Functional polymorphisms of innate immunity receptors are not risk factors for the non-SBP type bacterial infections in cirrhosis

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23rd of October, 2017

Gabriele Missale
Associate Editor

Mario U Mondelli
Editor-in-Chief
Liver International

Dear Professor **Gabriele Missale,**
Dear Professor **Mario U Mondelli,**

Please find attached the revised version of our manuscript entitled: **Functional polymorphisms of innate immunity receptors are not risk factors for the non-SBP type bacterial infections in cirrhosis"** (Manuscript ID: LIVint-17-01007.R1).

We are grateful to the reviewers for their in-depth and important comments as well as for the critiques that helped improving the manuscript. We revised the manuscript according to the concerns raised and inserted relevant additional information in the main text of the manuscript. Some information was also added to Supplementary Material. Following the valuable proposed changes by the Reviewers, we included more detailed descriptions and completely new data as well. Despite concision of the *Section of Discussion*, with the insertion of these significant pieces of information, and the 5 requested additional literature references the length of the manuscript exceeds the limits of the Journal to a minor degree (The original version of the manuscript was already close to limits of the Journal, 4936 words). We hope that these new data significantly enhance the overall quality of our research. We feel that the size of the article is still within the range that can keep the attention of the readers and sincerely hope that it is still acceptable for the Journal, even though it is slightly over of the official limits (5419 words instead of 5000, which includes 5 additional requested references).

Professional English editing requested by Associate Editor was also performed.

For Peer Review

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Reviewer 1
Major points

Comment 1: *"Please indicate the power of the study according to previous frequencies of bacterial infections in cirrhosis and the expected MAF in cirrhotic patients. Given the multiplicity of genetic associations tested here, the relatively low number of infections, I would expect the study to be somewhat underpowered."*

Mean allele frequencies (MAF) in our study are similar to those reported in Caucasian cohorts [*Lek M. et al, Nautre 2016*]. In our study, the probability of bacterial infections was 49.6 ± 4.1 % (Kaplan-Meier estimate ± SE) during the follow up period (median, IQR: 32 [12-60] months). This rate of infections is similar to those in previous reports [*Nahon P. Gut, 2017;66(2):330-341. doi: 10.1136/gutjnl-2015-310275., Borzio M. et al. Digest Liver Dis 2001;33:41-8*].
We did not perform a sample size calculation or power analysis in the design phase of the study that is a limitation to acknowledge. According the request of the *Reviewer 1* we performed a post-hoc power analysis in Stata (v13.0). The following factors were considered in the analysis:

- sample size: **243**
- allele frequencies (*NOD2* risk variant present: **15%**, *TLR2* (T-16934A) TT present: **25%**, *TLR4* (D299G) AG risk variant present: **7%**)
- Cumulative probability of bacterial infections in control group: **40%**
- Clinically relevant hazard ratio: **2**
- type I error: **0.05, two-sided**

We found that the power associated with *NOD2*, *TLR2* and *TLR4* for detecting a difference in the development of bacterial infections were 89.2%, 95.6% and 67%, respectively.

Command syntax for power analysis was the following:

```
stpower logrank 0.6, n(243) hratio(2) p1(0.85 0.75 0.93) table columns(p1 power)
```

Regarding *TLR4* polymorphism we mentioned this limitation in the *Discussion* section *Page 20*.
"**The limitation of the present study is that the association of bacterial infections and TLR4 polymorphisms warrants further evaluation in a larger cohort since our study was underpowered to detect such an association at this sample size (Supplementary Table 1).**"

Comment 2: *"The majority of infections are UTIs – UTIs (as well as erysipelas) are unlikely to be influenced by intestinal BT via NOD2 and are often asymptomatic in cirrhotic patients. I would suggest performing sensitivity analyses for spontaneous infections (SBP, spont. bacteremia, empyema), infections excluding UTI, skin infections, gastroenteritis and skin infections, and severe infections requiring hospitalisation. How many patients were treated with antibiotics?"*

We agree with both concerns of the *Reviewer 1*. that (1) UTI and skin infections are usually less severe than spontaneous infections, like SBP in cirrhosis, and (2) intestinal BT is also not

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a major contributor in the development of these infections. The rational why we considered evaluating non-SBP type bacterial infection of various locations altogether instead excluding some of their types were the followings:

In cirrhosis, UTIs are important and often solely precipitating factors of hepatic encephalopathy *[Strauss E, et al. Hepatogastroenterology. 1998 May-Jun;45(21):900-4.]* and can be the source of bacteraemia as well. Furthermore, the presence of UTI indicated an increased risk of 90-day mortality in patients with advanced cirrhosis *[Reuken PA, et al. Liver Int. 2013 Feb;33(2):220-30. doi: 10.1111/liv.12029.]*

In case of skin and soft tissue infections GPC, are the most prevalent pathogens, but *Enterobacteriaceae* and anaerob bacteria can also be the pathogens of these infections *[Christou L, et al. Am J Gastroenterol. 2007 Jul;102(7):1510-7.]* GNB (*Escherichia [E.] coli, Klebsiella [K.] pneumoniae*) should also be considered as potential etiologic agents. In these latter cases, supposedly, the source of the bacteria is the gut itself. As a result of BT, enteric bacteria reach the systemic circulation, cause bacteraemia and seed the tissues of the extremities *[Chang CM, et al. Infection. 2008 Aug;36(4):328-34. doi: 10.1007/s15010-008-7272-3.]*. The course of GNB cellulitis is usually rapid and can be fatal. Progression to septic shock is common *[Horowitz Y, et al. Gram-negative cellulitis complicating cirrhosis. Mayo Clin Proc. 2004 Feb;79(2):247-50.]*.

Finally, SBP can also occur in asymptomatic cirrhotic outpatients. *[Cadranel JF, et al. World J Hepatol. 2013 Mar 27;5(3):104-8. doi: 10.4254/wjh.v5.i3.104. and Mohan P, et al. Indian J Gastroenterol. 2011 Sep;30(5):221-4. doi: 10.1007/s12664-011-0131-7.]*

Subgroup analysis of spontaneous infections - that were SBPs - however, were reported in detail in *Page 13* (*Section of Results – Risk factor of SBP*) and *Figure 4*, acknowledging that intestinal BT is important process in the development of spontaneous infections in cirrhotic patients.

The pathogenic point of view why we considered it important to evaluate potential association of PRR gene variants with bacterial infections beyond SBP were the followings: (1) there is a universal function of PRR in innate host defense (detailed in *Section of Discussion*) (2) there are reports of association of functional polymorphisms of PRRs with systemic infections in various patients groups, which are devoid of pathologic intestinal BT.

We also accept the important remark of the *Reviewer 1* that it is worthy of evaluating the association of PRR variants with bacterial infections according to severity of infection. In *Section of Patients and Methods – Study design (Page 7)* we stated *"Outpatients at inclusion (n=243) were enrolled into an observational follow-up study where the attending gastroenterologist registered date and type of bacterial infection (BI) warranting hospital admission (diagnostic criteria are summarized in* ***Supplementary Material****) and development of disease specific complications (ascites, hepatic encephalopathy or variceal bleeding) during regular and extraordinary outpatient follow-up visits and inpatient stays prospectively."* This approach was really similar that was applied by *Senkerikova R, et al. (J Hepatol. 2014 Apr;60(4):773-81. doi: 10.1016/j.jhep.2013.12.011).* Based on these criteria, evaluated infectious episodes in the present cohort can be considered clinically significant bacterial infections. Concerning that local and systemic host defence mechanisms are compromised and local infections can easily be disseminated, ***all patients with a proved bacterial infection were treated with empiric antibiotic regimen*** and adjusted according to microbiologic results if it was necessary. Nevertheless, we are aware of the limitations of this

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definition as well. In the clinical practice when we face the acute deterioration of a patient with cirrhosis (episode of acute decompensation) with simultaneously diagnosed bacterial infection, it is difficult somehow to decide whether bacterial infection is a coincident problem/ complication or has a real causative effect.

In the revised version of the manuscript to be able to evaluate severity of bacterial infections, we determined infection-related mortality and its association with PRR gene variants. None of the examined PRR gene variants were associated with the risk of death during a subsequent bacterial infection.

We added these results into the *Section of Functional polymorphisms of PRR genes* and a new table into the *Supplementary Material*. None of the pattern recognition receptor gene variants were associated with the risk of death.

Supplementary Table 2. Infection-related mortality in cirrhosis according to *NOD2* risk alleles (L1007fsinsC -/C, R702W C>T or G908R G>C) (A) and *TLR2* (T-16934A) (B) or *TLR4* (D299G) (C) polymorphisms after the first bacterial infectious episode (n=85).

		Binary logistic regression analysis					
		30-day			90-day		
		OR	95% CI	P-value	OR	95% CI	P-value
NOD2 polymorphism (present)		0.14	(0.02-1.1)	0.061	0.24	(0.05-1.14)	0.073
TLR2	TT	Ref.		0.804	Ref.		0.352
	TA	0.67	(0.2-2.22)	0.509	0.43	(0.13-1.37)	0.153
	AA	0.85	(0.27-2.63)	0.773	0.64	(0.22-1.88)	0.414
TLR4 (AG)		4.07	(0.83-19.84)	0.082	3.13	(0.65-15.11)	0.155

OR: Odds ratio

Comment 3: *"How were genotyping experiments validated? I would suggest sequencing of PCR products for a subset of samples to prove that FRET genotyping is valid. Especially for rs4986790 as it is done by in-house primers. Alternatively you could show representative melting curves as supplemental material."*

For rs4986790 experiment synthesis primers were designed in-house, while FRET oligonucleotides were similar to *Hamann et al. (J Immunol Methods. 2004 Feb 15;285(2):281-91. PubMed PMID: 14980441.)* Representative melting curve genotyping results performed on a LightCycler 480 system and now it is shown in the Supplementary Figure 1. We modified the *Section of the Results – Gene analysis of NOD2, TLR2 and TLR4 (Page 8 and Page 9)* and *Supplementary Material* accordingly.

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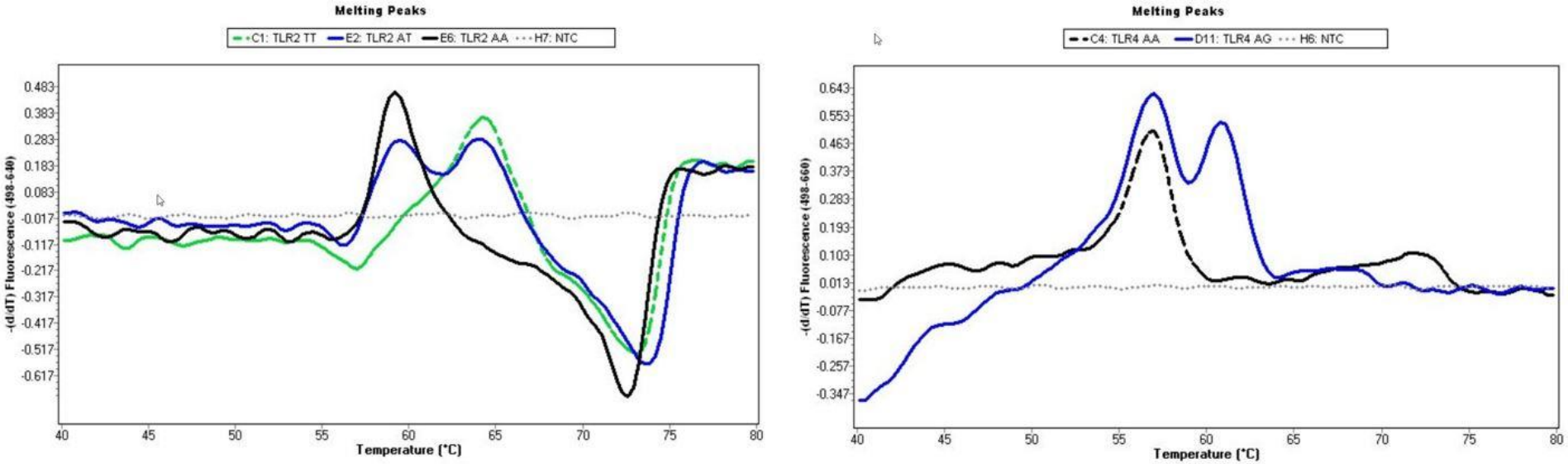
Figure 1. Representative melting curve genotyping results performed on a LightCycler 480 system.
NTC= "no template control"



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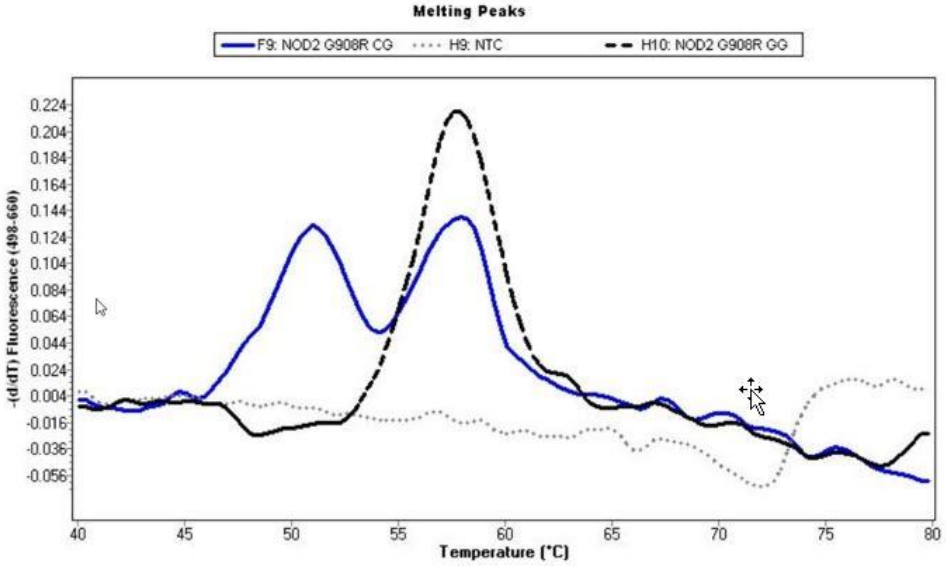
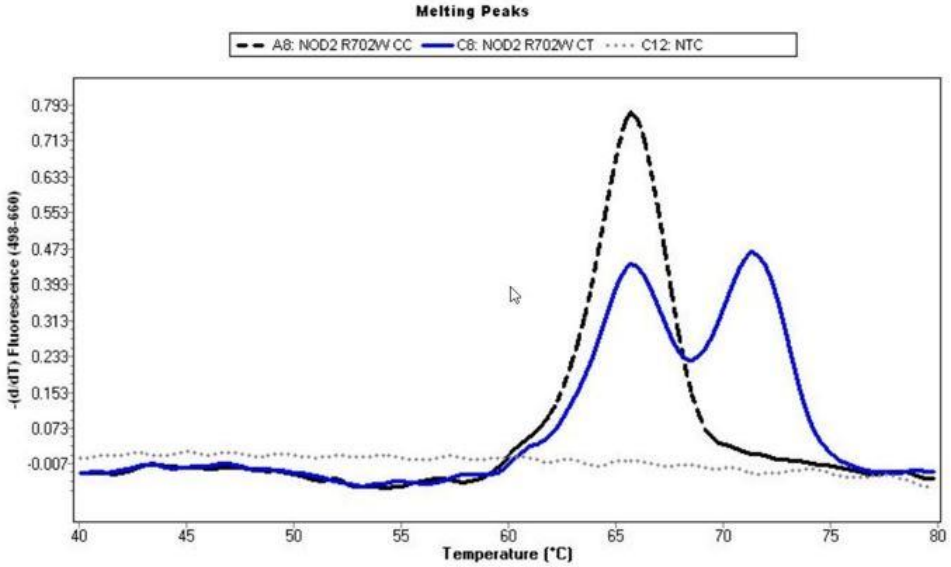
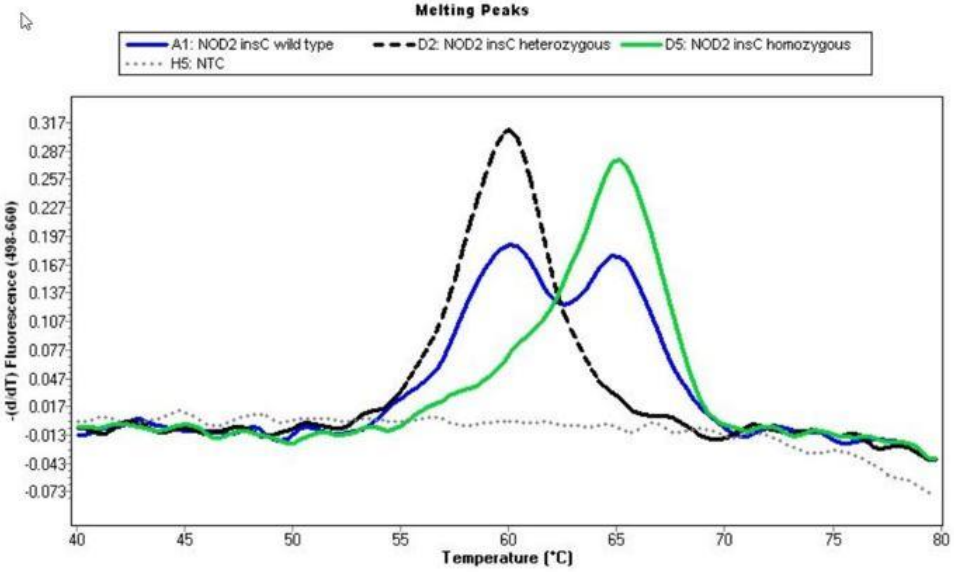

Melting Peaks
F9: NOD2 G908R CG
H9: NTC
H10: NOD2 G908R GG
-(d/dT) Fluorescence (498-660)
Temperature (°C)


Melting Peaks
A8: NOD2 R702W CC
C8: NOD2 R702W CT
C12: NTC
-(d/dT) Fluorescence (498-660)
Temperature (°C)


Melting Peaks
A1: NOD2 insC wild type
D2: NOD2 insC heterozygous
D5: NOD2 insC homozygous
H5: NTC
-(d/dT) Fluorescence (498-660)
Temperature (°C)

Comment 4: *"The discussion needs to be more concise and needs to recognize current investigations showing no association of NOD2 with SBP in large cohorts (Lutz et al. Dig Liver Dis. 2016. Bruns et al. Sci Rep 2017), association of NOD2 with BT (Harputluoglu Dig Dis Sci 2016, Bruns et al Dig Dis Sci 2016), the association of TLR4 with infections in cirrhosis (published in AP&T several years ago)."*

We took shortcut some degree the original version of the *Discusssion section* and added the suggested literature findings to the pertinent part.

Regarding SBP (Page 16 and 17): "Similar to most of the previous studies [8–10], the presence of *NOD2* allele variants was a risk factor for SBP in our cohort as well. **A recent large association study in patients with decompensated cirrhosis so far did not demonstrate a role of *NOD2* variant in mediating susceptibility for SBP.** ***(****Mai M, et al. Sci Rep. 2017 Jul 7;7(1):4914. doi: 10.1038/s41598-017-04895-z.)*

"The association of SBP with various *TLR2* genotypes is somewhat controversial in the published literature. In the studies of *Nischalke et al.* [10] **and *Lutz et al.*** *(Dig Liver Dis. 2016 Jan;48(1):62-8. doi: 10.1016/j.dld.2015.09.011.) TLR2* -16934 TT genotype but not *TLR2* R753Q and P631H mutations **[10]** were associated with SBP."

Regarding BT (Page 18 and 19): "**In patients with decompensated cirrhosis there was an increased translocation of bacterial DNA fragments into ascitic fluid in the presence of the *NOD2* risk variant p.G908R** *(Harputluoglu MM. et al. Dig Dis Sci. 2016 Jun;61(6):1545-52. doi: 10.1007/s10620-015-4024-y.)***. Moreover increased transition of pathologic BT to culture-positive SBP were reported in the case of the same *NOD2* variant**." *(Bruns T, et al. Dig Dis Sci. 2016 Jul;61(7):2142-4 doi: 10.1007/s10620-016-4151-0. Epub 2016 Apr 6. PubMed PMID: 27052012 and Bruns T, et al. Liver Int. 2016 Aug;36(8):1133-42. doi: 10.1111/liv.13095.)*

The *association of TLR4 with infections in cirrhosis* (published in AP&T) has been cited in the original version of the manuscript (Page 17) as follows: "In patients with advanced cirrhosis the *TLR4* (D299G, rs4986790) variant was recognized to increase overall BI rates in a single retrospective study (n=111). [22]"

Comment 5: ***"****The multivariate analysis of risk factors for infection is missing. I would suggest a multivariate model including MELD and the presence of ascites (well known factors) and a step wise forward inclusion to identify further factors."*

The rational why we did not show the results of multivariate analysis in the original version of the manuscript were the followings:

(1) None of the examined PRR genotypes (individually or in any combination) were associated with the risk of non-SBP type bacterial infections. Thus, there was no need to adjust the results with relevant clinical factors in the multivariate analysis and evaluate whether they are independent risk factor of bacterial infections.

(2) Previously we confirmed and reported in our patient cohort (*Foldi I, Liver Int. 2017 Jul;37(7):1023-1031. doi: 10.1111/liv.13368.*) that advanced disease stage (depicted either by Child-Pugh stage, presence of ascites or decompensated clinical stage) was

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associated with risk of bacterial infection development using multivariate Cox-regression analysis and the backward elimination procedure.

(3) Instead, we would like to emphasize, that the history of a prior bacterial infection and an advanced disease stage had similar impacts on the infectious risk. Prior history of a bacterial infectious episode significantly increased the probability of the development of a subsequent bacterial infection, regardless of disease severity. To summarize these results, KM curves were constructed (Figure 3).

We accept the suggestion of *Reviewer 1* that it would be important to present which clinical factors are independent risk factors of bacterial infections. According to this request of the reviewer, we added this information to the *Section – Results (Page 13)*:

"Multivariate analysis
Multivariate Cox-regression analysis and the forward inclusion procedure, taking all significant clinical co-variates of univariate analysis into account (see in Table 3), indicated that presence of ascites (HR [95%CI]: 1.71 [1.08-2.7], higher MELD score (1.08 [1.02-1.15]) and prior BI episode (2.02 [1.3-3.14]) were independently associated with the risk of a non-SBP type BI development during follow-up."

Variables in the Equation

		β	SE	Wald	df	p-value	Hazard Ratio	95% CI	
								Lower	Upper
Step 1	MELD score	0.115	0.028	17.379	1	<0.001	1.122	1.063	1.185
Step 2	MELD score	0.100	0.028	12.771	1	<0.001	1.105	1.046	1.168
	Prior BI	0.706	0.225	9.895	1	0.002	2.027	1.305	3.148
Step 3	MELD score	0.081	0.030	7.143	1	0.008	1.084	1.022	1.150
	Ascites	0.536	0.233	5.300	1	0.021	1.709	1.083	2.698
	Prior BI	0.704	0.225	9.840	1	0.002	2.023	1.303	3.141

Minor points

Comment 1: "Abstract: Please check spaces before parenthesis in the abstract section."

We inserted the missing spaces if needed.

Comment 2: *"Abstract: Please indicate whether the SBP frequencies are Kaplan-Meier estimates. If yes, please give standard errors."*

We agree, that reporting standard errors (SE) along KM estimates is more informative. We amended our reporting of KM estimates in the abstract, Table 3 and in the manuscript body as well and included SEs.

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Comment 3: *"Abstract: Please indicate the number of patients with ascites."*

Eighty-eight patients had ascites at inclusion; we added it to the abstract.

Comment 4: *"Methods: Please indicate why genotyping was not performed in 55 patients - what does unavailable mean? May this introduce a bias?"*

We agree with the *Reviewer 1* that this might introduce some bias. However, we had no possibility to organize our study otherwise.

As we stated in the *Section of Patients and Methods – Study design*, present study population was recruited consecutively between May 1, 2006 and December 31, 2010. At the time of inclusion we collected sera and whole-blood samples. Genomic DNA was extracted from whole-blood samples immediately and frozen -70 until testing.

Hungarian ethic legislations are rigorous, particularly in case of genetic studies and they went through substantial changes after 2010. First, they did not make possible to get a general permission for genetic analysis (testing certain gene variants in single study as a start and use DNA remnants for further different studies thereafter). Preservation of patients' genomic DNA is possible after a single study but resubmission and authorization is obligatory for every further study. As to see patients or his or her legal surrogates and inform them about nature of the new genetic study and to sign a new informed consent form. Additionally in 2011, National Scientific and Research Ethics Committee introduced a new legislation as well. In case of genetic studies permission of Regional and Institution Research Ethics Committee were not sufficient anymore even if it was a monocentric study. Since then, dual authorisation is required both from regional and national committees. In 2005, we got permission from regional committee for testing of *MPO*-463G/A polymorphism. Unfortunately, these results were never published due to lack of any association with either bacterial infections or progressive disease course. The idea of testing functional variants of PRR only came up after first publications in this field (*Appenrodt B, et al. Hepatology. 2010 Apr;51(4):1327-33. doi: 10.1002/hep.23440. and Nischalke HD, et al. J Hepatol. 2011 Nov;55(5):1010-6. doi: 10.1016/j.jhep.2011.02.022.*). Thereafter we had to resubmit the protocol of present genetic study in 2011 and also see each patient or legal surrogate after had getting ethical permissions (at the end of 2011 by regional and at the beginning of 2012 by national committees). Despite our efforts, it was not possible in case of above-mentioned 55 patients.

Clinical characteristics of these patients however, did not differ from the tested cohort. Furthermore, the follow-up time was also similar between the tested and non-tested patient groups. We hope that these data are convincing for the *Reviewer 1*, and that the missing 13.6% of the total cohort might not introduce substantial bias.

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		Outpatients			Acute Decompensation		
		not missing (n=243)	missing (n=34)	p-value	not missing (n=106)	missing (n=21)	p-value
Age		56 (50-63)	54 (46-66)	0.661	58 (51-64)	54 (48-66)	0.520
Child-Pugh score		6 (5-8)	7 (5-8)	0.403	9 (7-11)	10 (8-11)	0.173
MELD score		11 (8-14)	12 (9-15)	0.290	17 (13-22)	20 (15-22)	0.231
Creatinine µmol/L		67 (54-84)	63 (54-71)	0.282	77 (59-130)	99 (69-187)	0.217
Bilirubin µmol/L		26 (16-41)	31 (16-53)	0.298	55 (27-109)	63 (34-99)	0.534
INR		1.17 (1.09-1.32)	1.25 (1.15-1.34)	0.082	1.42 (1.2-1.74)	1.54 (1.34-1.73)	0.231
Albumin g/L		38 (33-42)	38 (32-41)	0.995	28 (24-32)	24 (22-33)	0.174
Follow-up time		988 (366-1825)	703 (85-1774)	0.107	147 (17-732)	63 (9-297)	0.069
Gender (female)		127 (52.3%)	13 (38.2%)	0.125	35 (33%)	11 (52.4%)	0.092
Etiology (alcohol)		152 (62.6%)	24 (70.6%)	0.362	90 (84.9%)	18 (85.7%)	0.924
Child-Pugh	A	137 (56.4%)	18 (52.9%)	0.771	14 (13.2%)	2 (9.5%)	0.422
	B	92 (37.9%)	13 (38.2%)		43 (40.6%)	6 (28.6%)	
	C	14 (5.8%)	3 (8.8%)		49 (46.2%)	13 (61.9%)	
Ascites		88 (36.2%)	10 (29.4%)	0.437	80 (75.5%)	18 (85.7%)	0.307

Comment 5: *"Methods: Why were clinical data determined by review of medical records? This was a prospective study with a designed CRF? Please clarify, which data were assessed retrospectively."*

At enrollment a structured interview was used for capturing clinical data about the period prior to the observational follow-up study (retrospective data collection by an in-depth review of patients' medical records), since mean disease duration from diagnosis of cirrhosis was 3.9 ± 4.2 years among patients at the time of the inclusion. These were the followings: age at diagnosis, etiology, presence of hepatocellular carcinoma, esophageal varices, extrahepatic co-morbidities, history of previous AD episode(s), and cirrhosis-related medication *were retrospectively analyzed for the period prior to the observational follow-up study*. At enrollment, laboratory parameters, disease severity – assessed by liver-oriented scores (Child-Pugh and MELD) and clinical stage of the disease (compensated/ decompensated) – was determined.

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All data collection after the inclusion was performed prospectively. Structured interview was used for capturing data again. Collected data were the follows: date and type of bacterial infection warranting hospital admission and the development of disease specific complications (ascites, hepatic encephalopathy or variceal bleeding).

Structured interview either at enrollment or later in the study ensured that data collection and data entering into database would be unified among attending physicians (senior hepatologist, n=3 and junior gastroenterologist, n=2) all the time, during regular and extraordinary outpatient follow-up visits and inpatient stays in a prospective manner. In Hungary, a regular outpatient follow-up visit is usually scheduled for every 3 months at a specialized gastroenterology center for patients with decompensated cirrhosis (a follow-up between 1-3 months may be scheduled if dictated by disease severity or the presence of certain disease specific complications) and for up to 6 months for patients with cirrhosis but without a prior episode of AD.
Collected data were transferred and stored in a database. At the end of the study period on December 31, 2013, all clinical data was extracted for further analysis.

Comment 6: *"Methods: Microbial analysis in SBP was performed in only 11 cases. A lack of bacteriological testing may also explain the low frequency of bacteriemia in follow up."*

We mentioned it as a drawback of present study in the *Section – Discussion* (Page 17). "**Present study** did not allow for an in-depth analysis of the association of PRR variants with different types of SBP (culture-negative, culture-positive or bacterascites), **which is a drawback. Since there was** twenty incident cases of SBP during follow-up **and only half of them was cultured.**"

Comment 7: *"Methods: How was censoring performed in KM-analysis? Were patients right-censored at death? How many patients were transplanted? Please indicate censored patients in the KM blots. Please indicate the patients at risk in Figure 2B."*

In KM-analysis for the development of non-SBP BI, SBP and decompensation event, censoring of patients (right-censoring) was performed in case of transplantation, death or loss of follow-up, whilst in survival analysis in case of transplantation or loss of follow-up. We added this information to the *Section of Patients and Methods – Statistical analysis (Page 11).*

During the study period, 4 patients were transplanted in this cohort that corresponds to the expected annual rate of transplantation/ inhabitants in Hungary: around fifty cases/year for 10 million-population between 2006 and 2013. 200,000 inhabitants belong to our university hospital and median follow-up lasted 1128 days (IQR: 469-1825) in the present study.

We indicated censored patients in the KM plots and patients at risk in Figure 2B.

Comment Awkward Phrases/Language:

We corrected the indicated language items and in accordance with the request of the Associate Editor a professional English editing was also performed and highlighted in the text.

- *Introduction: "can be like to"* - can be linked to
- *Introduction: "namely advent of decompensation events"* – such as development of decompensation events
- Methods: "clinical stage of the diseases" – clinical stage of the disease
- Methods: "genetic DNA" (it is genomic) – genomic DNA
- Results: "Mortality occurred in 82 subjects" – liver-related death occurred in
- *Page 14 and others: Please stick with nomenclature of human genes and symbols (italic, large capitals) consistently.* – We unified the nomenclature in the manuscript

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Reviewer 2

Comment 1: *"Did the authors analyse the intake of antibiotics, if yes how did they analyse "this influence". "Some patients with liver cirrhosis take rifaximin, an antibiotic treatment for prophylaxis for hepatic encephalopathy. These patients have to analysed separately."*

In the present cohort, 23 patients received norfloxacin, while 14 rifaximin at enrolment as a secondary prophylaxis of SBP or hepatic encephalopathy, respectively. The indication for rifaximin was the insufficient response to lactulose and was administered as an add-on-therapy to lactulose. None of the examined *NOD2*, *TLR2* and *TLR4* gene variants was different between patients with or without receiving secondary antibiotic prophylaxis. We completed the *Section – Results, Genotype distribution of various functional polymorphisms of PRRs in cirrhosis (Page 11-12) and Table 1* with these data.

"Co-medications at enrolment comprising use of proton pump inhibitor (PPI), non-selective beta blocker (NSSB) and secondary antibiotic prophylaxis either norfloxacin for prevention of SBP or rifaximin for prevention of HE were also not different among patients with genetic variants of *NOD2*, *TLR2* and *TLR4* and with wilde-type (Table 1)."

	Total	*NOD2* polymorphism[b]			*TLR2* 16934T>A polymorphism rs4696480				*TLR4* D299G A>G polymorphism rs4986790[×]		
	(N=243) *	Wild type (N=204)	Risk allele (N=37)	P-value	TT (N=64)	TA (N=104)	AA (N=74)	P-value	AA (N=225)	AG (N=17)	P-value
Secondary antibiotic prophylaxis											
Norfloxacin for prevention of SBP	9.5% (23)	8.8% (18)	13.5% (5)	0.372	6.3% (4)	13.5% (14)	6.8% (5)	0.189	9.3% (21)	11.8% (2)	0.742
Rifaximin for prevention of HE	5.8% (14)	5.4% (11)	8.1% (3)	0.457	7.8% (5)	3.8% (4)	6.8% (5)	0.515	5.8% (13)	5.9% (1)	0.986

We included secondary antibiotic prophylaxis as a clinical co-variate during exploring risk factors of non-spontaneous bacterial peritonitis type bacterial infections (non-SBP type BI) during the follow-up period (**Table 3**) such we did in the case of PPI and NSSB medication in the original version of the manuscript. Secondary antibiotic prophylaxis was not associated with an increased cumulative probability of non-SBP type of BI. We added these data to **Table 3**.

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Pertinent part of **Table 3.** Association of clinical factors with the development of non-spontaneous bacterial peritonitis type bacterial infections.

		Non-SBP type BI development				Univariate Cox regression		
		n of subjects	n of events	CP of BI ± SE	P-value*	HR	95%CI	P-value
Total cohort		243	85	49.6±4.1				
Secondary antibiotic prophylaxis								
Norfloxacin for prevention of SBP	no	220	75	49.0±4.3	0.108	1.71	(0.88 - 3.31)	0.112
	yes	23	10	52.5±12.0				
Rifaximin for prevention of HE	no	229	78	48.3±4.2	0.109	1.86	(0.86-4.04)	0.115
	yes	14	7	80.4±16.1				

Due to these findings we did not analyze further these small groups of patients separately.

Comment 2 *Part 1*: *"Patients with prior SBP take antibiotic prophylaxis. Did these patients developed also Non-SBP bacterial infections?"*

The requested data are presented in *Table 3*. Cumulative probability of a non-SBP type BI was similar between patients with or without a prior history of SBP. This finding might support the idea that the mechanisms of the development of these infections (SBP vs. non-SBP type BI) are different. In SBP, it is mainly related to intestinal bacterial translocation (BT), however it is not a major contributor in the development of non-SBP type BIs.

		Non-SBP type BI development				Univariate Cox regression		
		n of subjects	n of events	CP of BI ± SE	P-value*	HR	95%CI	P-value
Total cohort		243	85	49.6±4.1				
Prior SBP	absent	220	75	49.0±4.3	0.108	1.71	(0.88 - 3.31)	0.112
	present	23	10	52.5±12.0				

The **Comment 2 *Part 2*** and **Comment 3** are related to each other therefore we would like to answer for them simultaneously.

Comment 2 *Part 2*: *"We are learning more and more about the two sides of ßeta-blockers. We have understood a negative influence in patients with decompensated cirrhosis like patients with refractory ascites or with spontaneous bacterial peritonitis. On the other side, we know protective influences of ßeta-blockers in the cascade of bacterial translocation. The authors include patients with ß-blockers. Could the authors give us more details about the cirrhosis grade and the intake of ß-blockers. The patients should be analysed separately (groups like: no NSBB, NSBB and good liver function, NSBB and bad liver function)"*

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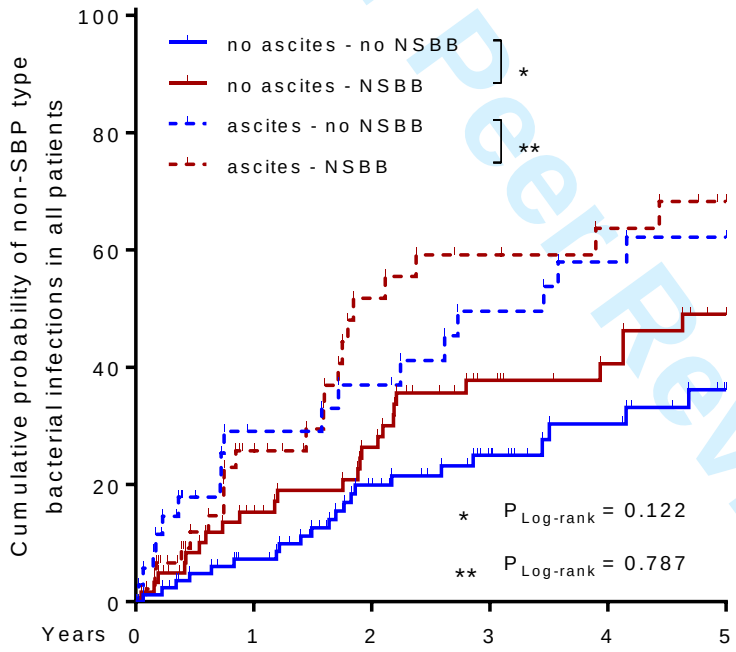
In the present cohort, NSBB use was significantly higher in the advanced diseases stage: 58% (51/88) vs. 41.9% (65/155) in patients with or without ascites (p=0.016). Corresponding data according to Child-Pugh stage were the followings: 71.4% (10/14) for Child C, 51.1% (47/92) for Child B and 43.1% (59/137) for Child A (p for trend= 0.039).

In *Table 3* in the original version of the manuscript, we reported that the CI of the development of non-SBP type BI did not differ between NSBB users and non-users. According to the suggestion of ***Reviewer 2*** we evaluated this association further taking into account disease severity (presence or absence of ascites). Likewise, there was also no association between NSBB use and the development of BI in KM analysis. In the subgroup of patients without ascites, the cumulative probability (±SE) of non-SBP type BI was 49.1±7.4% in NSBB users, while 36.2±6.8% in non-users (pLogRank=0.122). Corresponding data in the subgroup of patients with ascites were 68.2±9.1% and 62.2±9.7% (pLogRank=0.787).



Comment 3: *"The authors should analyse separately patients with and without NSBB and with and without PPI"*

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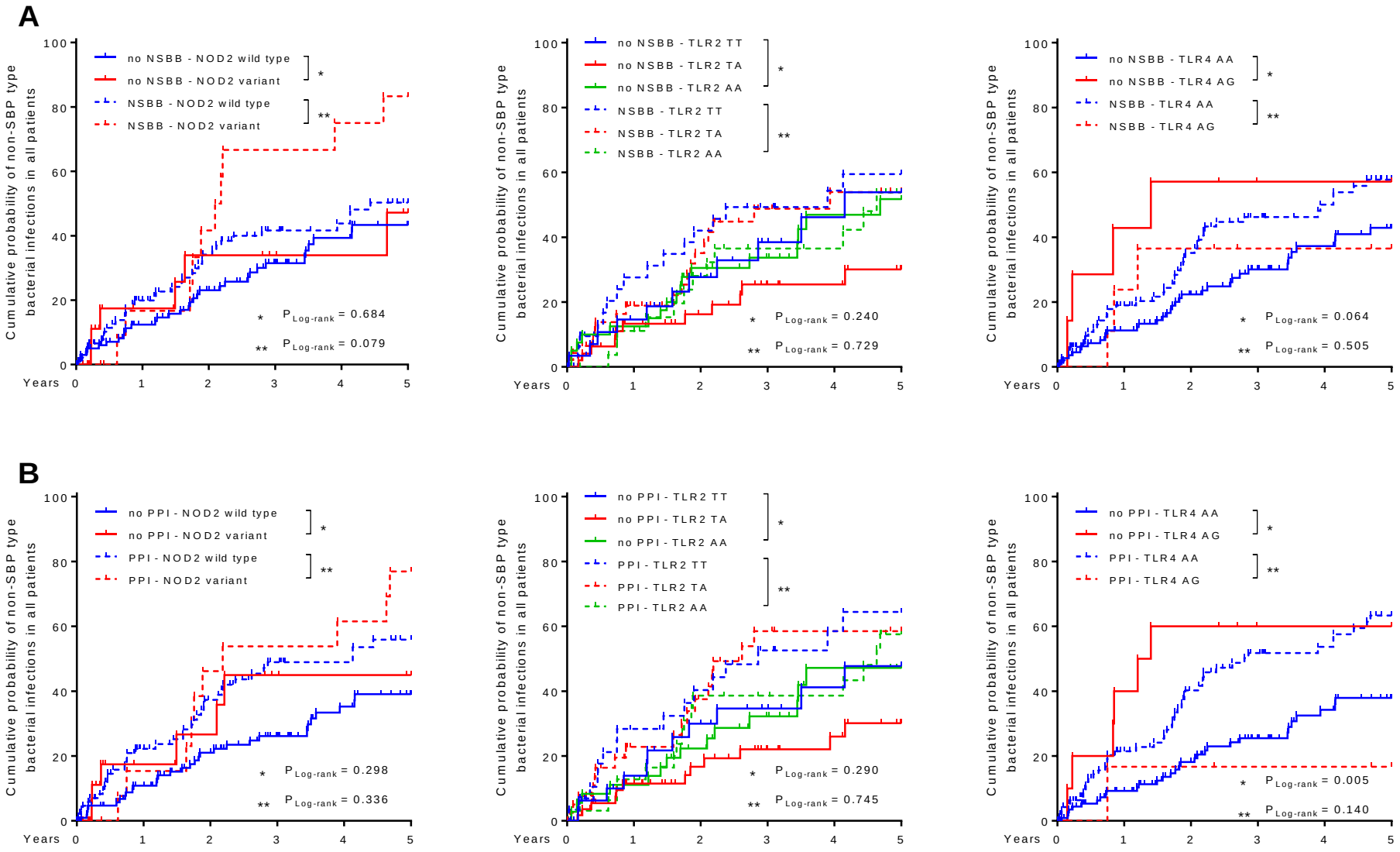
A
Cumulative probability of non-SBP type bacterial infections in all patients
Years
no NSBB - NOD2 wild type
no NSBB - NOD2 variant
NSBB - NOD2 wild type
NSBB - NOD2 variant
* P Log-rank = 0.684
** P Log-rank = 0.079
no NSBB - TLR2 TT
no NSBB - TLR2 TA
no NSBB - TLR2 AA
NSBB - TLR2 TT
NSBB - TLR2 TA
NSBB - TLR2 AA
* P Log-rank = 0.240
** P Log-rank = 0.729
no NSBB - TLR4 AA
no NSBB - TLR4 AG
NSBB - TLR4 AA
NSBB - TLR4 AG
* P Log-rank = 0.064
** P Log-rank = 0.505
B
no PPI - NOD2 wild type
no PPI - NOD2 variant
PPI - NOD2 wild type
PPI - NOD2 variant
* P Log-rank = 0.298
** P Log-rank = 0.336
no PPI - TLR2 TT
no PPI - TLR2 TA
no PPI - TLR2 AA
PPI - TLR2 TT
PPI - TLR2 TA
PPI - TLR2 AA
* P Log-rank = 0.290
** P Log-rank = 0.745
no PPI - TLR4 AA
no PPI - TLR4 AG
PPI - TLR4 AA
PPI - TLR4 AG
* P Log-rank = 0.005
** P Log-rank = 0.140

The requested data are summarized in the above series of KM curves; however, these are only provided for the ***Reviewer 2*****.** Interpretation of data about the effect of various drugs on long-term disease outcomes raises some problems. Distinctly to secondary prophylaxis of SBP or hepatic encephalopathy in case of certain drugs, like PPIs, episodic use is expected at least in some of the cases. In case of NSSB, though continuous long-term use is highly expected once it started. Drug discontinuation – either temporarily or permanently – however should also be required when contraindications arise. Thus, not only the administration of the drug used at inclusion but also their exact duration should be considered. This approach to the assessment of various medications was beyond scope of the present study.

In expert opinion (Beta-blockers in decompensated cirrhosis, Academic Debates – AASLD 2017, 20th of October), evaluating the effect of co-medications on various outcomes (e.g. bacterial infections, decompensation events or mortality) is somewhat questionable and results are hard to interpret in studies that were designed to evaluate other factors than co-medications.

Comment 4: *"The authors should specify the origin of SBP (nosocomial versus outpatient)"*

All the bacterial infections comprising SBP were community acquired. In the *Section – Patients and Methods, Study design (Page 7)* we stated the followings: "Outpatients at inclusion (n=243) were enrolled into an observational follow-up study where the attending gastroenterologist registered the date and type of bacterial infection (BI) warranting hospital admission (diagnostic criteria are summarized in Supplementary Material) and the development of disease specific complications (ascites, hepatic encephalopathy or variceal bleeding) during regular and extraordinary outpatient follow-up visits and inpatient stays in a prospective manner."

We added to the *Section – Results, Risk factors of SBP(Page 14)* that these episodes were community acquired.

"Of the patients with ascites 22.7% (20/88) developed **community acquired** SBP during the follow-up period."

Comment 5: *"Did the authors analyse the survival rates in patients with genetic variants of NOD2 and TLR2/4 and with wildtype?*

We provided these requested data in the *Section – Results (Page 15)* and *Supplementary Figure 3.* Unfortunately, during the PDF generation of the original manuscript, it did not cover the Supplementary material. Probably it was not available for the *Reviewer 2* during the review process. Now we presented the survival curves in patients with genetic variants of NOD2 and TLR2/4 and with wild type below.

"Functional polymorphisms of PRR genes and survival
In the total cohort, liver-related death occurred in 82 (33.7%) subjects. Median time to mortality was 660 (304-977) days. Kaplan-Meier survival analysis demonstrated a significantly worse survival in patients with advanced disease according to presence of ascites ($P_{LogRank}$<0.001), Child-Pugh stage B/C ($P_{LogRank}$<0.001), or decompensated clinical stage

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($P_{LogRank}$=0.033) and prior BI episode ($P_{LogRank}$=0.050). Neither NOD2 risk variants ($P_{LogRank}$= 0.785) nor *TLR2* (-16934A>T) and *TLR4* (D299G) polymorphisms ($P_{LogRank}$= 0.682 and 0.732) were associated with overall survival (**Supplementary Figure 3**)."

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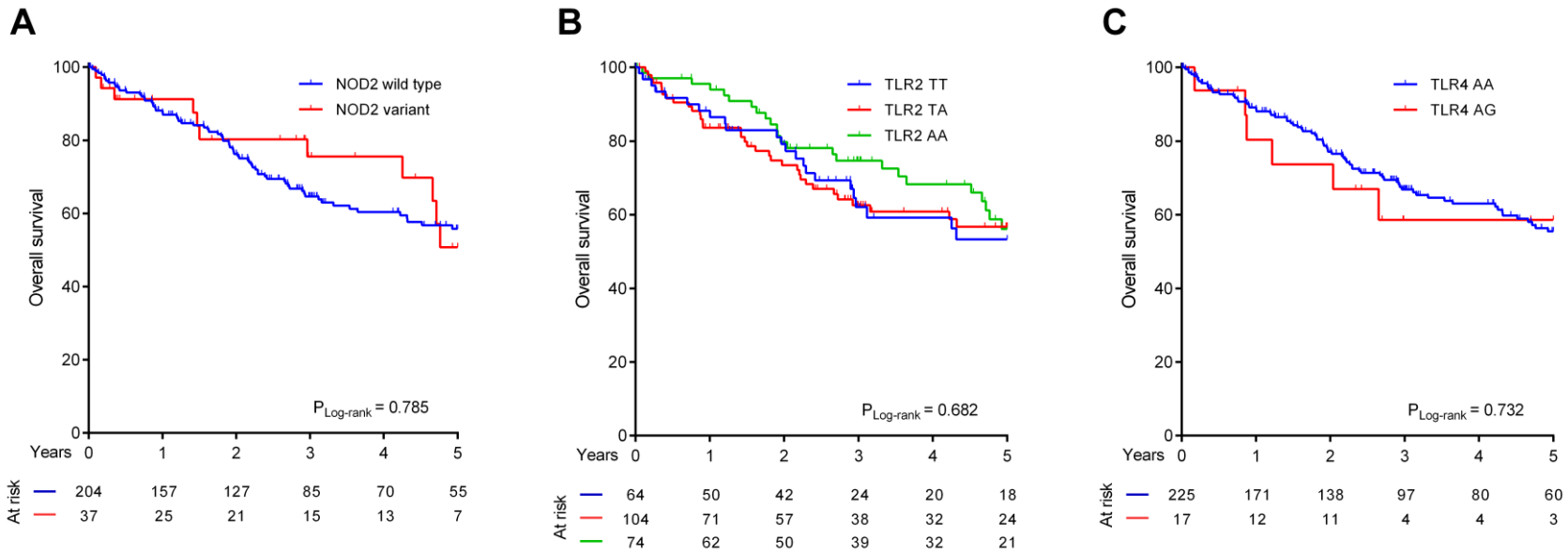
Supplementary Figure 3. Kaplan–Meier analysis of survival with respect to the presence of *NOD2* risk alleles (L1007fsinsC -/C, R702W C>T or G908R G>C) (A) and *TLR2* (T-16934A) (B) or *TLR4* (D299G) (C) polymorphisms in outpatients.



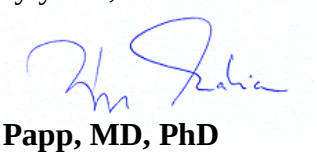
Comment 6: *"Could the authors please comment in more detail the different types of Non-SBP bacterial infection and their association to the genetic variants of NOD2, TLR2 and TLR4? For example, is there an association between genetic variants and the risk of e.g. pneumonia or urinary tract infection?"*

In our study we divided BIs into two groups: SBP and non-SBP type BIs. The rational of this categorization based on the established concept that the mechanisms of the development of these two groups of infections are different. Intestinal BT is an important mechanism in the development of SBP. And though systemic infections beyond SBP might also be related to BT (e.g. GNB caused skin infections – enteric bacteria reach the systemic circulation, cause bacteremia and seed the tissues of the extremities *[Chang CM, et al. Infection. 2008 Aug;36(4):328-34. doi: 10.1007/s15010-008-7272-3.]*) but it is not the major contributor in the development of these infections. In the present cohort evaluating first BI episodes after enrolment, eighty-five (35%) of the included outpatients encountered a non-SBP type BI episode. The incident cases were assigned into seven subgroups according to the location of the infection. (Summarized in *Section of Results – Page 12)*. We fully agree with this suggestion of the ***Reviewer 2***, however taking into account the low number of cases in seven various location subgroups and the occurrence of different PRR variants (16.4%, 24.8% and 6.6% for *any NOD2 variants*, TT genotype of *TLR2* [16934T>A] and AG genotype of *TLR4* D299G, respectively) did not make it possible to address this issue adequately, due to lack of statistical power. This is inevitably a drawback of our study. In the revised version of the manuscript we mentioned this limitation (*Section – Discussion* [Page 18]).

"The limited number of incident cases in the seven different location subgroups did not make possible the more subtle assessment of the potential role of PRR variants in the development of certain type of infection that is an inevitable drawback of the present cohort."

We hope that the quality of the revised version of the manuscript improved significantly and hope that this new version will reach the high standards of *Liver International*

Thank you very much for your kind helps and appreciation,
Sincerely yours,



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For Peer Review

Functional polymorphisms of innate immunity receptors are not risk factors for the non-SBP type bacterial infections in cirrhosis

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Electronic word count: 5420

Number of figures and tables: 4+4

List of abbreviations: AD: acute decompensation, ACLF: acute-on chronic liver failure, BT: bacterial translocation, BI: bacterial infection, LBP: lipopolysaccharide binding protein, MELD: model for end-stage liver disease, HBV: hepatitis B virus, HCV: hepatitis C virus, NOD: nucleotide-binding oligomerization domain, SBP: spontaneous bacterial peritonitis, SNP: single nucleotide polymorphism, TLR: toll-like receptor, PRR: pattern recognition receptor

Conflict of interest: none to declare

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ABSTRACT

Background&Aims: Pattern recognition receptors (PRRs) have a key role in the innate host defense. Functional polymorphisms of various PRRs have been established to contribute to an increased susceptibility to spontaneous bacterial peritonitis (SBP). Their role in the development of cirrhosis-associated bacterial infections (BI), beyond SBP or progressive disease course related to pathological bacterial translocation (BT) remains unknown. **Methods**: 349 patients with cirrhosis were genotyped for common *NOD2* (R702W, G908R and L1007PfsinsC), *TLR2* (-16934T>A), and *TLR4* (D299G) gene variants. Incidence of BIs, decompensating events (ascites, variceal bleeding and hepatic encephalopathy) and liver-related death were assessed in a 5-year follow-up observational study. Pathological BT was assessed based on the presence of anti-microbial antibodies or lipopolysaccharide-binding protein (LBP) level. **Results:** In patients with ascites (n=88) only *NOD2* gene variants were associated with an increased cumulative probability of SBP compared to wild-type (76.9%±19.9% vs. 30.9%±6.9%, $P_{LogRank}$=0.047). Neither individual polymorphisms, nor combined PRR genetic profiles were associated with the risk of non-SBP type BI. Advanced disease stage (HR,[95%CI]: 2.11 [1.38-3.25]) and prior history of a BI episode (HR: 2.42 [1.58-3.72]) were the major clinical risk factors of a subsequent BI. The risk of a non-SBP type BI in patients with advanced disease and a prior BI was even higher (HR: 4.74 [2.68-8.39]). The frequency of anti-microbial antibodies and LBP levels did not differ between various PRR genotypes. Correspondingly, PRR genetic profile was not able to predict the long-term disease course. **Conclusions:** In cirrhosis, functional polymorphisms of PRRs

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did not improve the identification of patients with high risk of BI beyond SBP or progressive diseases course.

Word count for abstract: 250

Key words: pattern recognition receptors, genetic polymorphisms, cirrhosis, bacterial infection, complications, mortality

Key Points

- In this 5-year follow-up study, we evaluated the role of functional polymorphisms of various PRRs (*NOD2*, *TLR2*, and *TLR4*) in the development of bacterial infections, clinical decompensation and mortality in patients with cirrhosis.
- We confirmed that *NOD2* variants were risk factors of SBP in patients with ascites.
- Clinical factors (advanced disease and history of a bacterial infection) were major determinants of non-SBP type bacterial infections.
- We found no association between PRR gene variants and serologic markers of pathological bacterial translocation. Concordantly, patients with PRR gene variants did not have an increased risk for clinical decompensation or mortality.

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INTRODUCTION

Pathological bacterial translocation (BT) is a characteristic feature of cirrhosis, mainly in the advanced disease stage, and it plays an essential role in the pathogenesis and the development of various complications of the disease. The most evidenced clinical consequence of BT is the spontaneous bacterial peritonitis (SBP) and bacteremia. [1] Systemic infections beyond SBP might also be related to BT. Even in the absence of an overt infection, sustained entry of various bacterial products into the hepato-splanchnic and systemic circulation can also have a deleterious effect by inducing an enhanced pro-inflammatory response. Failure to control invading bacteria and/or their products, together with an increased host susceptibility to infection, may result in the damage of the remote organ. [2] Development of consequential organ failure(s) is a major determinant of mortality in this patient population. [3]

Accurate identification and risk stratification of BT can efficiently aid the preventive strategies against bacterial infections and other complications of cirrhosis. Direct data on culturable BT to mesenteric lymph nodes and upstream compartments is not available in humans. Recently, various serologic markers (e.g. lipopolysaccharide binding protein [LBP], [4] bacterial DNA [5] or IgA type anti-microbial antibodies [6,7]) have been proposed to reflect sustained gut microbial exposure. Additionally, susceptibility genes for pathological BT have also been revealed. Functional polymorphisms of pattern recognition receptors (PRRs) alter the detection and clearance of bacterial pathogens, thus influencing the innate host defence mechanisms. Single nucleotide polymorphisms (SNPs) in the promoter and the encoding regions of nucleotide-binding oligomerization domain (NOD) [8,9] or toll-like

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receptors (TLR) [10,11] were reported to increase the risk of SBP. However, their comprehensive evaluation regarding non-SBP type bacterial infections, or various other aspects of progressive disease course in cirrhosis has not been fully elucidated so far.

In the present study, we aimed to investigate the clinical importance of functional polymorphisms of various PRRs in a large cohort of patients with cirrhosis. In a 5-year follow-up observational study, we evaluated whether certain genetic variants of *NOD2*, *TLR2* and *TLR4* (1) constitute a risk for the development of SBP or non-SBP type bacterial infections; (2) can be linked to the established serologic markers of bacterial translocation; (3) constitute a risk for the progressive disease course, such as development of decompensation events (ascites formation, hepatic encephalopathy or variceal bleeding), or liver-related mortality.

PATIENTS AND METHODS

Study design

We performed a cohort study among adult patients with an established diagnosis of cirrhosis of different etiologies, in a tertiary care referral center of Hungary (Division of Gastroenterology Department of Internal Medicine, Clinical Center, University of Debrecen). The present study population is a part of our entire patient cohort comprising a total of 404 patients with cirrhosis who were recruited consecutively between May 1, 2006 and December 31, 2010 from the outpatient clinic during regular, or extraordinary follow-up visits, and also from the inpatient ward, when hospitalized with an acute decompensation (AD) episode [12,13]. For the present study, blood

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samples from 349 patients were available (243 outpatients and 106 hospitalized subjects due to an AD episode) (**Figure 1**). Acute decompensation was defined by the acute development of large ascites (grade II/III) [14], acute hepatic encephalopathy [15], acute variceal bleeding [16] and/or the presence of systemic bacterial infection.

Clinical characteristics of patients at inclusion are presented in **Table 1**. Mean disease duration from diagnosis of cirrhosis was 3.9 ± 4.2 years among patients at the time of the inclusion. Blood samples, routine laboratory data and a detailed clinical phenotype were captured at inclusion. Clinical data was determined by an in-depth review of patients' medical records using a structured interview. Medical records that documented age at diagnosis, etiology, presence of hepatocellular carcinoma, esophageal varices, extrahepatic co-morbidities, history of previous AD episode(s), and cirrhosis-related medication were retrospectively analyzed for the period prior to the observational follow-up study. At enrollment, disease severity – assessed by liver-oriented scores (Child-Pugh and MELD) and clinical stage of the disease (compensated/ decompensated) – was always determined.

Outpatients at inclusion (n=243) were enrolled into an observational follow-up study where the attending gastroenterologist registered the date and type of bacterial infection (BI) warranting hospital admission (diagnostic criteria are summarized in **Supplementary Material**) and the development of disease specific complications (ascites, hepatic encephalopathy or variceal bleeding) during regular, and extraordinary, outpatient follow-up visits and inpatient stays in a prospective manner. In Hungary, a regular outpatient follow-up visit is usually scheduled for every 3 months at a specialized

gastroenterology center for patients with decompensated cirrhosis (a follow-up between 1-3 months may be scheduled if dictated by disease severity or the presence of certain disease specific complications) and for up to 6 months for patients with cirrhosis but without a prior episode of AD. Follow-up period lasted for 5 years, or until death/loss of follow-up. Eighty-two (34%) patients died during follow-up, median time to death was 660 days (IQR: 304-977). In the 181 patients without death occurring, median follow-up lasted 1128 days (IQR: 469-1825). Collected data were transferred and stored in a database. At the end of the study period on December 31, 2013, all clinical data was extracted for further analysis.

Gene analysis of NOD2, TLR2 and TLR4

Genomic DNA was extracted from whole-blood samples using the Gentra Puregene Blood Kit (Qiagen; Hilden, Germany) following the manufacturer's protocol. Three alleles of the *NOD2* gene variants rs2066844, (p.R702W:, NM_022162.2:c.2104C>T), rs2066845 (p.G908R; NM_022162.2:c.2722G>C), and rs2066847 (L1007Pfs; NM_022162.2:c.3019dupC) were genotyped using hybridization probes on fluorescence resonance energy transfer (FRET) on a LightCycler 480 (Roche) real-time PCR system, according to *Ferreiros-Vidal et a*l [17]. Gene variant of *TLR2* gene rs4696480 (NM_003264.4:c.-148+1614T>A) was also genotyped using oligonucleotides according to *Oh et al.* [18]. The gene variant of *TLR4* gene rs4986790 (p.D299G; NM_138554.4:c.896A>G) was genotyped using self-designed amplification oligos (TLR4-D299G F: CATCGTTTGGTTCTGGGAG and TLR4-D299G R: TTTACCCTTTCAATAGTCACACTCA), while FRET oligonucleotides were

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similar to *Hamann et al.* (TLR4-D299G SENS: CTACTACCTCGATGGTATTATTGACTTATT-6FAM, TLR4-D299G ANCH: Cy5.5 -AATTGTTTGACAAATGTTTCTTCATTTTCC-3'phosph) [19]. Representative melting curve genotyping results are shown in the **Supplementary Figure 1**. Genotyping was technically unsuccessful in two patient samples for *NOD2* analysis, and in one sample for *TLR2* and *TLR4* analysis.

Serologic analysis

Serum levels of total bilirubin, creatinine, and albumin, blood cell count and INR were determined by routine laboratory analysis.

Blood samples were obtained at enrollment from each patient and were frozen at -70°C until testing. All the serological assays were performed in a blinded fashion without prior knowledge of the patient's clinical information. Commercially available sandwich enzyme-linked immunosorbent assays (ELISA) were used according to the manufacturer's protocol to determine serologic markers of pathological BT, namely lipopolysaccharide-binding protein (LBP) (Hycult Biotechnology, Uden, Netherlands), endotoxin core IgA antibody (EndoCAb IgA) (Hycult Biotechnology, Uden, Netherlands) and anti-OMP Plus IgA antibody (QUANTA Lite®, Inova Diagnostics, San Diego, CA). EndoCAb directs against a mixture of incomplete endotoxins of 4 different species (*Pseudomonas aeruginosa, Salmonella typhimurium, Escherichia coli* and *Klebsiella aerogenes*), while anti-OMP Plus antibody does to a mixture of multiple bacterial proteins derived from two species of intestinal bacteria (one Gram-positive and one Gram-negative). Cut-off positivity was 195 AU/mL for

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EndoCAb IgA, defined by our group previously [19] as a value exceeding the 95th percentile level of the healthy control group, and 25 U for anti-OMP Plus IgA as recommended by the manufacturer.

Ethical considerations

The study protocol was approved by the Regional and Institutional Research Ethics Committee of University of Debrecen and by the National Scientific and Research Ethics Committee (DEOEC-RKEB/IKEB 5306-9/2011, 3885/2012/EKU [60/PI/2012]). Each patient or legal surrogate was informed of the nature of the study and signed an informed consent form.

Statistical analysis

Variables were tested for normality using Shapiro Wilk's W test. Continuous variables were summarized as means (standard deviation [SD]) or as medians (interquartile range [IQR, lowest 25%-highest 25%]) according to their homogeneity. Categorical variables were compared with Fisher's exact test or χ^2 test with Yates correction, as appropriate. Continuous variables were compared with Mann-Whitney U test or Kruskal-Wallis H test with Dunn's multiple comparison *post hoc* analysis. Allele frequencies of the respective SNPs were tested for deviations from the Hardy–Weinberg equilibrium and then compared for statistical differences with the Cochrane Armitage trend test (Helmholtz Center Munich, http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Kaplan–Meier (KM) analysis was used to calculate the cumulative probability (CP) of adverse outcomes (development of non-SBP BI, SBP, decompensation event and mortality). Right censoring of patients was

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performed in case of transplantation or loss of follow-up as appropriate. Differences in observed probabilities were assessed by the log-rank test. The association between categorical clinical variables, or different PRR genotypes, and adverse disease outcomes during follow-up was assessed by univariate Cox-regression analysis. Multivariate analyses were performed with a forward inclusion procedure and a likelihood ratio test to identify independent predictors. Binary logistic regression was used to assess the infection-related mortality at 28 and 90 days. Associations are given as a hazard ratio [HR] or odds ratio [OR] with 95% confidence intervals [CI]. For statistical analysis and graphical presentation, the SPSS 24.0 [SPSS, Chicago, IL], and GraphPad Prism 6 programs were used. A 2-sided probability value of <0.05 was considered statistically significant.

RESULTS

Genotype distribution of various functional polymorphisms of PRRs in cirrhosis

Frequencies of various PRR genotypes in cirrhosis are summarized in **Table 2**. None of the examined *NOD2*, *TLR2* and *TLR4* gene variants was different between outpatients and patients with AD. Further analysis of clinical and laboratory characteristics of outpatients revealed that age, gender, presence of a co-morbidity or HCC, etiology or severity of cirrhosis was not different across the various PRR genotype subgroups. Co-medications at enrolment comprising the use of proton pump inhibitor (PPI), non-selective beta blocker (NSSB) and secondary antibiotic prophylaxis either norfloxacin for prevention

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of SBP or rifaximin for prevention of HE were also not different among patients with genetic variants of *NOD2*, *TLR2* and *TLR4* and with wilde-type (**Table 1**).

Risk factors of non-SBP type BI

Eighty-five (35.0%) of the included outpatients encountered a non-SBP type BI episode during the follow-up period. The median time to development of a first BI episode was 581 (207-803) days. Urinary tract infection was the most commonly diagnosed BI, and accounted for 43.5% (n=37) of the events. Other sites of BI were as follows: pneumonia (18.8%), erysipelas (10.6%), acute bronchitis (5.9%), cholangitis (3.5%), bacteremia (3.5%), gastroenteritis (1.2%) and unidentified in 9 (10.6%) cases. 2.4% of the cases were multifocal. Microbiological analysis was performed in 35 (41.2%) cases. Bacteria were Gram-negative in 76.5% and Gram-positive in 23.5% of culture positive cases (n=17) (**Supplementary Material**).

Functional polymorphisms of PRR genes

Patients with any risk variants in *NOD2*, *TLR2* or *TLR4* genes did not have an increased cumulative probability of a non-SBP type BI episode during follow-up (**Figure 2A**), not even when stratifying according to presence of ascites (**Figure 2B**). Patients carrying both a *TLR2* variant and at least one *NOD2* risk variant (n=10) had also a similar rate of non-SBP type BI, than patients not carrying both variants ($P_{LogRank}$=0.397). There was no rationale for testing the potential effect of the *TLR4* and *NOD2 variant* combination. Only one patient carried both variant genotypes.

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The presence or absence of a *NOD2*, *TLR2* or *TLR4* variants did not affect the type of pathogen causing BI (Gram-negative or –positive), or the location of BI.

Furthermore, these PRR gene variants were not associated with the risk of mortality during a subsequent bacterial infection (**Supplementary Table 2).**

Clinical co-variates

Of the clinical factors: co-morbidity, PPI use, prior history of a BI episode and advanced disease stage were all associated with an increased cumulative probability of non-SBP type BI episodes during the follow-up period (**Table 3**). Of the patients with a prior history of BI, 66.5%±6.3% (standard error) developed another BI episode, compared to 39.7%±5.1 % of those with no such history ($P_{LogRank}$:<0.001). Regarding advanced disease stage, similar results were found if advanced disease stage was depicted either by the presence of ascites (65.2%±6.6% vs. 42.0%±5.1%, $P_{LogRank}$:<0.001), Child-Pugh stage B/C (68%±6.1% vs. 38.1%±5.2%, $P_{LogRank}$:<0.001) or by decompensated clinical stage (58.8%±5.7% vs. 40.8%±5.8%, $P_{LogRank}$=0.01). The combination of these two relevant clinical factors revealed important findings. First, prior history of a BI episode significantly increased the probability of the subsequent development of another BI event, regardless of disease severity. Furthermore, a prior history of a BI without ascites was associated with the same cumulative probability of a BI occurring, as the presence of ascites without prior history of a BI (57.3%±8.7% and 51.0%±9.9%, respectively). The combined presence of both clinical risk

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factors resulted in an even higher cumulative probability of BI (80.3%±7.7%) (**Figure 3 and Table 3**).

Multivariate analysis

Multivariate Cox-regression analysis and the forward inclusion procedure, taking all significant clinical co-variates of univariate analysis into account, indicated that presence of ascites (HR [95%CI]: 1.71 [1.08-2.7], higher MELD score (1.08 [1.02-1.15]) and prior BI episode (2.02 [1.3-3.14]) were independently associated with the risk of a non-SBP type BI development during follow-up.

Risk factors of SBP

Of the patients with ascites 22.7% (20/88) developed community acquired SBP during the follow-up period. Of the cases with microbiological investigation, 36.4% (4/11) was culture-positive SBP, while 63.6% (7/11) was culture negative. Bacteria were Gram-negative in 75% and Gram-positive in 25% of culture positive cases (**Supplementary Material**). The median time to the development of SBP was 340 (126-662) days. The presence of *NOD2* risk allele variants, but not of *TLR2* and *TLR4 variants,* were associated with an increased cumulative probability of SBP (**Figure 4**). Of the patients with any NOD2 risk variants 76.9%±19.9% developed SBP, compared to 30.9%±6.9% of those with *NOD2* wild type ($P_{LogRank}$=0.047). Patients with or without any *NOD2* risk allele variants had similar MELD scores (median [IQR]: 14 [9-16] vs. 13 [10-15], respectively, P=0.874). Prior SBP episode was also associated with the risk of SBP development ($P_{LogRank}$=0.048).

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Association of functional polymorphisms of PRR genes with serologic markers of BT

Serum level of LBP and frequencies of IgA type antibodies directed against various gut microbial components (anti-OMP Plus and EndoCab) were not different according to the examined PRR genotypes (**Table 4**).

Functional polymorphisms of PRR genes and development of decompensation events

Of the patients with a compensated clinical stage at enrolment 31.4% (38/121) developed any type of decompensation event (ascites, variceal bleeding or hepatic encephalopathy). The median time to the development of a first decompensation was 540 (140-913) days. Neither *NOD2* risk variants ($P_{LogRank}$=0.681) nor *TLR2* and *TLR4* polymorphisms ($P_{LogRank}$= 0.068 and 0.249) were risk factors of clinical decompensation (**Supplementary Figure 2**).

Functional polymorphisms of PRR genes and survival

In the total cohort, liver-related death occurred in 82 (33.7%) subjects. Median time to mortality was 660 (304-977) days. Kaplan-Meier survival analysis demonstrated a significantly worse survival in patients with advanced disease according to presence of ascites ($P_{LogRank}$<0.001), Child-Pugh stage B/C ($P_{LogRank}$<0.001), or decompensated clinical stage ($P_{LogRank}$=0.033) and prior BI episode ($P_{LogRank}$=0.050). Neither NOD2 risk variants ($P_{LogRank}$= 0.785) nor *TLR2* and *TLR4* polymorphisms ($P_{LogRank}$= 0.682 and 0.732) were associated

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with overall survival (**Supplementary Figure 3**).

DISCUSSION

Bacterial infections beyond SBP have significant prognostic implications in patients with cirrhosis [1]. Thus, individual risk stratification for BI is an important clinical issue, and it may be instrumental in identifying high-risk patients amenable to preventive measures and/or closer follow-up strategies as a part of the standard of care. Former clinical studies with functional PRR gene variants in cirrhosis primarily focused on the development of SBP in ascitic patients [8–11]. Distinctly, in the present study we comprehensively assessed the utility of various functional SNPs of three different PRR genes simultaneously in a large prospective cohort, comprising the whole severity spectrum of cirrhosis, with a special emphasis on the development of non-SBP type BI.

In our cohort, the frequencies of PRR gene variants were comparable within other cirrhotic patient cohorts and with healthy Caucasians [20,21].

Similar to most of the previous studies [8–10], the presence of *NOD2* allele variants was a risk factor for SBP in our cohort as well. A recent large association study in patients with decompensated cirrhosis however, did not demonstrate a role of *NOD2* variant in mediating susceptibility for SBP [23]. In our study development of SBP was not more frequent in patients with *TLR2* (-16934 T>A, rs4696480) and *TLR4* (D299G, rs4986790) polymorphisms. This latter finding is a novelty. At the same time, the association of SBP with various *TLR2* genotypes is somewhat controversial in the published literature. In the studies of *Nischalke et al.* [10] and *Lutz et al.* [22] *TLR2* (-16934 TT)

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genotype but not *TLR2* R753Q and P631H mutations were associated with SBP. Contrarily, *Bruns et al.* showed that not *TLR2* (-16934 T>A) but *TLR2* R753Q polymorphism increased the risk of SBP [11]. A limitation of our study compared to previous cohorts, was the relatively lower number of patients with ascites (n=88). The present study did not allow for an in-depth analysis of the association of PRR variants with different types of SBP (culture-negative, culture-positive or bacterascites), which is a drawback. Since there was twenty incident cases of SBP during follow-up and only half of them was cultured.

The PRR gene variants examined in our study were reported to have a special role in susceptibility to bacterial infections and sepsis in patients with acquired immune deficiency (i.e. acute leukaemia or allogeneic stem cell transplantation) [22,23]. Furthermore in critically ill patients, the *NOD2*/*TLR4* combination was associated with higher rate of bacteraemia [24]. In patients with advanced cirrhosis the *TLR4* (D299G, rs4986790) variant was recognized to increase overall BI rates in a single retrospective study (n=111) [25]. At the same time, another *TLR4* variant (c.+1196C/T, rs4986791) did not increase the risk of BI in a large retrospective cohort (n=336), including a validation cohort with same samples size [26]. In our study none of the examined PRR variants were associated with higher risk of non-SBP type BI. In spite of the known fact that two main members of the PRR family – TLRs and NOD like receptors – act synergistically in the initiation of host innate immune response to BI [27], neither of the *NOD2*, *TLR2* and *TLR4* gene variant combinations showed increased BI susceptibility. The limitation of the present study is that the association of bacterial infections and *TLR4*

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polymorphisms warrants further evaluation in a larger cohort since our study was underpowered to detect such an association at this sample size (**Supplementary Table 1**). The limited number of incident cases the seven different location subgroups did not make possible a more subtle assessment of the potential role of PRR variants in the development of certain types of infection.

The strength of the present study is that the whole disease severity spectrum of cirrhosis was represented, allowing for an in-depth evaluation of the interaction of PRR gene variants and BI development in various disease severity subgroups. Cirrhosis associated immunodeficiency syndrome (CAID) is a dynamic process evolving with the natural history of progression to end stage liver disease [28]. Therefore, the impact of an inherited risk for a BI might be different in early vs. advanced cirrhosis, owing to limited compensatory mechanisms. However, PRR gene variants were not associated with a higher risk of non-SBP type BI in any subgroups of various disease severities. These results confirm that acquired immune deficiency state in cirrhosis is more dominant of a risk factor than the presence of functional genetic polymorphisms in the development of BI.

The most notable discovery of the present study was that a prior episode of BI was a risk factor for the development of a subsequent BI episode. This finding suggests the presence of further persistent host factors that modulate an individual's susceptibility for BI. Interestingly, this association was present in early as well as in advanced disease stages. Remarkably, history of a prior BI episode and an advanced disease stage had similar impacts on the infectious risk.

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Pathological BT is associated with clinically relevant complications in cirrhosis [29]. There is evidence that variants of the *NOD2* gene [30,31], and various *TLR* [32] polymorphisms contribute to BT in patients with Crohn's diseases. Likewise in patients with decompensated cirrhosis an increased translocation of bacterial DNA fragments into ascitic fluid was found in the presence of the *NOD2* risk variant p.G908R [33]. Moreover there was increased transition of pathologic BT to culture-positive SBP in the case of the same *NOD2* variant [34,35]. Furthermore, *TLR2* (-16934 T>A, rs4696480) and *TLR4* (D299G, rs4986790) polymorphisms were associated with an increased systemic antigen burden as well, described by the serum level of lipoteichoic acid, LPS, and bacterial-DNA [32].

In our study we applied both serologic and clinical approaches to assess the impact of PRR genetic variants to BT. First, we examined the effect of *NOD* and *TLR* SNPs on the serological response to BT, but used different serologic markers than in the study of *Piñero et al.* [32]. The frequency of IgA type anti-microbial antibodies and LBP levels in our study did not differ between various PRR genotypes; neither in the entire cohort nor in the subgroup of patients with/ or without ascites. Second, we hypothesised that if PRR genetic variants were linked to BT, they would be associated with enhanced diseases progression, e.g. the advent of first decompensating event, or liver-related death. In accordance with our serologic results, different polymorphisms of the *NOD2* and *TLR2* and *TLR4* genes did not influence these adverse outcomes. It should be pointed out that our analysis is the first to consider the effect of PRR gene variants on the development of a decompensating event in cirrhosis.

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The effect of PRR gene variants on mortality was assessed previously but yielded conflicting results. *Appenrodt et al.* found four-fold increased risk in cirrhotic patients with *NOD2* risk alleles [9]. Concordantly to our findings *Bruns et al.* did not report an increased hazard of death related to the same variants of *NOD2* [8].

In conclusion, we were able to confirm the previous discovery that common *NOD2* gene variants increased the risk of SBP. However, *NOD2* and other SNPs of *TLR2* and *TLR4* did not influence the development of non-SBP type bacterial infections. Disease severity and a prior episode of bacterial infection were highly relevant clinical risk factors for a subsequent episode. PRR gene variants were neither associated with serological markers of bacterial translocation, nor were they associated with the development of clinical decompensation or liver-related death during follow-up. These results suggest a limited value of PRR genotyping in the prediction of a progressive disease course in cirrhosis.

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Functional polymorphisms of innate immunity receptors are not risk factors for the non-SBP type bacterial infections in cirrhosis

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List of abbreviations: AD: acute decompensation, ACLF: acute-on chronic liver failure, BT: bacterial translocation, BI: bacterial infection, LBP: lipopolysaccharide binding protein, MELD: model for end-stage liver disease, HBV: hepatitis B virus, HCV: hepatitis C virus, NOD: nucleotide-binding oligomerization domain, SBP: spontaneous bacterial peritonitis, SNP: single nucleotide polymorphism, TLR: toll-like receptor, PRR: pattern recognition receptor

Conflict of interest: none to declare

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ABSTRACT

Background&Aims: Pattern recognition receptors (PRRs) have a key role in the innate host defense. Functional polymorphisms of various PRRs have been established to contribute to an increased susceptibility to spontaneous bacterial peritonitis (SBP). Their role in the development of cirrhosis-associated bacterial infections (BI), beyond SBP or progressive disease course related to pathological bacterial translocation (BT) remains unknown. **Methods**: 349 patients with cirrhosis were genotyped for common *NOD2* (R702W, G908R and L1007PfsinsC), *TLR2* (-16934T>A), and *TLR4* (D299G) gene variants. Incidence of BIs, decompensating events (ascites, variceal bleeding and hepatic encephalopathy) and liver-related death were assessed in a 5-year follow-up observational study. Pathological BT was assessed based on the presence of anti-microbial antibodies or lipopolysaccharide-binding protein (LBP) level. **Results:** In patients with ascites (n=88) only *NOD2* gene variants were associated with an increased cumulative probability of SBP compared to wild-type (76.9%±19.9% vs. 30.9%±6.9%, $P_{LogRank}$=0.047). Neither individual polymorphisms, nor combined PRR genetic profiles were associated with the risk of non-SBP type BI. Advanced disease stage (HR,[95%CI]: 2.11 [1.38-3.25]) and prior history of a BI episode (HR: 2.42 [1.58-3.72]) were the major clinical risk factors of a subsequent BI. The risk of a non-SBP type BI in patients with advanced disease and a prior BI was even higher (HR: 4.74 [2.68-8.39]). The frequency of anti-microbial antibodies and LBP levels did not differ between various PRR genotypes. Correspondingly, PRR genetic profile was not able to predict the long-term disease course. **Conclusions:** In cirrhosis, functional polymorphisms of PRRs

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did not improve the identification of patients with high risk of BI beyond SBP or progressive diseases course.

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Key words: pattern recognition receptors, genetic polymorphisms, cirrhosis, bacterial infection, complications, mortality

Key Points

- In this 5-year follow-up study, we evaluated the role of functional polymorphisms of various PRRs (*NOD2*, *TLR2*, and *TLR4*) in the development of bacterial infections, clinical decompensation and mortality in patients with cirrhosis.
- We confirmed that *NOD2* variants were risk factors of SBP in patients with ascites.
- Clinical factors (advanced disease and history of a bacterial infection) were major determinants of non-SBP type bacterial infections.
- We found no association between PRR gene variants and serologic markers of pathological bacterial translocation. Concordantly, patients with PRR gene variants did not have an increased risk for clinical decompensation or mortality.

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INTRODUCTION

Pathological bacterial translocation (BT) is a characteristic feature of cirrhosis, mainly in the advanced disease stage, and it plays an essential role in the pathogenesis and the development of various complications of the disease. The most evidenced clinical consequence of BT is the spontaneous bacterial peritonitis (SBP) and bacteremia. [1] Systemic infections beyond SBP might also be related to BT. Even in the absence of an overt infection, sustained entry of various bacterial products into the hepato-splanchnic and systemic circulation can also have a deleterious effect by inducing an enhanced pro-inflammatory response. Failure to control invading bacteria and/or their products, together with an increased host susceptibility to infection, may result in the damage of the remote organ. [2] Development of consequential organ failure(s) is a major determinant of mortality in this patient population. [3]

Accurate identification and risk stratification of BT can efficiently aid the preventive strategies against bacterial infections and other complications of cirrhosis. Direct data on culturable BT to mesenteric lymph nodes and upstream compartments is not available in humans. Recently, various serologic markers (e.g. lipopolysaccharide binding protein [LBP], [4] bacterial DNA [5] or IgA type anti-microbial antibodies [6,7]) have been proposed to reflect sustained gut microbial exposure. Additionally, susceptibility genes for pathological BT have also been revealed. Functional polymorphisms of pattern recognition receptors (PRRs) alter the detection and clearance of bacterial pathogens, thus influencing the innate host defence mechanisms. Single nucleotide polymorphisms (SNPs) in the promoter and the encoding regions of nucleotide-binding oligomerization domain (NOD) [8,9] or toll-like

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receptors (TLR) [10,11] were reported to increase the risk of SBP. However, their comprehensive evaluation regarding non-SBP type bacterial infections, or various other aspects of progressive disease course in cirrhosis has not been fully elucidated so far.

In the present study, we aimed to investigate the clinical importance of functional polymorphisms of various PRRs in a large cohort of patients with cirrhosis. In a 5-year follow-up observational study, we evaluated whether certain genetic variants of *NOD2*, *TLR2* and *TLR4* (1) constitute a risk for the development of SBP or non-SBP type bacterial infections; (2) can be linked to the established serologic markers of bacterial translocation; (3) constitute a risk for the progressive disease course, such as development of decompensation events (ascites formation, hepatic encephalopathy or variceal bleeding), or liver-related mortality.

PATIENTS AND METHODS

Study design

We performed a cohort study among adult patients with an established diagnosis of cirrhosis of different etiologies, in a tertiary care referral center of Hungary (Division of Gastroenterology Department of Internal Medicine, Clinical Center, University of Debrecen). The present study population is a part of our entire patient cohort comprising a total of 404 patients with cirrhosis who were recruited consecutively between May 1, 2006 and December 31, 2010 from the outpatient clinic during regular, or extraordinary follow-up visits, and also from the inpatient ward, when hospitalized with an acute decompensation (AD) episode [12,13]. For the present study, blood

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samples from 349 patients were available (243 outpatients and 106 hospitalized subjects due to an AD episode) (**Figure 1**). Acute decompensation was defined by the acute development of large ascites (grade II/III) [14], acute hepatic encephalopathy [15], acute variceal bleeding [16] and/or the presence of systemic bacterial infection.

Clinical characteristics of patients at inclusion are presented in **Table 1**. Mean disease duration from diagnosis of cirrhosis was 3.9 ± 4.2 years among patients at the time of the inclusion. Blood samples, routine laboratory data and a detailed clinical phenotype were captured at inclusion. Clinical data was determined by an in-depth review of patients' medical records using a structured interview. Medical records that documented age at diagnosis, etiology, presence of hepatocellular carcinoma, esophageal varices, extrahepatic co-morbidities, history of previous AD episode(s), and cirrhosis-related medication were retrospectively analyzed for the period prior to the observational follow-up study. At enrollment, disease severity – assessed by liver-oriented scores (Child-Pugh and MELD) and clinical stage of the disease (compensated/ decompensated) – was always determined.

Outpatients at inclusion (n=243) were enrolled into an observational follow-up study where the attending gastroenterologist registered the date and type of bacterial infection (BI) warranting hospital admission (diagnostic criteria are summarized in **Supplementary Material**) and the development of disease specific complications (ascites, hepatic encephalopathy or variceal bleeding) during regular, and extraordinary, outpatient follow-up visits and inpatient stays in a prospective manner. In Hungary, a regular outpatient follow-up visit is usually scheduled for every 3 months at a specialized

gastroenterology center for patients with decompensated cirrhosis (a follow-up between 1-3 months may be scheduled if dictated by disease severity or the presence of certain disease specific complications) and for up to 6 months for patients with cirrhosis but without a prior episode of AD. Follow-up period lasted for 5 years, or until death/loss of follow-up. Eighty-two (34%) patients died during follow-up, median time to death was 660 days (IQR: 304-977). In the 181 patients without death occurring, median follow-up lasted 1128 days (IQR: 469-1825). Collected data were transferred and stored in a database. At the end of the study period on December 31, 2013, all clinical data was extracted for further analysis.

Gene analysis of NOD2, TLR2 and TLR4

Genomic DNA was extracted from whole-blood samples using the Gentra Puregene Blood Kit (Qiagen; Hilden, Germany) following the manufacturer's protocol. Three alleles of the *NOD2* gene variants rs2066844, (p.R702W:, NM_022162.2:c.2104C>T), rs2066845 (p.G908R; NM_022162.2:c.2722G>C), and rs2066847 (L1007Pfs; NM_022162.2:c.3019dupC) were genotyped using hybridization probes on fluorescence resonance energy transfer (FRET) on a LightCycler 480 (Roche) real-time PCR system, according to *Ferreiros-Vidal et a*l [17]. Gene variant of *TLR2* gene rs4696480 (NM_003264.4:c.-148+1614T>A) was also genotyped using oligonucleotides according to *Oh et al.* [18]. The gene variant of *TLR4* gene rs4986790 (p.D299G; NM_138554.4:c.896A>G) was genotyped using self-designed amplification oligos (TLR4-D299G F: CATCGTTTGGTTCTGGGAG and TLR4-D299G R: TTTACCCTTTCAATAGTCACACTCA), while FRET oligonucleotides were

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similar to *Hamann et al.* (TLR4-D299G SENS: CTACTACCTCGATGGTATTATTGACTTATT-6FAM, TLR4-D299G ANCH: Cy5.5 -AATTGTTTGACAAATGTTTCTTCATTTTCC-3'phosph) [19]. Representative melting curve genotyping results are shown in the **Supplementary Figure 1**. Genotyping was technically unsuccessful in two patient samples for *NOD2* analysis, and in one sample for *TLR2* and *TLR4* analysis.

Serologic analysis

Serum levels of total bilirubin, creatinine, and albumin, blood cell count and INR were determined by routine laboratory analysis.

Blood samples were obtained at enrollment from each patient and were frozen at -70°C until testing. All the serological assays were performed in a blinded fashion without prior knowledge of the patient's clinical information. Commercially available sandwich enzyme-linked immunosorbent assays (ELISA) were used according to the manufacturer's protocol to determine serologic markers of pathological BT, namely lipopolysaccharide-binding protein (LBP) (Hycult Biotechnology, Uden, Netherlands), endotoxin core IgA antibody (EndoCAb IgA) (Hycult Biotechnology, Uden, Netherlands) and anti-OMP Plus IgA antibody (QUANTA Lite®, Inova Diagnostics, San Diego, CA). EndoCAb directs against a mixture of incomplete endotoxins of 4 different species (*Pseudomonas aeruginosa, Salmonella typhimurium, Escherichia coli* and *Klebsiella aerogenes*), while anti-OMP Plus antibody does to a mixture of multiple bacterial proteins derived from two species of intestinal bacteria (one Gram-positive and one Gram-negative). Cut-off positivity was 195 AU/mL for

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EndoCAb IgA, defined by our group previously [19] as a value exceeding the 95th percentile level of the healthy control group, and 25 U for anti-OMP Plus IgA as recommended by the manufacturer.

Ethical considerations

The study protocol was approved by the Regional and Institutional Research Ethics Committee of University of Debrecen and by the National Scientific and Research Ethics Committee (DEOEC-RKEB/IKEB 5306-9/2011, 3885/2012/EKU [60/PI/2012]). Each patient or legal surrogate was informed of the nature of the study and signed an informed consent form.

Statistical analysis

Variables were tested for normality using Shapiro Wilk's W test. Continuous variables were summarized as means (standard deviation [SD]) or as medians (interquartile range [IQR, lowest 25%-highest 25%]) according to their homogeneity. Categorical variables were compared with Fisher's exact test or χ^2 test with Yates correction, as appropriate. Continuous variables were compared with Mann-Whitney U test or Kruskal-Wallis H test with Dunn's multiple comparison *post hoc* analysis. Allele frequencies of the respective SNPs were tested for deviations from the Hardy–Weinberg equilibrium and then compared for statistical differences with the Cochrane Armitage trend test (Helmholtz Center Munich, http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Kaplan–Meier (KM) analysis was used to calculate the cumulative probability (CP) of adverse outcomes (development of non-SBP BI, SBP, decompensation event and mortality). Right censoring of patients was

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performed in case of transplantation or loss of follow-up as appropriate. Differences in observed probabilities were assessed by the log-rank test. The association between categorical clinical variables, or different PRR genotypes, and adverse disease outcomes during follow-up was assessed by univariate Cox-regression analysis. Multivariate analyses were performed with a forward inclusion procedure and a likelihood ratio test to identify independent predictors. Binary logistic regression was used to assess the infection-related mortality at 28 and 90 days. Associations are given as a hazard ratio [HR] or odds ratio [OR] with 95% confidence intervals [CI]. For statistical analysis and graphical presentation, the SPSS 24.0 [SPSS, Chicago, IL], and GraphPad Prism 6 programs were used. A 2-sided probability value of <0.05 was considered statistically significant.

RESULTS

Genotype distribution of various functional polymorphisms of PRRs in cirrhosis

Frequencies of various PRR genotypes in cirrhosis are summarized in **Table 2**. None of the examined *NOD2*, *TLR2* and *TLR4* gene variants was different between outpatients and patients with AD. Further analysis of clinical and laboratory characteristics of outpatients revealed that age, gender, presence of a co-morbidity or HCC, etiology or severity of cirrhosis was not different across the various PRR genotype subgroups. Co-medications at enrolment comprising the use of proton pump inhibitor (PPI), non-selective beta blocker (NSSB) and secondary antibiotic prophylaxis either norfloxacin for prevention

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of SBP or rifaximin for prevention of HE were also not different among patients with genetic variants of *NOD2*, *TLR2* and *TLR4* and with wilde-type (**Table 1**).

Risk factors of non-SBP type BI

Eighty-five (35.0%) of the included outpatients encountered a non-SBP type BI episode during the follow-up period. The median time to development of a first BI episode was 581 (207-803) days. Urinary tract infection was the most commonly diagnosed BI, and accounted for 43.5% (n=37) of the events. Other sites of BI were as follows: pneumonia (18.8%), erysipelas (10.6%), acute bronchitis (5.9%), cholangitis (3.5%), bacteremia (3.5%), gastroenteritis (1.2%) and unidentified in 9 (10.6%) cases. 2.4% of the cases were multifocal. Microbiological analysis was performed in 35 (41.2%) cases. Bacteria were Gram-negative in 76.5% and Gram-positive in 23.5% of culture positive cases (n=17) (**Supplementary Material**).

Functional polymorphisms of PRR genes

Patients with any risk variants in *NOD2*, *TLR2* or *TLR4* genes did not have an increased cumulative probability of a non-SBP type BI episode during follow-up (**Figure 2A**), not even when stratifying according to presence of ascites (**Figure 2B**). Patients carrying both a *TLR2* variant and at least one *NOD2* risk variant (n=10) had also a similar rate of non-SBP type BI, than patients not carrying both variants ($P_{LogRank}$=0.397). There was no rationale for testing the potential effect of the *TLR4* and *NOD2 variant* combination. Only one patient carried both variant genotypes.

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The presence or absence of a *NOD2*, *TLR2* or *TLR4* variants did not affect the type of pathogen causing BI (Gram-negative or –positive), or the location of BI.

Furthermore, these PRR gene variants were not associated with the risk of mortality during a subsequent bacterial infection (**Supplementary Table 2).**

Clinical co-variates

Of the clinical factors: co-morbidity, PPI use, prior history of a BI episode and advanced disease stage were all associated with an increased cumulative probability of non-SBP type BI episodes during the follow-up period (**Table 3**). Of the patients with a prior history of BI, 66.5%±6.3% (standard error) developed another BI episode, compared to 39.7%±5.1 % of those with no such history ($P_{LogRank}$:<0.001). Regarding advanced disease stage, similar results were found if advanced disease stage was depicted either by the presence of ascites (65.2%±6.6% vs. 42.0%±5.1%, $P_{LogRank}$:<0.001), Child-Pugh stage B/C (68%±6.1% vs. 38.1%±5.2%, $P_{LogRank}$:<0.001) or by decompensated clinical stage (58.8%±5.7% vs. 40.8%±5.8%, $P_{LogRank}$=0.01). The combination of these two relevant clinical factors revealed important findings. First, prior history of a BI episode significantly increased the probability of the subsequent development of another BI event, regardless of disease severity. Furthermore, a prior history of a BI without ascites was associated with the same cumulative probability of a BI occurring, as the presence of ascites without prior history of a BI (57.3%±8.7% and 51.0%±9.9%, respectively). The combined presence of both clinical risk

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factors resulted in an even higher cumulative probability of BI (80.3%±7.7%) (**Figure 3 and Table 3**).

Multivariate analysis

Multivariate Cox-regression analysis and the forward inclusion procedure, taking all significant clinical co-variates of univariate analysis into account, indicated that presence of ascites (HR [95%CI]: 1.71 [1.08-2.7], higher MELD score (1.08 [1.02-1.15]) and prior BI episode (2.02 [1.3-3.14]) were independently associated with the risk of a non-SBP type BI development during follow-up.

Risk factors of SBP

Of the patients with ascites 22.7% (20/88) developed community acquired SBP during the follow-up period. Of the cases with microbiological investigation, 36.4% (4/11) was culture-positive SBP, while 63.6% (7/11) was culture negative. Bacteria were Gram-negative in 75% and Gram-positive in 25% of culture positive cases (**Supplementary Material**). The median time to the development of SBP was 340 (126-662) days. The presence of *NOD2* risk allele variants, but not of *TLR2* and *TLR4 variants,* were associated with an increased cumulative probability of SBP (**Figure 4**). Of the patients with any NOD2 risk variants 76.9%±19.9% developed SBP, compared to 30.9%±6.9% of those with *NOD2* wild type ($P_{LogRank}$=0.047). Patients with or without any *NOD2* risk allele variants had similar MELD scores (median [IQR]: 14 [9-16] vs. 13 [10-15], respectively, P=0.874). Prior SBP episode was also associated with the risk of SBP development ($P_{LogRank}$=0.048).

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Association of functional polymorphisms of PRR genes with serologic markers of BT

Serum level of LBP and frequencies of IgA type antibodies directed against various gut microbial components (anti-OMP Plus and EndoCab) were not different according to the examined PRR genotypes (**Table 4**).

Functional polymorphisms of PRR genes and development of decompensation events

Of the patients with a compensated clinical stage at enrolment 31.4% (38/121) developed any type of decompensation event (ascites, variceal bleeding or hepatic encephalopathy). The median time to the development of a first decompensation was 540 (140-913) days. Neither *NOD2* risk variants ($P_{LogRank}$=0.681) nor *TLR2* and *TLR4* polymorphisms ($P_{LogRank}$= 0.068 and 0.249) were risk factors of clinical decompensation (**Supplementary Figure 2**).

Functional polymorphisms of PRR genes and survival

In the total cohort, liver-related death occurred in 82 (33.7%) subjects. Median time to mortality was 660 (304-977) days. Kaplan-Meier survival analysis demonstrated a significantly worse survival in patients with advanced disease according to presence of ascites ($P_{LogRank}$<0.001), Child-Pugh stage B/C ($P_{LogRank}$<0.001), or decompensated clinical stage ($P_{LogRank}$=0.033) and prior BI episode ($P_{LogRank}$=0.050). Neither NOD2 risk variants ($P_{LogRank}$= 0.785) nor *TLR2* and *TLR4* polymorphisms ($P_{LogRank}$= 0.682 and 0.732) were associated

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with overall survival (**Supplementary Figure 3**).

DISCUSSION

Bacterial infections beyond SBP have significant prognostic implications in patients with cirrhosis [1]. Thus, individual risk stratification for BI is an important clinical issue, and it may be instrumental in identifying high-risk patients amenable to preventive measures and/or closer follow-up strategies as a part of the standard of care. Former clinical studies with functional PRR gene variants in cirrhosis primarily focused on the development of SBP in ascitic patients [8–11]. Distinctly, in the present study we comprehensively assessed the utility of various functional SNPs of three different PRR genes simultaneously in a large prospective cohort, comprising the whole severity spectrum of cirrhosis, with a special emphasis on the development of non-SBP type BI.

In our cohort, the frequencies of PRR gene variants were comparable within other cirrhotic patient cohorts and with healthy Caucasians [20,21].

Similar to most of the previous studies [8–10], the presence of *NOD2* allele variants was a risk factor for SBP in our cohort as well. A recent large association study in patients with decompensated cirrhosis however, did not demonstrate a role of *NOD2* variant in mediating susceptibility for SBP [23]. In our study development of SBP was not more frequent in patients with *TLR2* (-16934 T>A, rs4696480) and *TLR4* (D299G, rs4986790) polymorphisms. This latter finding is a novelty. At the same time, the association of SBP with various *TLR2* genotypes is somewhat controversial in the published literature. In the studies of *Nischalke et al.* [10] and *Lutz et al.* [22] *TLR2* (-16934 TT)

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genotype but not *TLR2* R753Q and P631H mutations were associated with SBP. Contrarily, *Bruns et al.* showed that not *TLR2* (-16934 T>A) but *TLR2* R753Q polymorphism increased the risk of SBP [11]. A limitation of our study compared to previous cohorts, was the relatively lower number of patients with ascites (n=88). The present study did not allow for an in-depth analysis of the association of PRR variants with different types of SBP (culture-negative, culture-positive or bacterascites), which is a drawback. Since there was twenty incident cases of SBP during follow-up and only half of them was cultured.

The PRR gene variants examined in our study were reported to have a special role in susceptibility to bacterial infections and sepsis in patients with acquired immune deficiency (i.e. acute leukaemia or allogeneic stem cell transplantation) [22,23]. Furthermore in critically ill patients, the *NOD2*/*TLR4* combination was associated with higher rate of bacteraemia [24]. In patients with advanced cirrhosis the *TLR4* (D299G, rs4986790) variant was recognized to increase overall BI rates in a single retrospective study (n=111) [25]. At the same time, another *TLR4* variant (c.+1196C/T, rs4986791) did not increase the risk of BI in a large retrospective cohort (n=336), including a validation cohort with same samples size [26]. In our study none of the examined PRR variants were associated with higher risk of non-SBP type BI.

In spite of the known fact that two main members of the PRR family – TLRs and NOD like receptors – act synergistically in the initiation of host innate immune response to BI [27], neither of the *NOD2*, *TLR2* and *TLR4* gene variant combinations showed increased BI susceptibility. The limitation of the present study is that the association of bacterial infections and *TLR4*

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polymorphisms warrants further evaluation in a larger cohort since our study was underpowered to detect such an association at this sample size (**Supplementary Table 1**). The limited number of incident cases the seven different location subgroups did not make possible a more subtle assessment of the potential role of PRR variants in the development of certain types of infection.

The strength of the present study is that the whole disease severity spectrum of cirrhosis was represented, allowing for an in-depth evaluation of the interaction of PRR gene variants and BI development in various disease severity subgroups. Cirrhosis associated immunodeficiency syndrome (CAID) is a dynamic process evolving with the natural history of progression to end stage liver disease [28]. Therefore, the impact of an inherited risk for a BI might be different in early vs. advanced cirrhosis, owing to limited compensatory mechanisms. However, PRR gene variants were not associated with a higher risk of non-SBP type BI in any subgroups of various disease severities. These results confirm that acquired immune deficiency state in cirrhosis is more dominant of a risk factor than the presence of functional genetic polymorphisms in the development of BI.

The most notable discovery of the present study was that a prior episode of BI was a risk factor for the development of a subsequent BI episode. This finding suggests the presence of further persistent host factors that modulate an individual's susceptibility for BI. Interestingly, this association was present in early as well as in advanced disease stages. Remarkably, history of a prior BI episode and an advanced disease stage had similar impacts on the infectious risk.

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Pathological BT is associated with clinically relevant complications in cirrhosis [29]. There is evidence that variants of the *NOD2* gene [30,31], and various *TLR* [32] polymorphisms contribute to BT in patients with Crohn's diseases. Likewise in patients with decompensated cirrhosis an increased translocation of bacterial DNA fragments into ascitic fluid was found in the presence of the *NOD2* risk variant p.G908R [33]. Moreover there was increased transition of pathologic BT to culture-positive SBP in the case of the same *NOD2* variant [34,35]. Furthermore, *TLR2* (-16934 T>A, rs4696480) and *TLR4* (D299G, rs4986790) polymorphisms were associated with an increased systemic antigen burden as well, described by the serum level of lipoteichoic acid, LPS, and bacterial-DNA [32].

In our study we applied both serologic and clinical approaches to assess the impact of PRR genetic variants to BT. First, we examined the effect of *NOD* and *TLR* SNPs on the serological response to BT, but used different serologic markers than in the study of *Piñero et al.* [32]. The frequency of IgA type anti-microbial antibodies and LBP levels in our study did not differ between various PRR genotypes; neither in the entire cohort nor in the subgroup of patients with/ or without ascites. Second, we hypothesised that if PRR genetic variants were linked to BT, they would be associated with enhanced diseases progression, e.g. the advent of first decompensating event, or liver-related death. In accordance with our serologic results, different polymorphisms of the *NOD2* and *TLR2* and *TLR4* genes did not influence these adverse outcomes. It should be pointed out that our analysis is the first to consider the effect of PRR gene variants on the development of a decompensating event in cirrhosis.

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The effect of PRR gene variants on mortality was assessed previously but yielded conflicting results. *Appenrodt et al.* found four-fold increased risk in cirrhotic patients with *NOD2* risk alleles [9]. Concordantly to our findings *Bruns et al.* did not report an increased hazard of death related to the same variants of *NOD2* [8].

In conclusion, we were able to confirm the previous discovery that common *NOD2* gene variants increased the risk of SBP. However, *NOD2* and other SNPs of *TLR2* and *TLR4* did not influence the development of non-SBP type bacterial infections. Disease severity and a prior episode of bacterial infection were highly relevant clinical risk factors for a subsequent episode. PRR gene variants were neither associated with serological markers of bacterial translocation, nor were they associated with the development of clinical decompensation or liver-related death during follow-up. These results suggest a limited value of PRR genotyping in the prediction of a progressive disease course in cirrhosis.

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Table 1. Epidemiological, clinical and laboratory characteristics of outpatients with cirrhosis at enrolment according to different pattern recognition receptors genetic variants

		Total	*NOD2* polymorphism[b]			*TLR2* 16934T>A polymorphism rs4696480				*TLR4* D299G polymorphism rs4986790[x]		
		(N=243)*	Wild type (N=204)	Risk allele (N=37)	P-value	TT (N=64)	TA (N=104)	AA (N=74)	P-value	AA (N=225)	AG (N=17)	P-value
Age, years[a]		56 (50-63)	55 (49-63)	59 (53-65)	0.082	55 (51-63)	57 (50-65)	55 (49-63)	0.172	56 (50-64)	53 (49-56)	0.151
Male sex		52.3% (127)	51.5% (105)	54.1% (20)	0.772	56.3% (36)	54.8% (57)	44.6% (33)	0.299	52.4% (118)	47.1% (8)	0.668
Alcoholic etiology		62.6% (152)	61.3% (125)	67.6% (25)	0.468	65.6% (42)	66.3% (69)	54.1% (40)	0.205	62.7% (141)	58.8% (10)	0.752
Child-Pugh stage	A	56.4% (137)	55.9% (114)	59.5% (22)	0.671	53.1% (34)	53.8% (56)	63.5% (47)	0.194	56.4% (127)	58.8% (10)	0.479
	B	37.9% (92)	37.7% (77)	37.8% (14)		37.5% (24)	43.3% (45)	29.7% (22)		38.2% (86)	29.4% (5)	
	C	5.8% (14)	6.4% (13)	2.7% (1)		9.4% (6)	2.9% (3)	6.8% (5)		5.3% (12)	11.8% (2)	
MELD score[a]		11 (8-14)	11 (8-14)	10 (7-14)	0.249	11 (8-14)	11 (8-14)	11 (8-14)	0.689	11 (8-14)	13 (8-15)	0.513
Ascites		36.2% (88)	36.3% (74)	35.1% (13)	0.894	35.9% (23)	39.4% (41)	31.1% (23)	0.520	35.6% (80)	41.2% (7)	0.641
Decompensated stage		50.2% (122)	49.0% (100)	56.8% (21)	0.386	54.7% (35)	50.0% (52)	45.9% (34)	0.592	49.8% (112)	52.9% (9)	0.801

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Prior VB	24.3% (59)	23.0% (47)	29.7% (11)	0.381	29.7% (19)	21.2% (22)	23.0% (17)	0.440	22.7% (51)	41.2% (7)	0.085
Prior BI	38.7% (94)	37.7% (77)	40.5% (15)	0.747	42.2% (27)	35.6% (37)	39.2% (29)	0.685	37.8% (85)	47.1% (8)	0.448
Prior SBP	9.5% (23)	8.8% (18)	13.5% (5)	0.372	6.3% (4)	13.5% (14)	6.8% (5)	0.189	9.3% (21)	11.8% (2)	0.742
Prior non-SBP type BI	34.2% (83)	34.3% (70)	29.7% (11)	0.587	39.1% (25)	28.8% (30)	36.5% (27)	0.338	33.3% (75)	41.2% (7)	0.510
Comorbidity	53.9% (131)	52.5% (107)	59.5% (22)	0.432	62.5% (40)	53.8% (56)	45.9% (34)	0.151	55.1% (124)	35.3% (6)	0.114
HCC	9.9% (24)	9.3% (19)	13.5% (5)	0.433	10.9% (7)	8.7% (9)	10.8% (8)	0.849	10.2% (23)	5.9% (1)	0.564
Creatinine (μmol/L)[a]	67 (54-84)	66 (55-82)	71 (52-98)	0.513	66 (57-89)	64 (53-83)	70 (56-84)	0.872	66 (54-83)	72 (55-85)	0.680
Bilirubin (μmol/L)[a]	26 (16-41)	27 (16-43)	22 (15-34)	0.278	27 (16-43)	23 (14-43)	28 (17-39)	0.529	26 (16-41)	29 (13-42)	0.693
INR[a]	1.2 (1.1-1.3)	1 (1.1-1.3)	1 (1.1-1.2)	0.074	1 (1.1-1.4)	1 (1.1-1.3)	1 (1.1-1.3)	0.514	1 (1.1-1.3)	1 (1.1-1.4)	0.204
Albumin (g/L)[a]	38 (33-42)	37 (32.5-42)	39 (34-43)	0.332	37 (33-42)	37 (32-42)	38 (34-42)	0.261	37 (33-42)	37 (29.5-44.5)	0.944
Leucocyte (G/L)[a]	5.4 (4.3-7.1)	5.4 (4.3-7.2)	5 (4.1-6.3)	0.246	6 (4.3-7.1)	5 (4.3-7.3)	5 (4-6.9)	0.452	5 (4.3-7.1)	5 (4.1-7.6)	0.868
Platelet (G/L)[a]	116 (76-171)	114 (75-172)	116 (93-158)	0.787	125 (80-170)	112 (76-182.5)	112 (72-163)	0.504	117 (78.5-170)	87 (66-171)	0.258
NSBB use	47.7% (116)	48.0% (98)	43.2% (16)	0.591	53.1% (34)	47.1% (49)	43.2% (32)	0.508	46.7% (105)	58.8% (10)	0.333
PPI use	44.9% (109)	44.6% (91)	43.2% (16)	0.878	46.9% (30)	41.3% (43)	47.3% (35)	0.671	45.3% (102)	35.3% (6)	0.422

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Secondary antibiotic prophylaxis											
Norfloxacin for prevention of SBP	9.5% (23)	8.8% (18)	13.5% (5)	0.372	6.3% (4)	13.5% (14)	6.8% (5)	0.189	9.3% (21)	11.8% (2)	0.742
Rifaximin for prevention of HE	5.8% (14)	5.4% (11)	8.1% (3)	0.457	7.8% (5)	3.8% (4)	6.8% (5)	0.515	5.8% (13)	5.9% (1)	0.986

BI, bacterial infection; HCC, hepatocellular carcinoma; HE: hepatic encephalopathy; INR, international normalized ratio; NSSB, non-selective beta blocker;
PPI, proton pump inhibitor; SBP, spontaneous bacterial peritonitis; VB: variceal bleeding;
[*] *NOD2* genotype were technically unsuccessful in 2 cases, while *TLR2* and *TLR4* for 1 case.
[a] median, IQR (lowest 25%-highest 25%) *p* values were calculated with Mann-Whitney U-test, χ^2-test or Fisher's exact test as appropriate
[b] *NOD2* risk variants were the followings: R702W C>T, rs2066844; G908R G>C, rs2066845 and L1007fsinsC -/C, rs2066847
[x] No cases with GG genotype of *TLR4* D299G polymorphism were detected in outpatients

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Table 2. Genotype distribution of functional polymorphisms of various pattern recognition receptors in patients with cirrhosis

		Total cohort		Outpatients		Acute Decompensation	
		n	%	n	%	n	%
NOD2 L1007fsinsC -/C, rs2066847[a]	-/-	326	93.9%	226	93.8%	100	94.3%
	-/C	21	6.1%	15	6.2%	6	5.7%
NOD2 R702W C>T, rs2066844[a]	CC	318	91.6%	220	91.3%	98	92.5%
	CT	29	8.4%	21	8.7%	8	7.5%
NOD2 G908R G>C, rs2066845[a]	GG	338	97.4%	238	98.8%	100	94.3%
	GC	9	2.6%	3	1.2%	6	5.7%
NOD2 polymorphism[b]	wild type	290	83.6%	204	84.6%	86	81.1%
	variant	57	16.4%	37	15.4%	20	18.9%
TLR2 (-16934T>A) rs4696480	TT	86	24.8%	64	26.4%	22	21.0%
	TA	154	44.4%	104	43.0%	50	47.6%
	AA	107	30.8%	74	30.6%	33	31.4%
TLR4 D299G rs4986790	AA	323	93.1%	225	93.0%	98	93.3%
	AG	23	6.6%	17	7.0%	6	5.7%
	GG	1	0.3%	0	0.0%	1	1.0%

[a] No homozygote mutant was found
[b] 2 patients were compound heterozygotes
NOD2 genotype were technically unsuccessful in 2 cases, while *TLR2* and *TLR4* for 1 case.

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Table 3. Association of clinical factors with the development of non- spontaneous bacterial peritonitis type bacterial infections.

		Non-SBP type BI development				Univariate Cox regression		
		n of subjects	n of events	CP of BI ± SE	P-value*	HR	95%CI	P-value
Total cohort		243	85	49.6±4.1				
Age	<65	198	65	46.1±4.5	0.039	1.02	(1 - 1.04)	0.045
	≥65	45	20	66.9±10.2				
Gender	male	116	39	44.8±5.6	0.486	1.16	(0.76 - 1.78)	0.487
	female	127	46	54.7±6.0				
Comorbidity	absent	112	34	41.5±5.8	0.38	1.58	(1.02 - 2.44)	0.04
	present	131	51	57.8±5.8				
HCC	absent	219	79	49.2±4.2	0.542	1.3	(0.56 – 3)	0.543
	present	24	6	40.8±15.6				
Etiology	other	91	25	40.5±6.8	0.083	1.51	(0.94 - 2.4)	0.085
	alcoholic	152	60	54.6±5.1				
Clinical stage	compensated	121	35	40.8±5.8	0.01	1.76	(1.14 - 2.71)	0.011
	decompensated	122	50	58.8±5.7				
Child-Pugh stage	A	137	38	38.1±5.2	<0.001	2.6	(1.68 - 3.98)	<0.001
	B/C	106	47	68.0±6.1				
Ascites	absent	155	47	42.0±5.1	<0.001	2.11	(1.38 - 3.25)	0.001
	present	88	38	65.2±6.6				
MELD score (per 1 point increase)		-	-	-	-	1.12	(1.06-1.19)	<0.001
Prior BI	absent	149	38	38.5±5.2	<0.001	2.42	(1.58 - 3.72)	<0.001
	present	94	47	66.4±6.2				
Ascites + Prior BI	none	100	24	33.7±6	<0.001	ref.		
	either	104	37	54.6±6.5		1.86	(1.11-3.11)	0.018
	both	39	24	80.3±7.7		4.74	(2.68-8.39)	<0.001
Prior SBP	absent	220	75	49.0±4.3	0.108	1.71	(0.88 - 3.31)	0.112
	present	23	10	52.5±12.0				
Prior non-SBP type BI	absent	160	42	39.7±5.1	<0.001	2.26	(1.48 - 3.46)	<0.001
	present	83	43	66.5±6.3				
NSBB use	no	127	39	44.0±5.7	0.084	1.45	(0.95 - 2.23)	0.086
	yes	116	46	55.9±5.8				
PPI use	no	134	37	40.2±5.5	0.006	1.81	(1.18 - 2.78)	0.007
	yes	109	48	60.5±5.9				
Secondary antibiotic prophylaxis								
Norfloxacin for prevention of SBP	no	220	75	49.0±4.3	0.108	1.71	(0.88 - 3.31)	0.112
	yes	23	10	52.5±12.0				
Rifaximin for prevention of HE	no	229	78	48.3±4.2	0.109	1.86	(0.86-4.04)	0.115
	yes	14	7	80.4±16.1				

**P*-values of the log-rank tests;

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CP, cumulative probability (Kaplan-Meier estimates); CI, confidence interval; HE: hepatic encephalopathy; HR: hazard ratio, BI; bacterial infection; SBP, spontaneous bacterial peritonitis; SE: standard error; HCC, hepatocellular carcinoma, NSBB, non-selective beta blocker; PPI, proton pump inhibitor

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Table 4. Association between serologic markers of bacterial translocation and various pattern recognition receptor genotypes

	NOD2 polymorphism[a]			*TLR2* 16934T>A polymorphism rs4696480				*TLR4* D299G polymorphism rs4986790		
	Wild type (N=204)	Risk allele (N=37)	P-value	TT (N=64)	TA (N=104)	AA (N=74)	P-value	AA (N=225)	AG (N=17)	P-value
EndoCab IgA	50.0% (96)	46.9% (15)	0.743	59.7% (37)	48.5% (47)	42.4% (28)	0.140	49.0% (102)	58.8% (10)	0.438
OMP IgA	60.9% (106)	61.5% (16)	0.952	68.5% (37)	61.8% (55)	53.4% (31)	0.259	61.7% (116)	53.8% (7)	0.574
LBP (mg/L)[b]	17.5 (12.4 - 24.3)	17.9 (12.7 - 34.6)	0.454	17.8 (12.1 - 27)	18.6 (12.8 - 25)	17.1 (12.2 - 27.6)	0.977	17.8 (12.8 - 27)	15.92 (9.4 - 23)	0.378

[a] *NOD2* risk variants were the followings: R702W C>T, rs2066844; G908R G>C, rs2066845 and L1007fsinsC -/C, rs2066847
[b] median, IQR (lowest 25%-highest 25%)

P-values were calculated with Mann-Whitney U-test, χ^2-test or Fisher's exact test as appropriate
NOD2 genotype were technically unsuccessful in 2 cases, while *TLR2* and *TLR4* for 1 case.

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Figure legends

Figure 1. Flowchart of the patients with cirrhosis in the cohort study

AD: acute decompensation

Figure 2. Development of non-spontaneous bacterial peritonitis type bacterial infections according to various pattern recognition genotypes in outpatients. Common *NOD2* risk variants (L1007fsinsC -/C, R702W C>T or G908R G>C) and *TLR2* (-16934T>A) or *TLR4* (D299G) polymorphisms were not associated with the risk of non-SBP type BI development either in the entire cohort (A) or in subgroups according to ascites (B).

Figure 3. Development of non-spontaneous bacterial peritonitis type bacterial infections according to clinical factors in outpatients. Prior history of a bacterial infection significantly increased the probability of the development of another bacterial infection episode during the follow-up independently of disease severity (presence or absence of ascites).

Figure 4. Development of spontaneous bacterial peritonitis according to various pattern recognition receptor genotypes in stable outpatients with cirrhosis. Any *NOD2* risk variants (L1007fsinsC -/C, R702W C>T or G908R G>C) but not the *TLR2* (-16934T>A) or *TLR4* (D299G) polymorphisms were associated with the risk of SBP development.

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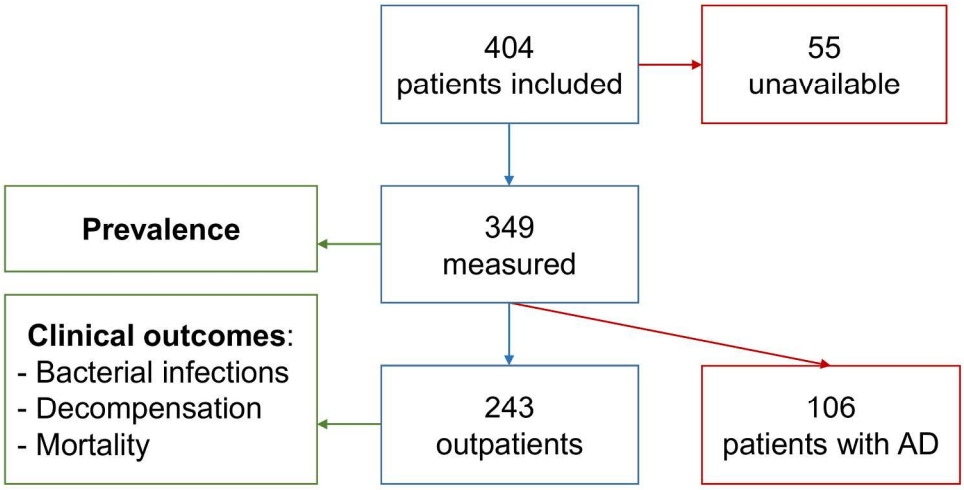

Flowchart of the patients with cirrhosis in the cohort study
AD: acute decompensation

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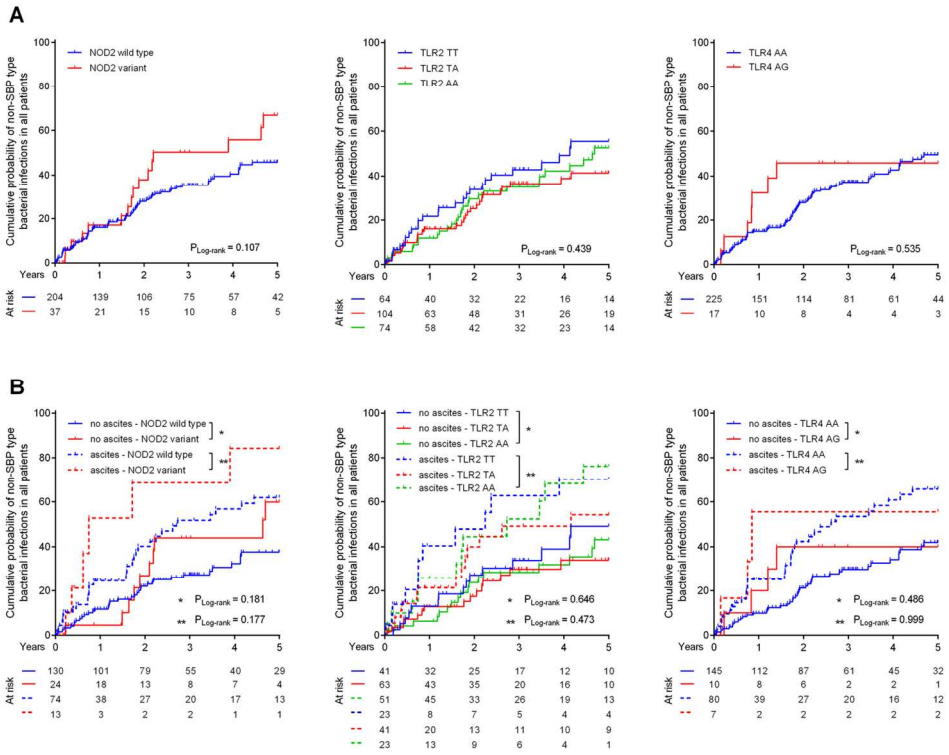

Development of non-spontaneous bacterial peritonitis type bacterial infections according to various pattern recognition genotypes in outpatients. Common NOD2 risk variants (L1007fsinsC -/C, R702W C>T or G908R G>C) and TLR2 (-16934T>A) or TLR4 (D299G) polymorphisms were not associated with the risk of non-SBP type BI development either in the entire cohort (A) or in subgroups according to ascites (B).

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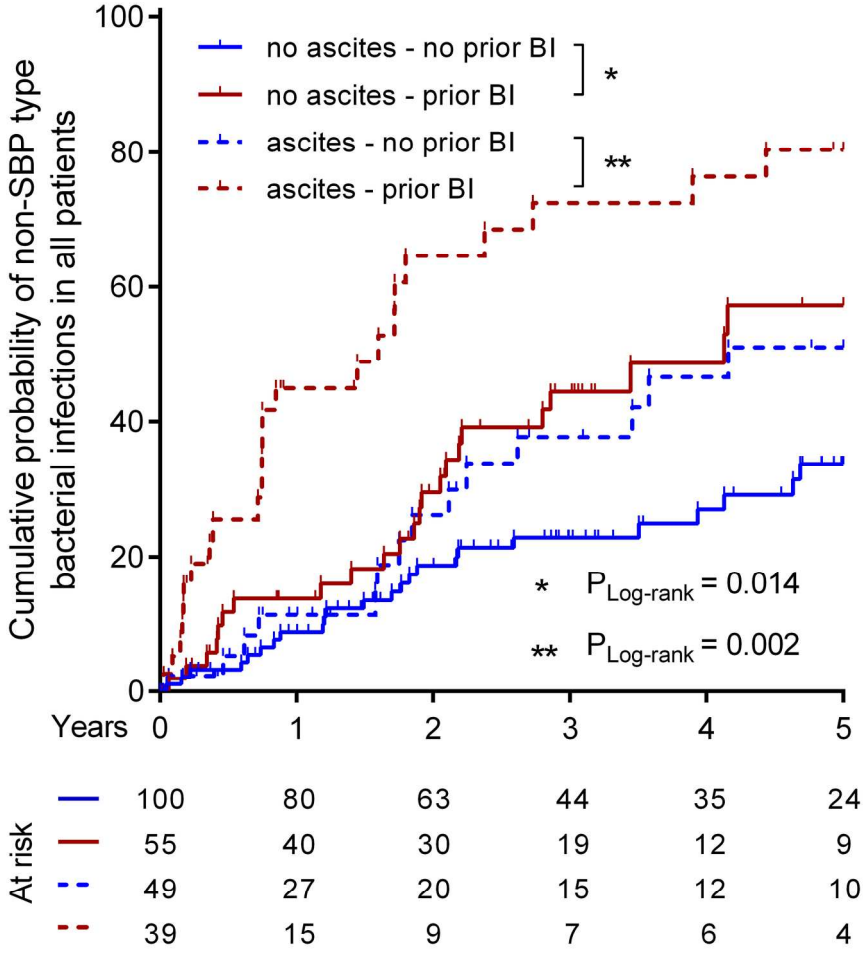

Development of non-spontaneous bacterial peritonitis type bacterial infections according to clinical factors in outpatients. Prior history of a bacterial infection significantly increased the probability of the development of another bacterial infection episode during the follow-up independently of disease severity (presence or absence of ascites).

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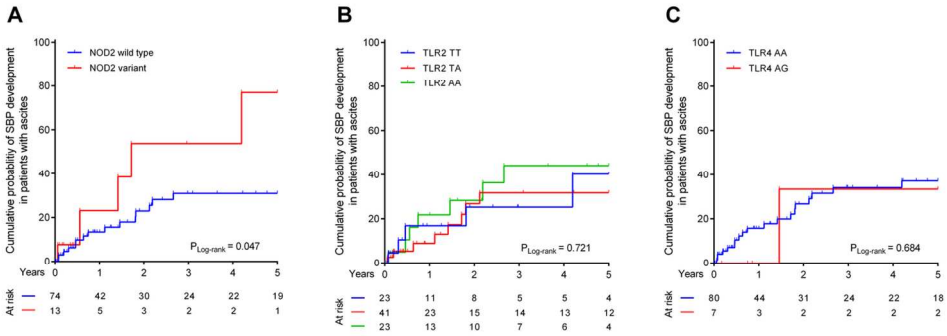

Development of spontaneous bacterial peritonitis according to various pattern recognition receptor genotypes in stable outpatients with cirrhosis. Any NOD2 risk variants (L1007fsinsC -/C, R702W C>T or G908R G>C) but not the TLR2 (-16934T>A) or TLR4 (D299G) polymorphisms were associated with the risk of SBP development.

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