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Chronic Heavy Alcohol Use is Associated with Upregulated Paneth Cell Antimicrobials in Gastric Mucosa

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OBJECTIVES: How alcohol consumption affects the integrity and the defense mechanisms of the mucosa in the upper gastrointestinal tract is largely unknown. We examined the effect of heavy alcohol use on gastric and duodenal Paneth-cell-derived and epithelial antimicrobial peptides (AMPs), cytokines, and the Wnt pathway, an important regulator of epithelial regeneration.
METHODS: In 22 patients with heavy alcohol use and 17 control subjects, biopsies from gastric corpus, antrum, and duodenum were examined for messenger RNA (mRNA) of AMPs, cytokines, and Wnt pathway factors using real-time PCR. The expression of the α-defensin HD5 was analyzed immunohistochemically. The effect of alcohol exposure on Wnt signaling and AMP production was also studied in a gastric cell line using mRNA and reporter gene assays.
RESULTS: Heavy alcohol use was associated with increased expression of Paneth cell HD5 and HD6 mRNA in the antrum, where these products are normally absent (HD5 mRNA in controls vs. patients: 2100 ± 900 and 365 500 ± 161 600, HD6 mRNA: 320 ± 130 and 58 300 ± 32 600 copies per 10 ng total RNA, means ± s.e.m., *P* value: 0.022 and 0.011). Upregulated HD5 was independent of intestinal metaplasia that was observed in a minority of patients. No significant differences were found for β-defensins and cytokines (interleukins IL1β, IL6, IL8, IL10). In patients, Wnt pathway factors showed a trend toward higher levels. *In vitro*, ethanol exposure induced the production of HD5 and HD6 and activation of the Wnt pathway.
CONCLUSIONS: Alcohol exposure can induce gastric Paneth cell AMP expression. This may be linked to Wnt pathway activation, which has an important role in the epithelial regenerative homeostasis.
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INTRODUCTION

Chronic heavy alcohol consumption is associated with alcoholic hepatitis, cirrhosis, and other forms of alcoholic liver disease (ALD), which have been responsible for 493,300 deaths worldwide in 2010.[1] However, the pathogenesis of ALD is not completely understood.[2] Apart from the widely discussed roles of the ethanol (EtOH) metabolite acetaldehyde, increased oxidative stress and epigenetic events, there is increasing evidence pointing to a diminished gut barrier integrity and microbial overgrowth and/or dysbiosis.[3–8] These mechanisms might contribute to increased translocation of bacteria or their products across the mucosal barrier into the portal circulation[4,9] and the subsequent activation of inflammatory processes in the liver.[5,10–12]

The exact mechanisms that may lead to a "leaky gut" and dysbiosis have not been extensively studied so far. This investigation therefore focusses on three aspects of the mucosal integrity in gastric and duodenal mucosa: the production of antimicrobial peptides (AMP), the induction of mucosal inflammation, and the activation of the Wnt pathway in response to alcohol exposure.

In gastric and duodenal biopsies obtained from two groups of individuals largely differing in alcohol consumption, we compared the messenger RNA (mRNA) expression of AMPs originating in large part from epithelial cells, such as β-defensins (HBD1, 2, 4) and elafin, and AMPs derived from Paneth cells. Paneth cells, which are normally found at the bottom of small intestinal crypts, abundantly produce the α-defensins HD5 and HD6 and numerous other AMPs, such as lysozyme or secreted phospholipase A2 with activity against a multitude of microbes.[13,14] In a rat model with experimental (non-alcoholic) cirrhosis we could previously show that a compromised small intestinal Paneth cell function predisposed some animals to bacterial translocation.[15]

To assess the inflammatory process in the upper gastrointestinal (GI) tract in response to alcohol exposure, we also compared the transcriptional expression of four cytokines, the pro-inflammatory interleukins IL8, IL6, IL1β, and the anti-inflammatory IL10, between the two groups.

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Third, we studied the mRNA expression of the Wnt pathway factors TCF4, TCF1, and Wnt3, as well as the classical Wnt target gene Axin-2. The Wnt pathway is involved in small intestinal Paneth cell maturation and governs the epithelial regenerative homeostasis within the whole GI tract.[16–18] Aberration in this pathway may compromise epithelial barrier integrity.[19,20]

In an additional part, we performed *in vitro* experiments in a human gastric epithelial cell line to examine the effect of varying EtOH concentrations on the induction of AMPs and on the activity of the Wnt pathway.

With this approach, we aimed at testing the hypothesis that heavy alcohol use might promote pro-inflammatory conditions, aberrations in innate epithelial antimicrobial defense, and/or changes in the Wnt pathway, and consequently in epithelial regeneration. Such effects could contribute to the "leaky gut" and dysbiosis, which is thought to promote ALD. In addition, shifts in GI mucosal homeostasis may be linked to other alcohol-related morbidities, such as the slightly increased gastric cancer risk, which is observed in heavy alcohol users.[21,22]

METHODS

Patients. The study was performed according to the guidelines of the Declaration of Helsinki of 1975, as revised in 1983, and was approved by the Ethical Review Board of the University of Tübingen (first approval 10 April 2003, renewal 20 April 2010). After informed consent, patients with heavy chronic alcohol use (consuming over 60 g of alcohol per day over a period of at least 6 months) and control individuals with none or only moderate (<20 g/day) alcohol consumption, scheduled for upper GI endoscopy for various reasons, that is, mild-to-moderate epigastric pain ($n=12$), exclusion of ulcers in patients with non-inflammatory cholecystolithiasis ($n=4$), and cervical globus sensation ($n=1$), were recruited for this study. The control group was not restricted to total abstainers, but also included subjects with low-to-moderate alcohol consumption (>1 g–<20 g/day) to reduce the influence of potentially confounding factors resulting from a pronounced health-conscious lifestyle often followed by total abstainers.

Exclusion criteria were signs of severe infection at the time of the study (that is, bacteremia and/or disease states meeting two or more criteria of the systemic inflammatory response syndrome, as previously defined,[23] as well as increased bleeding propensity due to impaired coagulation or overt portal hypertensive gastroduodenopathy.

During esophagogastroduodenoscopy, which was performed no later than 5 days after admission, three biopsy specimens were obtained from the descending duodenum, three from the gastric antrum and three from the corpus for mRNA analysis and histology (using a Radial Jaw 4 forceps, Boston Scientific, Natick, MA). Biopsies were taken from a total of 17 controls and 22 patients chosen for this study. In some instances due to low mRNA amount or quality, not all assays could be performed. The minimal number of patients includes 20 patients with heavy alcohol use at each location, 16 controls for comparisons of corpus or duodenum, and 11 controls for the antrum.

From additional biopsies of the antrum and corpus, a urease test was performed to determine colonization by *Helicobacter pylori*. Gastric pH was determined in gastric juice aspirated at the beginning of the endoscopy using a multiple indicator strip (pH0-14, Merck, Darmstadt, Germany). Also, on the day of endoscopy, blood was obtained for routine laboratory examinations.

Quantitative real-time PCR. Quantitative real-time PCR was performed using a system from Roche (LightCycler480, Roche Diagnostics, Indianapolis, IN) as described previously.[24] Single-stranded complementary DNA corresponding to 10 ng of RNA was used as a template with specific oligonucleotide primer pairs (Table 1). For copy number ascertainment we utilized specific plasmid standards for each product, which were designed using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and confirmed by sequencing analysis. For each product, levels of mRNA were normalized to β-actin expression.

Table 1 Oligonucleotides used in quantitative real-time PCRs

Product	Primer forward	Primer reverse
β-actin	AACACCAGGCGGAACGAA	AGTCTAAGGTATCCACGCATTT
hBD1	TTGTCTGAGATGGCCTCAGGTGGTAAC	ATACTTCAAAAGCAATTTTCCTTTAT
hBD2	ATCAGCCATCAGGGTCTTGT	GAGACCACAGGTGCCAATTT
hBD4	AGATCTTCCAGTGAGAAGCGA	GACATTTCTTCCGGCAACGG
Elafin	CGTGGTGGTGTTCCTCATC	CGTGGTGGTGTTCCTCATC
HD5	GCCATCCTTGCTGCCATTC	AGATTTCACACACCCCGGAGA
HD6	CCTCACCATCCTCACTGCTGTTC	CCATGACAGTGCAGGTCCCATA
Lyzozyme	GCTACAGGGGAATCAGCCTA	TGCTTCTGTCTCCAGCATTG
sPLA2	GCAGGAGCCCTTCTATACCC	GTTGAGGTGGAGGAGAGCAG
TCF4	ATCGTCCCAGAGTGATGTCG	CGGGCCAGCTCGTAGTATTT
TCF1	CTCATAAGTTGGACCAGAGGAAG	GGCGGACTGAATGCTGAAAGA
Wnt3	ATGCCACTGCATCTTCCACT	GTGCCCTACTTGCAGGGTGT
Axin-2	AACACCAGGCGGAACGAA	AGTCTAAGGTATCCACGCATTT
IL8	ATGACTTCCAAGCTGGCCGTGGC	TCTCAGCCCTCTTCAAAAACTTC
IL1β	ACGATGCACCTGTACGATCA	TCTTTCAACACGCAGGACAG
IL6	AATCATCACTGGTCTTTTGGAG	GCATTTGTGGTTGGGTCA
IL10	AAGCCTGACCACGCTTTCTA	ATGAAGTGGTTGGGGAATGA

HBD1,2,4, human beta-defensin1,2,4; HD5,6, human alpha-defensin5,6; sPLA2, secreted phospholipase A2; TCF1,4, T-cell factor1,4; IL1β,6,8,10, interleukin 1β,6,8,10.

Immunohistochemistry. We analyzed biopsies of six patients with heavy alcohol use (all negative for *H. pylori*) and six controls (one positive for *H. pylori*). Immunostaining for HD5 was performed using a two-step immunoperoxidase technique (EnVisionTM, Dako, Glostrup, Denmark). Three micrometer tissue slices of formalin-fixed paraffin-embedded sections were mounted. Slides were heated for 30 min at pH6 in a steamer for antigen retrieval and incubated for 1 h with the primary anti-HD5 (Anti-DEFA5, Sigma, St Louis, MO) antibody diluted 1:5000 in TBST (20 mM Tris-Base (pH 7.4), 0.14 M NaCl, 0.1% Tween 20). HD5 protein was visualized by a horseradish peroxidase-labeled secondary antibody (Dako), which was detected with 3′-diaminobenzidine tetrahydrochloride (Dako). Slides were counterstained with hematoxylin. Evaluation was performed by an expert pathologist (G.O.).

Cell line. For *in vitro* studies we used the gastric cell line SK-GT-2, which was purchased from the Leibniz-Institut DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. Incubation was carried out at 37 °C and 5% CO2 and cells were cultured in Dulbecco's modified Eagle's medium, 1% penicillin–streptomycin and 10% fetal calf serum.

Plasmids. The previously described HD5 and HD6 luciferase reporter constructs have been kindly provided by Béatrice Romagnolo and Pauline Andreu.[16] Vladimir Korinek kindly provided us with the Wnt responsive TopFlash luciferase reporter construct.[25] As a transfection control we utilized a standard pRL-TK *Renilla* vector.

Transfection and stimulation experiments. Cells were transfected using X-tremeGENE reagents (Roche) according to manufacturer's protocol. Owing to the strong activity of the TK Promotor, we only used 5 ng of the Renilla control plasmid, whereas 250 ng of the respective HD5, HD6 or TopFlash promotor constructs were utilized. For both, reporter gene assays and mRNA studies, stimulation of the cells was carried out for 20 h by adding respective volumes of EtOH to the media to achieve different final concentrations. To assess the effects of EtOH on cell viability, MTT assays (Sigma-Aldrich, St. Louis, MO) were performed according to the manufacturer's protocol (Supplementary Figure 1) using increasing EtOH concentrations (0%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%). In a second approach, we also used three different final concentrations of H_2O_2 (50, 100, and 200 mM) either without an additional EtOH stimulation or with an additional 5% EtOH in the media. To study promoter activity of HD5, HD6, TopFlash, and a mutated HD5 promoter with three altered Wnt response elements (HD5mut) 48 h after transfection and 20 h after EtOH (using either 0, 0.5, 1, or 5% final concentration in media) or H_2O_2 stimulation, luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Fitchburg, WI) and an *EnSpire* Plate Reader system (PerkinElmer, Waltham, MA). Firefly luciferase activities were normalized with respect to transfection efficiencies using the corresponding *Renilla* luciferase activity. Transfections were carried out in triplicates; six independent experiments using different EtOH concentrations, whereas four independent experiments using H2O2 with and without 5% EtOH were performed. For the quantification of transcription levels, a minimum of five independent experiments were performed and again carried out in triplicates. We isolated mRNA after 20 h of EtOH stimulation (with either 0, 0.5, 1, or 5% final EtOH concentration in media) using the RNeasy Plus Minikit (Roche), and analyzed corresponding complementary DNA, which was transcribed via the *iScript* cDNA synthesis kit (Bio-rad, Hercules, CA) via real-time PCR.

Statistics. Graphs that are presented as means ± s.e.m. were generated using GraphPad Prism V6 software. Differences in patient characteristics and in the mRNA analyses were analyzed for statistical significance by Mann–Whitney *U*-test (for non-normally distributed groups). To compare percentages or absolute cases, Fisher's exact test was performed. To adjust for multiple comparisons we used the Bonferroni correction. To test for significance of the luciferase reporter gene fold inductions, we used a matched, non-parametric one-way analysis (Friedman Test) and performed post analysis via Dunn's test for the induction by 1 and 5% EtOH (Figure 7a,b,c) and for the induction of HD5 and HD5mut by 5% EtOH under all H_2O_2 concentrations (Figure 7d). *P* values below 0.05 were considered statistically significant ($P<0.05=*$; $P<0.01=**$, $P<0.001=***$).

RESULTS

Patient characteristics. We compared 22 patients with heavy chronic alcohol use (111 ± 14 g alcohol/day (range: 60–220 g/day) over 8.6 ± 1.3 years, mean ± s.e.m.) vs. 17 controls with minor or no alcohol consumption (0–20 g/day). The control group comprised 10 persons with very low or zero alcohol consumption (≤1 g/day), four with >1–5 g/day and three with >5–<20 g/day. The basic characteristics of both groups are shown in Table 2. They were comparable in terms of age and body mass index with a higher percentage of females in the control group, which was, however, not statistically significant. Expectedly, the group with heavy alcohol use exhibited a significant elevation of liver enzymes. Among the studied patients with chronic heavy alcohol use, three exhibited sonographic and clinical signs of liver cirrhosis and one additionally had ascites, whereas control patients had no evidence of liver disease. No significant differences regarding the presence of *H. pylori* were noted between the groups. Achlorhydria that may increase bacterial density in the GI tract was only present in a minority of patients and controls.

Gastric and duodenal mRNA expression of Paneth cell products. Expectedly, the expression levels of Paneth cell products (HD5, HD6, secreted phospholipase A2) were highest in duodenal tissue. Here, no significant differences were detected between the two groups. However, heavy alcohol consumption was associated with increased transcript levels of Paneth cell-associated AMPs in the stomach (antrum and corpus, Figure 1a,b).

In the antrum, the HD5 expression level of controls was at 2100 ± 900 copies per 10 ng RNA, (mean ± s.e.m.), whereas patients with heavy alcohol use exhibited considerably higher

Table 2 Patient characteristics

	Controls ($n=17$) (mean ± s.e.m.)	Patients with heavy alcohol use ($n=22$) (mean ± s.e.m.)	*P* value
Antrhopometric and nutritional data			
Age (years)	43.47 (±3.78)	51.14 (±2.78)	ns*
Gender male/female (%)	64.7/35.3	86.4/13.6	ns
BMI	26.69 (±1.21)	24.16 (±1.08)	ns
Alcohol consumption (g/day)	4.97 (±2.27)	111.1 (±14.44)	<0.0001
Laboratory data			
Leukocytes (g/l)	7.29 (± 0.7)	8.54 (±0.7)	ns
C-reactive protein >0.5 mg/dl (%)	11.8	40.9	ns*
Aspartate transaminase (U/l)	28.77 (±4.38)	94.53 (±14.37)	0.0004
Alanine transaminase (U/l)	33.93 (±5.6)	170.3 (±113.56)	0.0187
Gamma-glutamyl transferase (U/l)	37.27 (±8.12)	701.9 (±361.84)	0.0004
Bilirubin (mg/dl)	0.75 (±0.12)	1.05 (±2.06)	ns
Comorbidities			
Sonographic chirrosis signs (*n*)	0	3	ns*
Ascites (*n*)	0	1	ns*
Achlorhydria (gastric pH≥6) (*n*)	3	3	ns*
H. pylori positive (*n*)	3	5	ns*

BMI, body mass index. *P* values were calculated by Mann–Whitney *U*-test or Fisher's exact test (as indicated by an asterisk).

copy numbers (365500 ± 161600). A similar difference was found for HD6 (320 ± 130 copies in controls and 58300 ± 32600 copies in heavy alcohol use). For both, HD5 and HD6, this was statistically significant (*P* value for HD5: 0.0014, Bonferroni-adjusted: 0.022; *P* value for HD6 0.0007, adjusted 0.011). Secreted phospholipase A2 was also increased in patients with heavy alcohol use, though this trend was not significant (Figure 1c). The mRNA expression of lysozyme, which, in the stomach, is not restricted to Paneth cells,[26] showed a different pattern. The highest lysozyme transcript levels were detected in the antrum, followed by duodenum and corpus (Figure 1d).

HD5 peptide in gastric tissue. As the results of the mRNA study suggest the increased occurrence of Paneth cells in heavy alcohol use, we aimed at confirming this via immunohistochemistry. These analyses were carried out in antrum biopsies of randomly selected heavy alcohol users and controls (six from each group, all patients with chronic alcohol use were *H. pylori* negative, one out of six control persons was *H. pylori* positive). Consistent with the fact that we universally detected low levels of HD5 mRNA in our samples, we found a weak and diffuse staining almost throughout all analyzed mucosal samples (Figure 2). In addition, two out of six patients, who were all negative for *H. pylori,* showed an even more clear-cut result. They displayed a distinct distribution of HD5-positive cells at the bottom of gastric glands (Figure 3b, lower left and lower middle). In addition to this occurrence of Paneth cell metaplasia, the evaluating pathologist also diagnosed the presence of general intestinal metaplasia (IM) in these two patients.

Comparing heavy alcohol users with and without IM (Supplementary Figure 2), it can be observed that HD5 expression was increased in both subgroups, whereas controls had nearly negligible HD5 transcript levels. This suggests that IM is not a prerequisite for the induction of HD5 and is present only in a minority of patients.

Gastric and duodenal mRNA expression of other epithelial AMPs. We not only found large inter-individual differences within both groups in the expression of AMPs, but also different trends between the groups (Figure 3). The constitutively produced HBD1 and the inducible β-defensin HBD2 showed higher levels in the group with heavy alcohol use at all locations, which was, however, not statistically significant. The antiprotease elafin showed a similar trend. HBD4, which was with few exceptions universally detected at low levels at the duodenal location, was only found in some of the studied gastric samples at negligible levels.

mRNA expression of cytokines. To study whether heavy alcohol use is associated with mucosal inflammation, we measured the mRNA expression of different pro-inflammatory cytokines, IL8, IL1β, IL6, and the anti-inflammatory IL10. As shown in Figure 4, no consistent differences in the mRNA expression of the four cytokines could be detected.

mRNA expression of Wnt pathway factors. Owing to the important role of Wnt signaling in epithelial homeostasis and Paneth cell biology, we analyzed mRNA levels of TCF1, TCF4, Wnt3, and the Wnt target gene Axin-2 in both groups (Figure 5). We found a trend toward increased transcript levels for all four factors, which, however, was not statistically significant.

Direct effects of EtOH exposure on a gastric cell line. To study whether the observed effects in patients might be directly attributable to the ingested alcohol, we analyzed a potential role of EtOH in the transcriptional regulation of HD5 and HD6 in a gastric cell line. In addition, we examined the effect of EtOH on the Wnt pathway target gene Axin-2 and the Wnt signaling factors TCF1, TCF4, Wnt3 while also studying elafin and IL8. As expected, HD5 and HD6 mRNA was not or only minimally expressed in SK-GT-2 cells. Interestingly however, after 20 h of stimulation with 5% EtOH, we observed a significant upregulation of both Paneth cell α-defensins

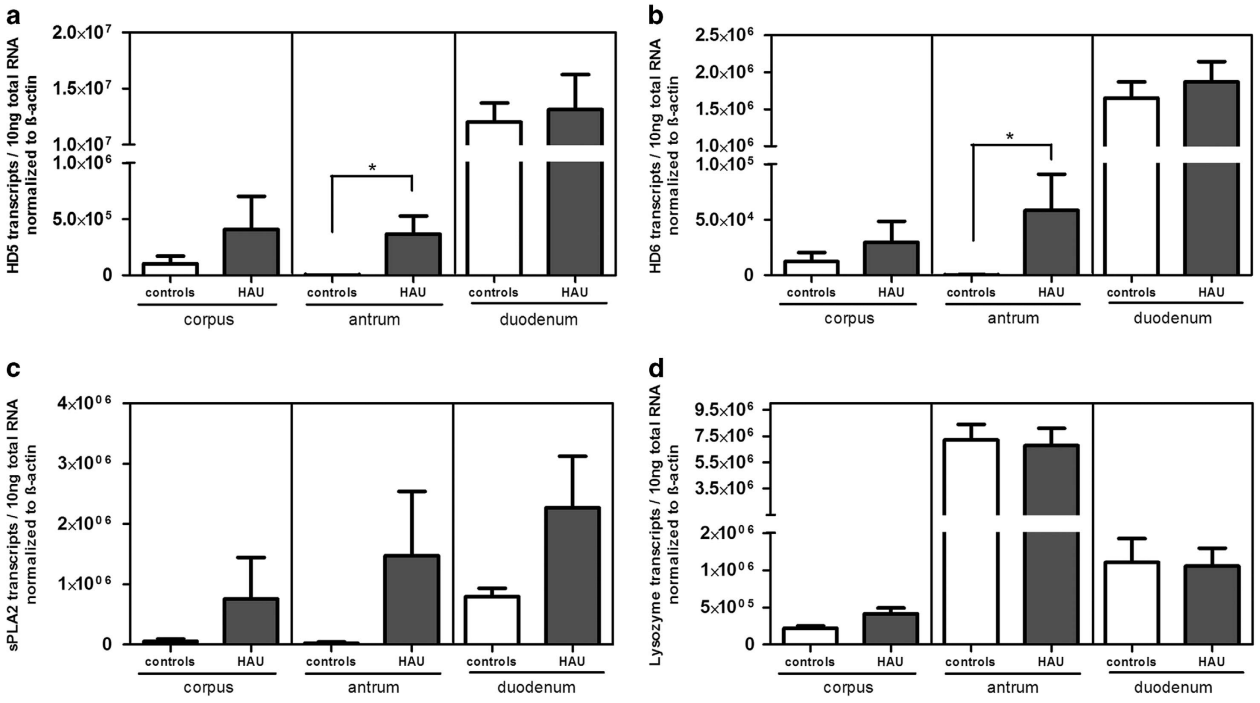

Figure 1 mRNA expression of Paneth cell antimicrobial peptides HD5 (**a**), HD6 (**b**), sPLA2(**c**), and lysozyme (which is not restricted to Paneth cells in gastric tissue) (**d**) in the corpus, the antrum, and the duodenum of patients with heavy alcohol use (HAU) and controls. Shown are means with the respective standard error (s.e.m.).

(Figure 6a, b) and the Wnt target gene Axin-2 (Figure 6c). Starting from very low basal levels, HD5 mRNA was significantly induced by a factor of 8 ($P=0.013$) and HD6 mRNA by a factor of 32 ($P=0.014$). Axin-2 mRNA was readily expressed in untreated cells and, after stimulation with 5% EtOH, exhibited a twofold increase ($P=0.015$).

In contrast to the data from gastric samples, the Wnt transcription factors TCF1 (Figure 6d) and TCF4 (Figure 6f) exhibited no such induction. Wnt3 (Figure 6e) showed a trend for, but no significant, upregulation after alcohol stimulation, so did IL8 and elafin (Figure 6g, h). To confirm a potential activation of the Wnt pathway by EtOH stimulation, we additionally performed reporter gene experiments using promotor constructs for HD5 and HD6 as well as the artificial Wnt responsive TOPFlash. After 20 h of stimulation, we noted a significant dose-dependent induction of the promotor activity of both studied α-defensins (Figure 7a, b) and the Wnt responsive TopFlash (Figure 7c). The inducibility of HD5 seemed to be highest compared with HD6 and the TOPFlash promotor. Consistency in the induction of all three promotors suggests that EtOH has at least partially a direct effect on β-catenin-dependent Wnt pathway activity.

This is also supported by the finding that a version of the HD5 promoter, which was mutated for all three potential TCF/Lef-binding sites (Wnt response elements) showed only a minor, statistically not significant induction after EtOH stimulation in a second approach using parallel hydrogen peroxide stimulation (Figure 7d). Alcohol has previously been shown to induce oxidative stress in gastric mucosa,[27] we therefore used H_2O_2 in these experiments to test whether this oxidative stressor can mimic the EtOH-mediated effect on HD5 promoter activity. Whereas the three different applied H_2O_2 concentrations had no effect on the transcriptional regulation of HD5, an additional stimulation with 5% EtOH again significantly induced the promotor, in large parts in a Wnt-dependent manner (Figure 7d).

DISCUSSION

This study represents the first report on an upregulation of Paneth cell-specific products as a consequence of chronic heavy alcohol abuse. In addition, it is the first to show a direct influence of EtOH on β-catenin-dependent Wnt signaling in a human gastric epithelial cell line.

Contrary to our initial hypothesis we found no evidence for defects in the production of AMPs in the stomach or duodenum. Thus, changes in the synthesis of mucosal defensins at these GI locations do not account for the translocation of bacterial products, which is thought to have a role in the pathogenesis of ALD. Also, the cytokine patterns observed in this study do not indicate a major role of inflammation.

Rather, the presented data suggest that the upregulation of AMPs in patients with heavy alcohol use is restricted to Paneth cell-specific antimicrobial products (HD5, HD6) in gastric mucosa. Interestingly, the occurrence of Paneth cells in gastric mucosa of some heavy alcohol users was also accompanied by IM, a phenomenon that has previously been linked to gastritis. The observed expression of Paneth cell AMPs in gastric tissue, where those products are normally absent, was paralleled by a trend toward induction of transcription factors of the Wnt pathway and its target gene Axin-2. Even though this

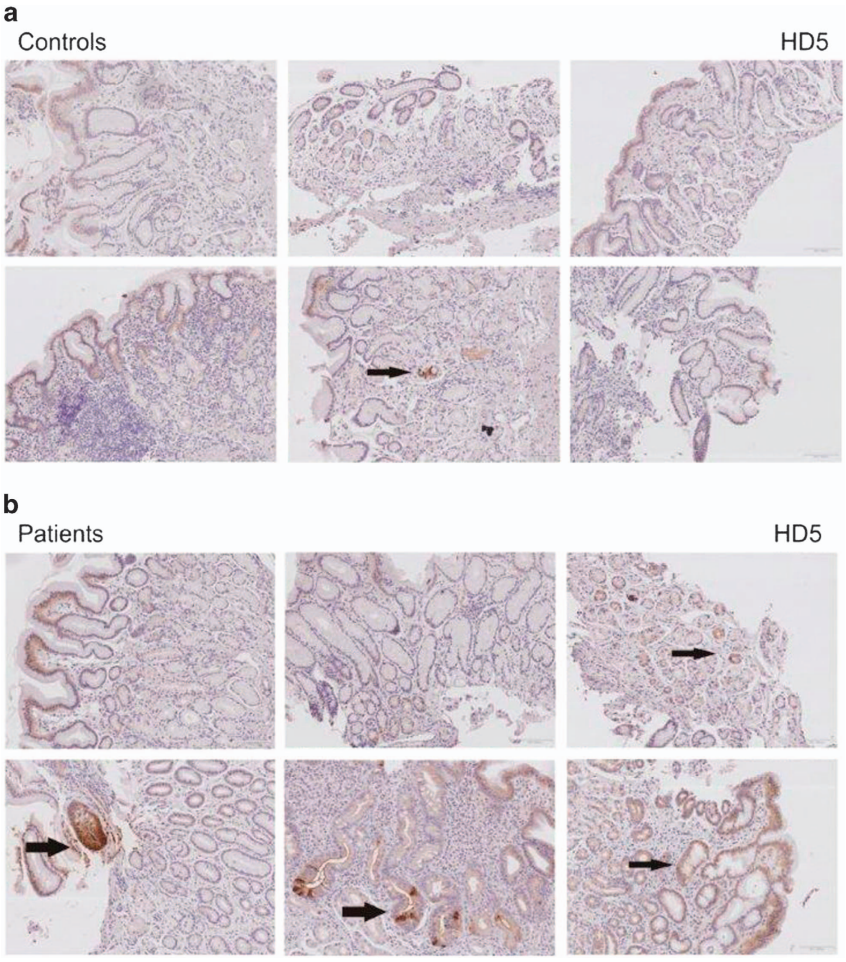

Figure 2 Immunohistochemical staining of HD5 (×20 magnification) in biopsies taken from the antrum of controls (**a**) and patients with heavy alcohol use (**b**). Specific staining is marked by large arrows. Examples for more unspecific staining are marked by thin arrows.

effect was not statistically significant, we hypothesized that a potential deregulation of this important signaling cascade could be one mechanism that might disturb the epithelial homeostasis in patients, promoting an expression of Paneth cell AMPs. As such a disturbance in cell homeostasis could have important clinical implications, we tested our hypothesis by performing different *in vitro* experiments in a human gastric epithelial cell line. These experiments indeed indicated a direct influence of EtOH exposure on Paneth cell α-defensin expression and β-catenin-dependent Wnt signaling.

The expression of Paneth cell products is normally restricted to the small intestine, where these specialized secretory cells are found at the bottom of crypts. Paneth cells represent the only differentiated epithelial cell in the gut, which is dependent on active Wnt for its maturation and function. In addition to their most abundant products HD5 and HD6, they generate a variety of mediators forming a broad arsenal of innate epithelial AMPs that combat bacteria, viruses and other microbial threats.[28] Paneth cell defects have been implicated in the pathogenesis of small intestinal Crohn's Disease, a multifactorial inflammatory disorder.[29] Multiple *in vivo* mouse models highlight the importance of Paneth cell AMPs in protecting the organism from pathogens and in controlling the intestinal homeostasis toward resident microbiota.[30–34] In an animal study investigating rats with chemically induced liver cirrhosis, we could previously show that bacterial translocation across the mucosal barrier was associated with reduced expression of Panth cell-derived antimicrobials in the small intestine.[15]

As mentioned, the cytokine patterns in both groups indicate that mucosal inflammation has no major role in the induction of Paneth cell products observed in patients with heavy alcohol use. We also examined whether relevant comorbidities contributed to the observed phenomena. As the three patients with liver cirrhosis did not display any gastric induction of Paneth cell HD5, and duodenal expression was not majorly affected (data not shown), we conclude that the observed induction of Paneth cell antimicrobials is independent of the degree of liver damage. The gastric pathogen *H. pylori* is another potential inducer of HD5 expression.[35] In our study, there was no significant difference regarding HD5 transcript levels in *H. pylori* positive and negative patients (data not shown), supporting again the assumption that heavy alcohol use is an independent mechanism for the induction

of Paneth cell AMPs. Notably, achlorhydria may increase bacterial density in the GI tract.[36,37] However, in both groups only a minority of patients (13.6%) and controls (22.2%) had a pH ≥ 6, and in both cases, these individuals did again not differ from those with a gastric pH < 6 regarding their HD5 level (data not shown).

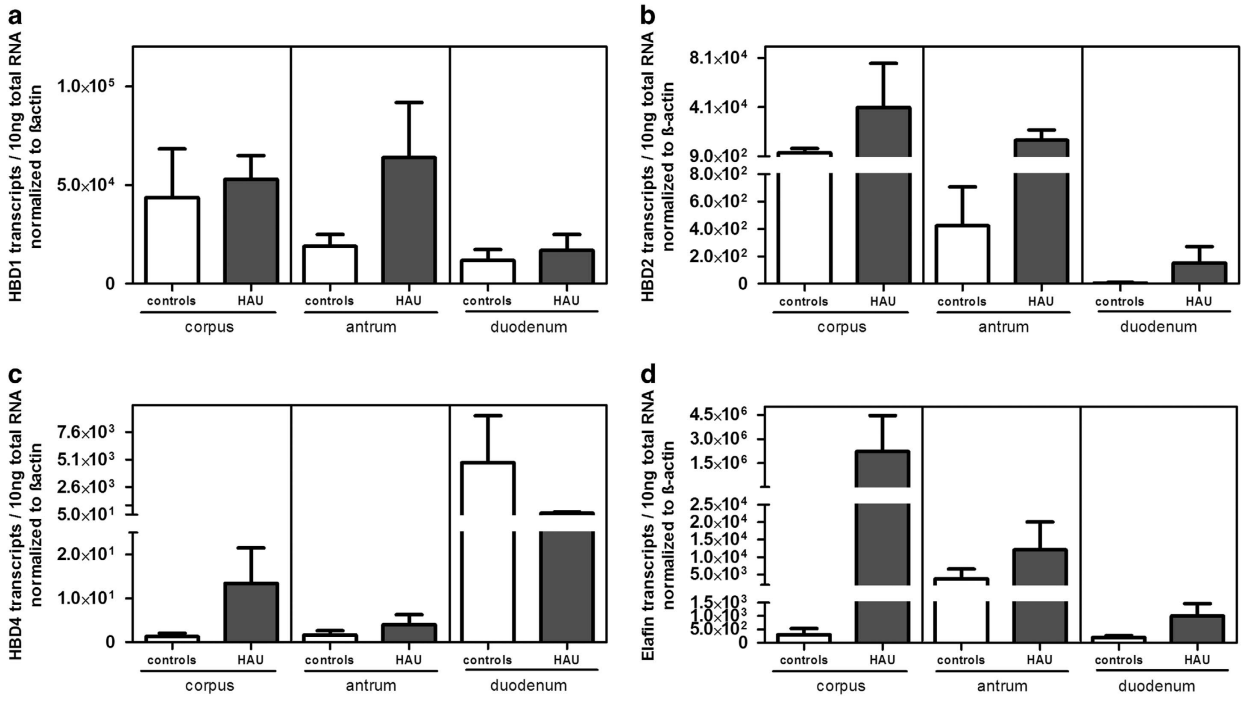

Figure 3 mRNA expression of the gastrointestinal epithelial antimicrobial peptides HBD1 (**a**), HBD2 (**b**), HBD4 (**c**), and elafin (**d**) in the corpus, the antrum, and the duodenum of patients with heavy alcohol use (HAU) and controls. Shown are means with the respective standard error.

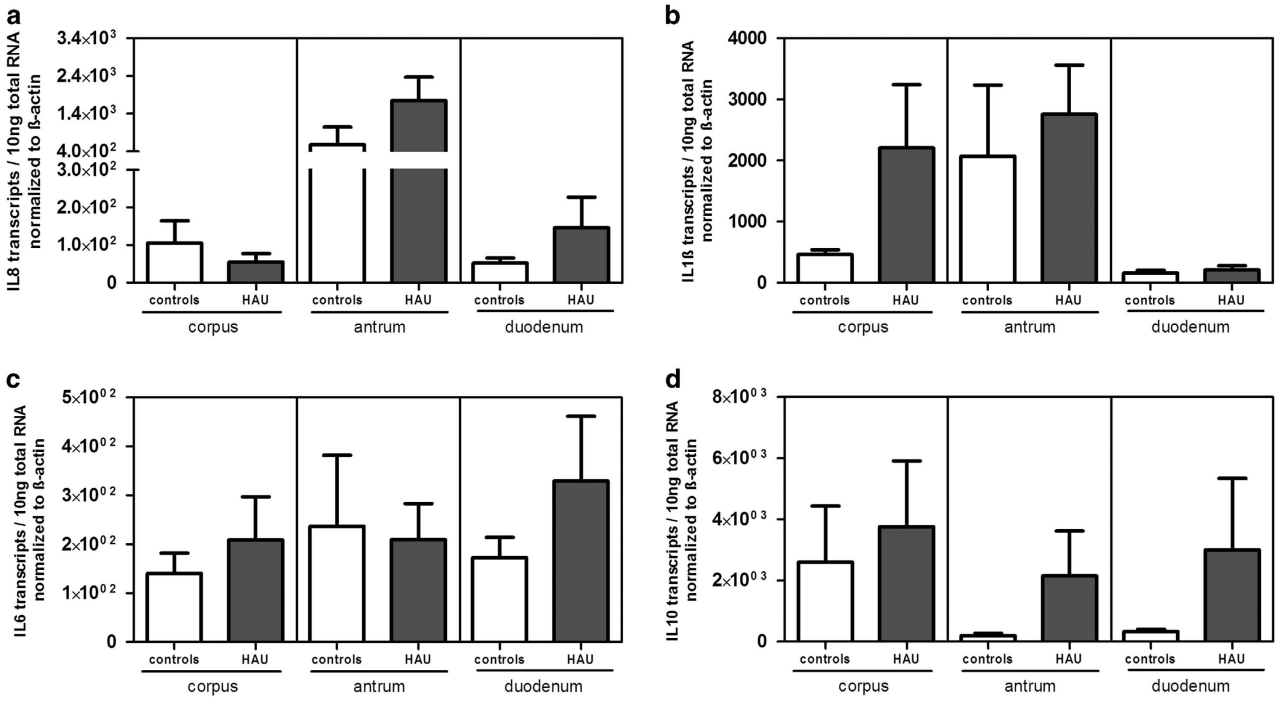

Figure 4 mRNA expression of pro-inflammatory cytokines IL8 (**a**), IL1β (**b**), IL6 (**c**) and the anti-inflammatory IL10 (**d**) in the corpus, the antrum, and the duodenum of patients with heavy alcohol use (HAU) and controls. Shown are means with the respective standard error (s.e.m.).

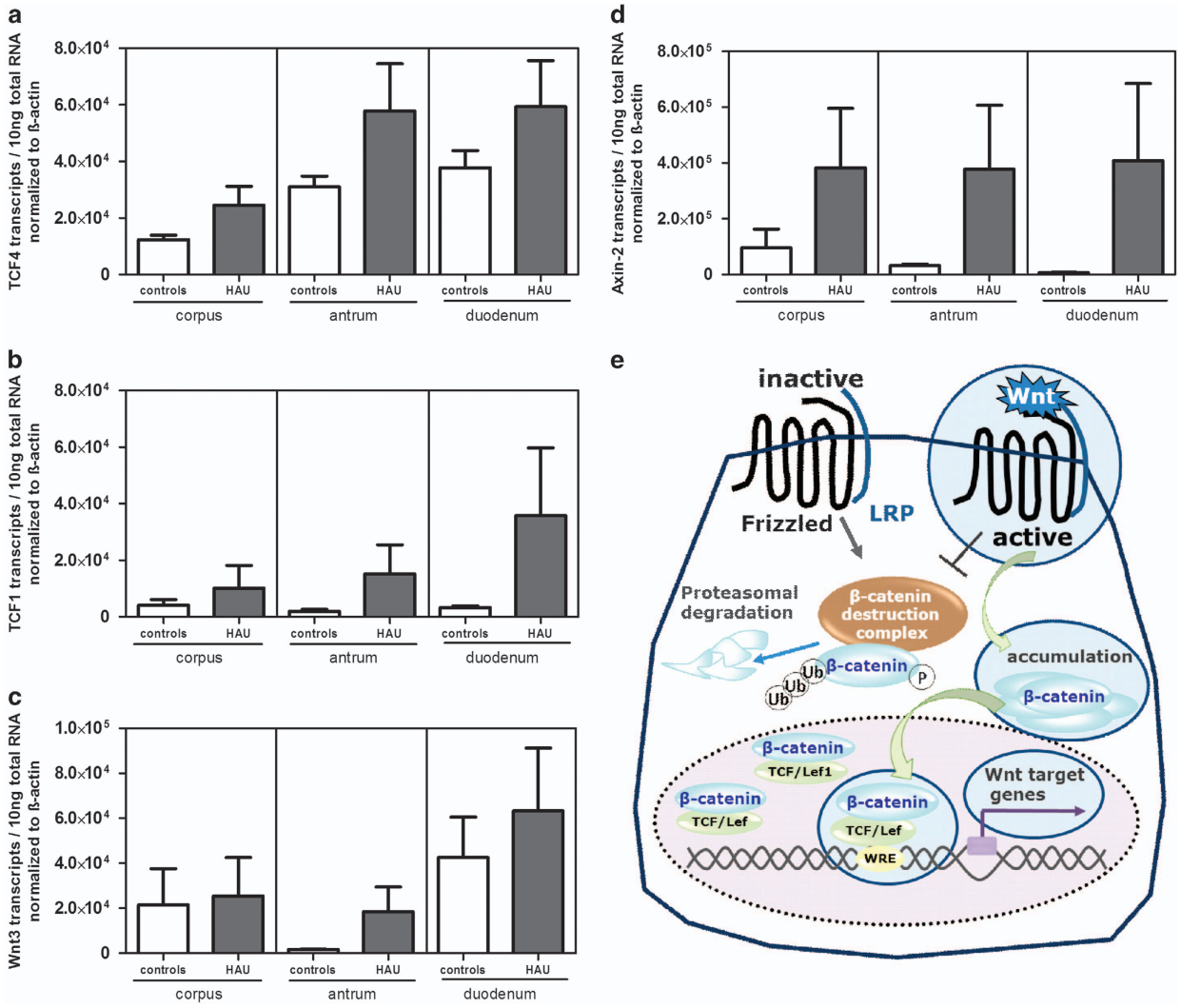

Figure 5 mRNA expression of the important Wnt pathway factors TCF4 (**a**), TCF1 (**b**), Wnt3 (**c**), and Axin-2 (**d**) in the corpus, the antrum, and the duodenum of patients with heavy alcohol use (HAU) and controls. Shown are means and the respective standard error (s.e.m.). The Wnt pathway (**e**) is characterized by two states of activity and multiple levels of control. In the absence of Wnt ligands, the central intracellular signaling molecule β-catenin undergoes phosphorylation and ubiquitination-dependent proteasomal degradation. Once Wnt ligands bind to their respective receptors, a protein complex that normally mediates the destruction of β-catenin is inhibited. This allows the accumulation and subsequent entry of β-catenin into the nucleus where it cooperates with transcription factors of the Lef/TCF family to promote target gene transcription.

Since alcohol has been shown to have direct necrotizing effects and can promote oxidative stress in gastric mucosal cells,[27,38] the induction of Paneth cell AMPs could be a protective mechanism, aiming at restoring a weakened epithelial defense during alcohol-associated damage. On the other hand, it could also represent a first symptom of epithelial transformation.[35] IM, which includes the occurrence of Paneth cells in upper GI tissues, may occur in the esophagus (Barrett's esophagus) and in the distal stomach during gastritis. Barrett's esophagus develops most likely due to prolonged exposure to gastric acid and bile salts, whereas gastric IM has been associated with *H. pylori* colonization. In the above-mentioned study,[35] the gastric form of IM has been shown to include the unequivocal presence of Paneth cells in some patients, whereas others only exhibit HD5-positive cells without typical Paneth cell features in standard histology. In the present study, all patients with histologically proven IM had no indication of *H. pylori* infection. Also, the induction of HD5 mRNA was not only observed in heavy alcohol users with IM, but also in a subgroup without this condition (Supplementary Figure 2). Thus, it appears that IM is not a prerequisite for the production of HD5 in the stomach.

The second group of AMPs studied in our investigations comprised the β-defensins HBD1, 2, and 4, which are of epithelial origin, but are also found in other cells such as macrophages, as well as the antiprotease elafin. For these factors, we found a high variability, but no significant differences.

As mentioned above, one might speculate that heavy alcohol consumption could promote a disturbance in Wnt and consequently in epithelial homeostasis. In the context of inflammation[39] and/or *H. pylori*[40] associated gastric cancer development, a role for upregulated Wnt is well accepted and extensively studied. With regard to alcohol, our data on Wnt pathway activation in a gastric cell line, represent a novel

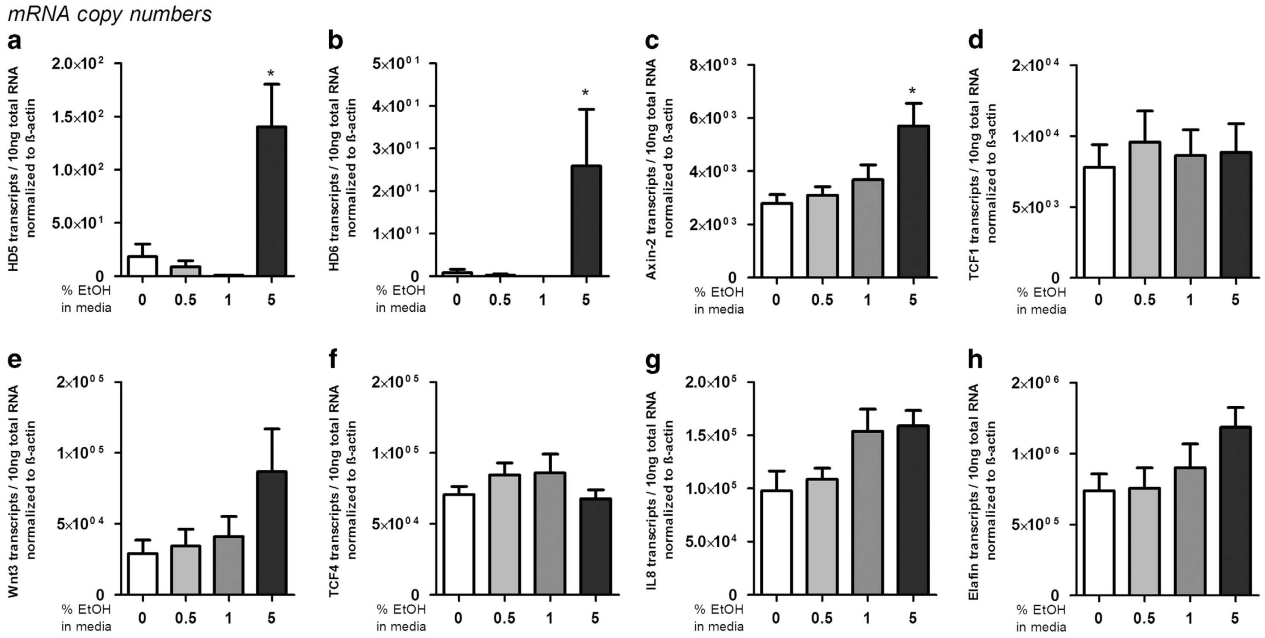

Figure 6 mRNA level of the Wnt target genes HD5 (**a**), HD6 (**b**), Axin-2 (**c**), and the Wnt pathway factors TCF1 (**d**), Wnt3 (**e**) and TCF4 (**f**) as well as IL8 (**g**) and elafin (**h**) in response to a 20 h stimulation with different ethanol (EtOH) concentrations in the culture media. Experiments were carried out in triplicates and repeated a minimum of five times. Shown are means of the independent experiments with the respective standard error (s.e.m.). Significances: Friedman test $P<0.0001$, *Dunn's post test $P<0.05$.

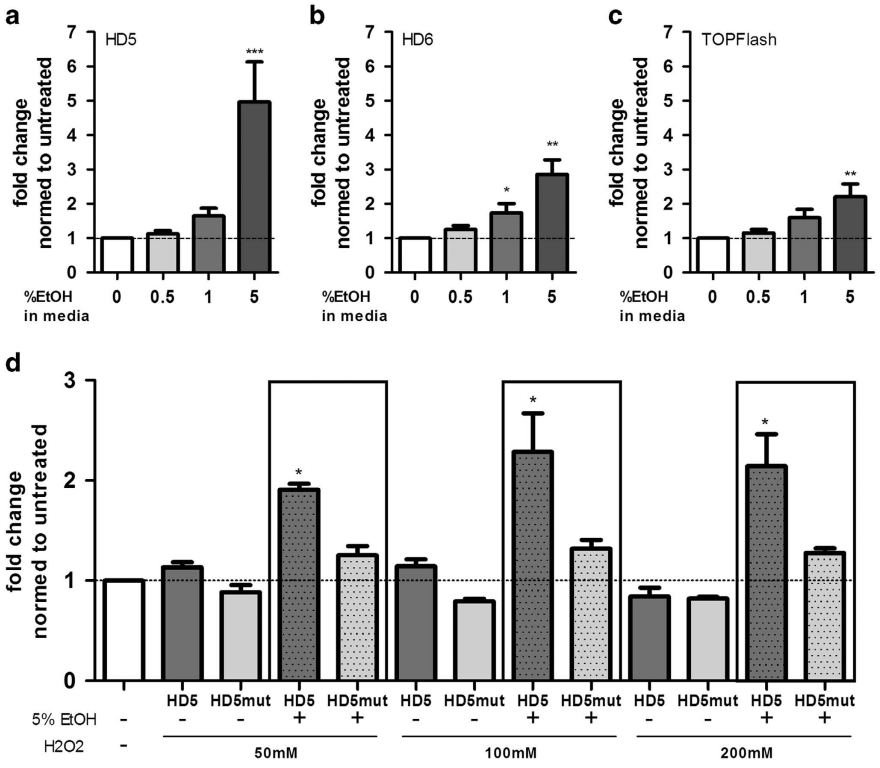

Figure 7 Fold induction of reporter gene activity of HD5 (**a**), HD6 (**b**), and the Wnt responsive TopFlash promotor (**c**) in response to a 20 h stimulation with different ethanol concentrations in the culture media. In addition, a regular as well as a mutated HD5 promotor, lacking three essential binding sites for TCF/Lef Wnt transcription factors, were stimulated 20 h by different concentrations of H2O2, with and without a parallel treatment of 5% EtOH (**d**). Luciferase reporter experiments were carried out in triplicates. Shown are means of independent experiments with the respective s.e.m. Significances: Friedman test $P<0.0001$ for (**a**), (**b**), and (**d**), $P=0.006$ for (**c**), Dunn's post test: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

mechanism. Our reporter gene assays, as well as the Axin-2 mRNA induction, demonstrated a direct activation of β-catenin-dependent Wnt in a short term *in vitro* setting. However, in the same approach, the Wnt transcription factors TCF1 and TCF4 exhibited no significant upregulation, suggesting that the trend for a higher expression found in the tissue of patients with heavy alcohol use may not be a direct but possibly a long-term effect. Although still speculative, the Wnt induction observed in this study may be mechanistically related to the slight increase in cancer risk, especially of non-cardia gastric cancer in heavy drinkers.[21,22] Although data on a connection between alcohol and Wnt signaling are rare, results obtained in another tissue support a link between the two. In the liver, it was recently shown that chronic alcohol feeding activates β-catenin-dependent Wnt to increase hepatocyte proliferation in mice, which ultimately promoted tumorigenesis in this model.[41] If such a relationship turned out to be true in gastric tissue of heavy alcohol users, screening for HD5 expression in gastric biopsies, as a likely symptom of induced Wnt activity, could constitute a potential prognostic marker.

A clear limitation of the study is the restriction to proximal GI tissue due to the lack of jejunal or ileal biopsies in our patient study. Another limitation is based on the fact that, owing to the restricted number of study participants, a further subdivision of controls into total abstainers vs. moderate alcohol consumers was not feasible. Also, as all data on alcohol intake were self-reported, we cannot exclude errors in the assessed levels of consumption. However, as according to our definition, the reported mean of daily alcohol consumption differed widely between the two groups, we are certain that such errors would not lead to a major bias.

The limited number of patients and controls might be a reason why many distinct differences in mRNA expression were lacking statistical significance. This was especially the case regarding TCF1, TCF4, Axin-2, HBD2, and elafin. However, the latter two factors are also highly regulated by different pathways, including NFκB, and MAPKinases, so a high inter-individual variability is to be expected.

Despite these limitations, the presented data suggest that chronic heavy alcohol consumption has the potential to promote the expression of Paneth cell AMPs, which subsequently may alter the innate gastric antimicrobial defense. Based on our mechanistic studies, this seems to be linked to changes in gastric β-catenin-dependent Wnt. Whether this is a potential protective, or rather a pathogenic mechanism, and whether similar observations can also be made in other tissues will require further studies.

CONFLICT OF INTEREST

Guarantor of the article: Jan Wehkamp, MD.
Specific Author contribution: M.J.O. and C.S. designed the study and analyzed the data. M.J.O. designed and planned the experimental work. C.S. phenotyped the patient cohort and organized sample collection. L.C. performed *in vitro* reporter gene experiments; S.R.D.S. performed *in vitro* mRNA experiments. E.F.S. and J.W. were involved in study design. M.J.O., C.S., E.F.S., and J.W. discussed the data and wrote the manuscript.

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Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Chronic heavy alcohol consumption is linked to the translocation of bacteria and their products across the mucosal barrier.
- ✓ This may be an essential mechanism in the pathogenesis of ALD.
- ✓ Paneth cell and other AMPs are important in maintaining mucosal integrity.
- ✓ The occurrence of Paneth cells and IM has been reported in Barrett's esophagus and helicobacter gastritis.

WHAT IS NEW HERE

- ✓ Chronic heavy alcohol use is linked to enhanced transcription of Paneth cell AMPs in the stomach.
- ✓ This is not exclusively related to the presence of IM, which occurs in a small subset of patients.
- ✓ Stimulation of the Wnt pathway is likely to be an important mechanism in this context, whereas inflammation is not.
- ✓ Translocation of toxins in ALD cannot be explained by a defect of defensin, secreted phospholipase A2, elafin, or lysozyme mRNA expression in the stomach and duodenum, but the upper GI tract may not necessarily reflect the more distal small intestine.

1. Rehm J, Samokhvalov AV, Shield KD. Global burden of alcoholic liver diseases. *J Hepatol* 2013; **59**: 160–168.
2. Tilg H, Moschen AR, Kaneider NC. Pathways of liver injury in alcoholic liver disease. *J Hepatol* 2011; **55**: 1159–1161.
3. Bjarnason I, Peters TJ, Wise RJ. The leaky gut of alcoholism: possible route of entry for toxic compounds. *Lancet* 1984; **1**: 179–182.
4. Parlesak A, Schafer C, Schutz T *et al.* Increased intestinal permeability to macromolecules and endotoxemia in patients with chronic alcohol abuse in different stages of alcohol-induced liver disease. *J Hepatol* 2000; **32**: 742–747.
5. Rao R. Endotoxemia and gut barrier dysfunction in alcoholic liver disease. *Hepatology* 2009; **50**: 638–644.
6. Bode C, Kugler V, Bode JC. Endotoxemia in patients with alcoholic and non-alcoholic cirrhosis and in subjects with no evidence of chronic liver disease following acute alcohol excess. *J Hepatol* 1987; **4**: 8–14.
7. Bode C, Bode JC. Effect of alcohol consumption on the gut. *Best Pract Res Clin Gastroenterol* 2003; **17**: 575–592.
8. Bode JC, Bode C, Heidelbach R *et al.* Jejunal microflora in patients with chronic alcohol abuse. *Hepatogastroenterology* 1984; **31**: 30–34.
9. Fukui H, Brauner B, Bode JC *et al.* Plasma endotoxin concentrations in patients with alcoholic and non-alcoholic liver disease: reevaluation with an improved chromogenic assay. *J Hepatol* 1991; **12**: 162–169.
10. Nanji AA, Khettry U, Sadrzadeh SM *et al.* Severity of liver injury in experimental alcoholic liver disease. Correlation with plasma endotoxin, prostaglandin E2, leukotriene B4, and thromboxane B2. *Am J Pathol* 1993; **142**: 367–373.

11. Schäfer C, Parlesak A, Schütt C *et al.* Concentrations of lipopolysaccharide-binding protein, bactericidal/permeability-increasing protein, soluble CD14 and plasma lipids in relation to endotoxaemia in patients with alcoholic liver disease. *Alcohol Alcohol* 2002; **37**: 81–86.
12. Orman ES, Odena G, Bataller R. Alcoholic liver disease: pathogenesis, management, and novel targets for therapy. *J Gastroenterol Hepatol* 2013; **28** (Suppl 1): 77–84.
13. Bevins CL. Paneth cell defensins: key effector molecules of innate immunity. *Biochem Soc Trans* 2006; **34** (Pt 2): 263–266.
14. Clevers HC, Bevins CL. Paneth cells: maestros of the small intestinal crypts. *Annu Rev Physiol* 2013; **75**: 289–311.
15. Teltschik Z, Wiest R, Beisner J *et al.* Intestinal bacterial translocation in rats with cirrhosis is related to compromised paneth cell antimicrobial host defense. *Hepatology* 2011; **55**: 1154–1163.
16. Andreu P, Colnot S, Godard C *et al.* Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. *Development* 2005; **132**: 1443–1451.
17. Andreu P, Peignon G, Slomianny C *et al.* A genetic study of the role of the Wnt/beta-catenin signalling in Paneth cell differentiation. *Dev Biol* 2008; **324**: 288–296.
18. van Es JH, Jay P, Gregorieff A *et al.* Wnt signalling induces maturation of Paneth cells in intestinal crypts. *Nat Cell Biol* 2005; **7**: 381–386.
19. Hoffman J, Kuhnert F, Davis CR *et al.* Wnts as essential growth factors for the adult small intestine and colon. *Cell Cycle* 2004; **3**: 554–557.
20. Kuhnert F, Davis CR, Wang HT *et al.* Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proc Natl Acad Sci USA* 2004; **101**: 266–271.
21. Tramacere I, Pelucchi C, Bagnardi V *et al.* A meta-analysis on alcohol drinking and esophageal and gastric cardia adenocarcinoma risk. *Ann Oncol* 2012; **23**: 287–297.
22. Tramacere I, Negri E, Pelucchi C *et al.* A meta-analysis on alcohol drinking and gastric cancer risk. *Ann Oncol* 2012; **23**: 28–36.
23. Bone RC, Balk RA, Cerra FB *et al.* Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 1992; **101**: 1644–1655.
24. Wehkamp J, Wang G, Kubler I *et al.* The Paneth cell alpha-defensin deficiency of ileal Crohn's disease is linked to Wnt/Tcf-4. *J Immunol* 2007; **179**: 3109–3118.
25. Korinek V, Barker N, Morin PJ *et al.* Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* 1997; **275**: 1784–1787.
26. Hidaka E, Ota H, Katsuyama T *et al.* Coexistence of gland mucous cell-type mucin and lysozyme in gastric gland mucous cells. *Histochem Cell Biol* 2000; **113**: 91–98.
27. Tamura M, Matsui H, Kaneko T *et al.* Alcohol is an oxidative stressor for gastric epithelial cells: detection of superoxide in living cells. *J Clin Biochem Nutr* 2013; **53**: 75–80.
28. Bevins CL. Antimicrobial peptides as effector molecules of mammalian host defense. *Contrib Microbiol* 2003; **10**: 106–148.
29. Wehkamp J, Stange EF. Paneth's disease. *J Crohns Colitis* 2010; **4**: 523–531.
30. Wilson CL, Ouellette AJ, Satchell DP *et al.* Regulation of intestinal α-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* 1999; **286**: 113–117.
31. Salzman NH, Gottsch JD, Huttner KM, Paterson Y, Bevins CL. Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature* 2003; **422**: 522–526.
32. Chu H, Pazgier M, Jung G *et al.* Human alpha-defensin 6 promotes mucosal innate immunity through self-assembled peptide nanonets. *Science* 2012; **337**: 477–481.
33. Vaishnava S, Behrendt CL, Ismail AS *et al.* Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci USA* 2008; **105**: 20858–20863.
34. Salzman NH, Hung K, Haribhai D *et al.* Enteric defensins are essential regulators of intestinal microbial ecology. *Nat Immunol* 2010; **11**: 76–83.
35. Shen B, Porter EM, Reynoso E *et al.* Human defensin 5 expression in intestinal metaplasia of the upper gastrointestinal tract. *J Clin Pathol* 2005; **58**: 687–694.
36. Friis-Hansen L. Achlorhydria is associated with gastric microbial overgrowth and development of cancer: lessons learned from the gastrin knockout mouse. *Scand J Clin Lab Invest* 2006; **66**: 607–621.
37. Naylor G, Axon A. Role of bacterial overgrowth in the stomach as an additional risk factor for gastritis. *Can J Gastroenterol* 2003; **17** (Suppl B): 13B–17BB.
38. Loguercio C, Tuccillo C, Federico A *et al.* Alcoholic beverages and gastric epithelial cell viability: effect on oxidative stress-induced damage. *J Physiol Pharmacol* 2009; **60** (Suppl 7): 87–92.
39. Oguma K, Oshima H, Oshima M. Inflammation, tumor necrosis factor and Wnt promotion in gastric cancer development. *Future Oncol* 2010; **6**: 515–526.
40. Ding SZ, Goldberg JB, Hatakeyama M. Helicobacter pylori infection, oncogenic pathways and epigenetic mechanisms in gastric carcinogenesis. *Future Oncol* 2010; **6**: 851–862.
41. Mercer KE, Hennings L, Sharma N *et al.* Alcohol consumption promotes diethylnitrosamine-induced hepatocarcinogenesis in male mice through activation of the Wnt/beta-catenin signaling pathway. *Cancer Prev Res (Phila)* 2014; **7**: 675–685.

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