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Journal

Gut, 74(1)

Authors

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Publication Date

2024-12-10

DOI

10.1136/gutjnl-2024-332730

Peer reviewed

HHS Public Access

Author manuscript

Gut. Author manuscript; available in PMC 2024 December 11.

Published in final edited form as:

Gut. ; 74(1): 103–115. doi:10.1136/gutjnl-2024-332730.

Fecal proteomics links neutrophil degranulation with mortality in patients with alcohol-associated hepatitis

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H.K. was responsible for processing of fecal samples, data analysis, and writing the manuscript; M.F.F. provided assistance processing fecal samples. C.G.G. and D.J.G. performed proteomics; C.L.H., P.H. and X.Z. provided assistance with statistical analysis; P.S., F.B., E.C.V., J.G.A., R.S.B., V.V., J.A., J.C., D.L.S., A.L., M.R.L., P.M., G.G., R.B. and AlcHepNet investigators (see Supplementary Material) were responsible for collection of fecal samples; B.S. was responsible for study concept and design, interpretation of data, editing the manuscript, and study supervision.

Conflicts of interest: B.S. has been consulting for Ferring Research Institute, HOST Therabiomics, Intercept Pharmaceuticals, Mabwell Therapeutics, Patara Pharmaceuticals, Surrozen and Takeda. B.S.'s institution UC San Diego has received research support from Axial Biotherapeutics, BiomX, ChromoLogic, CymaBay Therapeutics, NGM Biopharmaceuticals, Prodigy Biotech and Synlogic Operating Company. B.S. is founder of Nterica Bio. UC San Diego has filed several patents with C.L.H. and B.S. as inventors related to this work. D.L.S. has consulted for EnteroBiotix and delivered paid lectures for Norgine. J.G.A. received grants from Cook and Gilead (paid to the University of Alberta) and received consulting fees from Boehringer Ingelheim, AstraZeneca, Advanz and 89Bio.

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Abstract

Objective—Patients with alcohol-associated hepatitis (AH) have a high mortality. Alcohol exacerbates liver damage by inducing gut dysbiosis, bacterial translocation and inflammation, which is characterized by increased numbers of circulating and hepatic neutrophils.

Design—In this study, we performed Tandem Mass Tag (TMT) proteomics to analyze proteins in the feces of controls (n=19), patients with alcohol-use disorder (AUD; n=20) and AH (n=80) from a multicenter cohort (InTeam). To identify protein groups that are disproportionately represented, we conducted Overrepresentation Analysis using Reactome pathway analysis and gene ontology to determine the proteins with the most significant impact. A fecal biomarker and its prognostic effect were validated by ELISA in fecal samples from patients with AH (n=70), who were recruited in a second and independent multicenter cohort (AlcHepNet).

Result—Fecal proteomic profiles were overall significantly different between controls, patients with AUD and AH (Principal Component Analysis $P = 0.001$, dissimilarity index calculated by the method of Bray-Curtis). Proteins that showed notable differences across all three groups and displayed a progressive increase in accordance with the severity of alcohol-associated liver disease were predominantly those located in neutrophil granules. Overrepresentation and Reactome analysis confirmed that differentially regulated proteins are part of granules in neutrophils and the neutrophil degranulation pathway. Myeloperoxidase (MPO), the marker protein of neutrophil

granules, correlates with disease severity and predicts 60-day mortality. Using an independent validation cohort, we confirmed that fecal MPO-levels can predict short-term survival at 60 days.

Conclusions—We found an increased abundance of fecal proteins linked to neutrophil degranulation in patients with AH, which is predictive of short-term survival and could serve as a prognostic noninvasive marker.

Keywords

microbiome; microbiota; metagenomics; alcoholic liver disease

Introduction

Alcohol-associated liver disease is the leading cause for liver-transplantation in non-hepatocellular carcinoma patients in the United States [1] and the primary cause of liver-related mortality worldwide [2]. The clinical spectrum ranges from alcohol-associated fatty liver disease, steatohepatitis with fibrosis and cirrhosis to alcohol-associated hepatitis (AH), which 10–20% of patients with alcohol use disorder (AUD) develop [3]. Patients with AH present with rapid onset of jaundice and frequently have bacterial infections, liver- and multiorgan failure [4, 5]. Transplant free survival is low with survival rates of 81.1–94.6% at 28 days and 66–64.3% at 90 days [6].

Besides abstinence, the therapy of AH involves suppression of the immune system using steroids, which improve short-term, but not long-term survival [5, 7]. Early liver transplantation is beneficial in patients with severe AH, but is available only for few and highly selected patients with AH [8]. It remains a clinical challenge to identify patients at risk who would benefit from more intense therapy. Several scores have been published to predict short term mortality with the Model for End-Stage Liver Disease (MELD) score and MELD-Sodium (MELD-Na) showing highest accuracy [9, 10].

Alcohol is associated with gut dysbiosis including changes in bacteria, viruses and fungi [11–13]. In addition to directly damaging the liver, alcohol consumption activates macrophages and monocytes in the jejunum and increases tumor necrosis factor (TNF) production resulting in intestinal inflammation which initiates the process of epithelial barrier dysfunction [14]. This leads to translocation of microbial associated molecular patterns (MAMPs) which aggravates the systemic immune response and inflammation in the liver [5]. Alcohol and ischemic injury or trauma increase neutrophil migration to the intestine in animal models [15, 16]. Inflammation in AH patients is associated with increased numbers of circulating and hepatic neutrophils. Neutrophils are crucial innate immune responders and the first actors to fight pathogens, they produce antimicrobial peptides, reactive oxygen species, cytokines and other inflammatory mediators [17]. It is controversial if neutrophil hyperactivation aggravates liver damage or if they are beneficial for the resolution of AH [18, 19].

By performing high-quantitative fidelity Tandem Mass Tag (TMT) proteomics analysis using LC/MS analysis, we aimed to characterize the fecal proteome in patients with AH and

AUD and to elucidate its clinical importance. We correlated proteins with disease severity and outcome to determine new prognostic biomarkers.

Results

The fecal proteome is different between controls, patients with AUD and AH

To assess alterations in the fecal proteome among individuals with alcohol-associated liver disease, we performed TMT-based proteomics on stool samples from a cohort of 80 patients diagnosed with alcohol-associated hepatitis (AH), along with 20 patients diagnosed with alcohol use disorder (AUD), and compared their profiles. These profiles were further compared against an already published dataset of controls [20]. Patients with AH were enrolled through the InTeam multicenter cohort. Patients with AH displayed lower levels of serum albumin, and elevated levels of international normalized ratio (INR), bilirubin, and alkaline phosphatase (ALP), indicating compromised liver function (Table 1). The median Model for End-Stage Liver Disease (MELD) score among AH patients was 23.9 and the observed 60-day and 180-day mortality rate were 24% and 29%, respectively (Table 1).

The fecal host proteome showed significant differences between all three groups following PCA using a permutational multivariate analysis of variance (Permanova) [21]. AH and AUD groups exhibited a substantial separation from the healthy control group (adjusted $P = 0.001$), but no significant difference could be seen between AH and AUD (adjusted $P = 0.144$) (Fig. 1A, B). 51% and 42% of patients with AH received antibiotics and steroids, respectively (Table 1). No difference was found in the fecal proteome of patients with AH receiving antibiotics or not (Suppl. Fig. 1A), or patients with AH receiving steroids or not (Suppl. Fig. 1B).

To better characterize the proteins responsible for the separation in the PCA, we first identified all proteins exhibiting significant differences across all three groups (totaling 261 proteins, Kruskal-Wallis-Test) and those proteins which are different between AH and AUD patients (total of 149 proteins, Wilcoxon-Test) out of the total 680 human proteins. A subset of 101 proteins emerged as divergent across all three groups, showing altered levels in patients with AH when compared to patients with AUD and healthy controls (Fig. 1C). Twelve proteins exhibited a progressive increase or decrease in relation to the progression of alcohol-associated liver disease (Fig. 1D). Interestingly, 6 of the 8 increasing proteins were part of neutrophil function and granules, while all 4 of the decreasing proteins were associated with muscle metabolism (Fig. 1D). Following uptake of phagocytosed pathogens into vacuoles, pathogen-containing vacuoles fuse with neutrophil granules containing various antimicrobial mediators [22]. Lysosome-associated membrane glycoprotein 1 (LAMP1) is a lysosomal membrane protein, which is involved in phagolysosome formation and degranulation [23]. Granules are also being released into the extracellular space during neutrophil activation (degranulation), or during formation of neutrophil extracellular traps [22]. Primary or azurophilic granules contain the antibacterial enzyme myeloperoxidase (MPO) and serine proteases such as cathepsin G (CTSG) [22]. After release during neutrophil degranulation, MPO produces hypochlorous acid, which acts as a potent antimicrobial agent [24, 25]. Secondary granules contain large amounts of lactoferrin (LTF), which sequesters free iron to prevent bacterial growth and increases

permeability to lysozyme to facilitate breakdown of the bacterial cell wall [22]. Hemoglobin B (HBB) was identified as part of human neutrophil granules [26, 27]. On the other hand, skeletal muscle proteins nebulin (NEB), myosin light chain (MYL) 1, sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (ATP2A1), and MYL11, all decreased in AH patients compared with both controls and patients with AUD (Fig. 1D).

Faecal neutrophil, but not muscle related proteins, predict survival in patients with AH

Pearson's correlation analysis of the 12 differentially expressed proteins from figure 1D identified three clusters, one involving muscle protein, a second with neutrophil granule proteins and a third one with other proteins (figure 2A).

To evaluate whether any of these identified proteins exhibit clinical relevance in the context of AH, we conducted a correlation analysis between protein abundance, and clinical, histological, and laboratory parameters. MPO correlated with the MELD score, MELD-Na score and years of alcohol misuse and negatively with AST. CTSG associated with the MELD score and MELD-Na score, while HBB correlated with MELD score, but negatively with AST and ALT (Fig. 2B). All muscle proteins correlated with sodium levels (Fig. 2B). Nebulin displayed a negative correlation with the MELD-Na score. Furthermore, Nebulin and MYL1 showed negative correlations with histological attributes, including polymorphonuclear (PMN) infiltration and the presence of Mallory bodies on liver biopsy (Fig. 2B).

To further examine MPO's predictive ability, patients were divided into two categories according to their fecal MPO expression levels (cut-off = 6.38) using maximally selected rank statistics to discriminate between these groups according to survival at day 60. Patients with AH exhibiting MPO levels exceeding the threshold of 6.38 showed a higher MELD-Na score (Fig. 2C). AH patients with fecal MPO levels of 6.38 or greater had a significantly lower 60-day survival (53.8%) than to those with levels 6.38 or less (79.72%) (Fig. 2D). Long-term survival at 180 days, which is known to be primarily dependent on alcohol abstinence [5], was not significantly different in high vs. low MPO groups ($P = 0.83$; data not shown).

We performed a correlation analysis for MPO in patients with AUD for several markers. There was no significant correlation between MPO and AST (Pearson; R: −0.39, P=0.39, n=18), ALT (Pearson; R:−0.11, P=0.81, n=18), AST/ALT ratio (Pearson, R: −0.34, P=0.46, n=18), CAP-value (Pearson, R: −0.28, P=0.55, n=18), or liver stiffness in kPa (Pearson, R: 0.36, P=0.42, n=18).

Patients were also stratified into two groups based on their fecal nebulin expression levels, employing an optimized threshold of normalized protein abundance (cut-off = 4.33). AH patients with nebulin levels below the calculated threshold of 4.33 displayed a significantly higher MELD-Na score (Fig. 2E). However, 60-day mortality ($P = 0.14$) (Fig. 2F) and 180-day mortality ($P = 0.48$) (data not shown) were not significantly different between both groups.

Taken together, the abundance of fecal proteins associated with muscle metabolism are lower, while proteins involved in neutrophil degranulation are increased in patients with AH, and associated with disease severity and outcome.

Fecal proteins increased in patients with AH are associated with the neutrophil degranulation pathway

To better understand the biological pathways highlighted by the proteomic features, we used Overrepresentation Analysis (ORA), which is a statistical method to cluster proteins dependent on the functional roles of the proteins. Therefore, we defined a subset of proteins which were significantly upregulated in the comparison of the 3 groups with each other. Comprehensive functional enrichment analysis was performed with Reactome pathway annotation, and we observed similar changes when comparing control vs. AUD, control vs. AH, and AUD vs. AH conditions. 22.5 – 40% of all upregulated proteins were part of the *neutrophil degranulation* pathway compared between all three groups. Other important pathways included *platelet activation, signaling and aggregation, platelet degranulation, digestion and absorption,* and *digestion* and *diseases of metabolism* (Fig. 3).

We also used Gene Ontology (GO) annotation for information about the biological processes, cellular components, and molecular functions of differentially regulated proteins. In line with our data, most upregulated proteins are expressed in the lumen enclosed by a vacuolar membrane, including neutrophil granules. GO enrichment identified around 17.5% of upregulated proteins are associated with phagocytosis and 16.25% with the humoral immune response when comparing AH patients with controls. 25% of all proteins were associated with defense response against bacteria in patients with AH as compared with AUD patients, while 17.5% were part of the humoral immune response. When comparing AUD and control groups, the two main pathways with which the majority of proteins were associated are phagocytosis and detoxification of cellular oxidants (Fig. 4). Using gene ontology annotation for cellular component, azurophil granule lumen and primary lysosome are terms which are second and third when comparing AH with controls and AH with AUD. When comparing AUD and controls ficolin-1-rich granule and tertiary granules are among the terms upregulated proteins are associated with (Fig. 4). Gene ontology annotation for molecular function shows an enrichment of proteins mainly involved in antioxidant and peptidase activity in patients with alcohol-associated liver disease (Suppl. Fig. 2).

In conclusion, an unbiased analysis of the fecal proteome confirmed a strong association of increased proteins with the neutrophil degranulation pathway.

Fecal MPO levels are predictive of 60-day mortality in a validation cohort of patients with AH

To further analyze the predictive impact of our analysis, we compared AH patients who died with patients who survived at day 60. After filtering for proteins which were significantly upregulated, we performed ORA Reactome pathway analysis showing that the majority of proteins are part of the *neutrophil degranulation* pathway (Fig. 5A).

Given the prominent role of the *neutrophil degranulation* pathway for predicting mortality in patients with AH, we directed our subsequent analyses towards this particular pathway.

To validate the predictive potential of MPO and enhance the robustness of our proteomic findings, we assessed fecal MPO levels in an independent, second multicenter cohort (AlcHepNet) using ELISA. The median MELD Score in this cohort was 25 and the observed 60-day and 180-day mortality rate was 19% and 27%, respectively (Table 2). Using an optimal threshold of 0.074 ng MPO/mg protein, patients with high fecal MPO levels showed significantly lower survival than patients with low levels of MPO at 60 days (Fig. 5B, logrank: $P = 0.037$). Long-term survival at 180 days was not significantly different in high vs. low MPO groups ($P = 0.33$; data not shown). MPO levels in feces seem to be independent of neutrophils in the blood, as fecal MPO level and white blood cells did not correlate in the proteomic analysis (Suppl. Fig. 3A) or the ELISA analysis (Suppl. Fig. 3B). Furthermore, MPO level did not correlate with PMN-infiltration in the liver (Suppl. Fig 3C), or infection in the proteomic analysis (data not shown).

There was no significant difference in fecal MPO abundance in the proteomics cohort between patients who did not receive antibiotics or steroids, received either antibiotics or steroids, or received both (Suppl. Fig. 4A). This was confirmed in the validation cohort (Suppl. Fig. 4B). Fecal MPO levels were not significantly different in patients treated with steroids who were alive at day 60 as compared with those who died in the proteomics (Suppl. Fig. 4C) and validation cohort (Suppl. Fig. 4D). Patients with AH who received lactulose showed higher fecal MPO abundance in the proteomics (Suppl. Fig. 4E) and validation cohort (Suppl. Fig. 4F), although the difference was only significant in the validation cohort. AH patients with infections showed similar fecal MPO abundance (Suppl. Fig. 4G).

Taken together, using an independent validation cohort, we confirmed that fecal MPO-levels can predict short-term survival at 60 days.

Discussion

Our research focused on examining the fecal proteome in patients with alcohol-associated liver disease, specifically contrasting patients with alcohol-associated hepatitis (AH) with alcohol use disorder (AUD) and healthy individuals. We noted a clear proteomic distinction between healthy controls and those afflicted with alcohol-associated liver disease, with the most significant changes being the upregulation of proteins involved in neutrophil function and degranulation. Myeloperoxidase (MPO), indicative of azurophil granule presence, showed notably elevated levels in fecal samples of patients with AH. This elevation not only correlates with disease severity but it is also associated with higher 60-day mortality. Muscle proteins in the feces were reduced, which is in line with the prevalence of sarcopenia and muscle wasting in patients with AH.

Neutrophils are the first line of defense against invading pathogens and important for resolution of inflammation and tissue repair, but they can also contribute to tissue damage especially during chronic injury [17, 22]. Intrahepatic neutrophil activation and infiltration are often seen on liver biopsy in patients with AH [17], and high neutrophil counts in the liver were associated with higher MELD scores [28]. Due to translocation of microbial products into the enterohepatic circulation, neutrophils get activated, become

hyper-responsive and can contribute to liver damage [17, 29]. In addition to hepatic neutrophil infiltration, systemic neutrophil activation has been described in patients with AH and correlates with serum LPS and disease severity in humans [3, 30–32]. Liver injury models in rodents indicate that after activation, neutrophils migrate to the liver and release reactive oxygen species and proteases [3]. However, neutrophils also take part in the resolution of liver injury by phagocytosis of pathogens and cellular debris, polarization of inflammatory macrophages to anti-inflammatory macrophages, reduction of cytokine release and production of hepatocyte growth factor [18, 19, 31]. And indeed, neutrophil infiltration on liver biopsy was associated with favorable outcomes in patients with AH [33]. Thus, systemic or hepatic neutrophils can have a double-edged function, and their actions likely depend on spatial and temporal conditions. Our research introduces a further dimension to the observed elevation of liver and systemic neutrophils, revealing increased neutrophil degranulation in the feces of patients with AH. Neutrophil degranulation was significantly overrepresented not only between healthy controls and patients with AH or AUD, but also between patients with AUD and AH indicating a biomarker for disease progression and severity. Interestingly, upregulation of fecal neutrophil degranulation did not correlate with systemic or liver neutrophil counts.

The question emerges, how do neutrophils find their way into the luminal compartment of the gut? Neutrophil infiltration of the mucosa is a feature of intestinal inflammation during inflammatory bowel disease. Several fecal neutrophil-derived molecules have been proposed as biomarkers reflective of intestinal inflammation severity, and intestinal neutrophils are considered a therapeutic target for patients with inflammatory bowel disease [34]. Fecal calprotectin is clinically used as a marker for intestinal inflammation in patients with inflammatory bowel disease [35]. Fecal proteomics of patients with ulcerative colitis or Crohn’s disease revealed several biomarkers including MPO and lactoferrin predictive of disease severity [36], and fecal MPO correlates with intestinal inflammation in patients with inflammatory bowel disease [37]. Although the role of neutrophils specifically in the intestine has not been addressed in AH, intestinal inflammation is common in patients with alcohol-associated liver disease [14]. Reducing intestinal inflammation ameliorates ethanol-induced liver disease in preclinical models [14]. These findings raise the interesting question if fecal markers of neutrophil degranulation are merely reflective of intestinal inflammation in patients with alcohol-associated liver disease, or whether neutrophils or their released proteins perpetuate gut inflammation and hence liver disease. Addressing this issue necessitates further research, although the current experimental challenge lies in depleting intestinal neutrophils without impacting the systemic or hepatic compartment.

Several studies have performed proteomics analysis in AH patients in the liver and serum identifying marker and panels to predict inflammation [6, 38]. In a broader approach of proteomics in patients with alcohol-associated liver disease, a panel of six proteins in the plasma or liver could predict mild inflammatory activity [38]. In total 46 proteins were changed in the liver and plasma representing immune and inflammatory responses, cell adhesion, extracellular matrix organization, protease inhibitors and intracellular enzymes [38].The only protein which predicts inflammation in serum and liver and is upregulated in patients with AH is orosomucoid 1 (ORM1) [38]. ORM1 is a transport protein in the blood stream and modulates the immune system in the acute phase reaction. ORM2

shares 89.6% of the sequence with ORM1 and shows similar function, however it is less extensively studied. It is less frequent in the serum and seems to be associated with hepatocellular carcinoma [39]. ORM2 is mainly expressed in hepatocytes and secreted into the blood. However, the human protein atlas also shows an expression in neutrophils [26, 27]. Additional studies are necessary to explore the significance of the gradual increase in fecal ORM2 levels in relation to the progression of liver disease severity in individuals with alcohol-associated liver disease.

Limitations of the study include the cross-sectional design, which does not allow longitudinal assessment of fecal biomarkers. Given the limited size of our cohorts, future studies are required to confirm the predictive value of MPO. Major strengths of this study are the inclusion of patients from different hospitals and countries (United States, Europe, Mexico and Canada), and confirmation of our data in an independent validation cohort.

In summary, this is the first characterization of the fecal proteome in patients with alcohol-associated liver disease. Our study identified significant differences in protein expression related to neutrophil degranulation in controls, patients with AH and AUD, notably with a marked increase in proteins related to neutrophil degranulation. Fecal MPO emerged as a predictor of 60-day mortality.

Material and Methods

Patient cohorts

Cohorts derived from two NIH-funded, independent, multicenter observational studies are studied in this manuscript.

For the proteomics analysis, we included 119 patients, 80 with alcohol-associated hepatitis (AH), 20 with alcohol use disorder (AUD) and 19 controls. Patients with AH were from the Integrated Approaches for Identifying Molecular Targets in Alcoholic Hepatitis (InTeam) Consortium. The InTeam patient cohort has been described [11–13, 20]. Fecal samples were used from patients with AH who were enrolled from June 2014 to May 2017 in the InTeam Consortium in 11 centers of 5 different regions worldwide (ClinicalTrials.gov identifier number: NCT02075918). Baseline characteristics for AH patients are summarized in Table 1. Patients were eligible for the InTeam Consortium if they reported active alcohol abuse (> 50g/day for men and > 40g/day for women) in the last three months. Further inclusion criteria included aspartate amino transferase (AST) higher than alanine aminotransferase (ALT), total bilirubin higher than 3mg/dl in the past three months and liver biopsy and/or clinical picture consistent with AH. Liver biopsy was only performed when indicated as part of clinical routine. Exclusion criteria were autoimmune liver disease (ANA> 1/320), chronic viral hepatitis, hepatocellular carcinoma, complete portal vein thrombosis, extrahepatic terminal disease, pregnancy and lack of signed informed consent. Biospecimen were collected at admission to hospital. The protocol was approved by the Ethics Committee of each participating center and patients were enrolled after written informed consent was obtained from each patient.

Patients with AUD fulfilled the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition criteria admitted for elective alcohol rehabilitation in Brussels, Belgium. All patients reported long-term (>1 year) alcohol consumption (>60 g/day) and were actively drinking until the day of admission [40]. Patients with AUD did not use antibiotics or immunosuppressive medication 2 months preceding enrollment. Exclusion criteria included diabetes, inflammatory bowel diseases, liver diseases of any other etiology, clinically significant cardiovascular, pulmonary or renal comorbidities. Fecal samples were collected at the time of admission. Controls have been described [41].

For the ELISA validation cohort, data and samples of patients with AH from the Alcoholic Hepatitis Network (AlcHepNet) were used. We included 70 patients with AH who were enrolled between May 2019 and May 2022 from 8 clinical centers in the US (Cleveland Clinic, Indiana University, Mayo Clinic, University of Louisville, Beth Israel Deaconess Medical Center Boston & University of Massachusetts, University of Pittsburgh, University of Texas Southwestern, Virginia Commonwealth University). Patients with age greater or equal to 21 years with a clinical diagnosis of AH fulfilling the following criteria were included: Serum bilirubin > 3mg/dl; subject or guardian ability to understand and willingness to provide written consent; and re-enrollment of an AH patient was permissible up to 4 times if the donor presents with a new episode of AH 24 weeks or longer after the most recent enrollment in the study (no cases of re-enrollment are included in this current study). Patients were excluded when one of the following criteria were present: Hemochromatosis, autoimmune liver disease, Wilson disease, NAFLD, and acute viral hepatitis; pregnant or breast feeding; and based on the judgment of the investigator, subject was not capable of understanding or complying with the study requirements. Chronic hepatitis B, hepatitis C or HIV infection did not exclude patients in the absence of significant liver disease. One patient was HCV antibody positive but RNA negative; and three patients had positive HBV surface antibody but not antigen. Patients were not involved in the development of research questions, the design of the study and recruitment, and there are no plans to disseminate study results to the patients.

Protein extraction and Tandem Mass Tag (TMT) proteomics

Stool samples were thawed on ice and 100 mg was aliquoted into a 96-well bead-beating plate (Omni International, 27–6006) for further processing in a randomized order to avoid grouped plating effects. To each well, 500 mL lysis buffer was added (7% SDS, 6M urea, 50 mM TRIS, Roche PhosStop tablet (#4906845001), Roche protease inhibitor tablet (#11836170001), pH 8.1)). Plates were then sealed and bead beat for 1 minute for 3 rounds, with 3 minutes between rounds on ice (Qiagen TissueLyzer II). After homogenization, plates were spun down and 200 µL supernatant was collected and placed in a new deep well plate. Proteins were then reduced using dithiothreitol (DTT, 5 µL of 500 µg/mL, Sigma-Aldrich #D0632) and placed in 37°C for 30 min, cooled to room temperature (RT) and alkylated using 15 µL 500 uL/mL iodoacetamide in the dark for 45 min. To each well, 1500 µL “binding buffer” was added in order to trap proteins for further processing and purification. This solution was put through an “S-trap’ 96 well plate in order to isolate proteins and washed 5x with binding buffer (Protifi S-trap, C02–96well-1). After washing, proteins were digested using 10 µg trypsin and digestion buffer (125 µL 50 µM triethyl ammonium

bicarbonate - TEAB) for 4 hours at 47°C. Samples were then eluted sequentially by adding 150 μL 50 mM TEAB, then 5% formic acid, and 50% acetonitrile/5% formic acid via centrifuge (1000 RCF, RT). into a clean 96 well plate and dried using a SpeedVac (Thermo Scientific).

Dried down peptides were desalted using SepPak tC18 96-well plates (Waters #186002321) with centrifugation at 500–100 RCF. Briefly, plates were primed using 1 mL 100% acetonitrile, then 3 × 1 mL 0.1% formic acid. Samples were resuspended in 1 mL 0.1% formic acid and loaded into the SepPak plates, washed 4x with 1 mL 0.1% formic acid, and eluted using 40% acetonitrile, then 80% acetonitrile and dried down using a SpeedVac.

Samples were resuspended in 1000 μL 50% acetonitrile. 20 μL of resuspended peptide was then taken to quantify peptide abundance using Pierce Quantitative Colorimetric Assay (# 23275). After quantification, 50 ug peptide was taken from the initial peptide resuspension, placed in a new plate, and dried down for further processing.

Dried down peptides were then resuspended in 50 μL 50% anhydrous acetonitrile, 20% HEPES for TMT labeling (TMT-16, # A44520). As previously stated, samples were randomized in order to avoid batch effects. Briefly, 8 μL of 20 μg/μL TMT reagent was used to label peptides for 1 hr at RT, then quenched with 5% hydroxylamine for 15 min, and finally acidified with 50 μL 1% trifluouroacetic acid. After labeling, plexed samples were combined and dried down. As before, samples were then desalted using C18 columns (Waters #WAT054955) and dried down. After sample-plexes were dried, each plex was subjected to basic reverse-phase HPLC fractionation and collected into 12 fractions over the course of 72 minutes, and dried down. Samples were finally resuspended in 20 μL 5% formic acid, 5% acetonitrile and loaded into the HPLC (Thermo Easy nLC-1000) for subsequent data acquisition. HPLC was programmed with the following parameters and material: aqueous phase (A) reagent = 97% water, 2.9% acetonitrile, and 0.125% formic acid, while organic phase (B) was 99.9% acetonitrile, 0.125% formic acid. Samples were loaded onto a house pulled and packed column with an inner diameter of 100 μm and outer diameter of 360 μm. Column was initially packed with 0.5 cm of 5μm c4 (frit), followed by 0.5 cm of 3 μm C18, and finally packed with 29 cm 1.8 μm c18. Each fraction was subjected to a gradient with the following parameters: Total run time = 180 minutes, ramping from 6% B (t = 0) to 25% B (t = 165), then ramping to 100% B for 5 minutes (t = 170).

Mass Spectrometry Data Acquisition—LC-MS spectral data was collected on an Orbitrap Fusion (Thermo Scientific) mass spectrometer using settings previously described [42]. The mass spectrometer acquired spectra in data-dependent mode, with a survey scan performed in the ranges of 500–1200 at a resolution of 60,000 in the Orbitrap. MS1 settings were as follows: automatic gain control (AGC) = 5 × 105, max. injection time = 100 ms., S-lens RF = 60. Top Speed mode was enabled with a 5 s. duty cycle. Data collected at MS1, MS2, and MS3 levels were centroided, no profile data collected.

Acquired data was processed using Proteome Discoverer 2.4 (Thermo Scientific). MS2 fragment data was searched against an HMP-generated metaproteome database concatenated with Uniprot Human database using the Sequest searching algorithm [43] in order to align

spectra to peptides for AH and AUD groups. For healthy controls, we used a metagenome-matched/generated protein database[41]. Precursor mass tolerance was set to 50 ppm and 0.6 Da tolerance for MS2 fragments. Included in the search parameters was static modification of TMTpro 16-plex tags on lysine and peptide n-termini (+304.2071 Da). Additional modifications to peptides were set as: carbamidomethylation (+57.0214 Da). Dynamic modifications searched for: oxidation (+15.995 Da) and N-terminal protein acetylation (+42.011 Da) and phosphorylation (+79.996 Da). Only high-confidence proteins ($q < 0.01$) were used for analysis. All .RAW data and .MZML data is deposited at massive.ucsd.edu, Study ID: MassIVE MSV000094610.

Proteomics Analysis

In total, 96,094 proteins were detected in the stool samples of both AH and AUD patients, whereas 108,081 proteins were identified in the stool samples of the healthy control group. Initially, we combined the two datasets and subsequently removed any unmatched proteins. To ensure statistical robustness, we focused on proteins that were present in more than 50% of the patients. This yielded 9,175 proteins with diverse origins, including bacterial, host, and those of unknown origin. Subsequently, we conducted annotation and narrowed down our focus to human proteins, resulting in a subset of 680 proteins (Suppl. Fig. 5A). For these proteins, we carried out logarithmic transformation and normalization of run intensities. Notably, while data was available for almost all proteins in AH and AUD patients, some proteins were missing in healthy controls (Suppl. Fig. 5B, C).

MPO ELISA

Stool samples were frozen at −80°C until usage. MPO ELISA was performed according to manufacturer's instructions (KTR-880 Quantitative Fecal/Urine Myeloperoxidase ELISA Kit, Epitope Diagnostics). In brief, approximately 50–100mg stool was diluted with Extraction Buffer in two steps. For every mg of stool 40µl of Buffer was added, after homogenization, samples were diluted 1:9. 100µl of the sample was added to the microwell plates and samples were run in duplicates. Protein was measured by BCA following manufacturing instructions. MPO was normalized to protein amount in feces.

Statistical Analysis

Results are expressed as median and range unless stated otherwise. Two groups were compared using Mann-Whitney-Wilcoxon rank sum test or the Fisher's exact test for categorial variable as appropriate. Three or more groups were compared using Kruskal Wallis Test or Fisher's exact test for categorial variable followed by false discovery rate or Benjamin-Hochberg adjustment to correct for multiple comparison. All statistical tests were two sided.

Annotation of proteins, normalization, logarithmic transformation and difference was performed using the protti "package" in R. PCA was performed using the "base" and "forcats" package in R and p values were calculated using the dissimilarity index calculated by the method of Bray-Curtis and Permutational Multivariate Analysis of Variance Using Distance Matrices.

Heatmap was calculated using hierarchical clustering using the ComplexHeatmap package in R. Pearson correlation was performed using the “Hmsic” package in R. Correlation > 0.25 and < −0.25 were regarded as clinically relevant when significant. Correlation Matrix was calculated using hierarchical clustering method by the “corrplot” package in R. For survival analysis we calculated Kaplan Meier Curves and the logrank test. Patients were censored at time point when last seen alive. Time point of liver transplantation was regarded as time point of death. Patients were divided into two groups classified as high and low abundance of a protein when significant using maximal selected rank statistics. Kaplan Meier Curves were used to compare 60-day survival between groups. Upregulated proteins were defined as a differential abundance > 0.6 with a *P*-value < 0.05. GO Annotation, Reactome Pathway analysis and Overrepresentation Analysis was performed with the “ClusterProfiler” or “protti” package in R [44, 45].

Statistical Analysis was performed using R statistical software, R version 4.3.1 (2023).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

H.K. is supported by the Walter-Benjamin-Fellowship of the Deutsche Forschungsgemeinschaft (KR 5843 1–1). M.F.F. is supported by the Galicia Regional Government & Fulbright Fellowship (ED481B-2022–030). This work was supported by University of California San Diego Altman Clinical and Translational Research Institute (ACTRI)/National Institutes of Health (NIH) grant KL2TR001444, Pinnacle Research Award in Liver Diseases Grant #PNC22–159963 from the American Association for the Study of Liver Diseases (AASLD) Foundation, and Pilot/Feasibility Grant P30 DK120515 from the San Diego Digestive Diseases Research Center (SDDRC) (to P.H.), by Grant #CTORA23–208366 from the American Association for the Study of Liver Diseases Foundation, a Pilot and Feasibility Award from the Southern California Research Center for ALPD and Cirrhosis funded by the National Institute on Alcohol Abuse and Alcoholism of the National Institutes of Health P50AA011999, and T32 DK007202 (to C.L.H), by grants from the Fond national de recherche scientifique Belgium (J.0146.17, T.0217.18 and J.0195.24) and Action de recherche concertée (ARC), Université Catholique de Louvain, Belgium (to P.S.), in part by NIH grants R01 AA024726, R01 AA020703, U01 AA026939, U01 AA026939–04S1, by Award Number BX004594 from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development (to B.S.) and services provided by NIH centers P30 DK120515 and P50 AA011999. We thank the Collaborative Center for Multiplexed Proteomics (D.G.). C.G.G. is also funded by Institutional Research and Career Development Award (K12, K12GM068524).

Abbreviations:

ATP2A1	sarcoplasmic/endoplasmic reticulum calcium ATPase 1
AH	alcohol-associated hepatitis
ALT	alanine amino-transferase
ALP	alkaline phosphatase
AST	aspartate amino-transferase
AUD	alcohol use disorder
CTSG	cathepsin G

ELISA	enzyme-linked Immunosorbent assay
GO	gene ontology
HBB	Hemoglobin beta
INR	international normalized ratio
LTF	lactoferrin
MAMP	microbial associated molecular patterns
MELD	Model for End-Stage Liver Disease
MPO	Myeloperoxidase
MYL	myosin light chain
NEB	nebulin
ORA	overrepresentation analysis
PCA	principal component analysis
PMN	polymorphonuclear
TMT	Tandem Mass Tag
TNF	tumor necrosis factor

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Box

What is already known on this topic

Alcohol exacerbates liver damage by inducing gut dysbiosis, bacterial translocation and inflammation. Inflammation in AH patients is associated with increased numbers of circulating and hepatic neutrophils.

What this study adds

We found an increased abundance of proteins linked to neutrophil degranulation in patients with AH. Myeloperoxidase (MPO), the marker protein of neutrophil granules, correlates with disease severity and predicts 60-day mortality.

How this study might affect research, practice or policy

Fecal MPO could be used as prognostic marker. The role of intestinal and fecal neutrophils for disease pathogenesis deserves further research.

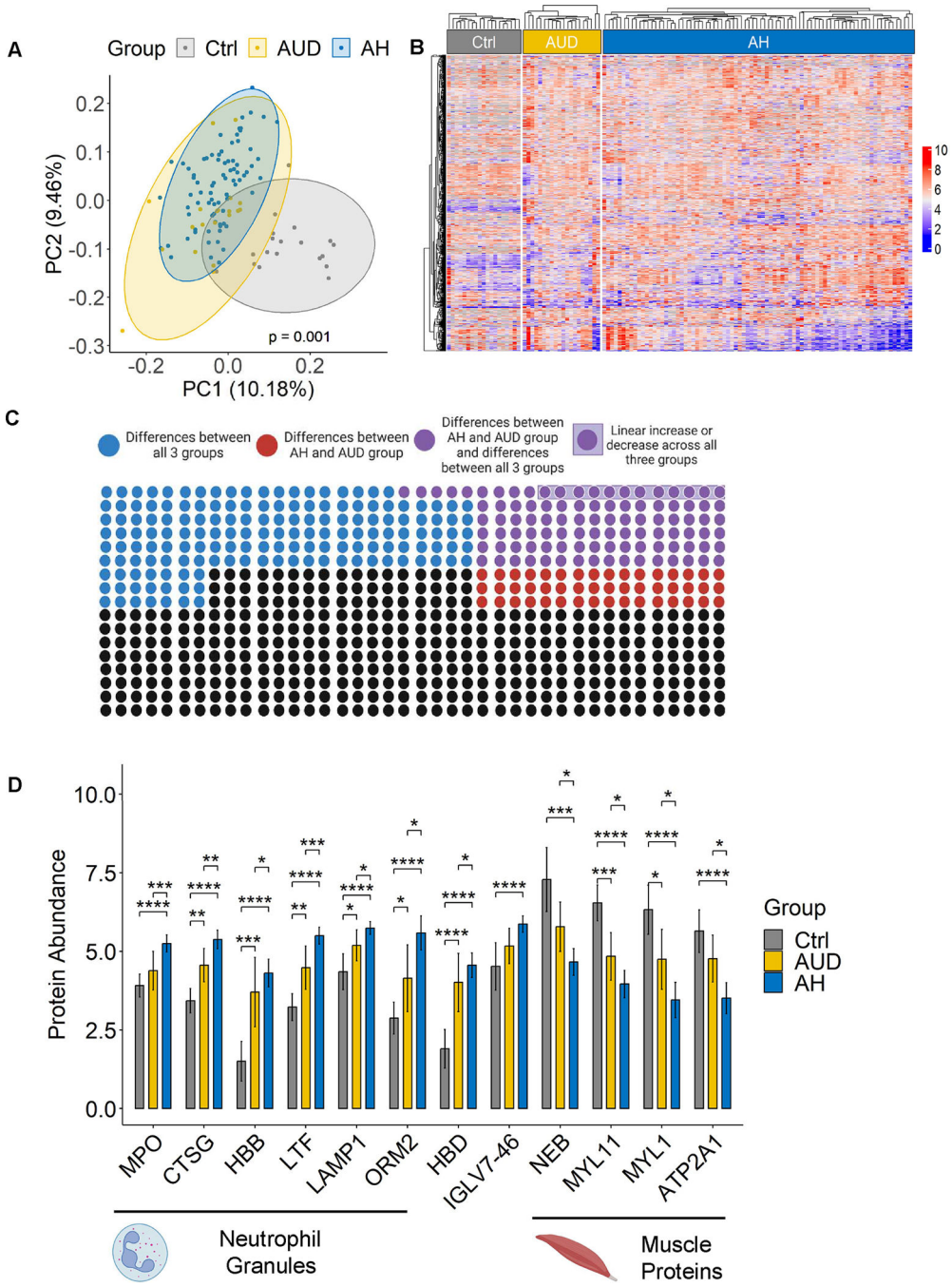

Figure 1. Fecal protein patterns are different between controls, patients with alcohol use disorder (AUD) and alcohol-associated hepatitis (AH)

Proteomics was performed from fecal samples of controls (Ctrl, n=19), patients with alcohol use disorder (AUD; n=20) and alcohol-associated hepatitis (AH, InTeam; n=80).

A: Human proteins in fecal samples in Ctrl, AUD, and AH (P = 0.001 PERMANOVA test [18]); AH vs. AUD P_{adj} = 0.132, AH vs. healthy P_{adj} = 0.003, AUD vs. control P_{adj} = 0.003. P-values were calculated using the dissimilarity index calculated by the method of Bray-Curtis and Permutational Multivariate Analysis of Variance Using Distance Matrices.

B: Heatmap of human proteins in fecal samples was clustered using hierarchical clustering. **C:** All detected 680 human proteins were tested for significant differences between groups using Kruskal-Wallis test (n=261, blue). Afterwards they were tested for significant differences between AH and AUD patients using Kruskal Wallis Test (n=149, red). 101 proteins overlapped in both groups (violet) and were selected if they showed a progressive increase or decrease between all three groups (n=12, violet with violet background). *P* value adjustment by Benjamin-Hochberg.

D: Protein abundance of the 12 detected proteins of Fig. 1C. Significant difference in between groups using Wilcoxon Test is marked in the plot (* $P<0.05$; ** $P<0.01$; *** $P< 0.001$, **** $P<0.0001$). Kruskal-Wallis-Test: MPO, myeloperoxidase (*P = 9.41e-7*); CTSG, cathepsin G (*P = 7.52e-8*); HBB, hemoglobin beta (*P = 3.66e-7);* HBD, hemoglobin delta (*P = 7.20e-8*); LTF, lactoferrin (*P = 1.62e-8*); LAMP1, lysosome-associated membrane glycoprotein 1 (*P = 6.37e-5*); ORM2, orsomucoid 2 (*P* = 1.12e-6); IGLV7–47, immunoglobulin light chain 7–47 (*P = 5.47e-5*); NEB, nebulin (*P = 4.57e-4*); MYL11, myosin light chain 11 (*P = 1.92e-6*); MYL1, myosin light chain 1 (*P = 9.92e-6*); ATP2A1, sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (*P = 7.59e-5*)

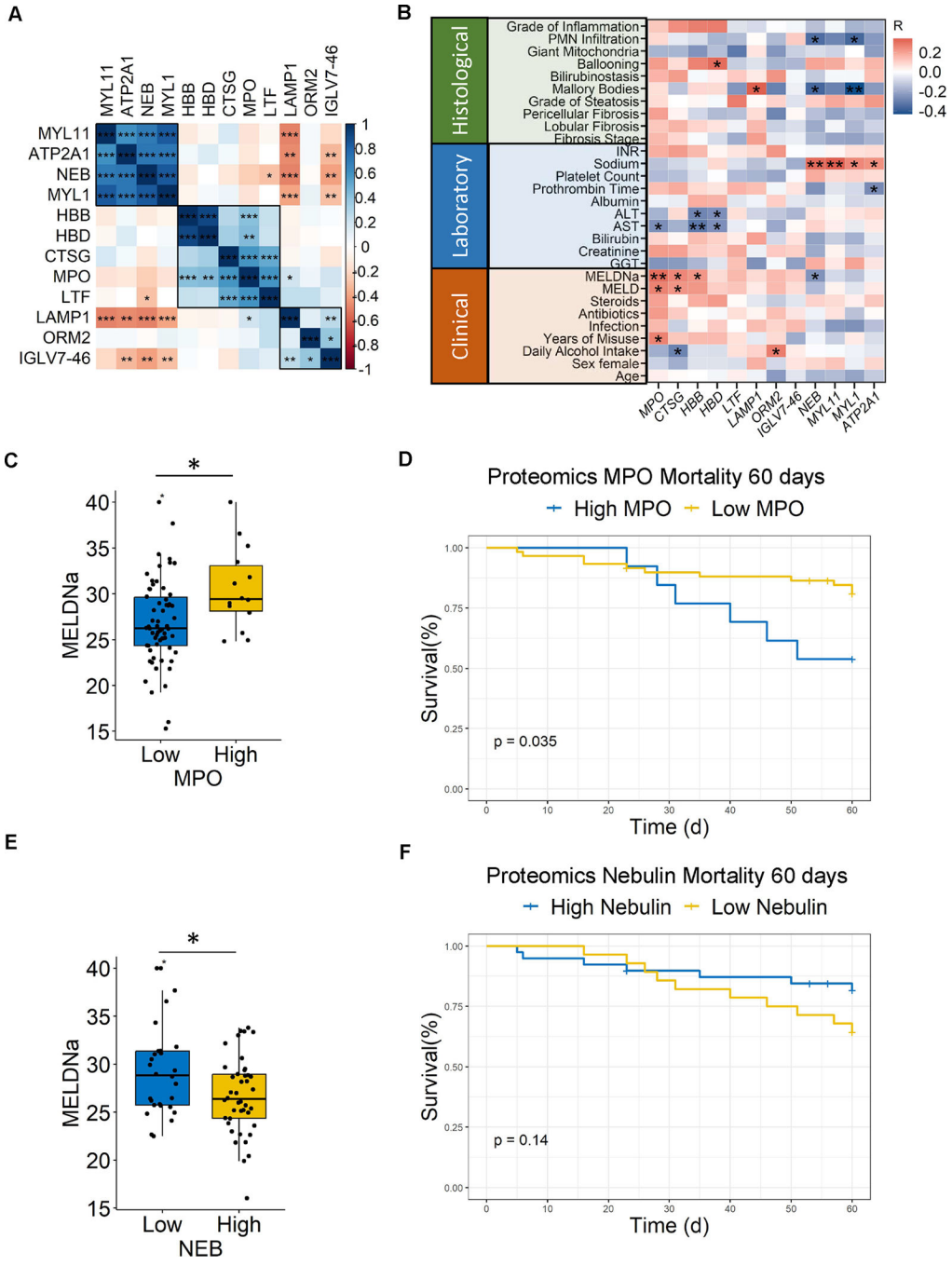

Figure 2. Fecal proteins are associated with disease severity and survival in patients with alcohol-associated hepatitis (AH)

A: Correlation heatmap of differentially expressed proteins from Fig. 1C clustered by hierarchical clustering. *P* values are calculated using Pearson correlation. * $P<0.05$; ** $P<0.01$; *** $P<0.001$

B: Pearson correlation of the 12 differentially expressed proteins from Fig. 1C with histological, clinical and laboratory parameter. * $P<0.05$; ** $P<0.01$; *** $P<0.001$

C and E: Model for End-Stage Liver Disease-Sodium (MELD-Na) scores of patients with AH divided into a low and high group according to their fecal MPO ((**C**), *P = 0.012*) or nebulin (NEB) protein level ((**E**), *P = 0.044*). Optimal cut-off values (cut-off for NEB = 4.33; cut-off for MPO = 6.38) were determined using maximally selected rank statistics (maxstat.test in R package maxstat). *P*-value was calculated using Wilcoxon-Test.

D and F: Kaplan-Meier curve of 60-day mortality for 72 patients with AH. Patients were grouped according to their fecal level of MPO or nebulin, which was determined in (**C**) or (**E**), respectively.. Date of liver transplantation was regarded as death date for patients receiving transplantation (n=3), censored patients n=3. MPO: log rank *P* = 0.035; Nebulin: log rank *P* = 0.14.

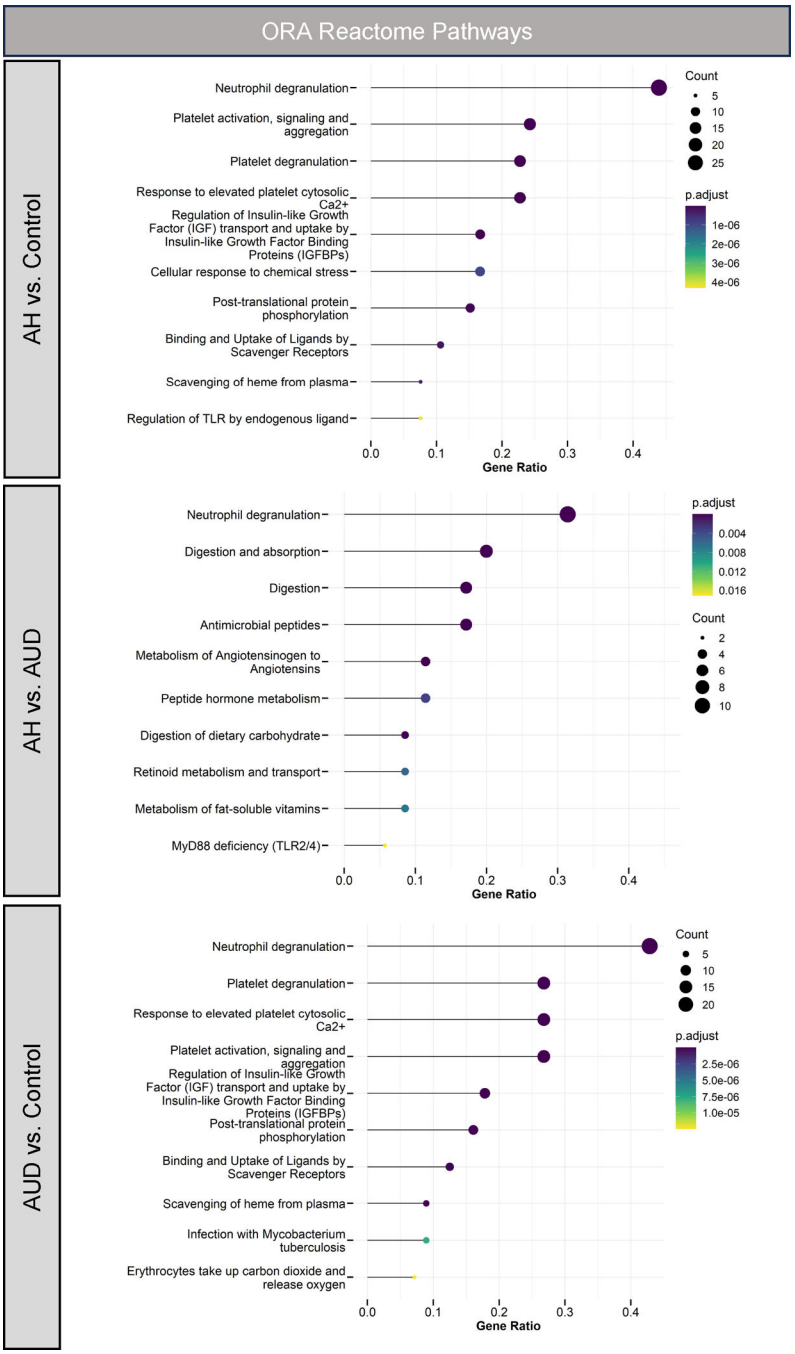

Figure 3. Fecal proteins are enriched in the neutrophil degranulation pathway in patients with alcohol-associated hepatitis (AH)

Difference in protein abundance was calculated between AH vs. controls, AH vs. AUD patients, and AUD vs. controls using the moderated t-test. A protein was regarded as significantly upregulated when the abundance in one group was 1.5 times higher than in the compared group and the *P*-value <0.05. Afterwards all proteins with a significantly different abundance >0.6 (p<0.05) were selected and over-representation analysis (ORA) using Reactome pathways was performed. *P*-values were adjusted using the Benjamin-Hochberg method.

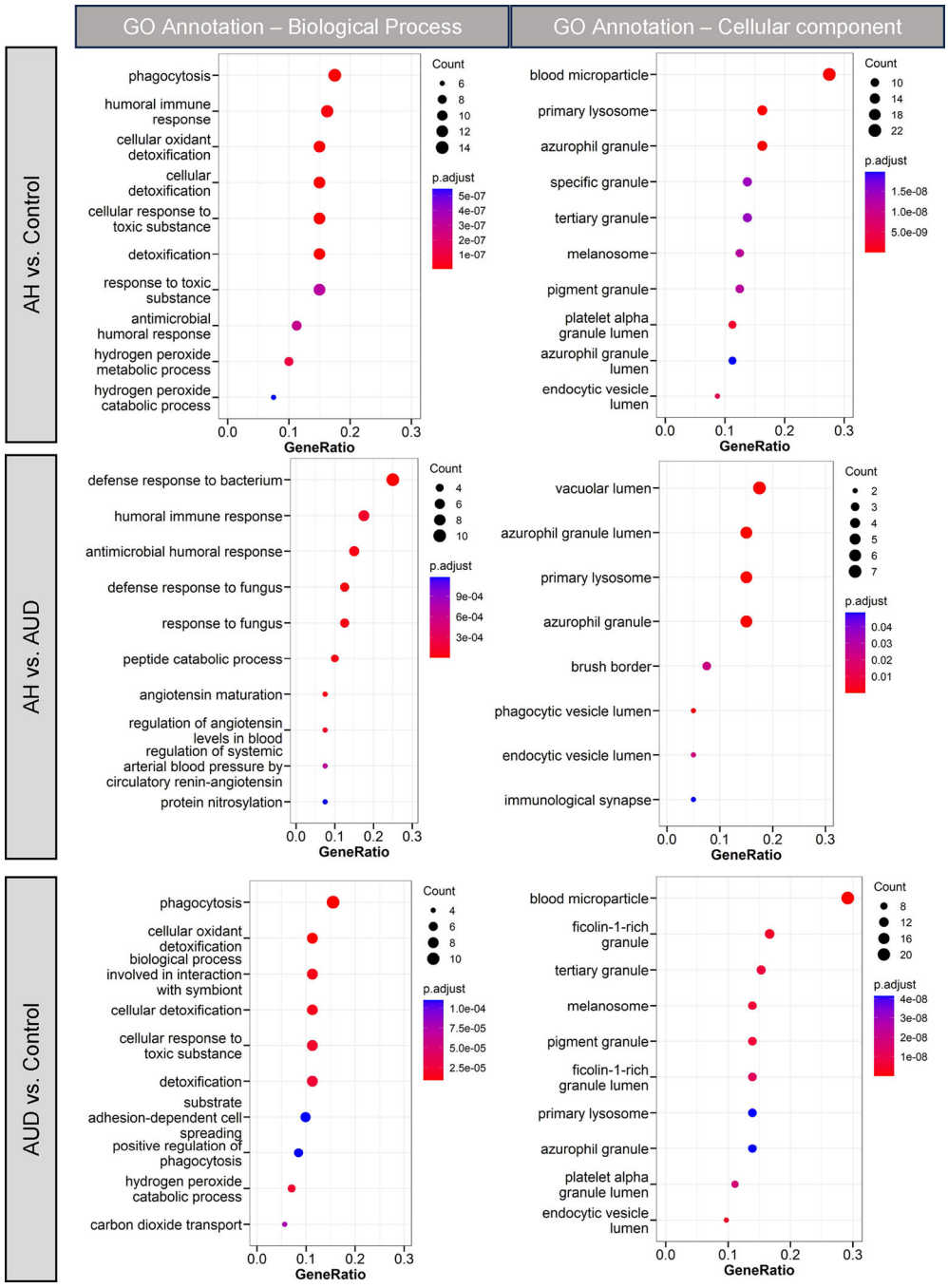

Figure 4. Overrepresentation Analysis using gene ontology enrichment terms associates increased fecal proteins with immune response and azurophilic granules in patients with alcohol-associated hepatitis (AH)

Difference in protein abundance was calculated between AH vs. controls, AH vs. AUD patients, and AUD vs. controls using the moderated t-test. A protein was regarded as significantly upregulated when the abundance in one group was 1.5 times higher than in the compared group and the *P*-value < 0.05. Afterwards all proteins with a significant different abundance of >0.6 (*P* <0.05) were selected and Overrepresentation Analysis (ORA) using

gene ontology (GO) terms was performed. *P*-values were adjusted using the Benjamin-Hochberg method.

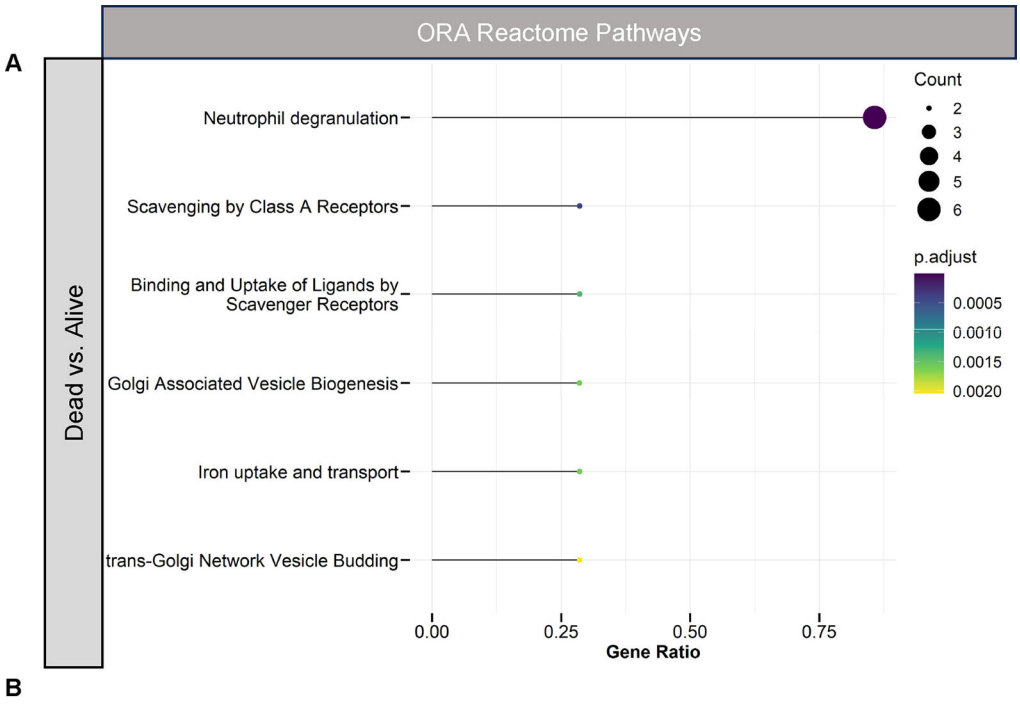

Figure 5. The association between mortality and fecal MPO levels can be validated in an independent cohort of patients with alcohol-associated hepatitis (AH)

A: Difference in protein abundance was calculated between dead and alive patients with AH at day 60 using the moderated t-test. A protein was regarded as significantly upregulated when the abundance in the alive group was 1.5 times higher than in the compared group and the *P*-value < 0.05. A protein was regarded as significantly downregulated when the abundance in the alive group was 1.5 times lower than in the compared group and the *P*-value <0.05. Afterwards all proteins with a significant different abundance of >0.6 (*P*

<0.05) were selected and Overrepresentation Analysis (ORA) using gene ontology (GO) terms was performed. P-values were adjusted using the Benjamin-Hochberg method.
B: Fecal MPO was measured by ELISA and then normalized for total protein content in feces of patients with alcohol-associated hepatitis (AH) (n=70 in a validation cohort from AlcHepNet). An optimal cut-off value of 0.074 ng MPO/mg protein was calculated using maximally selected rank statistics (maxstat.test in R) to divide the cohort into two groups with low and high fecal MPO. Kaplan-Meier curve of 60-day mortality for patients with AH. (log rank $P = 0.037$).

Table 1.

Clinical characteristics of patients with AH (InTeam) and AUD in the proteomics analysis

Variable	Patients with alcohol-associated hepatitis n=80	Patients with alcohol use disorder n=20	*P*-value
Clinical parameters			
Age (years), n=92	50 (40-60)	43 (41-52)	0.22
Sex (% Female), n (%),	25 (32%)	6 (30%)	0.18
Weight (kg), n=81	78 (68-94)	78 (66-87)	0.5
Height (cm), n=82	170 (165 – 177)	169 (167 – 178)	0.9
BMI (kg/m2), n=80	28 (24 – 30)	25 (23 – 30)	0.4
Laboratory parameters			
Albumin (g/dl), n=90	2.4 (2 – 2.8)	4.55 (4.28 – 4.63)	<0.001
ALP (IU/L), n=91	173 (112 – 278)	93 (66 – 155)	<0.001
ALT (IU/L), n=92	43 (29 – 65)	63 (40 – 114)	0.084
AST (IU/L), n=92	128 (99 – 178)	87 (43 – 170)	0.089
Bilirubin (mg/dl), n=92	17 (10 – 22)	1 (0 – 1)	<0.001
Creatinine (mg/dl), n=92	0.8 (0.6 – 1.03)	0.75 (068 – 0.87)	0.5
GGT (IU/L), n=52	189 (120 – 393)	237 (121 – 540)	0.9
INR, n=89	1.9 (1.6 – 2.3)	0.98 (0.91 – 1.02)	<0.001
Cholesterol (mg/dl), n=27	100 (57 – 135)	216 (179 – 239)	0.004
Triglycerides (mg/dl), n=27	85 (42 – 132)	181 (117 – 266)	0.053
Non-invasive fibrosis assessment			
Fibroscan n=15			
F0-F1		10 (67%)	
F2		3 (20%)	
F4		2 (13%)	
Clinical presentation and treatment at admission			
Steroids, n (%), n=77	32 (42%)		
Antibiotics, n (%), n=72	37(51%)		

Variable	Patients with alcohol-associated hepatitis n=80	Patients with alcohol use disorder n=20	*P*-value
Infections, n (%), n=58	12 (21%)		
Abstinent at day 30, n=41			
Abstinent, n (%)	38 (93%)		
Reduced alcohol consumption, n (%)	2 (4.9%)		
Continued alcohol abuse, n (%)	1 (2.4%)		
Stage of fibrosis, n (%), n=37 0/1/2/3/4	1 (2.5) / 0 (0) /5 (13) /6 (15) /28 (70)		
Clinical scores and outcome			
MELD, median (range), n=76	23.9 (21.7 – 27.9)		
MELDNa, median (range), n=76	26.7 (24.9 – 30.5)		
Child-Pugh stage (A/B/C), n (%), n=75	1 (1.3) /18 (24) /56 (75)		
60-day-mortality, n (%) n=72	17 (24%, 95% CI 15-35%)		
180-day-mortaltiy, n (%) n=72	21 (29%, 95% CI 19-41%)		

Values are presented as median and interquartile range in parentheses. The number of patients for whom data was available is indicated in the first column. P-values were calculated with Wilcoxon-Test for continuous variables and Exact Fisher's Test for categorial variable.

BMI, body mass index; ALP, Alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl-transferase; INR, international normalized ratio; MELD, model of end-stage liver disease; 95% CI, 95% Confidence Interval

Table 2.

Clinical characteristics of patients with AH in the validation cohort (AlcHepNet)

Variable	Alcohol-associated hepatitis n=72
Clincal parameters	
Age (years), n= 72	45 (45-50)
Sex (% Female), n =70	23 (32)
Weight (kg), n=93	84 (71-96)
Height (cm), n=91	178 (169 – 183)
BMI (kg/m2), n=84	27.5 (24.8 – 31.1)
Laboratory parameters	
Albumin (g/dl), n=95	2.9 (2.3 – 3.2)
ALP (IU/L), n=95	152 (121 – 225)
ALT (IU/L), n=95	41 (27 – 71)
AST (IU/L), n=95	98 (75 – 149)
Bilirubin (mg/dl), n=93	18 (9 – 28)
Creatinine (mg/dl), n=95	0.95 (0.66 – 1.45)
INR, n=94	1.8 (1.5 – 2.35)
Cholesterol (mg/dl), n=32	122 (90 – 159)
Triglycerides (mg/dl), n=32	139 (102 – 163)
Clinical presentation and treatment at admission	
Steroids, n (%), n=22	13 (59%)
Antibiotics, n (%), n=22	13 (59%)
Clinical scores and outcome	
MELD n=70	25 (21-32)
Child-Pugh stage (A/B/C), n (%), n=68	2 (2.9) /24 (34.29) /42 (60.0)
60-day-mortality, n (%), n=70	13 (19%, 95% CI 11-30%)
180-day-mortality, n (%), n=70	19 (27%, 95% CI 18-39%)