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ORIGINAL ARTICLE

Influence of *NOD2* risk variants on hepatic encephalopathy and association with inflammation, bacterial translocation and immune activation

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Abstract

Background: Nucleotide-binding oligomerization domain containing 2 (*NOD2*) risk variants lead to impaired mucosal barrier function, increased bacterial translocation (BT), and systemic inflammation.

Aim: To evaluate the association between the presence of *NOD2* risk variants, BT, inflammation, and hepatic encephalopathy (HE).

Patients and Methods: This prospective multicenter study included patients with cirrhosis and testing for *NOD2* risk variants (p.R702W, p.G908R, c.3020insC, N289S, and c.-958T>C). Patients were evaluated for covert (C) and overt (O) HE. Markers of systemic inflammation (leukocytes, CRP, IL-6, LBP) and immune activation (soluble CD14) as well as bacterial endotoxin (hTRL4 activation) were determined in serum.

Results: Overall, 172 patients (70% men; median age 60 [IQR 54–66] years; MELD 12 [IQR 9–16]; 72% ascites) were included, of whom 53 (31%) carried a *NOD2* risk variant. In this cohort, 11% presented with OHE and 27% and CHE. Presence and severity of HE and surrogates of inflammation, BT, and immune activation did not differ between patients with and without a *NOD2* risk variant, also not after adjustment for MELD. HE was associated with increased ammonia and systemic inflammation, as indicated by elevated CRP (w/o HE: 7.2 [2.7–16.7]; with HE 12.6 [4.5–29.7] mg/dL; $p < 0.001$) and elevated soluble CD14 (w/o HE 2592 [2275–3033]; with HE 2755 [2410–3456] ng/mL; $p = 0.025$).

Conclusions: The presence of *NOD2* risk variants in patients with cirrhosis is not associated with HE and has no marked impact on inflammation, BT, or immune activation.

Abbreviations: ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; BCLC, Barcelona Clinic Classification for Liver Cancer; CI, cardiac index; CO, cardiac output; CRP, C reactive protein; CVP, central venous pressure; DAMPS, damage associated molecular patterns; FHVP, free hepatic venous pressure; HCC, hepatocellular carcinoma; HF, heart frequency; HIV, human immunodeficiency virus; HVPG, hepatic venous pressure gradient; INR, international normalized ratio; MAP, mean arterial pressure; MELD, model for end-stage liver disease; NF-κB, nuclear factor kappa B; NOD2, nucleotide-binding oligomerization domain containing 2; PAMPS, pathogen associated molecular patterns; PAP, pulmonary arterial pressure; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; SBP, spontaneous bacterial peritonitis; SVR, systemic vascular resistance; TIPS, transjugular intrahepatic portosystemic shunt; VCI, vena cava inferior pressure; WHVP, wedged hepatic venous pressure.

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In contrast, the presence of HE was linked to ammonia, the acute phase response, and myeloid cell activation.

KEYWORDS
bacterial translocation, end-stage liver disease, genetic risk factor, immune response, liver insufficiency, portal hypertension

1 | INTRODUCTION

The nucleotide-binding oligomerization domain containing 2 (*NOD2*) protein acts as a pattern recognition receptor with affinity for the bacterial muramyl dipeptide and assumes important functions in the immune system. Among others, it is involved in the intestinal recognition of bacteria and their by-products.

Certain *NOD2* variants (p.R702W, p.G908R, c.3020insC, p.N289S, and c.-958T>C) are considered risk variants and are associated with impaired mucosal barrier function,[1] leading to increased intestinal permeability which is one of the bases for bacterial translocation (BT) from the gut.[2–4]

The presence of *NOD2* risk variants was initially associated with Crohn's disease.[5–7] In this patient population, the presence of these variants led to increased detection of bacterial DNA (bactDNA) and increased TNF-α levels as a sign of BT and inflammation.[8]

BT plays a central role in the development of classic complications in patients with liver cirrhosis according to the current systemic inflammation hypothesis.[9] Indeed, patients with liver cirrhosis and ascites show an increased presence of bactDNA in the blood, which is associated with inflammation and poor prognosis.[10–13] Furthermore, BT is essential for the development of spontaneous bacterial peritonitis (SBP).[3,14,15]

The *NOD2* risk variants have been identified as genetic risk factors for the occurrence of SBP in patients with liver cirrhosis and ascites.[16,17] To date, associations between the presence of the *NOD2* variants and hepatic encephalopathy (HE) are not known. In the pathogenesis of HE, ammonia has a cardinal role.[18] Nevertheless, the neurotoxic effect of ammonia can be modulated by inflammation and its mediators.[19] Pro-inflammatory cytokines such as IL-6, IL-18 and TNF-alpha have been associated with the presence of HE and its severity.[20–24] Finally, the presence of infection and the systemic inflammatory response syndrome (SIRS) have been associated with the presence of cognitive dysfunction and severity of HE in patients on the ICU[25,26] has been associated with HE.

Therefore, the hypothesis of the present study was that patients with cirrhosis and a *NOD2* risk variant may have increased blood levels of bacterial products and/or immune mediators and as a consequence an increased prevalence of cognitive dysfunction. The aim of the study is to examine whether the presence of the *NOD2* risk variants (p.R702W, p.G908R, c.3020insC, p.N289S, and c.-958T>C) is associated with HE and covert hepatic encephalopathy (CHE) and the pathophysiological mechanisms involved including ammonia, inflammation, and BT.

Lay Summary

Nucleotide-binding oligomerization domain containing 2 (*NOD2*) risk variants are genetic mutations that lead to an impaired mucosal barrier in the gut and increased blood levels of bacterial products. Their presence has been associated to infections in cirrhosis in previous studies. Infections and bacterial products can lead to hepatic encephalopathy (HE). However, in this study, we saw no association between *NOD2* risk variants and HE. Furthermore, no association was seen between *NOD2* risk variants and markers of inflammation suggesting that the effect of *NOD2* risk variants on intestinal permeability is overridden by other factors in decompensated cirrhosis.

2 | PATIENTS AND METHODS

2.1 | Study design and study population

The study was designed as a multi-center prospective cross-sectional study (University of Jena, University of Halle, Saarland University), which was performed between 07.2017 and 11.2020. Consecutive patients who were identified in the in-hospital or outpatient setting were considered for inclusion. Inclusion criteria included (a) age 18 years or older, (b) liver cirrhosis with and without ascites, (c) genetic testing for *NOD2* risk variants (p.R702W, p.G908R, c.3020insC, N289S, and c.-958T>C), and (d) informed consent for the participation in the study. Patients were excluded if they had (a) other neurological diseases with impairment of cognition (e.g., dementia or Parkinson disease), (b) ophthalmological diseases such as colour blindness and/or severe visual impairment, (c) psychiatric medication that may influence cognition (e.g., benzodiazepines, antidepressants, neuroleptics), (d) presence of other non-hepatic encephalopathies, and (e) antibiotic therapy due to an infection within 2 weeks before inclusion. Patients who were administered antibiotics for prophylaxis (SBP or HE) were not excluded.

All patients underwent physical examination for signs of HE and were assessed for minimal HE by means of critical flicker frequency (CFF) and psychometric portal encephalopathy scores (PHES), and blood sampling. Patients from Homburg (Saarland) underwent evaluation of minimal HE exclusively by CFF and blood tests. If the patient was admitted due to HE, the tests were

performed once the attending physician considered that the episode had resolved.

2.2 | *NOD2* genotyping

After isolation of genomic DNA from EDTA-anticoagulated blood using a membrane-based extraction kit (Qiagen), the NOD2 variants rs2066844 (p.R702W), rs2066845 (p.G908R), rs2066847 (c.3020insC), rs72796367 (c.-958T>C) and rs5743271 (p.N289S) were genotyped using Taqman PCR-based allelic discrimination assays (LifeTechnologies). The assays utilized were: p.R702W: C__11717468_20; p.G908R: C__11717466_20; c.-958T>C: C__97921071_10; p.N289S: C__26935007_10. For c.3020insC, the following specifically designed primer and probe sequences were used: MGB_F CCAGGTTGTCCAATAACTGCATC; MGB_R CCTTACCAGA-CTTCCAGGATGGT; VIC TGCAGGCCCCTTG; FAM CTGCAGGCCCTTG. This determination was offered as part of the routine work-up after informed consent and was used in the context of prescreening for the randomized controlled trial "Impact of *NOD2* genotype-guided antibiotic prevention on survival in patients with liver Cirrhosis and Ascites" (*INCA*).[27] The genetic assays were performed at Saarland University. All technicians performing the genotyping were blinded to clinical data.

2.3 | Portosystemic encephalopathy (PSE) syndrome test

The PSE syndrome test consists of five psychometric tests designed to identify cognitive limitations and deficits common in CHE.[23] Number Connection Test A, Number Connection Test B, Dot Circle Test, Line Trace Test and Number Symbol Test were included. The result scale ranges from 1 to −3 for each individual test. The total value of the individual tests is called psychometric hepatic encephalopathy score (PHES). The PHES correlates with the metabolically caused cerebral changes in HE patients,[28] has a good prognostic predictive value with regard to the development of overt hepatic encephalopathy (OHE) and mortality in cirrhotic patients[29] and shows an improvement with adequate treatment of HE.[30] So far, the test has been validated for Germany, Italy, India, Spain, Korea and Great Britain, since standardized versions with age- and education-specific corrected values for the respective populations are available for above mentioned countries.[31–33] A total score of <−4 to <−6, depending on the country, indicates the presence of a CHE. The test was carried out by appropriately trained personnel.

2.4 | Critical flicker frequency

The determination of the CFF is a test in which the frequency of a flickering light is reduced progressively until perceived as a flickering light by the patient. This frequency is the CFF. The CFF is a measure of cortical function and is used in a wide variety of neurological disorders,[34,35] as well as in the diagnosis of low-grade HE and CHE.[29,36,37] It takes only up to 10 min to perform and interpret the test, which requires no special training. The investigation is limited because it is not applicable to patients with certain ophthalmic conditions such as colour blindness. The CFF was determined using the Hepatonorm Analyser (R&R Medi-Business). After an introductory phase in the study, 10 measurements were taken and the mean value of the results was calculated. For this study, a cut-off of 39 Hz was used in line with previous studies.[36] Lower values were associated with greater cognitive impairment. CFF correlates with psychometric tests such as the PSE.[29,36,37]

2.5 | Definition of hepatic encephalopathy

Given the lack of a standard diagnostic test for the diagnosis of CHE, different analyses using the different definitions of HE were included (only OHE, OHE + CHE as defined by PHES, OHE + CHE as defined by CFF, only CHE as defined by PHES, only CHE as defined by CFF). Diagnosis of OHE was based on clinical judgement as done by evaluating orientation in time, space and person as well as the presence of flapping.

2.6 | Evaluation of pathophysiological mechanisms

Routine laboratory tests including white blood cell counts, C- reactive protein and serum ammonium were performed. Further analysis including interleukin-6 (Human Interleukin 6 ELISA, MyBiosource; cat. number: MBS772136), soluble IgG endotoxin antibodies (EndoCab® IgG ELISA, Hycult Biotech; cat. number: HK504-IGG), lipoprotein binding protein (Human LBP ELISA, Hycult Biotech; cat. number: HK315-01), sCD14 (ELISA, Hycult Biotech; cat. number: HK320-02), and hTRL4 were determined. The first four parameters were determined at the University of Jena, and the latter biomarker was measured at the University of Vienna. Here, a commercially available reporter gene assay with TLR4 transfected HEK293 cells was used to determine the levels of TLR4 ligands (InvivoGen). In brief, cells were grown up to 80% confluence and challenged with 20 μL of serum for 12 h. Colour changes of medium, being indicative of ligand concentration, were determined at 655 nm.

2.7 | Statistical analysis and sample size calculation

Data are presented as medians and interquartile ranges (IQR). Categorical data are presented as proportions, while continuous variables are presented with medians and IQR. Mann–Whitney-Wilcoxon tests or ANOVA were used for comparison between groups. The association between continuous variables was assessed using the Spearman rank correlation. Logistic regression

analysis was performed to evaluate the association between the detection of a *NOD2* risk variant and the presence of OHE and/or CHE adjusted by model for end-stage liver disease (MELD) score categories.

The prevalence of HE (OHE + CHE) in patients with cirrhosis in the Halle University was 60%. There are no data on the prevalence of HE in patients with *NOD2* risk variants. The hypothesis was that the prevalence of HE in patients with *NOD2* mutations is higher than that in the wild-type population. Since poor liver function, measured by the MELD score, is a risk factor for the presence of HE,[38] the analysis will be adjusted by the MELD score (categories: MELD 6–10, MELD 10–20, MELD 20–30, MELD >30). To detect a 20% difference in prevalence, 86 patients (wild type 55% and *NOD2* variant 75%) and 91 (wild type 50% and *NOD2* variant 70%) per group would be required. Inclusion of 100 patients per group was planned.

The study was conducted according to the Declaration of Helsinki and Good Clinical Practice (European guidelines). Institutional review board approval was obtained prior to the initiation of the trial (Halle 2017-85; Homburg: 271/11). All participants provided written informed consent prior to inclusion. The study was specifically funded by the Schwiete Stiftung (NOD2-HE 01/2017), funding was obtained prior to the initiation of the study.

3 | RESULTS

3.1 | Baseline characteristics

A total of 172 patients were included in the study, of which 53 patients (31%) carried a *NOD2* risk variant. Since homozygosity/double heterozygosity was infrequent ($n = 6$), homo- and heterozygous carriers of *NOD2* risk variants were grouped together.[39–41] As expected, most patients were men ($n = 121$, 70%) with a median age of 60 years (IQR 54–66 years). The median MELD score was 12 (IQR 9–16), and the distribution among the predefined MELD categories was: MELD 6–10: 54 pts (32%), MELD 10–20: 89 (52%), MELD 20–30: $n = 25$ (15%), and MELD >30: $n = 3$ (2%). Most patients ($n = 148$, 86%) were in the decompensated stage of the disease. The majority of patients suffered from alcohol associated liver disease ($n = 112$, 65%). OE was present in 19 patients (11%) and previous history of HE was documented in 45 (26%). The prevalence of CHE varied according to the test used: CFF $n = 44/165$ (27%) and PHES $n = 63/136$ (46%). Table 1 summarizes further baseline characteristics of the study population.

3.2 | Association between *NOD2* risk variants and HE according to MELD score

No association was observed between the presence of HE (including all definitions, see Table 2, Table S1) and the presence or absence of *NOD2* risk variants. Remarkably, the proportion of HE/non-HE (independent of the definition) is very similar in patients with wild-type alleles or *NOD2* risk variants across the different HE definitions. Furthermore, no association was observed between MELD score and the presence of *NOD2* risk variants (see Table 3). As expected, increasing MELD score categories were associated with a higher proportion of HE (including CHE and OHE or just OHE) (Table S2). The proportion of patients undergoing treatment with prophylactic antibiotics and lactulose was similar in patients with wild-type alleles or *NOD2* risk variants. A subanalysis was performed to evaluate the association between presence of HE (as defined by OHE and CHE determined by PHES) and NOD2 risk variants in patients without signs of an acute event (CRP < 10 mg/dL), and again no association could be observed (wild-type alleles 38% with HE, *NOD2* risk variants 30% with HE, $\chi^2 = 0.450$).

A bivariate logistic regression was performed to evaluate the association between the presence of any *NOD2* risk variant (independent variable) and HE (dependent variable), adjusted by MELD score categories. Again, no association was observed between genetic risk variant and HE (including all definitions of HE, Table 4). Not unexpectedly, MELD score was associated with the presence of HE.

3.3 | Association of the presence of HE and possible pathophysiological mechanisms

Characteristics of patients with and without HE were compared (Table 5, Tables S3–S6). As expected, patients with HE had more advanced liver insufficiency, as indicated by lower serum albumin and higher bilirubin concentrations as well as higher INR and MELD scores (Tables S3, S4 and S6).

Of note, patients with HE presented with lower haemoglobin levels (Table 5, Tables S3–S5). Foreseeably, patients with HE also had lower serum sodium (Table 5, Tables S3–S6) and higher serum ammonium concentrations (Table 5, Tables S3–S5). Patients with HE showed signs of greater systemic inflammation, as reflected by higher CRP and sCD14 levels (Table 5, Tables S3–S6), although no differences were observed for leucocytes, IL-6, LBP, endotoxin, or hTRL4. The results regarding albumin, haemoglobin, sodium, ammonium, CRP and sCD14 were robust, as these parameters were associated with HE in all five (albumin, sodium, CRP, sCD14) or four out of five HE definitions (haemoglobin, ammonium) (Table 5, Tables S3–S6).

Multivariate analysis was performed with these variables to identify the independent variables associated with the presence of HE (OHE and CHE identified by PHES). In this analysis, serum ammonium levels (RR 1.025 [1.007–1.043]) and sCD14 (RR 1.001 [1.000–1.002]) were independently associated with HE (Figure 1). No multiplicative interaction between sCD14 and ammonium could be detected in the analysis.

3.4 | Association of the presence of *NOD2* risk variants and possible pathophysiological mechanisms

Patients with a *NOD2* risk variant and controls were compared regarding baseline characteristics (including the degree of liver

TABLE 1 Baseline characteristics of the study population ($N = 172$) and according to the presence of *NOD2* risk variants.

	Total ($N = 172$)	Wild type ($N = 119$)	*NOD2* risk variant ($N = 53$)	*p* value
Male sex, *n* (%)	121 (70%)	83 (70%)	38 (72%)	0.796
Age, median (IQR)	60 (54–66)	60 (53–66)	62 (54–66)	0.354
Years of school, median (IQR)	10 (9–10)	10 (8–10)	10 (10–10)	0.155
MELD score, median (IQR)	12 (9–16)	12 (9–16)	13 (10–16)	0.748
MELD categories				0.588
6–10	54 (32%)	39 (33%)	15 (28%)	
10–20	89 (52%)	59 (50%)	30 (57%)	
20–30	25 (15%)	17 (14%)	8 (15%)	
>30	3 (1.8%)	3 (3%)	0	
Aetiology of cirrhosis, *n* (%)				0.327
Alcohol	112 (65%)	79 (66%)	33 (63%)	
NASH	12 (7%)	7 (6%)	5 (9%)	
Viral	14 (8%)	9 (8%)	5 (9%)	
AIH	9 (5%)	9 (8%)	0	
Cholestatic	7 (4%)	3 (2%)	4 (8%)	
Hemochromatosis	4 (2%)	3 (2%)	1 (2%)	
Cryptogenic/other	14 (8%)	9 (8%)	5 (9%)	
Current OHE, *n* (%)	19 (11%)	13 (11%)	6 (11%)	0.939
CFF, median (IQR)	44 (39–51)	43 (39–51)	44 (39–52)	0.917
PHES, median (IQR)	−4 (−9 to 0)	−4 (−10 to 0)	−3 (−8 to 0)	0.280
Previous OHE, *n* (%)	45 (26%)	31 (26%)	14 (26%)	0.984
CHE as defined by CFF, *n* (%)	44/165 (27%)	32 (27%)	12 (26%)	0.917
CHE as defined by PHES, *n* (%)	63/136 (46%)	46 (49%)	17 (40%)	0.280
Varices, *n* (%)	120 (71%)	85 (71%)	35 (66%)	0.381
Previous variceal bleeding, *n* (%)	35 (20%)	**20 (17%)**	**15 (28%)**	**0.084**
TIPS, *n* (%)	24 (14%)	16 (13%)	8 (15%)	0.773
Ascites, *n* (%)				0.811
No ascites	47 (27%)	34 (29%)	13 (25%)	
Diuretic responsive	66 (38%)	44 (37%)	22 (42%)	
Refractory	59 (34%)	41 (34%)	18 (34%)	
Lactulose, *n* (%)	92 (54%)	66 (55%)	27 (51%)	0.704
Prophylactic antibiotics, *n* (%)	34 (20%)	22 (18%)	12 (23%)	0.301
Betablockers, *n* (%)	100 (58%)	69 (58%)	31 (58%)	0.950
Leucocytes (Gpt/L), median (IQR)	6.03 (4.40–7.99)	6.4 (4.40–8.40)	5.6 (4.4–7.0)	0.299
Haemoglobin (mmol/L), median (IQR)	7.3 (5.9–8.3)	7.3 (5.9–8.4)	7.1 (6–0-8.3)	0.902
Platelets (Gpt/L), median (IQR)	118 (82.25–175.75)	126 (85–185)	101 (75–155)	0.144
INR, median (IQR)	1.3 (1.14–1.50	1.30 (1.17–1.50)	1.30 (1.12–1.51)	0.427
PTT (s), median (IQR)	33 (29–37)	33 (30–37)	34 (29–39)	0.990
Bilirubin (μmol/L), median (IQR)	22 (14–44)	22.1 (13.5–44.4)	24.0 (15.7–46.7)	0.500
Albumin (g/dL), median (IQR)	33 (29–39)	33 (29–39)	33 (29–39)	0.992
AST (μkat/L), median (IQR)	0.58 (0.73–1.19)	0.72 (0.55–1.19)	0.73 (0.60–1.18)	0.565
ALT (μkat/L), median (IQR)	0.48 (0.34–0.70)	0.45 (0.34–0.67)	0.50 (0.33–0.72)	0.837
GGT (μkat/L), median (IQR)	2.1 (1.1–4.2)	2.1 (1.1–4.2)	2.1 (1.1–4.5)	0.962
AP (μkat/L), median (IQR)	1.5 (2.1–2.1)	2.0 (1.5–3.0)	2.3 (1.6–3.4)	0.265

(Continues)

TABLE 1 (Continued)

	Total ($N=172$)	Wild type ($N=119$)	*NOD2* risk variant ($N=53$)	*p* value
Serum sodium (mmol/L), median (IQR)	138 (135–141)	139 (135–141)	138 (135–142)	0.992
Potassium (mmol/L), median (IQR)	4.1 (3.9–4.5)	**4.1 (3.9–4.4)**	**4.3 (3.9–4.7)**	**0.047**
Creatinine (μmol/L), median (IQR)	84 (66–117)	80 (66–115)	88 (65–123)	0.434
NH_4 (μmol/L), median (IQR)	44 (33–60)	44 (34–61)	42 (30–59)	0.461
CRP (mg/L), median (IQR)	8.9 (3.0–18.3)	8.7 (2.7–18.1)	9.7 (3.7–18.5)	0.772
IL-6 (pg/mL), median (IQR)	18.2 (10.7–55.9)	18.2 (10.6–67.2)	18.3 (10.9–33.8)	0.249
LBP (ng/mL), median (IQR)	17 106 (12223–23 260)	17 053 (12528–21 398)	17 183 (11858–24 794)	0.683
IgG Ab endotoxin (GMU/mL), median (IQR)	141.1 (81.2–240.1)	130.3 (80.9–212.9)	147.4 (82.5–319.3)	0.560
sCD14 (ng/mL), median (IQR)	2647.59 (2307.54–3088.58)	2626.26 (2307.54–3073.26)	2723.83 (2229.43–3099.86)	0.576
hTRL4 (OD), median (IQR)	0.545 (0.341–0.895)	0.545 (0.370–0.918)	0.537 (0.325–0.830)	0.918

Abbreviations: AIH, autoimmune hepatitis; ALT, alanine transaminase; AP, alkaline phosphatase; AST, aspartate aminotransferase; CFF, critical flicker frequency; CHE, covert hepatic encephalopathy; CRP, C reactive protein; GGT, gamma-glutamyl transferase; INR, international normalized ratio; IQR, interquartile ranges; LBP, lipopolysaccharide binding protein; MELD, model for end-stage liver disease; NASH, non-alcoholic steatohepatitis; NOD2, nucleotide-binding oligomerization domain containing 2; OHE, overt hepatic encephalopathy; PHES, psychometric hepatic encephalopathy score; PTT, partial thromboplastin time; TIPS, transjugular intrahepatic portosystemic shun.

TABLE 2 Association between hepatic encephalopathy (OHE + CHE defined by PHES) and NOD2 risk variants (chi-squared test $p=0.334$).

NOD2 mutation	No HE	HE (OHE + CHE defined by PHES)	Total
Wildtype	67 (56%)	52 (44%)	119
NOD2 risk variant	34 (64%)	19 (35%)	53

Abbreviations: CHE, covert hepatic encephalopathy; HE, hepatic encephalopathy; NOD2, nucleotide-binding oligomerization domain containing 2; OHE, overt hepatic encephalopathy; PHES, psychometric hepatic encephalopathy score.

TABLE 3 Association between MELD score categories and NOD2 status (chi-squared test $p=0.588$).

MELD score categories	Wildtype	*NOD2* risk variant	Total
6–10	39 (72%)	15 (28%)	54
10–20	59 (66%)	30 (34%)	89
20–30	17 (68%)	8 (32%)	25
≥30	3 (100%)	0	3

Abbreviations: MELD, model for end-stage liver disease; NOD2, nucleotide-binding oligomerization domain containing 2.

failure as reflected by the presence of complications of cirrhosis) and laboratory values. No significant differences could be observed. Interestingly, no differences were observed in inflammatory markers such as leucocytes, CRP and IL-6 either. Finally, no differences were detected measuring markers of BT (such as LBP or endotoxin) or immune response (soluble CD14 or hTRL4) between patients with and without *NOD2* risk variants (Table 1).

TABLE 4 Bivariate analysis with MELD score and presence of *NOD2* risk variants. All analyses show that NOD2 has no association on the presence of hepatic encephalopathy, even when considering the MELD score category.

Variables included in the bivariate analysis	Dependent variable	Outcome of the analysis, RR (95% CI)
MELD NOD2 mutation	Total hepatic encephalopathy (CHE defined by PHES)	MELD 1.046 (0.996–1.099)
MELD NOD2 mutation	Total hepatic encephalopathy (CHE defined by CFF)	MELD 1.086 (1.030–1.145)
MELD NOD2 mutation	Overt hepatic encephalopathy	MELD 1.089 (1.021–1.162)
MELD NOD2 mutation	Covert hepatic encephalopathy defined by PHES	No association
MELD NOD2 mutation	Covert hepatic encephalopathy defined by CFF	MELD 1.077 (1.021–1.136)

Abbreviation: CFF, critical flicker frequency; CHE, covert hepatic encephalopathy; MELD, model for end-stage liver disease; NOD2, nucleotide-binding oligomerization domain containing 2; PHES, psychometric hepatic encephalopathy score.

4 | DISCUSSION

The main aim of this study was to evaluate the association of the presence of *NOD2* risk variants and the presence of HE, both CHE and OHE, respectively. The hypothesis was that patients with *NOD2* risk variants would have increased BT, and therefore increased

TABLE 5 Comparison of patients with and without HE (OHE + CHE as defined by PHES).

	No hepatic encephalopathy	Hepatic encephalopathy (OHE + CHE-PHES)	*p* value
Male sex, *n* (%)	**68 (67%)**	**53 (75%)**	**0.024**
Age, median (IQR)	**58 (53–65)**	**64 (55–67)**	**0.054**
Years of school, median (IQR)	**10 (9–10)**	**10 (8–10)**	**0.075**
MELD score, median (IQR)	11 (9–16)	14 (9–19)	0.166
Aetiology of cirrhosis, *n* (%)			0.377
Alcohol	64 (63%)	48 (68%)	
NASH	7 (7%)	5 (7%)	
Viral	11 (11%)	3 (4%)	
AIH	4 (4%)	5 (7%)	
Cholestatic	4 (4%)	3 (4%)	
Hemochromatosis	4 (4%)	0	
Cryptogenic/other	7 (7%)	7 (10%)	
CFF, median (IQR)	**44.6 (39.7–51.0)**	**41.5 (38.0–51.1)**	**0.088**
Previous OHE, *n* (%)	**20 (20%)**	**25 (36%)**	**0.020**
CHE as defined by CFF, *n* (%)	**19 (20%)**	**25 (36%)**	**0.024**
Varices, *n* (%)	76 (75%)	44 (62%)	0.107
Previous variceal bleeding, *n* (%)	**27 (27%)**	**8 (11%)**	**0.013**
TIPS, *n* (%)	15 (15%)	9 (13%)	0.685
Ascites, *n* (%)			0.585
No ascites	26 (26%)	21 (30%)	
Diuretic responsive	42 (42%)	24 (34%)	
Refractory	33 (32%)	26 (37%)	
Lactulose, *n* (%)	53 (53%)	40 (56%)	0.600
Prophylactic antibiotics, *n* (%)	16 (16%)	19 (71%)	0.275
Betablockers, *n* (%)	60 (60%)	40 (56%)	0.688
Leucocytes (Gpt/L), median (IQR)	5.85 (4.31–7.96)	6.45 (4.50–8.01)	0.317
Haemoglobin (mmol/L), median (IQR)	**7.5 (6.1–8.6)**	**6.6 (5.8–7.9)**	**0.032**
Platelets (Gpt/L), median (IQR)	116 (82.5–162.5)	120 (75–212)	0.344
INR, median (IQR)	1.27 (1.13–1.51)	1.30 (1.20–1.50)	0.353
PTT (s), median (IQR)	33.1 (28.6–36.7)	33.7 (30.2–38.0)	0.395
Bilirubin (μmol/L), median (IQR)	22.6 (12.5–43.9)	22.1 (15.0–54.7)	0.595
Albumin (g/dL), median (IQR)	**35 (29–40)**	**31.2 (26–35.3)**	**0.008**
AST (μkat/L), median (IQR)	**0.67 (0.50–1.10)**	**0.79 (0.61–1.35)**	**0.043**
ALT (μkat/L), median (IQR)	0.45 (0.34–0.67)	0.50 (0.33–0.75)	0.796
GGT (μkat/L), median (IQR)	1.96 (0.99–4.13)	2.18 (1.12–5.25)	0.385
AP (μkat/L), median (IQR)	**1.96 (1.50–2.82)**	**2.45 (1.59–3.21)**	**0.061**
Serum sodium (mmol/L), median (IQR)	**139 (136–142)**	**137 (134–140)**	**0.014**
Potassium (mmol/L), median (IQR)	4.1 (3.9–4.4)	4.2 (3.9–4.5)	0.786
Creatinine (μmol/L), median (IQR)	79 (64–106)	87 (67–138)	0.115
NH_4 (μmol/L), median (IQR)	**39.5 (29.5–56.9)**	**49.0 (38.0–68.0)**	**0.005**
CRP (mg/L), median (IQR)	**6.55 (2.03–16.4)**	**10.9 (5.96–27.0)**	**0.002**
IL-6 (pg/mL), median (IQR)	17.9 (10.8–37.6)	21.8 (10.4–68.7)	0.496
LBP (ng/mL), median (IQR)	16 345 (12623–21 804)	17 644 (11610–24 159)	0.492
IgG Ab endotoxin (GMU/mL), median (IQR)	149 (80–251)	128 (83–238)	0.542
sCD14 (ng/mL), median (IQR)	**2478 (2204–2872)**	**2876 (2473–3347)**	**<0.001**
hTRL4 (OD), median (IQR)	0.496 (0.336–0.809)	0.576 (0.348–0.972)	0.622

Abbreviations: AIH autoimmune hepatitis; ALT, alanine transaminase; AP alkaline phosphatase; AST, aspartate aminotransferase; CFF, critical flicker frequency; CHE, covert hepatic encephalopathy; CRP, C reactive protein; HE, hepatic encephalopathy; GGT gamma-glutamyl transferase; INR, international normalized ratio; IQR, interquartile ranges; LBP lipopolysaccharide binding protein; MELD, model for end-stage liver disease; NASH, nonalcoholic steatohepatiti; OHE, overt hepatic encephalopathy; PHES, psychometric hepatic encephalopathy score; PTT partial thromboplastin time; TIPS, transjugular intrahepatic portosystemic shunt.

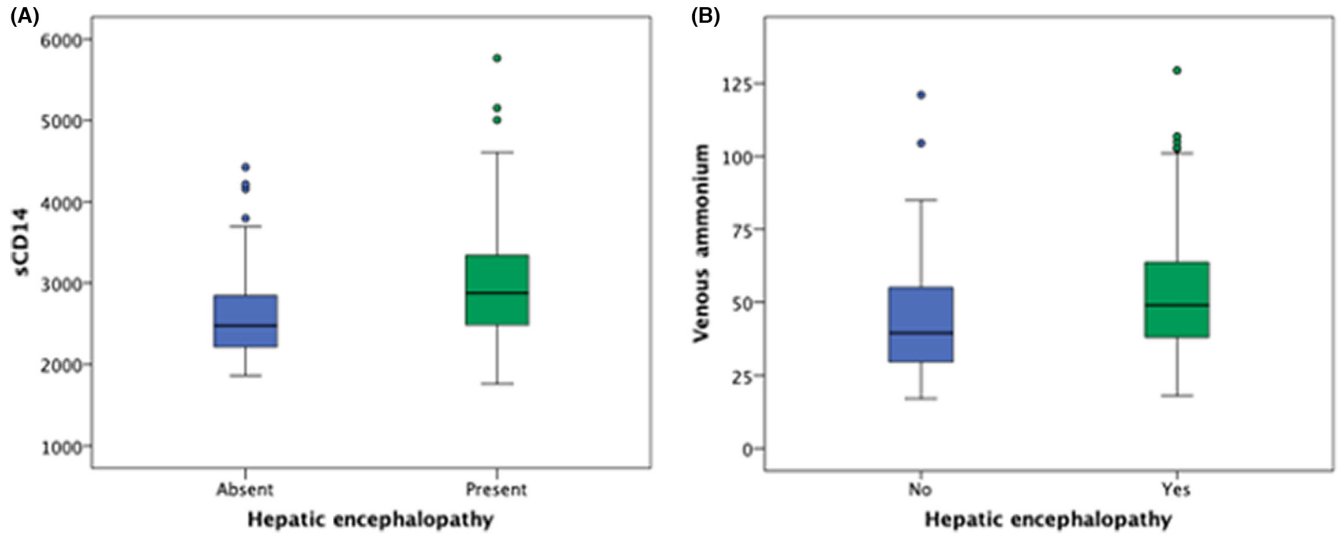

FIGURE 1 Box-plots of sCD14 (A) and venous ammonium (B) according to the presence or absence of hepatic encephalopathy (including overt hepatic encephalopathy and covert hepatic encephalopathy as defined by psychometric hepatic encephalopathy score).

systemic inflammation due to this and hence a greater prevalence of HE.

In the present study, we did not observe a different prevalence of HE according to the presence or absence of *NOD2* risk variants. This finding was consistent when evaluating the definition of HE using the whole spectrum (OHE and CHE as defined by PHES or CFF) or OHE and CHE (defined by PHES of CFF) individually. There were also no differences regarding the previous history of HE. In agreement with this finding, no differences in BT or systemic inflammation were observed according to the presence or absence of *NOD2* risk variants as evaluated by different biomarkers.

These results are in line with previous results from the study group in which the influence of the presence of *NOD2* risk variants was mainly relevant in patients with compensated cirrhosis, while in patients with decompensated cirrhosis, this mechanism was overridden by other possible factors that have an influence on intestinal permeability such as portal hypertension.[41,42] In this study, most patients had decompensated disease (86%) and therefore clinically significant portal hypertension. In this group of patients, the presence of *NOD2* risk variants did not lead to further systemic inflammation or BT. The results are consistent, as the same result was obtained despite using different definitions of HE and different markers of inflammation and BT.

We further analysed the association of BT and systemic inflammation and the presence of HE. Not unexpectedly, patients with HE had an increase in serum ammonium and decrease in serum sodium and worse liver function (as determined by albumin, bilirubin and/or INR). In accordance with the present hypothesis of the development of complications of cirrhosis, patients with HE had an increase in CRP and soluble CD14, a marker of macrophage–monocyte activation. Contrary to previous published data[43] in which no association was observed between neither the presence of CHE as measured by PHES and ammonium (in this case as measured in arterial blood) nor markers of BT or systemic inflammation, we show an association between CHE and ammonium, CRP and CD14.

The interaction between inflammatory mediators and ammonium in HE is complex. On one hand, clinical studies have shown that induced hyperammonemia leads to worsening of neuropsychological score, when patients had SIRS, but on the other hand this association could not be observed after resolution of SIRS.[19] In the present study, we observed no multiplicative interaction between inflammatory mediators and ammonia in regression analysis.

The main limitation of the study is the fact that the original sample size was not achieved. The initial study design was planned to have 100 patients with and without NOD2 risk variants in order to be able to detect a 20% difference in the prevalence of HE. This number of patients was not achieved due to several factors: (1) After the termination of the INCA trial, the complimentary determination of the presence NOD2 risk variants was no longer available, (2) the prevalence of *NOD2* risk variants is 11% in the general population[39,40] and 22% in patients with cirrhosis.[41] In the present study, although a greater number of patients with *NOD2* risk variants were included as would be expected by chance in patients with cirrhosis, the planned study sample in this subgroup was not achieved. However, given the similarity of the prevalence of HE as well as the lack of a trend in the explanatory mechanisms after achieving more than 50% in this group, we consider that it is unlikely that further inclusion of patients would change the results substantially. A further limitation is the cross-sectional design of the study and that most patients were recruited in the hospitalized setting. Although the presence of acute infections was an exclusion criterium, other acute precipitating events could override a potential genotype–phenotype relationship of a life-long risk factor. However, there were no hints that patients with *NOD2* risk variants were younger than patients without. Another limitation was the inclusion of mainly decompensated patients. The actual definition of decompensation is the current or past presence of variceal bleeding, OHE and/or ascites. The

presence of CHE does not identify decompensation. The prevalence of CHE among compensated patients is around 12%,[44] however the impact of this entity including effects on progression of cirrhosis and mortality is not well characterized.[45] Whether the presence of a *NOD2* risk variant has a pathogenetic role on the development of CHE in patients with compensated cirrhosis cannot be answered with the present study.

In conclusion, the presence of *NOD2* risk variants is not associated with an increase in the prevalence of HE. The presence of *NOD2* risk variants does not lead to an increase in markers of BT and systemic inflammation in a population of predominantly decompensated cirrhosis. Finally, the presence of HE is associated with an increase in serum ammonium, acute phase response, and myeloid cell activation.

AUTHOR CONTRIBUTIONS

Cristina Ripoll developed the idea, achieved funding, designed the study, included patients, did the statistical analysis, and wrote the paper. Robin Greinert developed the idea, included patients, wrote the paper, and provided valuable intellectual input. Philipp Reuken and Matthias Christian Reichert included patients, and provided valuable intellectual input. Yvonne Hupfer, Raphaela Staltner, and Susanne N. Weber performed the blood analysis. Frank Lammert and Tony Bruns contributed to the development of the idea and study design. Alexander Zipprich developed the idea, helped to achieve funding, designed the study, included patients, and provide valuable intellectual input. All authors have approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data is available upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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