further, we aimed to confirm and independently evaluate PLP's ability as a prognostic factor to predict LT-free survival over and above current risk models for PSC. Finally, we evaluate PLP's association with recurrence-free and re-transplantation-free survival in a post-LT PSC cohort.

Patients and methods

Study design and patients

We included a cross-sectionally sampled cohort consisting of plasma samples from 191 LT-naïve people with PSC prospectively recruited at admission to Oslo University Hospital (Oslo, Norway) in 2008–2015 (NO-1; Fig. 1A). We recruited 48 healthy controls from the Norwegian Bone Marrow Donor Registry.

A cross-sectional cohort with plasma samples from 41 people with PSC admitted to Haukeland University Hospital (Bergen, Norway; NO-2) in 2017 to 2018 and 52 healthy controls (also from the Norwegian Bone Marrow Donor Registry) was used to replicate the case-control analyses. Time-to-event data were not available in this cohort.

An external cohort consisting of serum samples from people with PSC recruited from Karolinska University Hospital (n = 141, Stockholm, Sweden; SE) between 2008 and 2012 was used to evaluate the added predictive value of PLP in an independent PSC population. Baseline characteristics of the healthy controls and the total PSC population sampled pre-transplant are shown in Table 1.

A longitudinal Norwegian cohort consisting of 158 people who had received a LT for PSC at Oslo University Hospital and for whom plasma samples were available from more than one routine visit from 2006 onwards was recruited; 51 of these individuals also participated in the NO-1 cohort. Follow-up data were collected from medical records and outcomes defined as described below. The participants in this cohort were prospectively sampled, once shortly prior to their first LT (median±IQR: 57±64 days before surgery), and serially at 3 months, 1, 2, 3, 5 and 10 years, totaling 567 samples.

Fifty-one serum samples from participants in the prospective, population-based IBD cohort (IBSEN[14]) were also included in the study. The only inclusion criteria were that the samples were collected 20 years after the participants were diagnosed with IBD and that there was no evidence of a PSC diagnosis by magnetic resonance cholangiopancreatography at the time of sample draw.

A cross-sectional cohort with plasma from 100 people with PBC collected between 2016 and 2020 at Aarhus University Hospital, Denmark, was included as a cholestatic disease control population.

Clinical follow-up data for all cohorts except for the PBC (DK) and IBD (IBSEN) were collected up until 31.12.2019. The PSC cohort sizes were determined by sample availability. Routine blood biochemistry results were collected from medical records. Study reporting adhered to the REMARK guidelines.[15] All study participants were prospectively recruited, signed informed consent forms and had a minimally invasive blood sampling. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the institution's human research committee. The study was approved by the Regional Committee for Medical and Health Research Ethics in South-Eastern Norway (2015/2140 and 2016/1690), as well as 2010/1540 with project amendment 2019/1256, the Regional Ethical Review Authority Stockholm (2018/1111-32), and the ethical committee of the Central Denmark Region (1-10-72-146-16).

Liquid chromatography-tandem mass spectrometry

Plasma and serum samples were collected and stored according to standardized procedures at each center. A panel for B vitamins and kynurenines was analyzed by BEVITAL (www.bevital.no) using liquid chromatography-tandem mass spectrometry according to Midttun *et al.*[16]

Scores and ratios

To evaluate the added prognostic value of PLP in predicting LT-free survival, we calculated the PSC Mayo risk score (MRS),[17] Amsterdam-Oxford PSC model (AOM score),[18] and the PSC risk estimate tool (PREsTo)[19] 5-year risk score as benchmarks. APRI (aspartate aminotransferase-to-platelet ratio index)[20] and FIB-4 (Fibrosis-4)[21] were used as generic measures of liver disease severity in the post-transplant population, as the PSC-specific MRS, AOM and PREsTo scores have to our knowledge not been studied post-transplant. The HKr index was calculated as: hydroxykynurenine/(kynurenine+anthranilic acid + xanthurenic acid + 3–hydroxyanthranilic acid).[13] The PAr index was calculated as: pyridoxic acid/(pyridoxal + PLP).[22] Clinically meaningful categories of PLP were defined as (i) deficient: 0–20 nmol/L, (ii) marginally deficient: 20–40 nmol/L, and sufficient: 40 and higher.[13,23]

Outcome, definitions and diagnostic criteria

PSC was defined according to accepted criteria, with presence of typical large-duct changes on cholangiography and

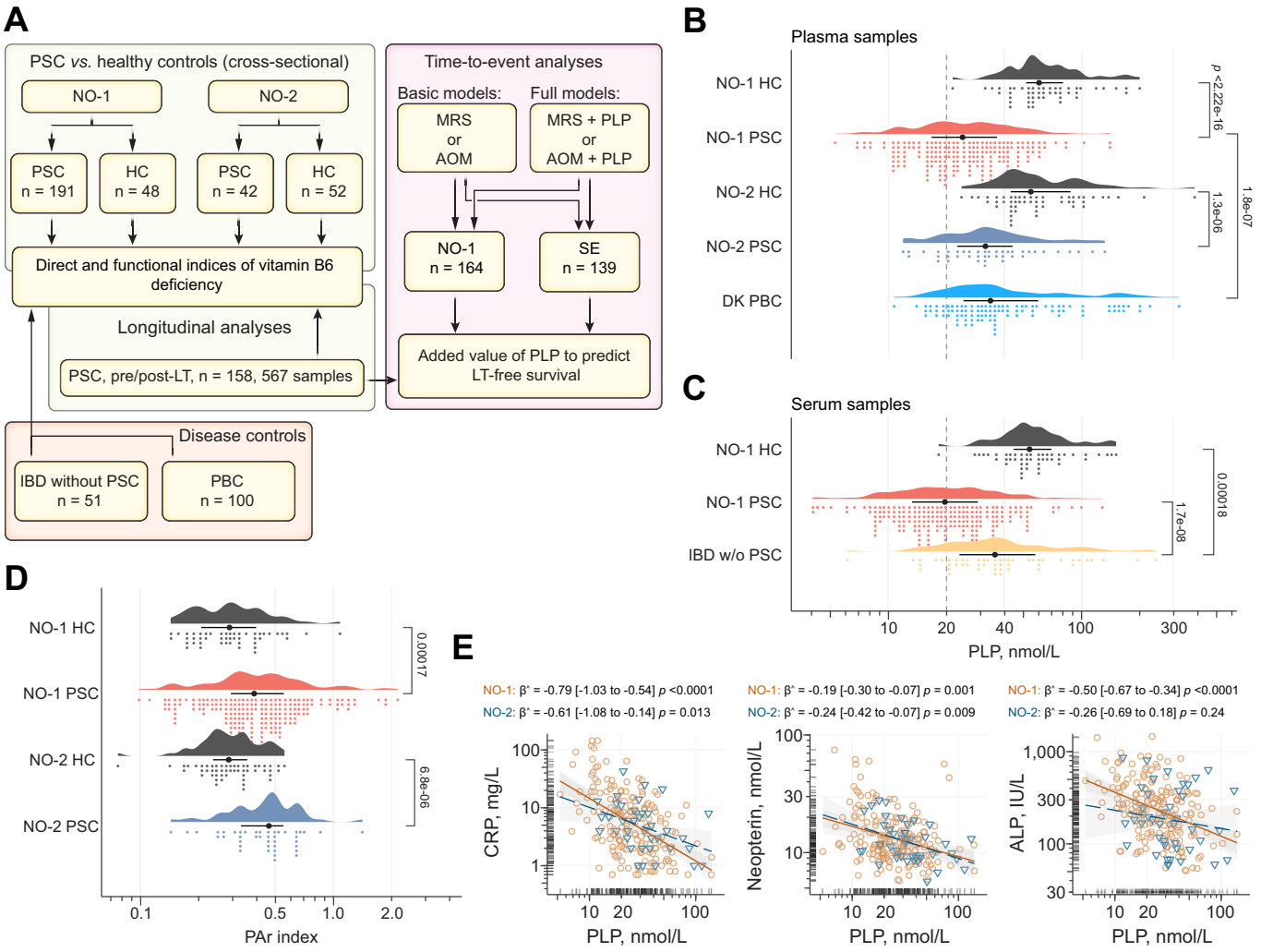

Fig. 1. Vitamin B6 deficiency prevalence in PSC. (A) Cohort overview and flow chart. (B) PLP concentrations in HC, PSC in NO-1[3] and NO-2, and PBC samples from the DK cohort. Dashed lines: B6 deficiency threshold. Horizontal black lines with dots: IQR and median. Statistical significance was tested using Mann-Whitney *U* tests. (C) PLP concentrations in serum samples from healthy controls and PSC in NO-1 and people with IBD confirmed not to have PSC (IBSEN cohort). Dashed lines: B6 deficiency threshold. Horizontal black lines with dots: IQR and median. Statistical significance was tested using Mann-Whitney *U* tests. (D) Levels of PAr indexes in NO-1 and NO-2. Horizontal black lines with dots: IQR and median. Statistical significance was tested using Mann-Whitney *U* tests. (E) Log-log plots of PLP *vs.* CRP, neopterin and ALP. Solid and dotted lines: Linear fits with 95% CIs (circles: NO-1; triangles: NO-2). Age-adjusted linear regression estimates, 95% CIs and *p* values for log-transformed PLP are shown above each panel. ALP, alkaline phosphatase; AOM, Amsterdam-Oxford PSC model; CRP, C-reactive protein; HC, healthy control; IBD, inflammatory bowel disease; LT, liver transplantation; MRS, PSC Mayo risk score; PBC, primary biliary cholangitis; PLP, pyridoxal 5'-phosphate; PSC, primary sclerosing cholangitis.

exclusion of secondary causes.[24] The outcome for non-transplanted people with PSC was the composite event of LT or all-cause mortality. LT-free survival was calculated as the time from sampling until the outcome or otherwise until time of censoring. Exclusion criteria for Cox regression were i) sampling made less than 3 months prior to a LT, as we reasoned these samplings were made in conjunction with referrals for LT and hence their times to LT were determined by donor availability (n = 21 and n = 1 in NO-1 and SE, respectively) and ii) participants who had a hepatobiliary cancer at time of blood draw were excluded (n = 8 and n = 1 in the NO-1 and SE cohorts, respectively).

For recipients of a LT in the longitudinal cohort, two outcomes were evaluated, namely rPSC, defined according to Graziadei,[25] and liver re-transplantation. All available protocol and clinically indicated liver biopsies were retrieved from routine pathology archives and re-evaluated individually by two pathologists experienced with gastrointestinal and transplant-related pathology as further described in the supplementary methods. All available magnetic resonance cholangiography protocols and clinically indicated magnetic resonance cholangiography examinations were also re-evaluated as described in the supplementary methods. Recurrence-free survival was defined as the time from sampling (after the first LT) until a rPSC diagnosis or death, or censoring due to loss-to-follow-up. Re-transplantation-free survival was calculated as the time from sampling made in conjunction with (defined as a sample taken ≤90 days before the diagnosis), or the first sample any time after, a rPSC diagnosis, until re-LT, death or last date of follow-up. Eligibility criteria for the latter analysis were that the recipients must have

Table 1. Baseline (time of sampling) characteristics of the healthy controls and people with PSC pre-transplant.

Variable	Healthy controls (n = 100)	PSC (n = 374)
Cohort, N		
NO-1	48	191
NO-2	52	42
SE	0	141
Sex, female	41 (41%)	94 (25%)
Age at sampling	40 ± 9	42 ± 23
IBD, any	—	282 (75%)
Ulcerative colitis	—	206 (56%)
Crohn's disease	—	57 (15%)
Indeterminate colitis	—	16 (4.3%)
Unknown	—	3 (1%)
Ursodeoxycholic acid	—	175 (47%)
Unknown	—	7 (2%)
HPB cancer, any	—	9 (2.4%)
Cholangiocarcinoma	—	7 (1.8%)
Gallbladder cancer	—	1 (<1%)
HCC	—	1 (<1%)
Variceal bleeding	—	7 (1.8%)
Ascites	—	20 (5.3%)
Encephalopathy	—	2 (<1%)
ALP, IU/L	—	193 ± 236
ALT, IU/L	—	70 ± 92
AST, IU/L	—	60 ± 71
Bilirubin, μmol/L	—	15 ± 24
Albumin, g/L	—	40 ± 7
Platelets, $x10^9$/L	—	244 ± 134
Sodium, UNIT	—	140 ± 3
Hemoglobin, UNIT	—	134 ± 31
MRS	—	0.13 ± 1.52
AOM score	—	1.74 ± 1.08
PREsTo 5-year risk score	—	4.52 ± 7.18

Continuous variables: median ± IQR. IBD and HPB cancer: counts (% of any).
ALT, alanine aminotransferase; ALP, alkaline phosphatase; AOM, Amsterdam-Oxford PSC model score; APRI, AST-to-platelet ratio index; AST, aspartate aminotransferase; HPB, hepatobiliary; HC, healthy control; HCC, hepatocellular carcinoma; IBD, inflammatory bowel disease; MRS, PSC Mayo risk score; PREsTo, PSC risk estimate tool; PSC, primary sclerosing cholangitis.

had ≥1 sample taken in conjunction with or after their rPSC diagnosis and had only undergone one prior LT.

Handling of missing data

Of variables used to compute composite risk scores in NO-1 and SE, 2.7% and 0.9% of the values were missing, respectively. C-reactive protein (CRP) was missing for 1.8% of the samples, while sodium and haemoglobin values were missing in SE. Among variables measured using the BEVITAL panel there were no missing values. Under the assumption that data were missing completely at random, we applied single imputation using multivariate imputation by chained equations with ursodeoxycholic acid use, inflammatory bowel disease status, the outcome, routine blood biochemistry and the targeted metabolomics panel as predictors. The median adjusted hazard ratio (HR) of PLP from multiple imputation (n = 100) of NO-1 was similar to the apparent HR from the single imputation (0.44±0.01 *vs.* 0.45).

In the longitudinal cohort, there was a theoretical maximum of 1,106 samples from 158 donors at seven time points during routine clinical follow-up. Since it was not clear why about half were not available, we considered them possibly missing-not-at-random, and hence we did not impute missing data in this dataset.

Statistical analyses

Surviving distributions were visualized by plotting Kaplan-Meier survival curves of strata defined by established, clinically meaningful categories for PLP.

To test whether PLP added value over and above established risk scores to predict LT-free survival, we fitted both "basic" Cox proportional hazards models containing either MRS, AOM or PREsTo scores alone and "full" models where PLP was nested together with either risk score. The risk scores were modeled as continuous variables, while PLP was modeled multiplicatively (log, base 2) (Fig. 1A). The Cox models were fitted on all eligible individuals with PSC in the NO-1 and SE cohorts separately using the *rms::cph* function.[26] We estimated the added prognostic value of PLP using likelihood ratio χ^2 tests of the nested Cox models (*rms::lrtest*) and by calculating resampling-validated concordance statistics (optimism-corrected *C*-statistic), calculated using the *rms::validate* function. This latter method was performed using bootstrapping with 200 repetitions to calculate a shrinkage factor that takes overfitting into account. To estimate variability of the resampled *C*-statistic estimates, we bootstrapped the aforementioned procedure 100 times and calculated intervals containing 95% of the estimates. The proportional hazards assumption was checked by inspection of Schoenfeld residuals for the covariates (data not shown).

Hepatobiliary cancer may influence the potential of reaching the main endpoint (*e.g.* cholangiocarcinoma is normally a contraindication for LT), which when not accounted for can yield biased estimates. To obtain subdistribution HRs for the main endpoint (LT or death) without hepatobiliary cancer, we fitted competing risk models using the Fine & Gray method[27] using the *cmprsk::crr* function. The subdistribution HR can be interpreted as the instantaneous rate of LT or death for individuals who have not experienced the outcome or have experienced the competing event.[28]

To test whether PLP changed longitudinally directly before and serially after LT, we fitted a generalized least squares model with PLP (log-transformed) as outcome and time as a continuous covariate using *nmle::gls* in the longitudinal cohort. We specified an autoregressive correlation structure since we assumed that observations closer in time would be more correlated than more spaced-out observations, and included patient ID in the correlation structure to account for the fact that each individual's result would be related to their previous result. To test the longitudinal associations between PLP and functional indices of vitamin B6 deficiency, ALP and CRP, we fitted similar models with time and PLP (log-transformed) as additive, fixed covariates, a correlation matrix as described above, and each of the mentioned markers (log-transformed) as outcomes.

To test for differences in distributions of continuous variables we used the Mann-Whitney *U* test. Distributions are given as median±IQR unless otherwise stated. Statistical analyses and data visualizations were carried out in R.

Results

Vitamin B6 deficiency is common in PSC

As described in the methods section, we included two cross-sectional PSC cohorts from two liver centers in Norway (NO-1; Fig. 1A and NO-2) along with 100 healthy controls. In both cohorts, plasma levels of PLP were lower in people with PSC than in

healthy controls (Fig. 1B). Using a published, biochemical threshold for vitamin B6 deficiency (20 nmol/L[13]), 38% and 17% of the people with PSC were vitamin B6 deficient in the NO-1 and NO-2 cohorts, respectively. In contrast, none of the healthy controls in either cohort were vitamin B6 deficient.

In the next steps, we assessed the relationship to relevant disease conditions and confounders. There was no clear evidence of PLP degradation over time in storage at -80 °C (Fig. S1A). We re-analysed PLP in serum samples from the NO-1 cohort and observed a near perfect correlation between serum and plasma levels of PLP, although PLP was on average 16% lower in serum (Fig. S2). We next measured PLP in serum samples from an IBD population without PSC (Table S2). Serum PLP levels were higher in IBD compared to PSC, but lower than in healthy controls (Fig. 1C). However, within PSC, levels of PLP were similar in serum samples taken from people with and without IBD (Fig. S3A). In neither PSC nor IBD without PSC was there clear evidence of a difference in PLP by IBD subtype (Fig. S3A-B).

Further, in a PBC cohort (Table S), plasma PLP levels were higher than in the NO-1 PSC cohort, but lower than in healthy controls (Fig. 1B), and 13% were biochemically B6 deficient.

The PAr index, which is a proposed marker of inflammation-related breakdown of PLP, was higher in PSC than in healthy controls in both cohorts (Fig. 1D). There were moderate, negative associations between PLP and the inflammation markers CRP and neopterin, and the cholestasis marker ALP (Fig. 1E), the latter of which is known to degrade PLP to PL. However, the sum of PLP and PL was, like PLP alone, lower in PSC than in healthy controls (Fig. S1B), overall suggesting that reduced PLP in PSC was not primarily caused by *in vivo* degradation of PLP to PL. Further, PLP levels were also similar in people with PSC reporting to be fasting compared to those reported to be non-fasting at the time of blood sampling ($p = 0.49$).

We next cross-sectionally investigated the association between PLP and MRS in the NO-1 and NO-2 cohorts and in a set of samples in the longitudinal cohort that were collected shortly before LT. We found that PLP and MRS were negatively associated in all sets of samples (Fig. S4). This finding was supported by linear regressions, which indicated that a 50% increase in PLP was associated with lower MRS in the pooled NO cohorts and the longitudinal cohort (0.38, 95% CI 0.21–0.56 and 0.42, 95% CI 0.31–0.53, respectively). Thus, the circulating PLP levels reflected the stage of PSC, as evaluated by MRS. However, when stratifying PSC by fibrosis evaluated by transient elastography (above *vs.* below 9.5 kPa[29]), available in a subset of 42 and 57 people with PSC in the NO-2 and SE cohorts, there was no difference in PLP levels (Mann-Whitney *U* tests, both $p > 0.29$).

PSC-associated vitamin B6 deficiency causes functional impairment of PLP-dependent reactions

The HKr index, which relates the level of 3-hydroxykynurenine to four kynurenine products of PLP-dependent enzymatic reactions within the kynurenine pathway (Fig. 2A),[13] was higher in PSC than in healthy controls (Fig. 2B), suggesting that the activity of this pathway was reduced in PSC. There was a steep increase in the HKr index in PSC samples with biochemically determined B6 deficiency (Fig. 2C), which was similar to what has been observed in a population cohort study.[13] In this B6 deficiency range, a ~50% reduction in PLP corresponded to a ~50% increase in the HKr index (unadjusted slope for PLP = -0.98, 95% CI -1.24 to -0.72). Plasma cystathionine, which is dependent on the activity of transsulfuration pathway enzymes that utilize PLP as a cofactor,[23] was also higher in PSC and was negatively associated with PLP levels (unadjusted slope for PLP = -0.40, 95% CI -0.55 to -0.26) (Fig. S5A-C).

Vitamin B6 deficiency persists after LT for PSC

In a longitudinal cohort of 158 people with PSC with blood samples drawn shortly prior to and serially after LT (Table S1) followed for a median of 13±6.8 years, 80 participants (51%) were diagnosed with rPSC 10 years after LT after a median of 3.1±6.5 years.

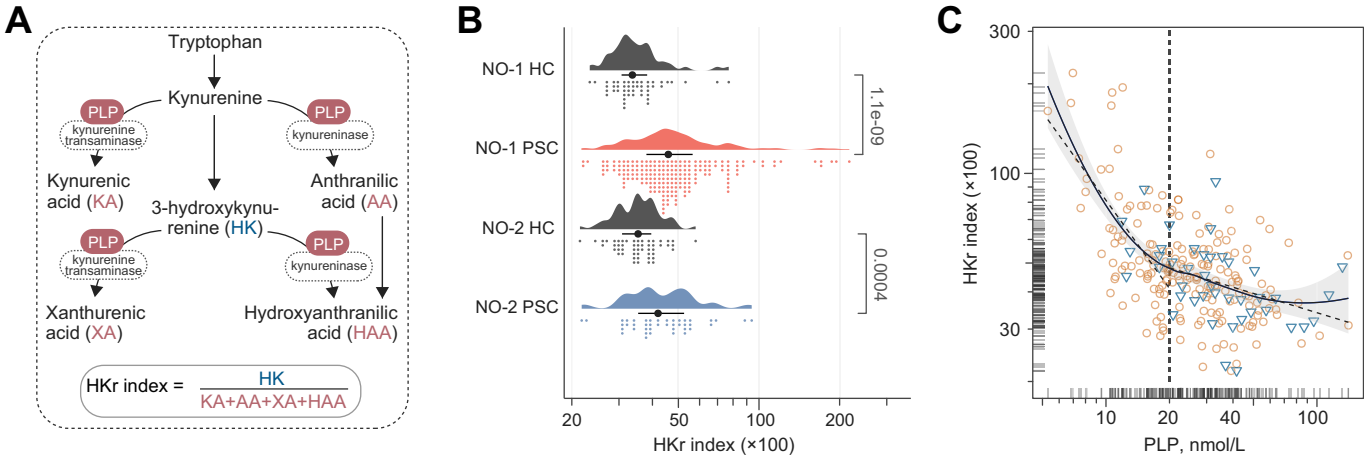

Fig. 2. Vitamin B6 deficiency impairs PLP-dependent reactions in people with PSC. (A) The kynurenine pathway and the basis for the calculation of the HKr index. (B) HKr index in HC and PSC in NO-1 and NO-2. Horizontal black lines with dots: IQR and median. Statistical significance was tested using Mann-Whitney *U* tests. (C) HKr index (x100) *vs.* PLP in PSC samples from NO-1 and NO-2. A loess line (solid line) with 95% CI (gray shade) was fit to pooled data. Dashed lines: Linear fits above/below the biochemical B6 deficiency threshold. Circles: NO-1 PSC; triangles: NO-2 PSC. HC, healthy control; HKr index, HK-ratio index; PLP, pyridoxal 5'-phosphate; PSC, primary sclerosing cholangitis.

In the samples taken shortly prior to LT, 52% were biochemically B6 deficient. After LT, median PLP levels increased until the 1-year mark, after which they plateaued (Fig. 3A). Notably, at the 1- and 2-year marks after LT, 21% and 19% of participants with PSC sampled were biochemically vitamin B6 deficient. When excluding samples from individuals diagnosed with rPSC within those time points, 19% and 21% were vitamin B6 deficient. We next investigated individual trajectories, which showed that PLP levels varied over time but increased overall (Fig. 3B). The increase was similar when excluding samples taken in conjunction with or after a rPSC diagnosis (data not shown). Expectedly, markers of both cholestasis (ALP) and systemic inflammation (CRP) dropped abruptly after LT (Fig. S6A-B). Thus, although there was some improvement in the B6 status after LT, the effect was modest compared to that of ALP and CRP, and the prevalence of biochemically determined B6 deficiency remained high.

We next investigated whether functional impairment of PLP-dependent biochemical pathways also was evident after LT. HKr index levels were on average nearly unchanged after LT, while there was a drop in cystathionine levels (Fig. S6C-D), thus reflecting the modest increase in PLP observed after LT. Furthermore, the negative association between PLP and the HKr index in post-transplant samples appeared to be similar to that in the pre-transplant cohorts, with a more prominent elevation in the HKr index in the biochemical B6 deficiency range (Fig. 3C). Also, cystathionine was negatively associated with PLP in post-transplant samples (Fig. S5D). These findings were supported by generalized least squares analyses, where PLP levels were negatively associated with both the HKr index and cystathionine when controlling for the effect of time. This indicated that, on average, an increase in PLP was associated with a decrease in HKr and cystathionine (unadjusted slopes for PLP to predict HKr = -0.32, 95% CI -0.37 to -0.28 and to predict cystathionine = -0.31, 95% CI -0.40 to -0.22). Also, ALP and CRP were negatively associated with PLP in the post-transplant samples (slope for ALP = -0.51, 95% CI -0.61 to -0.40; slope for CRP = -0.86, 95% CI -1.02 to -0.69).

PLP adds value to predict future LT or death from PSC in two independent cohorts

The metabolic pathway deficit in B6 deficiency, which was not resolved by LT, suggested a disease-modifying role and formed a rationale for studying PLP as a prognostic factor. The 164 people with PSC eligible for time-to-event analyses in NO-

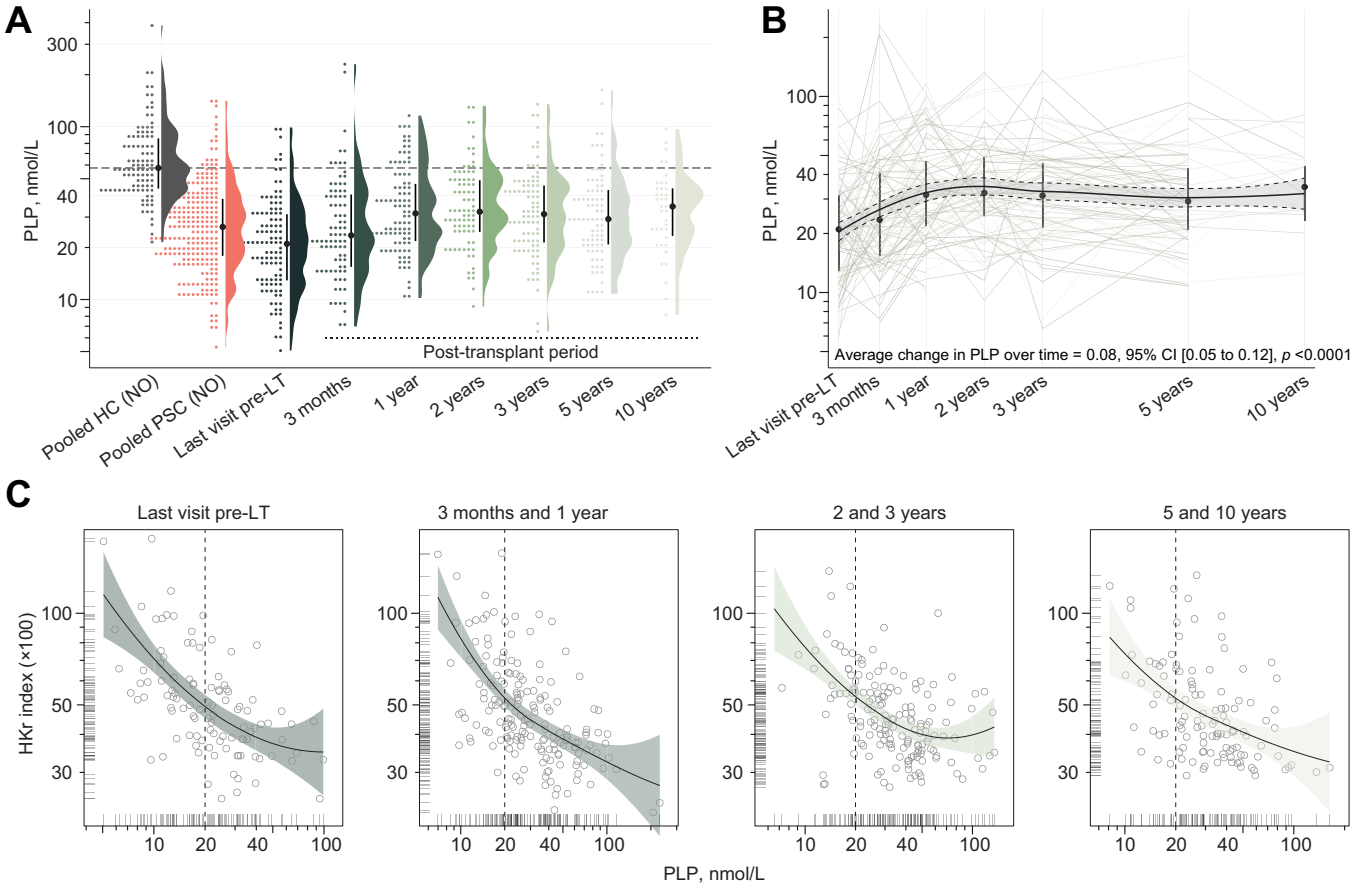

Fig. 3. Persistency of vitamin B6 deficiency after liver transplantation for PSC. (A) PLP levels are shown for HC and PSC in a pooled Norwegian cohort (same data as in Fig. 1B), and in the serial samples from the longitudinal cohort. The horizontal dashed line indicates the median PLP concentration in HC. Vertical black lines with dots: IQR and median. (B) Individual longitudinal trajectories of PLP in the longitudinal cohort. The black dots and vertical lines indicate the median and IQR, respectively, of all blood samples for each time point. A loess smoother was fit to all the data points, with the dotted lines indicating the 95% CIs. The longitudinal change in PLP was assessed using a generalized least squares model as described in the material and methods and the model estimates are shown in the plot. (C) Log-log plots of the HKr index (x100) *vs.* PLP at various merged time points in the longitudinal cohort. Loess lines with 95% CI (shaded areas) are shown. Vertical dashed lines: B6 deficiency threshold. HC, healthy control; HKr index, HK-ratio index; LT, liver transplantation; PLP, pyridoxal 5'-phosphate.

1 (Table S4) had a median follow-up of 6.9±7.1 years. At 6- and 8-years follow-up, 38% (n = 62) and 44% (n = 72) had undergone LT or died. When categorized according to clinically meaningful categories of PLP, a clear association between vitamin B6 deficiency and marginal deficiency and shorter LT-free survival was seen (Fig. 4A). This association was also evident when modeling PLP as a continuous marker (Table 2). Low PLP was also associated with shorter LT-free survival in people with low-to-intermediate-risk PSC (Fig. S7), which indicated that PLP may also help predict outcomes in this population. When holding MRS constant, a doubling in PLP was compatible with a 40% to 67% lower hazard of LT or death (Table 2). Similar estimates were found for PLP when holding either AOM or PREsTo scores constant. Compared to current risk scores alone, adding PLP improved both model goodness-of-fit and discrimination (Table 3), with similar results obtained using serum PLP concentrations (data not shown). Finally, using competing risk regression, low PLP associated with an increased risk of LT or death without hepatobiliary cancer.

We next refitted our univariable and multivariable Cox models to the SE cohort (Table 1) of whom 139 were eligible for Cox analyses (Table S4). This cohort had fewer diagnoses of hepatobiliary cancer during follow-up (median follow-up 7.5±4.0 years), and after 6 and 8 years, 27% (n = 38) and 32% (n = 44) had undergone LT or died. The SE cohort included fewer individuals with PSC with biochemically determined vitamin B6 deficiency than NO-1 (27% *vs.* 52% in serum, respectively, Fisher's exact test p <0.0001) and higher PLP overall (26±23 *vs.* 20±16 in serum, Mann-Whitney U test p <0.0001; Fig. S8). The distributions of MRS were overall similar between the two cohorts (Table S1 and S4). There was also discrimination of the survival curves in the SE cohort

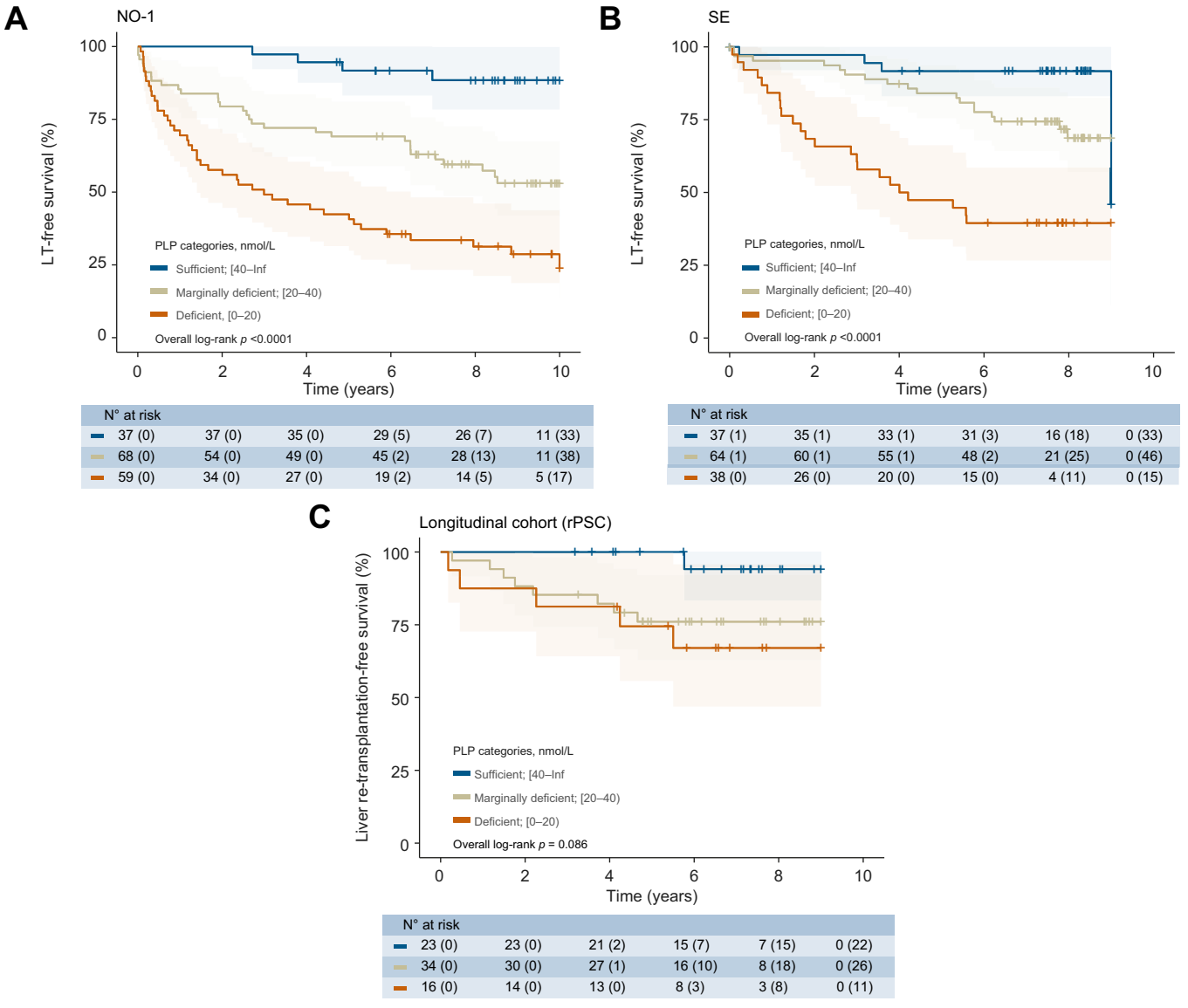

Fig. 4. Apparent associations between PLP and LT-free and re-transplantation-free survival in PSC. (A-B). LT-free survival curves in NO-1 (A) and SE (B) using published categories for PLP (censored at 10 and 9 years, respectively). (C) Liver re-transplantation-free survival curves among individuals diagnosed with rPSC after LT (censored at 9 years). PLP concentrations in samples taken in conjunction with or the first sample after a rPSC diagnosis were used to categorize into groups. The number at risk tables indicate the number of individuals (censored). Shaded areas around the survival curves indicate 95% CIs. Differences in the overall survival distributions were tested using log-rank tests (p values shown in plot). Inf, infinity; LT, liver transplantation; PLP, pyridoxal 5'-phosphate; rPSC, recurrent PSC.

Table 2. The relation of pre-defined variables to the risk of liver transplantation or death in people with PSC in NO-1 and SE that were eligible for time-to-event analyses.

		Cox proportional hazards		Competing risk regression	
Cohort	**Model[a]**	**HR (95% CI)**	***p* value**	**SHR (95% CI)**	***p* value**
Univariable					
NO-1 n = 164, 76 events	PLP	0.38 (0.29–0.50)	<0.0001	0.44 (0.33–0.59)	<0.0001
	MRS	1.74 (1.47–2.07)	<0.0001	1.38 (1.15–1.65)	<0.0001
	AOM	2.28 (1.77–2.94)	<0.0001	1.98 (1.49–2.62)	<0.0001
	PREsTo[b]	1.04 (1.03–1.05)	<0.0001	1.02 (1.00–1.03)	0.013
SE n = 139, 41 events	PLP	0.32 (0.22–0.46)	<0.0001	0.32 (0.22–0.46)	<0.0001
	MRS	2.14 (1.65–2.78)	<0.0001	2.23 (1.69–2.93)	<0.0001
	AOM	2.30 (1.59–3.33)	<0.0001	2.30 (1.51–3.51)	<0.001
Multivariable					
NO-1 n = 164, 76 events	PLP + MRS	0.45 (0.33–0.60)	<0.0001	0.49 (0.36–0.68)	<0.0001
	PLP + AOM	0.43 (0.32–0.57)	<0.0001	0.52 (0.38–0.70)	<0.0001
	PLP + PREsTO[b]	0.43 (0.32–0.59)	<0.0001	0.46 (0.34–0.63)	<0.0001
SE n = 139, 41 events	PLP + MRS	0.44 (0.28–0.69)	0.0003	0.45 (0.27–0.74)	0.016
	PLP + AOM	0.37 (0.24–0.56)	<0.0001	0.39 (0.25–0.62)	<0.0001

Results of univariable and multivariable Cox proportional hazards and competing risk models examining the relation of pre-defined variables to the risk of liver transplantation or death in people with PSC in NO-1 and SE that were eligible for time-to-event analyses.
AOM, Amsterdam-Oxford PSC model score; HPB, hepatobiliary; HR, hazard ratio; MRS, PSC Mayo risk score; PLP, pyridoxal 5'-phosphate; PREsTo, primary sclerosing cholangitis risk estimate tool; PSC, primary sclerosing cholangitis; SHR, subdistribution hazard ratio.
[a]In the multivariable models, only estimates for PLP are reported. PLP was log (base 2) transformed in all models.
[b]PREsTO 5-year risk score.

Table 3. Performance metrics of the multivariable Cox proportional hazards models examining the relation of PLP (log_2) to the risk of liver transplantation or death in NO-1 and SE.

Cohort	Model	Optimism-corrected *C*- statistic (95% CI)[a]	Δ*C*-statistic[b]	LR χ^{2}[c]	LR test *p* value[c]
Univariable					
NO-1	PLP	0.720 (0.655–0.766)	—	—	—
	MRS	0.717 (0.657–0.768)	—	—	—
	AOM	0.712 (0.648–0.753)	—	—	—
	PREsTo	0.697 (0.633–0.774)			
SE	PLP	0.739 (0.668–0.811)	—	—	—
	MRS	0.750 (0.693–0.804)	—	—	—
	AOM	0.698 (0.625–0.782)	—	—	—
Multivariable					
NO-1	PLP + MRS	0.765 (0.714–0.809)	0.048	30.2	<0.0001
	PLP + AOM	0.762 (0.698–0.820)	0.050	33.4	<0.0001
	PLP + PREsTo	0.744 (0.700–0.801)	0.047	28.6	<0.0001
SE	PLP + MRS	0.772 (0.680–0.833)	0.022	12.9	<0.001
	PLP + AOM	0.761 (0.715–0.831)	0.063	21.7	<0.0001

AOM, Amsterdam-Oxford PSC model score; LR, likelihood ratio; MRS, PSC Mayo risk score; PLP, pyridoxal 5'-phosphate; PREsTo, primary sclerosing cholangitis risk estimate tool; PSC, primary sclerosing cholangitis.
[a]Resampling-validated C-statistic and 95% bootstrapped confidence intervals (0.025 to 0.975 percentiles).
[b]Change in optimism-corrected C-statistic of full model relative to simple model (either MRS or AOM score alone).
[c]Likelihood ratio test $\chi 2$ statistic and *p* value of nested models.

(Fig. 4B; log-rank for all comparisons p <0.0001), and PLP appeared prognostic across risk groups (Fig. S7). Confidence intervals of both unadjusted and adjusted HRs for PLP were largely overlapping in NO-1 and SE (Table 2), and PLP also added value over and above both the contemporary risk scores in the SE cohort (Table 3). PLP was also associated with a higher risk of LT or death without hepatobiliary cancers using competing risk regression (Table 2).

In both cohorts, bias-corrected predicted risks of the PLP + MRS model corresponded well with actual (observed) risks at both the 6- and 8-year time points (Fig. S9), suggesting no problems with overfitting. Using decision curve analysis on the same time points, the PLP + MRS model gave a higher net benefit than MRS alone in the 5% to 15% risk threshold range (Fig. 5; Supplementary methods).

PLP may also predict future events in transplant recipients with recurrent PSC

The persistently high prevalence of vitamin B6 deficiency in transplant recipients motivated us to study whether PLP could be predictive of recurrent disease and re-transplantation-free survival. Using either PLP levels in plasma samples taken directly prior to LT or at the 1-year mark (excluding those who developed rPSC before the 1-year mark), we found no associations with recurrence-free survival (data not shown). However, among individuals who had been diagnosed with rPSC (Table S5), low plasma PLP in samples taken in conjunction with, or the first sample after, a rPSC diagnosis was associated with an increased risk of re-transplantation or death (Fig. 4C and Table S6). In these individuals, a doubling in PLP was associated with a lower risk of re-transplantation or death

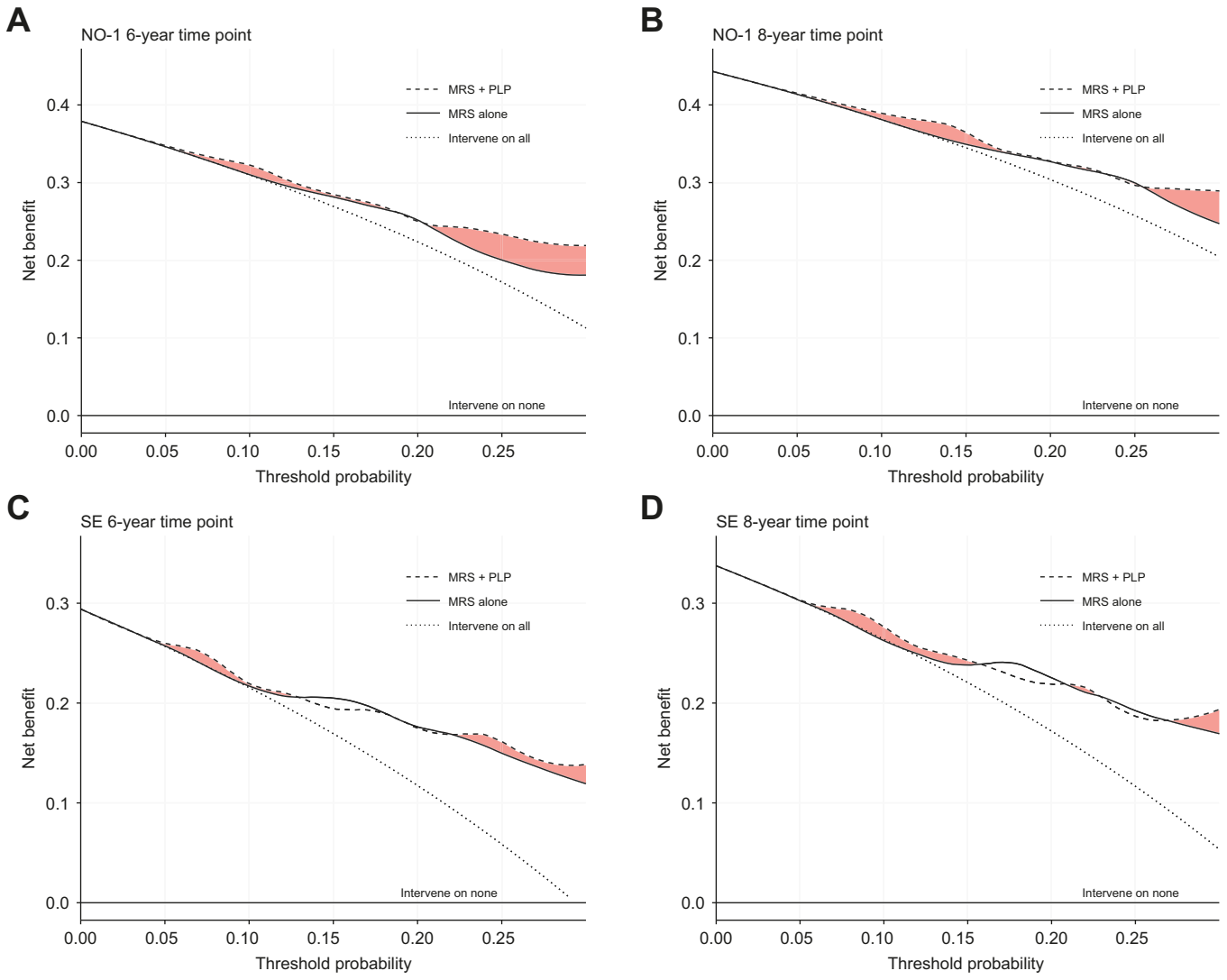

Fig. 5. Clinical utility of PLP. Decision curve analyses at the 6- and 8-year time horizons for NO-1 (A-B) and SE (C-D). The net benefit is plotted for a range of clinically relevant thresholds for decision-making. The solid and dashed lines indicate the smoothed net benefit of the MRS and MRS + PLP models, respectively, at various probability thresholds. Where the MRS + PLP model has an added net benefit compared to the MRS alone model, the area between the two lines is colored. The "intervene on all" reflects the benefit of a non-informative model, and crosses the y-axis at the prevalence of liver transplantation or death at the indicated time horizons. The "intervene on none" line reflects the net benefit of no interventions. MRS, PSC Mayo risk score; PLP, pyridoxal 5'-phosphate.

(unadjusted HR = 0.33; 95% CI 0.14–0.79), with a *C*-statistic of 0.69, despite the low number of events (n = 14). Adjusted for either APRI, FIB-4 or MRS, PLP's estimates were nearly unchanged compared to when modeling PLP alone. Of these risk scores, only MRS had a notable predictive value in univariable models (unadjusted HR = 2.67; 95% CI 1.38 to 5.17; *C*-statistic = 0.62). The model estimates were similar when using time-dependent covariates and time-invariant covariates (supplementary methods).

Collectively, these results showed that PLP was not prognostic for rPSC. However, like in non-transplanted PSC, a low PLP predicted a higher risk of liver re-transplantation or death in individuals who had developed recurrent disease.

Discussion

In the present study including more than 1,000 peripheral blood samples taken from people with PSC at various disease stages, and both before and after LT, as well as healthy donors and relevant disease controls, we demonstrate that vitamin B6 deficiency, evaluated both directly and functionally in peripheral blood, is prevalent in PSC. Further, PLP levels were prognostic for LT-free survival and added significant value over and above established prognostic models in two independent, geographically distinct cohorts. In addition, LT modestly improved vitamin B6 status, but circulating PLP levels remained suppressed and functional consequences on PLP-dependent reactions persisted. In a small sample of people with rPSC after LT, PLP levels were prognostic for liver re-transplantation-free survival, while low PLP did not predict recurrence *per se*, and is not disease specific, which may suggest that low vitamin B6 may be a modifier and not a cause of PSC.

Isolated B6 deficiency is rare in the general population,[23] perhaps related to the high dietary availability and the high

capacity of gut microbiota to supply their hosts.[30] However, low circulating vitamin B6 is prevalent in people with IBD,[31–33] rheumatoid arthritis[34] and other conditions characterized by systemic inflammation.[31,35,36] In addition, a high prevalence of vitamin B6 deficiency in people with advanced chronic liver disease of various etiologies is well known.[37–40] While underlying causes of the deficiency in PSC remain unclear, our observation of reduced PLP in both IBD without PSC and PBC suggest that the deficiency is not disease specific but may indicate a shared mechanism possibly involving the gut. On the other hand, PLP was particularly depleted in PSC, and we found no differences in PLP according to IBD status in these individuals.

Increased degradation and tissue-specific uptake are plausible known factors that may contribute to the deficiency.[23,37] Indeed, blood samples from people with PSC showed signs of increased degradation of PLP into pyridoxic acid, as indicated by an increase in the PAr index, and blood samples with elevated CRP and ALP had decreased PLP; both compatible with systemic inflammation-driven degradation or altered distribution of active vitamin B6. On the other hand, we found that reduced circulating PLP was associated with deficits in PLP-dependent pathways known to reflect a deficiency largely not confounded by inflammation and ALP activity, particularly in individuals with PLP <20 nmol/L; this is in line with a study in people with cardiovascular disease.[13] Furthermore, while LT reduced cholestasis and systemic inflammation, transplant recipients showed signs of persistent vitamin B6 deficiency with functional impairment of PLP-dependent biochemical reactions. Hence, our results suggest that liver function is not a major bottleneck in the production of PLP from its dietary precursors in PSC, which is further supported by our finding that PLP levels were similar in PSC with and without advanced fibrosis.

Our results demonstrate a *true* B6 deficiency reflecting functional consequences, which is a major strength of our study. In keeping with the reduced bacterial diversity and loss of gut bacterial capacity to produce PLP,[3–5] we speculate that B6 deficiency in the context of PSC may be related to loss of microbially derived vitamin B6,[3] although the present study does not shed further light on the link to the gut. However, we recently showed that gut dysbiosis persists in the post-transplant period,[6] which aligns with the observed high prevalence of B6 deficiency in post-LT PSC. The gut microbiota can synthesize vitamin B6 *de novo* with a particularly high capacity,[30] and B6 vitamers are recognized as microbial metabolites,[41,42] but further data are needed to establish whether gut microbial alterations are a contributing cause of B6 deficiency.

Our results do not verify a causal link between low vitamin B6 and rapid disease progression, and this apparent association may be limited by residual or unmeasured confounding. However, we showed that circulating PLP levels add value to existing prognostic models to predict future events across two geographically distinct cohorts and may offer clinical utility and aid in decision-making in individuals with a low-to-intermediate risk of LT or death within 6 and 8 years. Furthermore, both internal and external measures of discrimination and validation show that our results may generalize to other PSC populations. Importantly, the observed association with re-transplantation-free survival in people with rPSC and the lack of association with recurrent disease *per se*, lend further support to our hypothesis of reduced PLP negatively affecting PSC but not causing disease. Further, B6 has multifactorial and widespread roles in human health, including in regulation of one-carbon metabolism,[41] immune modulation and the inflammatory response,[23] and the prognostic value of PLP may be due to its ability to represent the activities of these processes.

The major strength of our study is the inclusion of a high number of people (>1,000 patient samples) with PSC at various disease stages, and both before and after LT, as well as healthy donors and relevant disease controls. All cohorts are well characterized and included from high-volume PSC centers. However, our findings are still limited by the lack of validation in large cohorts from other care settings and non-Nordic countries. Other clinical characteristics such as dietary data, malnutrition, supplements and alcohol consumption[12] were not available which may limit the interpretability of the results. The analyses on liver re-transplantation-free survival were limited by a small sample size, which was reflected in confidence intervals around the HR, and should be further explored in larger cohorts. A major limitation involves the use of LT as an outcome, since time to LT will likely vary depending on clinical practice and donor availability. Another limitation is that we did not analyze vitamin B6 in non-cholestatic liver disease controls, as it is well known that vitamin B6 deficiencies exist in advanced liver disease of different etiologies.[37–40] This study therefore provides an obvious rationale for studying vitamin B6 homeostasis in other liver diseases before end-stage, and illustrates the utility of a complete assessment by measuring PLP and functional B6 markers. Large cohorts with proper follow-up to define effects on disease severity and progression in other diseases were not available to us, and we are thus unable to define to what degree the predictive power of vitamin B6 deficiency is PSC-specific.

In conclusion, we found that low PLP and functional B6 deficiency were prevalent in people with PSC, and B6 deficiency was prevalent also in LT recipients. PLP added value to predict transplant-free survival regardless of whether PSC was diagnosed in the innate or transplanted liver, suggesting that it is a promising candidate variable in prediction model development. Our study does not confirm a causal link between gut microbial alterations and reduced vitamin B6 in PSC, which should be investigated in studies applying an integrated metabolomic and metagenomics study design with temporally matched blood and stool samples from large cohorts. However, our findings align with the possibility that restoration of vitamin B6 homeostasis may positively influence the disease course, warranting analysis of vitamin B6 supplementation in a randomized trial.

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Abbreviations

ALP, alkaline phosphatase; AOM, Amsterdam-Oxford model; CRP, C-reactive protein; HC, healthy control; IBD, inflammatory bowel disease; LT, liver transplant(ation); MRS, PSC Mayo risk score; PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5'-phosphate; PSC, primary sclerosing cholangitis; PREsTo, PSC risk estimate tool; rPSC, recurrent PSC.

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Conflict of interest

All authors report no conflicts of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study concept and design: PRB, AB, MK, MCS, AU, PMU, EM, THK, MV, JRH. Acquisition of data: PRB, AB, MK, LKE, HMR, IB, KG, AA, TF, AU, PMU, EM, PDL, THK, MV, LB, HG, MLH, JRH. Analysis and interpretation of data: PRB, AB, MK, LKE, MCS, AU, LB, MLH, PMU, JRH. Drafting of the manuscript: PRB, MCS, AU, PMU, JRH. Critical revision of the manuscript: All authors. Statistical analysis: PRB, MCS, AU, LB, JRH. Obtained funding: JRH.

Data availability statement

Data, analytic methods and study materials will be made available to other researchers upon reasonable request.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2023.05.038.

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Author names in bold designate shared co-first authorship

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