Specific alterations of the intestinal microbiome in autoimmune hepatitis show partially hepatopathy-specific patterns being related to the extent of liver parenchymatous tissue remodeling

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**ABSTRACT**

Background & Aims

Precise aetiology of autoimmune hepatitis (AIH) has not been fully recovered. Besides established concepts of the disease, the intestinal microbiome (IM) is discussed as one considerable resource and modulator of auto-immunologically mediated inflammation. Moreover, the IM has been implicated to be indispensable for immune regulatory mechanisms in general as well as a key player within the close interaction of the gut-liver axis, where bacterial translocation (BT) is considered a crucial component.

In this context, we analyzed the IM in patients with AIH using a next-generation sequencing (NGS) approach, compared these data with healthy individuals as well as alterations in non-AIH hepatopathy controls and finally associating these data with additionally measured markers of BT as well as investigating these data in the context of liver parenchymatous tissue remodeling (LPTR).

Methods

Fresh fecal and EDTA-plasma samples were collected. For detection of BT we measured intestinal fatty acid binding protein (i-FABP), LPS-binding protein (LBP), and soluble CD14 (sCD14). After genomic DNA extraction, IM detection was performed by 16 rRNA-based NGS (Illumina MiSeq platform). Finally, data were studied with state-of-the-art computational biology approaches.

Results

In AIH individuals IM alpha-diversity (detected-(OTUs and Chao-1 index levels) showed significantly decreased levels, being even more distinct in non-AIH hepatopathy controls. Investigating the IM, we were able to detect AIH-specific alterations on class as well as on genus level. In comparison to very parallel findings in the non-AIH hepatopathy control cohort, on class level *Erysipelotrichi* showed contrarious expression patterns with an increase in AIH and a corresponding decrease in non-AIH individuals. On genus level we detected a broad resemblance of alterations between AIH and non-AIH hepatopathy controls. Additionally, *Acinetobacter*, *Butyricimonas*, *Clostridium*, *Dialister*, *Janthinobacterium*, *Lachnospira*, *Lactobacillus*, and *Pseudomonas* showed significant increases in AIH and interestingly corresponding decreases in non-AIH hepatopathy controls. Furthermore, PCoA analysis showed separation of healthy from all hepatopathy samples and proceeding with a constrained approach we were able to detect a clear separation of AIH from healthy but also from non-AIH hepatopathy samples further supporting the significance of our results.

Turning our data perspective referring LPTR, we were able to detect relevant associations with BT as i-FABP, LBP and sCD14 showed proceeding increases dependent on liver fibrosis progression. Moreover, IM alpha diversity markers displayed inverse associations as d-OTUs as well as Chao-1 index levels showed advancing decreases. In line, we found BT markers to be negatively associated with IM alpha diversity alterations. Performing constrained PCoA in this context, we saw a clear separation between different stages of LPTR additionally being clearly marked of from healthy samples.

Conclusion:

For the first time, we were able to detect specific IM alterations in AIH individuals based on a NGS-approach. Compared to alterations of non-AIH hepatopathy controls showing broadly paralled results, we additionally suppose an IM impairement being to some extent related to hepatopathy in general but not only being disease-specific. This is further supported, as we detected IM alterations as well as measured BT to be considerably associated with the corresponding progress of LPTR.

**INTRODUCTION**

Autoimmune hepatitis (AIH) represents a chronically proceeding liver disease with auto-immunologically mediated inflammation of not fully uncovered aetiology (Heneghan et al., 2013; Krawitt, 2006; Manns et al., 2015). Concepts of the disease provenance include a combination of genetic background, environmental factors as well as disturbed self-tolerance mechanisms of both the adaptive and innate immune system (Doherty, 2016). Another hypothesis considers mechanisms of the so-called “molecular mimicry” as one important step in the development of auto-immunity in in general (Albert and Inman, 1999; Wucherpfennig, 2001) but also in the context of AIH (Doherty, 2016). By providing plethoras of potential antigens, the immensely complex ecosystem of the intestinal microbiome (IM) seems to be especially important in this context (Floreani et al., 2016; Proal et al., 2013). Moreover, the relevance of the IM for shaping and modulating immunological mechanisms taking mainly place in the gut and the gut-associated lymphoid tissues (GALT) has achieved fast growing appreciation in recent years (Honda and Littman, 2016; Hooper et al., 2012; Thaiss et al., 2016) and has been addressed to be relevant in the context of AIH (Ma et al., 2015; Trivedi and Adams, 2013).

In patients with end-stage liver disease and associated liver cirrhosis alterations of the IM have already been described (Aly et al., 2016; Bajaj et al., 2014; Chen et al., 2011a; Qin et al., 2014a). Additionally, the so-called gut-liver axis has been defined to characterize the outstanding interaction between these two pathophysiologically relevant compartments (Seo and Shah, 2012; Szabo et al., 2010). Bacterial translocation (BT) – the passover of whole bacteria or bacterial components to extra-intestinal sites – has been shown to be increased in a pathological manner (pathological BT) in patients with liver cirrhosis and is seen to play one key part for immunological interactions in hepatic diseases (Bellot et al., 2013; Giannelli et al., 2014; Seki and Schnabl, 2011; Wiest et al., 2014).

Besides one only cursory study indicating alterations of the IM without using a next-generation sequencing approach and detecting elevated markers of BT being both somehow associated with the degree of liver damage (Lin et al., 2015), to our current knowledge these issues have not been carefully characterized in the setting of AIH, elsewhere. Moreover, in relation to culture- and solely PCR-based approaches, nowadays the complexity and diversity of the human IM could be defined in formerly unknown precision by next-generation sequencing methods such as amplicon pyrosequencing of the bacterial 16S rRNA gene (Huttenhower et al., 2012; Lozupone et al., 2012; Qin et al., 2010). Based on this, we combined a 16S rRNA survey of the IM containing gut microbiota with state-of-the-art computational biology approaches to investigate differences in the structure of fecal microbiota of individuals with AIH comparing to non-AIH hepatopathy controls as well as healthy individuals. Furthermore, we measured established BT markers (Brenchley and Douek, 2012; Koutsounas, 2015; Wang et al., 2015) and tried to link these data with the information of the IM.

Finally, we turned our data perspective and looked at the found alterations in the context of LPTR as there is emerging data of relevant associations in this context (Heidrich et al., 2017; Loomba et al., 2017).

**MATERIAL AND METHODS**

Study cohort and biomaterial sampling

During their clinical stay or outpatient visits, a total of 58 individuals (healthy, n=19; AIH, n=16; and non-AIH hepatopathy controls, n=23; for detailed information see ***Table 1***) were enrolled into this study. Written informed consent was collected of all participants upon enrollment. A detailed questionnaire regarding general clinical and especially specific IM relevant informations (e.g. body-mass-index (BMI) status of antibiotics age status of immuno-suppressive therapy, and proton-pump inhibitor (PPI) medication) was answered of the participants. Individuals who received antibiotics within at least 1 month before study enrollment were excluded.

The design and aim of this study was developed in accordance to and conforms the *Declaration of Helsinki* and has been approved by the local Ethic´s Committee of the Medical Faculty, University Bonn, Germany (internal processing Nr.: 275/13) in front of enrollment.

Of every study participant EDTA blood (for plasma) as well as one fresh fecal sample (max. 6h after defecation; storage at 4°C until freezing) were collected. Plasma (after centrifugation of EDTA blood) and fecal samples were portioned in sterile aliquots and immediately frozen at -80°C (both max. 6h after collection) until thawing only once before analysis.

Evaluation of liver parenchymatous tissue remodeling

The extent of LPTR was carefully assessed based on histological findings, measurements of tissue stiffness (FibroScan® method (Castera et al., 2008)and/or shear-wave elastography), radiological imaging results (sonography, computer and magnetic resonance tomography), and clinical findings (e.g. classical esophageal varices indicating existing liver cirrhosis) – weighting of data significance was applied in the foregoing order as well as referring data were available for each individual case. Four different grades were classified: (i) healthy; hepatopathy (ii) without LPTR, (iii) with related liver fibrosis and (iv) with related liver cirrhosis.

Measuring bacterial translocation markers

Intestinal fatty acid binding protein (i-FABP), LPS-binding protein (LBP) and soluble CD14 (sCD14) were measured using commercially available ELISA-kits detecting the referring human isotypes (Hycult Biotech, Plymouth Meeting, USA). All assays were performed with plasma samples following the manufacturer´s protocol.

Fecal sample processing and gDNA extraction

Fecal aliquots were carefully thawed on ice. Then a small amount of fecal material (~10-20mg) was transferred to a glass beads containing tube of the *tough micro-organism lysing VK05 Kit* (Bertin Corp., Rockville, MD, USA) prefilled with sterile PBS (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Taking into account the importance for reliable and stable detection of especially gram positive bacteria (Santiago et al., 2014), mechanical bead beating was performed on a *Precellys 24 instrument* (Bertin Instruments, Montigny-le-Bretonneux, France) with a multi-step sequence: (i) 5000 oscillations/minute for 75 seconds; (ii) 90 seconds on ice; (iii) another 5000 oscillations/minute for 45 seconds with again following (iv) cooling on ice. Afterwards, RNAse A (Promega, Madison, Wisconsin, USA) was added in a concentration of 5µl/1000µl and after gentle vortexing RNA digestion took effect for 25 minutes at 37°C under strict protection of light. Genomic DNA (gDNA) extraction was performed using a bead-based technology. Therefore, 150µl of each processed sample was transferred to a ready-made cartridge of the *Maxwell® 16 Extraction Kit*; final extraction was performed on a *Maxwell® 16 instrument* (both Promega, Madison, Wisconsin, USA). From the timepoint of sample insertion into the cartridge the extraction protocol was performed following the manufacturer´s instructions. Extracted gDNA was terminally diluted in sterile water (RNAse/DNAse free, part of the kit) and all samples were immediately stored at -20°C until forwarding and analysis.

16S rRNA Amplicon Seq (V4 region)

The 16S rRNA gene was amplified using uniquely barcoded primers flanking the V4 hypervariable regions (515f-806r, barcoded) with fused MiSeq adapters in a 25 μl PCR. We used 0.5μl of each forward and reverse primer (10 μM), 0.5 μl dNTPs (200 μM each), 0.25 μl Phusion Hot Start II High-Fidelity DNA Polymerase (0.5 Us), 5 μl of HF buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1 μl of undiluted DNA. PCRs were conducted with the following cycling conditions (94 °C—180s, 30 × [94 °C—60s, 50 °C—60s, 72 °C—105s], 72 °C—10 min) and checked on a Bioanalyzer 2100 High sensitivity chip. The concentration of the amplicons was measured with the Qubit dsDNA br Assay Kit (Life Technologies GmbH, Darmstadt, Germany). The samples were pooled into approximately equimolar subpools. Sequencing was performed on the Illumina MiSeq platform (San Diego, CA, USA) with v3 chemistry (Paired end, 2x250 cycles).

Availability of data and material

16S sequence data are submitted to the Sequence Read Archive (SRA) and have been assigned accession numbers SAMNXXXXX through SAMNXXXXX (Bioproject PRJNAXXXXX).

16S data analysis

16S data analysis was done using a combination of QIIME 1.9.0 (Caporaso et al., 2010a) and 64-bit usearch version 8.0 (Edgar, 2010). After removal of barcode and primer sequences, reads were truncated to a length of 290bp, and only reads with a quality score Q above 15 and no ambiguous bases were retained for the analysis. 16S rRNA gene sequence reads were subjected to demultiplexing, quality filtering using QIIME. Dereplication was done using usearch. De novo OTU identification and chimera detection was done on the remaining 6.705.959 reads using OUT clustering algorithm of UPARSE (Edgar, 2013) with the ‘minsize’ option set to 2 to remove singletons. Furthermore, we use a reference based approach to remove singletons in usearch using Greengenes (GG) das reference database (version 13.8) (DeSantis et al., 2006) clustered at 97% identity with parameters ‘strand plus’. OTU were renamed and reads were mapped back to OTUs to generate the OTU table with usearch8 with ‘-strand plus’ and ‘-id 0.97’ as options followed by a modified version of uc2otutab.py from drive5. We used ‘biom convert’ script with parameters --table-type="OTU table" --to-json to generate a table in biom format. Finally, we assigned taxonomy using QIIME and the RDP classifier trained on GG clustered at 97% identity to obtain taxonomic classification of the representative sequences.

Diversity analysis

We aligned the representative OTU sequences using PyNAST (Caporaso et al., 2010b) and generated a phylogenetic tree with QIIME. We used QIIME to determine the Shannon (OTU evenness) and the Chao1 (OTU richness) indices as well as the total number of observation on the OTU table. Statistical analysis (ANOVA) was performed in R using aov function and the TukeyHSD function to perform Tukey Honest Significant Differences. All R scripts to reproduce figures are accessible at github.com/hzi-bifo/AIH\_metagenome.

Constrained PcoA

Constrained PCoA was done in R using the capscale and anova.cca function of package VEGAN (Dixon, 2003) on Bray-Curtis (Bray and Curtis, 1957), unweighted Unifrac and weighted normalized Unifrac distance (Lozupone et al., 2011) matrix with 5.000 permutations. Permutational multivariate analysis of variance using distance matrices (ADONIS) was done in R using the adonis function of package VEGAN with 5.000 permutations. In each case we controlled for false discovery rate (FDR) using the Benjamini and Hochberg procedure (Benjamini and Hochberg, 1995) with alpha set to 0.05. Between-class analysis was performed by a principal component analysis using bca function of the ade4 package (Dray and Dufour, 2007) to support the clustering. All R scripts to reproduce figures are accessible at github.com/hzi-bifo/AIH\_metagenome.

Identification of significant different OTUs

After normalization using cumNormStat and cumNorm functions of package metagenomeSeq we fitted a linear model for each OTU or taxa using the lmFit function of the limma R package and perfomed a moderated t-statistics, moderated F-statistics and log-odds of the differential expression by empirical Bayes moderation of the standard errors.

ical bayes statistics for differential expression using the eBayes function of the limma package.  All R scripts to reproduce figures are accessible at github.com/hzi-bifo/AIH\_metagenome.

Statistics on bacterial translocations markers

For calculating statistics and creating figures of BT markers GraphPad PRISM software (GraphPad Software, Inc., La Jolla, CA, USA) has been applied. Differences were seen and indicated statistically significant at reaching P-values <0.05. For differences between groups unpaired, two-sided t-tests were applied. Associations between BT markers and the degree of LPTR was investigated by linear regression.

**RESULTS**

We collected and subjected total DNA of fecal samples from 58 individuals either healthy, AIH and non-AIH hepatopathy (***Table 1***) to selective amplification of the 16S rRNA gene with PCR primers flanking the V4 hypervariable region. After in silico removal of error-containing sequences and chimeras we identified operational taxonomic unites (OTUs) at 97% sequence similarity at the remaining 6,705,959 reads (see Methods).

Taxonomic classification of the OTU resentative sequences to class level highlighted that AIH-specific alterations of the IM are very similar to that found in non-AIH hepatopathy control samples and that all samples are dominated by *Bacteroidia* and and *Closridia* with a mean relative abundance (RA) of 43% and 42%, respectively (***Figure 1 A,B***). By comparing the AIH cohort with the healthy control group we found significant increases in OTUs classified to *Gammaproteobacteria and Bacteroidia* (moderated t test, false discovery rate-adjusted [FDR], P value <0.001, **Figure 1a, Supplementary Table 2**) irrespectively from the cohorts we have tested (AIH, non-AIH hepatopathy control, healthy control). Other members of the IM such as *Verrucomicrobiae* and *Gammaproteobacteria* are significantly enriched in both, the AIH (FDR corrected P values < 0.001) and non-AIH hepatopathy control cohort (FDR corrected P values < 0.05 and < 0.01 with an RA of 4% and 5%, respectively) in respect to the healthy controls (RA 1%) **Figure 1a**, **Supplementary Table 1, 2**). On family level (**Supplementary Table 3**) we found 14 taxa such as *Streptococcaceae*, *Veillonellaceae* and *Prevotellaceae* that are highly significant different in the AIH cohort compared to healthy cohort (FDR corrected P values < 0.001, **Supplementary Table 3, Supplementary Figure 2**). On the same significance level, three taxa namely *Erysipelotrichaceae*, *Ruminococcaceae* and *Lachnospiraceae* are significant when comparing the AIH with non-AIH hepatopathy control samples while 13 taxa are significant for the healthy vs. non-AIH hepatopathy control comparison. Of note, the families *Erysipelotrichaceae*, *Rikenellaceae* and *Clostridiaceae* are significant different when comparing both, AIH vs. healthy samples as well as when comparing non-AIH hep. cohort with the healthy cohort. Taken together, these results highlight a shift in IM composition in AIH and non-AIH hep. diseased patients comparing to healthy individuals with a distinct composition of the AIH cohort compared to other non-AIH hepatopathy dieseases.

To gain insights into the richness of the microbiota of AIH, healthy and non-AIH hepatopathy control samples, we compared the number of detected operational taxonomic units (OTUs), Chao1- and Shannon-index values (Hamady and Knight, 2009; Morgan and Huttenhower, 2012). Observed OTUs as well as Chao1 index values showed a significant reduction of the bacterial diversity in AIH samples (TukeyHSD, FDR corrected P value < 0.05, Figure 1b, Supplementary Figure 1) and non-AIH hepatopathy control samples (P value < 0.001) compared to healthy individuals while AIH samples displayed intermediate index levels between healthy and control samples (***Figure 1 C, Supplementary Figure 1***) indicating a reduced abundance of bacterial taxa in AIH samples and an even more aggravated state in non-AIH hepatopathy controls. For Shannon-index values we could not detect such differences, nor for the AIH, neither for the non-AIH hepatopathy control cohort (***Supplementary Figure 1*** ).

This showed a separation of healthy from AIH and non-AIH- associated microhabitats, but no clear separation between the AIH and non-AIH hepatopathy control cohort (***Figure X***) supporting our findings of broad resemblance of IM alterations between these two cohorts on class, but also to some extent on genus level.

To check whether the differences in composition of the IM communities correlated or are independent from the disease status, we used the OTU count data to construct Bray-Curtis dissimilarity metrics and performed a principal coordinates analysis (PCoA). While healthy controls emerged to be tight clustered, samples of non-AIH hepatopathy were clearly separated by principal component 1 and showed an expanded pattern within the principal companent 2 with a pattern of successive transition corresponding to the degree of LPTR, respectively (see ***Figure 2A***).

Permutational multivariate ANOVA based on distance matrices (ADONIS) revealed a contribution of the disease status (**Figure 2B**) as well as immunosuprressive-dependent contributions to the composition of the IM (**Figure 2C**). We used canonical analysis of principal coordinates (CAP, Anderson and Willis, 2003) to quantify the influence of the disease status to the IM composition. According to this analysis, 5.4% (CI = [4.6%, 6.4%], P (PERMANOVA) < 0.001) of the IM composition variance can be explained by the disease status (AIH, non-hep., healthy).

The constrained ordination showed a clear clustering of the three cohorts (Figure **2B**). Further, we repeated the PARMANOVA and CAP analysis to check for the influence of the hepatopathy status among the samples to the IM composition. This revealed that 7.4% of the total variance (CI=[6.5%, 8.5%], P (PERMANOVA) < 0.002) is explained by the hepatopathy score (list here the for groups). On the constrained ordination, a clear clustering of the samples without hepatopathy and samples with zirrhose can be seen, but no clear clustering between hepatop\_ohne\_veraenderung and mit fibrose .

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Together, these resultus further support the hypothesis that the IM community of AIH patients and the IM of non-AIH hepatopathic patients are colonized by communities with taxonomically distinct profiles.

To identify OTUs responsible for the diversification between the three disease-assciated microhabitsa we employed a linear model analysis to determine bacterial OTUs significantly enriched in either AIH-, non-AIH hep. Control and healthy IM. With this approach we identified three distinct sub-communities (designated *AIH\_OTUs*, *hep\_control\_OTUS*, *healthy\_OTUs*) for the three cohorts (**Figure 3, Database D1**).

In contrary to the similar change pattern of AIH and non-AIH hepatopathy control in comparison to healthy samples, OTUs associated to the *Erysipelotrichi* class showed contrariuos alterations with a significant increase in AIH (p <0.05) and a corresponding decrease in non-AIH hepatopathy controls (p <0.001, both ***Figure 1 C***). In addition to the mentioned alterations within the AIH sub-cohort (see above), OTU-representative sequences associated with *Bacteroidia*, *Epsilonproteobacteria*, *Fusobacteria*, *TM7-3*, and *Verrucomicrobiae* showed no differing expression between non-AIH hepatopathy and healthy controls (***Figure 1 C***).

Moreover, examining changes in overall OTUs associated to the class level without a single-class based perspective (***Figure 1 D***), relative expression of *Gammaproteobacteria* best characterized AIH (**test xx corrected with xx** showing **x %** of d-OTUs/reads, respectively), while *Alphaproteobacteria* do so for healthy (**x %**)and *Verrucomicrobiae* for non-AIH hepatopathy controls (**x %**).

We further examined differences in RA of OTUresentative sequences to the genus level to generate a more distinct picture of AIH-specific alterations as well as discriminating the three cohorts (***Figure 1 E+F***). Here, we found specific alterations which showed a distinct pattern in AIH samples compared to non-AIH hepatopathy controls. In comparison to healthy individuals and in relation to alterations of the non-AIH hepatopathy control cohort, only AIH samples showed alterations with significant increases of *Prevotella*, *Streptococcus* (ZIG mixture model, FDR corrected P value <0.001, respectively), *Veillonella* (p <0.01), *Atopobium*, *Bulleidia*, *Christensella*, *Escherichia*, and *Oribacterium* (p <0.05, respectively) as well as a significant decrease of *Actinomyces* (p <0.01; ***Figure 1 E***).

Moreover, in contrary to the rather similar change patterns on class level (see above), on genus level we additionally detected alterations with an inverse pattern comparing AIH and non-AIH hepatopathy controls with healthy samples. Thus, we saw contrarious changes between AIH and non-AIH hepatopathy in comparison to healthy controls as OTU-representative sequences associated with *Acinetobacter*, *Butyricimonas*, *Clostridium*, *Dialister*, *Janthinobacterium*, *Lachnospira*, *Lactobacillus*, and *Pseudomonas* were all positively correlated with AIH and showed a corresponding decrease in non-AIH hepatopathy control samples (ZIG mixture model, FDR corrected P values of at least <0.05, respectively; ***Figure 1 E***). In addition, in both AIH and non-AIH hepatopathy control samples d-OTU associated with *Defluvitalea and Granulicatella* are significantly increased while those associated with *Ruminococcus*, *Alistipes*, *Bacteroides*, *Butyricicoccus*, *Faecalibacterium*, *Odoribacter*, *Oscillospira*, *Parabacteroides*, *SMB53*, *Sphingomonas*, and *Sutterella* were significantly decreased in comparison to healthy controls (all at least p <0.05, respectively; ***Figure 1 E***).

Again, examining changes in overall OTUs associated to the genus level without a single-genus based perspective (***Figure 1 F)***, we found relative expression of *Haemophilus* (**test xx corrected with xx** showing **x %** of d-OTUss/reads, respectively), [*Ruminococcus*] (**x %**), *Succinivibrio* (**x %**) relating to the class Gammaproteobacteria and Clostridia, respectively characterized best AIH; while *Escherichia* (**x %**), and in a less extent *Veillonella*(**x %**), *Prevotella* (**x %**) relating to the class Gammaproteobacteria, Clostridia and Bacteroidia, respectively did so for non-AIH hepatopathy controls and *Turicibacter*(**x %**), *Phascolarcotobacterium*(**x %**), *Dialister*(**x %**), *Roseburia*(**x %**) relating to the class Bacilli and Clostridia, respectively for healthy individuals.

Beta-diversity analysis showed clear separation of AIH samples:

**Searching for entity-unrelated but generally liver-specific intesinal microbiome alterations in our data set**

Besides the exposed findings between our three sub-cohorts showing specific alterations in the AIH sub-cohort as well as separating these samples from the non-AIH hepatopathy control cohort, we recognized that there might be alterations of the IM with a liver-disease specific pattern in both AIH and non-AIH hepatopathy control samples. Based on this, we further asked if there exists influence of liver-disease specific parameters on the IM in general, at least partially irrespective of the causing liver disease entity. Therefore, we assessed the corresponding liver-parenchymatous status of each individual included in the study and classified them regarding the respective degree of existing LPTR, grading healthy and hepatopathy (i) without change, (ii) with related liver fibrosis, (iii) with related liver cirrhosis (see ***Material and Methods***, above). Moreover, as BT has been associated with alterations of the IM as well as it has been clearly defined to play a pivotal role in the immunological interaction of the so-called gut-liver axis (Bellot et al., 2013; Giannelli et al., 2014; Seki and Schnabl, 2011; Wang et al., 2015; Wiest et al., 2014, 2017), we measured markers of BT and tried to integrate these data with the found IM alpha diversity alterations.

Bacterial translocation markers are dependent on the status of LPTR:

We detected elevated levels of the examined BT markers in AIH as well as in non-AIH hepatopathy controls (***Figure 2 A***). Levels of sCD14 (applying unpaired t-tests with a two-tailed P-value, repectively; p <0.01) and i-FABP (p <0.05) were increased in liver cirrhosis individuals, whereas LBP (p = NS) showed only a trend. Regarding the related remodeling status, expression of i-FABP displayed only elevated levels in liver cirrhosis and seems to be of a different pattern in comparison to LBP and sCD14. Correspondingly, LBP and sCD14 exhibited elevated levels also in lower stages of LPTR with sCD14 (p <0.01) and LBP (p <0.05) being both significantly elevated in individuals with liver fibrosis and sCD14 even showing a trend towards elevation in individuals with hepatopathy but without existing parenchymatous tissue remodeling (p = NS). A suggested trend towards a decrease of LBP levels in individuals with liver cirrhosis in comparison to individuals with liver fibrosis seems to be affected specifically by AIH samples displaying lower LBP levels than non-AIH hepatopathy controls (p = NS; ***SUPP-1***). By investigating relevant associations (***Figure 2 B***), we were able to detect linear regression of sCD14 (r2 = 0.18, p < 0.01) as well as i-FABP levels (r2 = 0.18, p < 0.01) with corresponding degrees of LPTR; wheras LBP levels did not show such associations (r2 = 0.08, p = NS; data not shown).

Detected alterations of the intestinal microbiome showed associations with the degree of LPTR:

We found that the different degrees of LPTR were inversely associated with the measured IM alpha-diversity parameters (***Figure 2 C***). Thus, compared to healthy controls, individuals with hepatopathy and liver fibrosis (TukeyHSD, FDR corrected P value <0.05) as well as with liver cirrhosis (p <0.001) showed significantly decreased levels for both d-OTUs and Chao1-index values. In line with the found alterations of AIH and non-AIH hepatopathy control samples, for Shannon-index values we could not detect such associations for the different degrees of LPTR (not significant,; data not shown).

Markers of bacterial translocation are associated with intestinal microbiome alpha diversity:

By further investigating BT markers in the context of IM alpha diversity (***Figure 2 D***) we found a strong inverse correlation between especially sCD14 levels with both d-OTUs (R = -0.52, p < 0.001) as well as Chao1-index values (R = -0.49, p < 0.001) within the complete cohort, but also within the AIH sub-cohort (d-OTUs: R = -0.60, p < 0.05; Chao-1: R = -0.55, p < 0.05). For i-FABP levels we also detected such inverse association with d-OTUs (R: -0.28, p = 0.05) and with Chao-1 index values (R: -0.31, p < 0.05). For sCD14 levels we could also find relevant correlation with shannon index levels but only within the AIH sub-cohort (R: -0.53, p < 0.05). For LBP levels we could not detect such associations, neither within the complete cohort nor within both sub-cohorts (data not shown).

Beta diversity of the intestinal microbiome shows clear differences between different stages of LPTR and healthy samples:

By again investigating IM beta diversity applying constrained PCoA, we were able to detect a clear separation of the different stages of LPTR and non-hepatopathy healthy controls (7.4% of variance, p < 0.001, 95% CI = 6.5%, 8.5%; see ***Figure 2 E***).

**DISCUSSION**

Examining patients with AIH and comparing these results with data of non-AIH hepatopathy as well as healthy controls, we were able to detect (i) AIH-specific alterations of the IM, but (ii) also a broad pattern of variation in a liver-disease specific aspect, with the latter reflecting known associations of elevated markers of BT with progressed liver diseases in general (Giannelli et al., 2014; Gómez-Hurtado et al., 2016; Hackstein et al., 2016; Koutsounas, 2015; Tilg et al., 2016; Wiest et al., 2014, 2017).

First, we detected reduced markers of IM alpha-diversity in AIH samples in comparison to healthy controls, with decreased numbers of both d-OTUs as well as Chao1-index values strongly indicating a diminished alpha-diversity of the IM in AIH, which has also been assumed before by Lin and coworkers (Lin et al., 2015), but based on data of a far less detailed level of IM examination and not on NGS as it has been applied in our study. Next, non-AIH hepatopathy controls of our cohort showed alterations in the same characteristic but in an even more intensified manner. This is in line with former findings (Aly et al., 2016; Bajaj et al., 2014; Chen et al., 2011a) further supporting an association of liver diseases with an anyway pronounced IM dysbiosis usually resulting in decreased alpha-diversity levels (Tilg et al., 2016). Aggravation of IM alpha-diversity decrease in individuals of our hepatopathy control cohort could be additionally influenced by the fact, that this sub-group consists of a higher proportion of individuals with liver cirrhosis (16/23 vs. 5/16 in the AIH cohort).

Onwards, by examining IM alterations on the class level, we observed significant differences in class RAs of AIH samples in comparison to healthy controls, what to our knowledge have not been described using a NGS approach, before. Lin and coworkers investigated IM alterations in AIH individuals, but used realtime (q)PCR measurements of 4 different bacterial classes with specific 16S rDNA primers, respectively (Lin et al., 2015). In our data set, we were able to detect Erysipelotrichi belonging to the Firmicutes phylum to be contrariously changed in comparison to healthy contols, with an increase in AIH and a decrease in non-AIH hepatopathy control samples. Interestingly, Tomas and coworkers saw over-representation of Erysipelotrichi to be associated with decreased mucosal barrier functions of the small intestine in mice (Tomas et al., 2016). This could maybe relevant in the context of AIH immuno-pathology as disease-relevant leucocyte priming as well as associated host-microbe interactions have been postulated to take particularly place within the mucosa and the GALT of the small intestine (Trivedi and Adams, 2013). In line with this, Lin et al. also detected decreased integrity of tigh-junctions within the duodenum of AIH individuals (Lin et al., 2015). Overall, in comparison to healthy and non-AIH hepatopathy controls, over-representation of Gammaproteobacteria was somehow characteristic for AIH samples on class level in our cohort. In comparison to the unveiled specific changes in AIH samples of our study, we saw a rather similar pattern of RA differences on class level in the examined non-AIH hepatopathy controls. Having limitations of comparability between different approaches of IM detection in mind (Hamady and Knight, 2009; Morgan and Huttenhower, 2012; Tyler et al., 2014), our data in the non-AIH hepatopathy control sub-cohort is nicely supported by rather likewise results of (i) Chen and coworkers (Chen et al., 2011a) as well as (ii) Qin et al. (Qin et al., 2014a) as patterns of IM alterations fitted nicely in this frame. Both investigated IM alterations in the context of liver cirrhosis in patients with different entities of liver damage using (i) a combination of qPCR and 16S rRNA pyrosequencing as well as (ii) a metagenomic approach. This could further underpin the robustness of our data.

Proceeding to the genus level, we could specifically detect *Prevotella*, *Veillonella*, *Atopobium*, *Bulleidia*, *Christensella*, *Escherichia*, *Oribacterium* and *Streptococcus* to be increased as well as *Actinomyces* to be decreased in AIH samples, while these genus showed no alterations in the non-AIH hepatopathy cohort. Moreover, in the AIH cohort alterations of the IM on genus level in general showed parralel as well as contrarious patterns compared to alterations found in non-AIH hepatopathy controls. Accordingly, *Acinetobacter*, *Butyricimonas*, *Clostridium*, *Dialister*, *Janthinobacterium*, *Lachnospira*, *Lactobacillus*, and *Pseudomonas* showed contrarious changes between AIH and non-AIH hepatopathy controls in comparison to healthy samples. Interestingly, all of these contrariously expressed genus displayed an increase in AIH while being decreased in non-AIH hepatopathy controls in comparison to healthy samples. In addition, by investigating relative changes on genus level within the whole cohort, AIH samples were found to be characterized by over-representation of *Haemophilus*, [*Ruminococcus*], and *Succinivibrio* further supporting our finding of Gammaproteobacteria over-representation in AIH on class level. Over-representation of *Veillonella*, *Prevotella*, and *Escherichia* belonging to the class of Clostridia, Bacteroidia, and Gammaproteobacteria, respectively, in the non-AIH hepatopathy control group again nicely fits with as well as supports former data in this context (Chen et al., 2011a; Qin et al., 2014a; Tyler et al., 2014). Together, these findings suggests that found relevant and AIH-specific alterations of the IM on genus level. On the other side, the overall pattern of IM alterations additionally seems to display hepatopathy-specific alterations within an aspect of IM dysbiosis showing proceeding decreases in line with hepatopathy progression more than with disease-specificity. This further rises the question for the influence of liver-disease specific parameters on the IM in general, at least partially irrespective of the causing liver disease entity.

Our findings in the PCoA analyses showing a clear separation of the three sub-cohorts in the constrained version further supports our findings of relevant and cohort-specific alterations of the IM in AIH in comparison to non-AIH hepatopathy control but also healthy samples. Moreover, the non-constrained version showed a separation of healthy samples from AIH and non-AIH controls, but no such clear separation between the AIH and non-AIH hepatopathy control sub-cohort as was found in the constrained version. This could deliver further clues that there are other factors than the specific liver disease (e.g. the status of LPTR) influencing the IM in hepatopathy. In line with this, very recently Loomba et al. could find IM signatures to specifically detect advanced liver fibrosis in individuals with Non-alcoholic fatty liver disease (NAFLD) (Loomba et al., 2017). In addition, Heidrich et al. found specific alterations of the IM in individuals with hepatitis C virus (HCV) depending on the corresponding degree of liver fibrosis stages (Heidrich et al., 2017). Moreover, the latter work could also see negative correlation of alpha and beta diversity levels of the IM with measurements of liver parencyhamtous tissue remodeling progression.

Further asking if there are hints for entity-unrelated but liver-disease specific alterations of the IM in general and by examining the influence of liver-disease specific parameters, we also evaluated the found IM changes within our data set in the context of LPTR. Here, we were able to find BT markers to be dependent on the status of LPTR with elevation of i-FABP levels only in cirrhosis, but LBP and especially sCD14 levels showed also increased values in earlier stages of the liver tissue remodeling process. Moreover, we could detect linear regression between sCD14 but also i-FABP levels with the progression of liver fibrosis. An association between liver diseases and elevated markers of BT has been revealed before as well as it is acknowledged as a relevant aspect in etiology, immunological interactions and infectious complications of liver diseases in general (Giannelli et al., 2014; Gómez-Hurtado, 2014; Sacchi et al., 2015; Szabo et al., 2010; Tilg et al., 2016; Wiest et al., 2014). The found difference in the pattern of i-FABP and LBP as well sCD14 levels could be explained by the fact, that i-FABP has been associated with a real gut-barrier dysintegrity (Piton and Capellier, 2016), whereas LBP and sCD14 are related to the LPS-signalling cascade. Thus especially LBP, as an acute phase protein, might more likely reflect increased immunological and inflammatory states (Zweigner et al., 2006). This is further supported by our finding of again decreasing levels of LBP in our AIH patients with liver cirrhosis receiving immuno-suppressive therapy in a higher amount than cirrhotic individuals of the non-AIH hepatopathy control cohort (10/16 vs. 3/23 patients; ***Table 1***). Based on this point of view, we additionally could not fully rule out that some of the IM alterations in the AIH sub-cohort were somehow influenced by the action of immuno-suppressive therapy, as there exist very limited murine data of such effects (Viaud et al., 2013; Xu and Zhang, 2015). Contrariously to this speculation and aside of the stated AIH-specific alterations, our AIH subgroup in general broadly corresponded with referring IM alterations of the non-AIH hepatopathy control sub-cohort what might devitalize relevant interactions.

By further examining the found alterations of the IM in reference to liver corresponding parenchymatous tissue remodeling, we detected parameters of alpha-diversity being highly associated. So, we found both Chao-1 index as well as d-OTU levels to be progressively decreased with advancing states of LPTR. These findings are paralleled by very recent results of Heidrich et al. showing comparable associations in patients with HCV infection (Heidrich et al., 2017). By further investigating relevant associations with BT markers, we also detected levels of sCD14 as well as i-FABP to be negatively correlated with IM alpha diversity markers within our complete study cohort. This association hints towards potentially relevant interactions between the IM and immune-mechanisms in liver diseases, also adding a new aspects to the known relevance of pathological BT in this context (Bellot et al., 2013; Brenchley and Douek, 2012; Giannelli et al., 2014; Gómez-Hurtado et al., 2016, 2016; Wiest et al., 2014). Besides, performing also constrained PCoA analysis, we saw a clear separation of healthy from all hepatopathy samples, irrespective of their disease entitiy. Interestingly, hepatopathy samples displayed a successive transition pattern corresponding to their degree of LPTR from hepatopathy without, to hepatopathy with fibrosis, to hepatopathy with cirrhosis. This suggests potentially relevant relationships between an advancing dysbiosis of the IM in general (in our case referring to IM alpha- and beta diversity) and generally called dysbiosis (Gorham and Gleeson, 2016) with progressive LPTR states. As our data nicely fits as well as it adds more body to very recently found interactions in this context showing LPTR-related IM alterations in NAFLD and HCV infected individuals (Heidrich et al., 2017; Loomba et al., 2017), this might be at least partially irrespective of the causing damage to the liver. Moreover, one might speculate, this could be more likely connected to pathophysiological changes in the gut-liver axis in general. Our findings of BT-related associations in this context further support the relevance of pathological BT. In this regard, also other consequences of LPTR (e.g. portal hypertension) could be relevant. Moreover, this is of interest, as besides very recent and limited data (Heidrich et al., 2017; Loomba et al., 2017), the topic of IM alterations has not really been properly investigated in this context before and is only starting to be recognized. Furthermore, almost all existing data of IM alterations and LPTR depend on results of patients with liver cirrhosis (Aly et al., 2016; Bajaj et al., 2014; Chen et al., 2011b; Gorham and Gleeson, 2016, 2016; Qin et al., 2014b). Moreover, former existing data of IM alterations regarding the generation of and effects on liver fibrosis until now has almost exclusively been relying on animal experiments (Macpherson et al., 2016; Tilg et al., 2016). If this, admittedly speculative, drafted association of IM alterations and BT in the context of LPTR represents a relevant contributor or only displays an epiphenoma still remains unexplained and will be an issue for further and much-needed research in this context (Wiest et al., 2017).

**CONCLUSION**

We were able to detect specific alterations of the IM (alpha-, and beta-diversity as well as on class and genus level) in patients with AIH, also in delineation of existing and already known alterations in hepatopathy controls of non-AIH etiology. As our data of non-AIH hepatopathy controls nicely fits into known alterations, our specific AIH-based data can only serve as a first spotlight in this context but adds important body to very sparse evidence in the field being additionally not based on a NGS approach as it did our work (Lin et al., 2015). Moreover, our results could serve as a starting point for further IM-based research in the field of AIH. Here, pathophysiologically relevant (e.g. induction and regulation of autoimmunological processes, immuno-regulation, generation of impairment mechanisms etc.) as well as diagnostical information (e.g. diagnosis classifier, evaluation of disease severity etc.) could result from further research in this context. Moreover, this might help to close existing gaps in the understanding of AIH generation as well disease-modulating factors helping to discover new therapeutic as well as preventive approaches. Especially in an immunological context, our data could serve as a fruitful starting point for further identification of IM-specific immuno-modulatory mechanisms in AIH (Ahern et al., 2014; Geva-Zatorsky et al., 2017). The additionally unveiled relation between markers of BT and IM alterations further fosters the importance and relevance of the gut-liver axis in hepatopathies in general. Additionally these findings encourage more research to better understand relevant connections in this context as well as to recover the therapeutic potential by modulating these interactions (Wiest et al., 2017).

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**FIGURE / TABLE LEGENS**

**Figure 1: (A)** Average relative abundance (RA) in percent and SD of the top 5 most represented phyla on class level as revealed by the 16S rRNA gene ribotyping. Stars indicate FDR corrected significance level of fecal microbiota composition by disease status (AIH: black; non-AIH hepatopathy controls; grey; healthy individuals: green). **(B)** ternary plot of all OTUs detected in the data set with RA > 1% in at least one sample; each circle represents one class; the size of each circle represents its relative abundance of the OTUs associated with this class; the position of each circle is determined by the contribution of the indicated disease status (AIH, non-AIH hepatopathy controls, healthy) to the total relative abundance; **(C)** mean and SD of alpha diversity of fecal microbiota composition by disease status (AIH: black; non-AIH hepatopathy controls; grey; healthy individuals: green);

**Figure 2**: **(A)** PCoA analysis (Bray-Curtis dissimilarity) indicating disease status (AIH: black; non-AIH hepatopathy controls; grey; healthy individuals: green); **(B)** PCoA of Bray-Curtis dissimilarities constrained by disease status (AIH: black; non-AIH hepatopathy controls; grey; healthy individuals: green) with explained variance of 5.4 (P < 0.001 based on PERMANOVA analysis) **C)** PCoA of Bray-Curtis dissimilarities constrained by hepathopathy status.

**Figure 3:** Tenary plot of all OTUs (lgiht grey) detected in dataset with RA > 1%. OTUs that are not present in at least 5 sample are not shown. Each circle represents one OTU. The size of the circle is determined by the contribution of the disease status (AIH, non-AIH hepatopathy controls, healthy controls) to the total relative abundance. Black colored circles mark OTUs that are significantly enriched in the AIH cohort (AIH\_OTUs, P value < 0.05); green colored cirles mark OTUs that are significantly enriched in the healthy cohort (healthy\_OTUs, P value < 0.05); dark grey colored cirlces mark OTUs that are significantly enriched in the non-AIH hepatopathy control cohort (hep\_control\_OTUs, P value < 0.05). Circle diagrams show taxnomic annotations of these three OTU datasets. **Supplementary Figure 1:** mean and SD of alpha diversity of fecal microbiota composition by disease status (AIH: black; non-AIH hepatopathy controls; grey; healthy individuals: green); **(A)** for observed OTUs **(B)** for Shannon index

**Supplementary Figure 2:** Average relative abundance (RA) in percent and SD of the top 7 most represented phyla on family level as revealed by the 16S rRNA gene ribotyping.

**Supplementary Figure 3:** Average relative abundance (RA) in percent and SD of the top 8 most represented phyla on genus level as revealed by the 16S rRNA gene ribotyping. **Supplementary Database 1:** OTUs singificantly enrichted in one of the tree cohorts designated AIH\_OTUs, hep\_control\_OTUs and healthy\_OTUs with annotation down to species level.**Supplementary Table 1**: mean RA of IM composition of three cohorts on class level.

**Supplementary Table 2:** FDR corrected P value on class level of cohort comparisions (1) helathy vs. non-AIH hepatopathy control; (2) AIH vs. health; (3) AIH vs. non-AIH hepatopathy control

**Supplementary Table 3:** FDR corrected P value on family level of cohort comparisions (1) helathy vs. non-AIH hepatopathy control; (2) AIH vs. health; (3) AIH vs. non-AIH hepatopathy control**Supplementary Table 4:** FDR corrected P value on genus level of cohort comparisions (1) helathy vs. non-AIH hepatopathy control; (2) AIH vs. health; (3) AIH vs. non-AIH hepatopathy control

Table 1:

x