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Blood Biomarkers of Intestinal Epithelium Damage Regenerating Islet-derived Protein 3α and Trefoil Factor 3 Are Persistently Elevated in Patients with Alcoholic Hepatitis

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Abstract

Background—Alcohol abuse disrupts gut epithelial integrity, leading to increased permeability of the gastrointestinal tract and subsequent translocation of microbes. Regenerating islet-derived protein 3α (REG3α) and Trefoil factor 3 (TFF3) are mainly secreted to the gut lumen by Paneth and Goblet cells, respectively, and are functionally linked to gut barrier integrity. Circulating levels of REG3α and TFF3 have been identified as biomarkers for gut damage in several human diseases. We aimed to identify whether plasma levels of REG3α and TFF3 were dysregulated and correlated with conventional markers of microbial translocation (MT) and pro-inflammatory mediators in heavy drinkers with and without alcoholic hepatitis (AH).

Methods—Cross-sectional and longitudinal studies were performed to monitor plasma levels of REG3α and TFF3 in 79 AH patients, 66 heavy drinkers without liver disease (HDC), and 46 healthy controls (HC) at enrollment, 6- and 12-month follow-ups. Spearman correlation was

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CONFLICT OF INTEREST

The authors declare no conflicts of interest that pertain to this manuscript.

carried out to study the relationships of REG3α and TFF3 levels with MT, disease severity, inflammation, and alcohol abstinent effects.

Results—At enrollment, AH patients had significantly higher levels of REG3α and TFF3 than HDC and HC. The elevated REG3α levels positively correlated to the 30-day fatality rate. Plasma levels of REG3α and TFF3 in AH patients differentially correlated with conventional MT markers (sCD14, sCD163, and LBP) and several highly up-regulated inflammatory cytokines/chemokines/growth factors. At follow-ups, REG3α and TFF3 levels were decreased in AH patients with alcohol abstinence, but did not fully return to baseline levels.

Conclusions—Circulating levels of REG3α and TFF3 were highly elevated in AH patients and differentially correlated with AH disease severity, MT, and inflammation, thereby serving as potential biomarkers of MT and gut epithelial damage in AH patients.

Keywords

REG3α; TFF3; alcoholic hepatitis; gut epithelial damage; microbial translocation; inflammation; immune activation

INTRODUCTION

Long-term heavy drinkers develop a spectrum of severe alcoholic liver disease (ALD), ranging from alcoholic hepatitis (AH) and fibrosis/cirrhosis to hepatocellular carcinoma (HCC) (O'Shea et al., 2010). Up to 35% of chronic heavy drinkers develop AH, a severe and progressive liver inflammatory disease associated with significant morbidity, mortality, and economic burdens (Liangpunsakul, 2011, Bruha et al., 2012). Animal and human studies have revealed that alcohol and its metabolites cause gut barrier dysfunction at multiple interconnected levels including (1) damage of the intestinal epithelial cells (Lambert et al., 2003, Lippai et al., 2014), (2) interruption of gap junction integrity of gut mucosal epithelial cells, leading to increased permeability of the gastrointestinal (GI) tract (Elamin et al., 2014, Bode and Bode, 2005, Gao and Bataller, 2011), (3) gut dysbiosis caused by altering gut microbiota composition and function (Engen et al., 2015, Mutlu et al., 2012, Puri et al., 2018, Bjorkhaug et al., 2019), (4) dysregulation of gut mucosal cell function by suppressing IL-22 production from mucosal immune cells, leading to loss of IL-22-mediated protection of intestinal stem cells against alcohol-mediated insults and stress (Rendon et al., 2013, Szabo and Petrasek, 2017), (5) reduction of the amount of anti-microbial molecules, resulting in microbial imbalance and an impaired gut mucosal barrier (Hendrikx and Schnabl, 2019, Hartmann et al., 2015), and (6) inhibition of mucosal signal molecules and immune cells, causing suppression of the intestinal mucosal immune response and bacterial clearance (Szabo and Saha, 2015, Riva et al., 2018). Alcohol-induced gut hyperpermeability causes translocation of microbes and their components, such as lipopolysaccharides (LPS), from the GI tract into the blood and liver (Elamin et al., 2014, Bode and Bode, 2005, Gao and Bataller, 2011). Translocated LPS and other microbial components profoundly trigger immune activation and inflammation by activating a number of receptors, including Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors, that are expressed in many types of immune cells, such as circulating monocytes/macrophages and liver-resident Kupffer cells (Franchi et al., 2009, Prajapati et al., 2014, Wheeler, 2003,

Stahl et al., 2018). Thus, alcohol-induced microbial translocation (MT) represents a major driver of chronic immune activation and inflammation in AH patients (Pasala et al., 2015, Gao et al., 2011, Miller et al., 2011).

The circulating levels of soluble CD14 (sCD14), soluble CD163 (sCD163) and LPS in the peripheral circulation have been used as non-invasive surrogate biomarkers of MT in a wide spectrum of human diseases including AH (Liangpunsakul et al., 2017, Li et al., 2019, Saha et al., 2019). Circulating sCD14 and sCD163 are mainly shed from their membrane compartments during activation of monocytes and macrophages (Kirkland and Viriyakosol, 1998, Galea et al., 2012). A variety of stimuli including inflammatory cytokines, LPS, and non-LPS TLR ligands can effectively activate monocytes/macrophages to produce sCD14 and sCD163 in *vitro* and in *vivo* (Shive et al., 2015). Therefore, circulating sCD14 and sCD163 are nonspecific activation markers of monocytes and macrophages, and their alterations in the peripheral blood do not precisely reflect the spectrum of gut damage and dysbiosis present in AH. Because LPS is an endotoxin derived from the gram-negative bacteria found in gut microbes, LPS levels in the peripheral blood are not able to accurately reflect MT of more highly abundant gram-positive bacteria and may, therefore, under-evaluate the severity of gut damage in AH. Thus, there is an urgent demand for novel non-invasive biomarkers for evaluating MT, gut epithelium damage, and gut dysbiosis in AH patients.

Recently, regenerating islet-derived protein 3 alpha (REG3α) has been identified as a novel marker of gut epithelial damage in several inflammatory diseases, such as nonalcoholic steatohepatitis (NASH) (Bluemel et al., 2018, Wang et al., 2016) and graft-versus-host disease (GVHD) (Ferrara et al., 2011, Zhao et al., 2018). REG3α is a member of the antimicrobial REG3 C-type lectin family (Shin and Seeley, 2019). REG3α is selectively and constitutively produced and secreted into the gut lumen by Paneth cells, a principal epithelial cell type of small intestinal crypts found to be critically involved in the homeostasis of the gut microbiota, mucosal tissues and survival of small intestine stem cells. REG3α affects the intestinal bacterial community through its bactericidal activities against gram-positive bacteria. In addition, REG3α plays an important role in maintaining gut integrity by reducing apoptosis of intestinal epithelial cells (Chen et al., 2019). Trefoil factor 3 (TFF3), a small peptide of the TFF regulatory protein family, is another protein that functions to protect and repair GI epithelia (Aihara et al., 2017). TFF3 is mainly secreted by the intestinal epithelium, specifically via goblet cells that are highly enriched in the mucosal layer of the GI tract (Aihara et al., 2017). Both REG3α and TFF3 can be detected in the peripheral blood of healthy humans. The circulating levels of REG3α and TFF3 are highly elevated and correlate with damage to the intestinal crypts and disease severity in patients with inflammatory gut diseases, such as inflammatory bowel disease (IBD) (Gironella et al., 2005), gastric cancer (Matsumura et al., 2011), and ulcerative colitis (UC) (Marafini et al., 2014), thereby serving as markers of gut epithelium damage and MT.

Herein, we used a well-known cohort of AH patients, heavy drinking controls without overt liver disease (HDC), and healthy controls (HC) from the consortium of the Translational Research and Evolving Alcoholic Hepatitis Treatment (TREAT, NCT02172898) to conduct cross-sectional and longitudinal studies of plasma REG3α and TFF3 levels at the time of

enrollment, 6 month follow-up, and 12 month follow-up. We also analyzed the relationship of plasma REG3α and TFF3 levels with AH liver severity, conventional MT biomarkers, and inflammatory factors. We found that plasma REG3α and TFF3 levels were highly elevated in AH patients and differentially correlated with AH disease severity, MT, and inflammation, thereby potentially serving as novel biomarkers of MT and gut epithelial damage in AH patients.

MATERIALS AND METHODS

Study Subjects

The study subjects, including 79 AH patients and 66 HDC at baseline, 30 AH patients and 33 HDC at 6 month follow-up, and 18 AH patients and 27 HDC at 12 month follow-up, were among the participants enrolled in the prospective multicenter observational TREAT 001 study (NCT02172898). These participants also served as the study cohort in our previous studies (Li et al., 2017, Li et al., 2019, Xia et al., 2020, Li et al., 2020). Detailed definitions for AH and HDC have already been reported (Liangpunsakul et al., 2016). In brief, AH was defined as the onset of aspartate aminotransferase (AST) >50 IU/L and elevated total bilirubin (initially >2 mg/dL, later amended to >3 mg/dL) in long-term alcoholics who were drinking heavily within the 6 weeks prior to enrollment. HDC were age- and gender-matched participants with a similar history of alcohol abuse as the AH patients but without overt clinical liver disease (AST <50 U/L, alanine aminotransferase [ALT] <50 U/L, total bilirubin within normal range). All participants were advised to completely stop drinking and were followed-up at 6 months and 12 months or until death. The demographic and clinical characteristics and drinking patterns of the alcoholic cohort was summarized in Table 1 and Supplementary Table 1.

Blood Collection and Isolation of Plasma

Peripheral blood was collected in tubes coated with heparin (BD Biosciences, Franklin Lakes, NJ). Blood samples were centrifuged within 2 h of collection at 700 g for 20 min at room temperature without brake. The top layer (plasma) was harvested and stored at −80°C. Plasma were also prepared from 46 HCs matched for age and gender with AH patients and HDC.

ELISA and Multiplex Immunoassays

Levels of REG3α, TFF3, I-FABP (intestinal fatty acid binding protein), and IL-22 in plasma samples were quantified using the Human REG3α DuoSet ELISA Kit, Human TFF3 DuoSet ELISA kit, Human I-FABP DuoSet ELISA kit, and Human IL-22 Quantikine ELISA kit (R&D Systems, Minneapolis, MN), respectively, according to the manufacturer's instructions. Plasma levels of sCD14, sCD163, LPS-binding protein (LBP), and IL-6 were quantified using the Human CD14 Quantikine Kit, the Human CD163 Quantikine Kit, the Human LBP DuoSet ELISA Kit, and the IL-6 High Sensitivity Quantikine ELISA kit (R&D Systems, Minneapolis, MN), respectively, as previously described (Li et al., 2017, Li et al., 2019, Xia et al., 2020). Plasma LPS levels were determined using the PYROGENT-5000 Kinetic Turbidimetric Limulus Amebocyte Lysate Assay (Lonza, Walkersville, MD) as previously described (Li et al., 2019). Plasma concentrations of 45 cytokines/chemokines/

growth factors were measured using the Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex Panel 1 (ThermoFisher Scientific, Waltham, MA) as previously described (Li et al., 2019).

Statistical Analysis

Mann-Whitney test and Kruskal-Wallis test with Dunn's corrections were performed to calculate differences in continuous variables between 2 groups and among 3 groups, respectively. Chi-square test was used to compare categorical variables. Spearman correlation test was used to analyze the linear association of REG3α, TFF3, I-FABP, and IL-22 with clinical parameters and other factors. Friedman rank sum test with Dunn's corrections was used to calculate the differences in longitudinal analysis. Log-Rank (Mantel-Cox) test was used to compare survival curves. $p < 0.05$ was considered statistically significant.

Ethical Considerations

This study was performed with the approval of the Institutional Review Boards at Indiana University School of Medicine, Mayo Clinic, and Virginia Commonwealth University. Blood samples were drawn after each participant provided written informed consent.

RESULTS

Characteristics of Study Participants

The study subjects, including 79 AH patients and 66 HDC at enrollment (baseline), 30 AH patients and 33 HDC at 6 month follow-up, and 18 AH patients and 27 HDC at 12 month follow-up, were among the participants enrolled in the TREAT 001 cohort (NCT02172898) (Li et al., 2017, Li et al., 2019, Xia et al., 2020, Li et al., 2020). The demographic and clinical characteristics of these participants at enrollment and follow-ups were summarized in Table 1. There were no differences in age and gender distributions between AH patients and HDC at enrollment, 6 month and 12 month follow-ups. HDC had significantly more drinks than AH patients during the last 30 days prior to enrollment. At follow-ups, both AH patients and HDC reported much less drinking with no significant differences found in alcohol consumption between the 2 groups. As expected, the AH patients had higher levels of biochemical markers of liver injury and function (ALT, AST, and total bilirubin), higher MELD (model for end stage liver disease) score, and longer prothrombin time than HDC at recruitment. Levels of baseline creatinine were similar between AH patients and HDC. Compared to HC, HDC had slightly higher levels of AST, ALT, and total bilirubin. At follow-ups, ALT, AST, total bilirubin, MELD score, and prothrombin time improved in AH patients, but were still significantly higher than in HDC. ALT levels became similar between AH patients and HDC at the 12 month follow-up. For patients who achieved complete alcohol cessation at follow-ups, AST, total bilirubin, the MELD score, and prothrombin time remained higher in AH patients than HDC (Supplementary Table 1). Peripheral blood samples (plasma and PBMCs) from 46 healthy donors, matched for age and gender with AH patients and HDC, were used as HCs.

Plasma Levels of REG3α and TFF3 Were Elevated in AH Patients

To determine and compare the levels of REG3α and TFF3 in the peripheral blood in AH patients vs HDC vs HC, we used ELISA assays to measure their plasma levels. Cross-sectional analysis showed that AH patients had higher baseline levels of REG3α (median 18.9 ng/ml, interquartile range [IQR] 11.0–70.8 ng/ml) when compared to HDC (median 8.1 ng/ml, IQR 5.1–11.3 ng/ml) or HC (median 7.34 ng/ml, IQR 6.0–9.8 ng/ml) (Fig. 1A). Plasma levels of TFF3 were also higher in AH patients (median 9.0 ng/ml, IQR 6.4–13.9 ng/ml) when compared to HDC (median 5.3 ng/ml, IQR 4.3–7.5 ng/ml) or HC (median 4.7 ng/ml, IQR 4.1–6.8 ng/ml) (Fig. 1B). There were no differences in the plasma levels of either REG3α or TFF3 between HDC and HC. (Fig. 1A, 1B).

Longitudinal assessment was conducted to determine whether elevated plasma levels of REG3α and TFF3 were decreased at follow-ups. Plasma levels of REG3α, but not TFF3, declined in AH patients at 6 months (median 10.3 ng/ml, IQR 6.9–14.8 ng/ml, p = 0.0005) and 12 months (median 10.9 ng/ml, IQR 7.7–22.6 ng/ml, p = 0.04) from the baseline value. Plasma levels of REG3α and TFF3 in HDC remained unchanged except for slightly increased TFF3 levels at 12 months (median 6.9 ng/ml, IQR 5.5–9.9 ng/ml, p = 0.02) compared to the baseline level. We next compared the plasma levels of REG3α and TFF3 in AH patients vs HDC at 6 month and 12 month follow-ups. At 6 months, plasma levels of both REG3α and TFF3 remained elevated in AH patients when compared to HDC (p = 0.0016 and p = 0.001, respectively) (Fig. 1A, B). At 12 months, plasma levels of REG3α were still higher in AH patients when compared to HDC (p = 0.04) (Fig. 1A), however plasma levels of TFF3 did not show a significant difference between AH patients and HDC (Fig. 1B).

We next examined plasma levels of I-FABP, an established blood biomarker of intestinal epithelial damage produced by gut epithelial cells and released into the circulation upon gut epithelium damage (Schoultz and Keita Å, 2020, Grootjans et al., 2010). There were no significant differences in plasma levels of I-FABP between AH patients, HDC, and HC at enrollment or between AH patients and HDC at follow-ups (Fig. 1C). However, plasma levels of I-FABP at the 6 month follow-up (median 1.9 ng/ml, IQR 1.5–3.0 ng/ml) were higher than the baseline level (median 1.2 ng/ml, IQR 0.5–1.7 ng/ml, p = 0.0003). I-FABP levels in HDC remained unchanged throughout the study.

Spearman correlation analysis was performed to identify any associations between plasma levels of the 3 gut epithelium damage markers. There was a positive correlation between plasma levels of REG3α and TFF3 in AH patients at recruitment (r = 0.54 and p<0.0001) (Fig. 1D). Baseline I-FABP levels also correlated with TFF3 levels (r = 0.35 and p = 0.002) (Fig. 1E), but not with REG3α levels (Fig. 1F). There were no correlations between REG3α, TFF3, or I-FABP at either 6 month or 12 month follow-ups (data not shown).

Since IL-22, a member of the IL-10 cytokine family, plays an important role in ameliorating alcoholic liver injury in a murine model of ALD (Ki et al., 2010) and can directly regulate the production of the REG family proteins by epithelial cells (Zheng et al., 2008), we also measured plasma levels of IL-22 in the cohort of AH patients, HDC, and HC. In agreement with results previously reported (Liu et al., 2017), we found that plasma levels of IL-22 at

recruitment were elevated in AH patients (median 20.9 pg/ml, IQR range 11.7–46.1 pg/ml) when compared to HDC (median 11.3 pg/ml, IQR 8.1–20.9 pg/ml) or HC (median 7.1 pg/ml, IQR 5.0–15.4 pg/ml) (Supplementary Fig. S1A). There was no significant difference in the plasma levels of IL-22 between HDC and HC at recruitment (Supplementary Fig. S1A). Plasma levels of IL-22 in AH patients decreased significantly at the 12 month (median 7.6 pg/ml, IQR 0–18.0 pg/ml, p = 0.0007), but not the 6 month follow-up as compared to the baseline IL-22 levels, whereas IL-22 levels in HDC remained unchanged (Supplementary Fig. S1A). IL-22 levels only had a negligible positive correlation with REG3α (r = 0.27 and $p = 0.027$) (Supplementary Fig. S1B), and no significant correlations with either TFF3 or I-FABP (data not shown) in AH patients at recruitment. This association between IL-22 and REG3α persisted at the 6 month follow-up (r = 0.43 and $p = 0.019$).

Together, our data indicates that plasma levels of REG3α, TFF3 and IL-22 are elevated in AH patients when compared to HDC or HC at baseline and that REG3α and TFF3 levels correlated with each other. At follow-ups, REG3α and TFF3 levels remained higher in AH patients.

Plasma Levels of REG3α, TFF3, and IL-22 Correlated with AH Disease Severity

We next determined whether plasma levels of REG3α, TFF3, I-FABP, and IL-22 might be related to AH pathogenesis by examining their correlations with clinical parameters, including MELD scores and the concentrations of creatinine, total bilirubin, AST, ALT, prothrombin time and C-reactive protein (CRP) from the peripheral blood in AH patients. As shown in Table 2, levels of REG3α, TFF3, and IL-22, but not I-FABP, had positive correlations with MELD scores and the concentrations of creatinine and total bilirubin. Plasma levels of REG3α were also positively correlated with CRP (Table 2). REG3α, TFF3 and IL-22 showed no correlations with concentrations of prothrombin time, ALT, or AST, whereas I-FABP had a slight negative correlation with AST (Table 2). TFF3 levels at 6 months still correlated with MELD scores (r = 0.50, p = 0.005) and total bilirubin (r =0.45, p = 0.01). However, REG3α, TFF3, I-FABP, and IL-22 showed no correlation with AH disease severity at the 12 month follow-up (data not shown). Since MELD score has been widely used as an indicator of ALD severity, the positive correlations of REG3α and TFF3 with MELD scores suggest that these two factors may serve as biomarkers of ALD severity.

Plasma Levels of REG3α Predicted for 30-day Mortality in AH Patients

In order to further clarify the correlation of REG3α and TFF3 with severity and prognosis of AH, we compared the plasma levels of REG3α and TFF3 in AH patients who survived over 30 days from enrollment (survivors, n=73) and those who died within 30 days (deceased, n=6). Plasma levels of REG3α were higher in the deceased group when compared to the survivor group ($p = 0.001$) (Fig. 2A), whereas plasma TFF3 levels did not reach a significant difference between the two groups (Fig. 2B). Further, we examined whether plasma levels of REG3α and TFF3 could predict survival by performing Kaplan-Meier survival analysis. Elevated plasma levels of REG3α predicted 30-day mortality (Fig. 2C). Plasma levels of TFF3 trended to predict 30-day mortality. Because of the limited numbers of deceased individuals, the differences were not significant (Fig. 2D). Plasma levels of IL-22 and I-FABP did not show any correlation with the 30-day survival rate or mortality of AH patients

(data not shown). Thus, plasma levels of REG3α have a negative correlation with patients' 30-day survival rate and can predict 30-day mortality of AH patients.

Correlation of Plasma Levels of REG3α and TFF3 with Conventional Markers of Microbial Translocation

Microbial translocation (MT) represents one of the critical mechanisms in the development of ALD including AH (Ponziani et al., 2018). Conventionally, the levels of sCD14, sCD163, and LPS in the peripheral blood of AH patients are highly upregulated (Supplementary Table 2) and used as surrogate biomarkers of MT. We determined whether plasma levels of REG3α and TFF3 correlated with plasma levels of these conventional MT biomarkers in AH patients. The correlation analysis results were summarized in Table 3. REG3α had a positive correlation with sCD14, an activation marker of monocytes and macrophages. REG3α also correlated with plasma levels of LBP, a soluble acute-phase protein that binds to LPS to activate immune cells. In comparison to REG3α, TFF3 showed a different correlation profile with the conventional MT markers. As shown in Table 3, TFF3 had a weak correlation with plasma levels of sCD163, but no correlation with sCD14 and LBP. Interestingly, neither REG3α nor TFF3 correlated with plasma levels of LPS in AH patients. Plasma levels of IL-22 and I-FABP did not correlate with any of these conventional MT markers (Table 3). Thus, plasma levels of REG3α and TFF3 are differentially associated with certain conventional MT markers, but not LPS, in AH patients.

REG3α and TFF3 Levels Were Associated with Systemic Inflammation

Plasma concentrations of 45 cytokines/chemokines/growth factors in our study cohort were measured using the Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex Panel 1 (ThermoFisher Scientific, Waltham, MA) as described in our recent report (Li et al., 2019). Given that both inflammatory responses and MT are primary contributors to the development and progression of AH and that several cytokine signals directly regulate expression of REG3α and TFF3 in ALD (Wu et al., 2016, Baus-Loncar et al., 2004, Dudakov et al., 2015), we analyzed the correlations of plasma levels of REG3α and TFF3 with inflammatory cytokines/chemokines and growth factors that were elevated in AH patients (Supplementary Table 2). The inflammatory factors that showed significant correlations with REG3α and/or TFF3 were summarized in Table 3. Circulating levels of REG3α and TFF3 correlated with multiple pro-inflammatory cytokines/chemokines, including IL-1α, IL-6, and IL-8. In addition, both REG3α and TFF3 correlated with IL-10, an anti-inflammatory cytokine, as well as several growth factors including HGF, SDF-1α, and VEGF-A. Additional correlations were detected between REG3α and several chemokines including IP-10, MCP-1, and MIP-1β and between TFF3 and IL-21, PIGF-1, and SCF. IL-22 levels weakly correlated with IL-8, IP-10, and HGF, whereas I-FABP only showed a negative correlation with HGF. These results suggest dysregulated production of REG3α and TFF3 are associated with inflammatory responses in AH patients.

Plasma Levels of REG3α and TFF3 Were not Completely Reversed after Alcohol Abstinence in AH Patients

To examine whether elevated levels of REG3α and TFF3 in AH patients might be reversed or normalized after alcohol abstinence, we analyzed their plasma levels in abstinent AH

patients and HDC at 6 and 12 month follow-ups. Plasma levels of both REG3α and TFF3 were still higher in AH patients than HDC at the 6 month follow-up, but not at the 12 month follow-up (Fig. 3A, B). However, both REG3α and TFF3 levels remained elevated at 12 months compared to HC (Fig. 3A, B). Interestingly, HDC at the 12 month follow-up had a higher TFF3 level than HC. Longitudinal analysis showed that plasma levels of REG3α and TFF3 did not significantly change throughout the study period in either abstinent AH patients or HDC (Fig. 3C, D). Plasma levels of IL-22 and I-FABP at follow-ups were not different among abstinent AH patients, HDC, or HC (data not shown) and did not change significantly during the longitudinal study period (data not shown). Our data indicate that elevated plasma levels of REG3α and TFF3 in AH patients were not completely reversed by alcohol abstinence.

To further characterize the effect of alcohol abstinence, we compared the clinical parameters and the 4 soluble gut integrity markers (REG3α, TFF3, I-FABP, and IL22) between the drinking and abstinent AH or HDC subjects at 6 and 12 month follow-ups. One of the AH patients at the 6 month follow-up was excluded from this analysis due to a lack of data on alcohol use. These results are shown in Supplementary Tables 3 and 4. For AH patients, the drinking subjects had significantly higher levels of AST at 6 months and 12 months, ALT at 6 months, and total bilirubin at 12 months than their abstinent counterparts. Other clinical parameters, including the MELD scores, and REG3α, TFF3, I-FABP and IL22 were not significantly different between the drinking and abstinent subjects at follow-ups. For the HDC subjects, the only significant difference between drinking and non-drinking was the prothrombin time at 12 months. These results are consistent with the notion that alcohol abstinence could not completely reverse the abnormalities found in AH patients.

DISCUSSION

In the present study, we performed cross-sectional and longitudinal analyses of REG3α and TFF3 as potential noninvasive biomarkers for gut barrier impairment and microbial translocation in a large cohort of AH patients, HDC, and healthy controls (Table 1). We found that AH patients had elevated plasma levels of REG3α and TFF3 (Fig. 1), which were positively correlated with MELD scores, up-regulated conventional MT markers (sCD163, sCD14, and LBP), and multiple elevated pro-inflammatory cytokines/chemokines/growth factors (IL-1α, IL-6, IL-8, IL-10, IL-21, IP-10, MCP-1, MIP-1β, HGF, PIGF-1, SCF, SDF-1α, and VEGF-A) in AH patients (Tables 2 and 3). Based on the survival analysis, lower REG3α plasma levels had a better prognosis than higher REG3α plasma levels in AH patients (Fig. 2). We also found that alcohol abstinence considerably improved but did not completely reverse REG3α and TFF3 abnormalities in AH patients (Fig. 3). In contrast, plasma levels of IL-22 in AH patients reversed to levels similar to those found in HC.

As it is challenging to get access to gut tissues for routine testing, blood biomarkers are more practical to assess gut damage in AH patients. The levels of LPS, sCD14 and sCD163 in the peripheral circulation have been used as surrogate biomarkers of MT and gut damage in a wide spectrum of human diseases including AH (Liangpunsakul et al., 2017, Li et al., 2019, Saha et al., 2019). These biomarkers are highly elevated in the peripheral blood of AH patients, which generally correlate with AH severity and/or mortality (Liangpunsakul et al.,

2017, Li et al., 2019, Saha et al., 2019). However, none of these biomarkers are able to indicate which specific cell-type network in the gut is impaired or provide insights into the mechanisms of alcohol-impaired intestinal epithelial barriers in AH patients.

The intestinal epithelium is composed of multiple cell types which differentiate from small intestinal stem cells, such as Paneth and goblet cells (van der Flier and Clevers, 2009). REG3α is secreted to the gut lumen by Paneth cells in the crypts of the intestinal epithelium and plays a critical role in maintaining gut barrier integrity through its bactericidal and anti-apoptotic properties (Ayabe et al., 2004). REG3α can translocate to blood through intestinal permeability changes, thereby serving as a soluble marker for gut epithelial integrity. Circulating levels of REG3α have been identified as a biomarker of gut damage in GVHD (Ferrara et al., 2011, Zhao et al., 2018), celiac disease, Crohn disease, and ulcerative colitis (Marafini et al., 2014). Paradoxically, GVHD patients have elevated levels of REG3α in the peripheral blood, but reduced REG3α production in the gut tissues (Zhao et al., 2018). In a mouse model of ALD, alcohol feeding leads to down-regulation of intestinal Reg3γ (the homolog of human REG3α), which is associated with overgrowth of gut bacteria and enteric dysbiosis (Yan et al., 2011, Wang et al., 2016). Currently, it is not known whether intestinal REG3α expression is also suppressed in ALD patients. Our study, for the first time, reported circulating levels of REG3α were elevated and correlated to disease severity in AH patients. Future studies on the effects of alcohol and its metabolites on intestinal REG3α production and the activation/function of Paneth cells is warranted. Particularly, small intestinal biopsy samples from AH patients vs HDC will be highly valuable for future histological evaluations of Paneth cell dysregulation.

REG3α can be produced by CD4 T cells, innate lymphoid cells, natural killer T cells (NKT) and dendritic cells (Zheng et al., 2008). The expression of REG3α can be regulated by several inflammatory cytokines, including IL-22. IL-22 directly targets epithelial cells to prevent bacterial invasion and damage via REG family induction (Ouyang and Valdez, 2008). Consistent with a previous study (Liu et al., 2017), we found that AH patients had elevated circulatory levels of IL-22 when compared to HDC and HC (Supplementary Fig. 1). As REG3α and IL-22 both had correlations with several clinical parameters (MELD score, creatinine, and total bilirubin), they are likely involved in AH disease progression.

TFF3 is mainly secreted by goblet cells and the circulating levels of TFF3 are elevated in several GI diseases (Vestergaard et al., 2002) (Srivastava et al., 2015, Huang et al., 2014). Currently, the role of TFF3 in ALD has not been well studied. Alcohol and its metabolites can activate TFF3 expression in intestinal cell lines, suggesting TFF3 gene expression can be modulated by alcohol consumption (Ludeking et al., 1998). In addition, reduced intestinal TFF3 expression is associated with alcohol-induced gut barrier dysfunction in a mouse model of ALD (Shao et al., 2018). We found that AH patients had higher plasma levels of TFF3 than HDC and HC, while HDC and HC had comparable plasma levels of TFF3 (Fig. 1), suggesting that alcohol-induced gut barrier breakdown is likely responsible for elevated plasma levels of TFF3 in AH patients. In addition, we found that TFF3 levels in AH correlated with multiple elevated inflammatory cytokines/chemokines/growth factors; most interestingly with two highly up-regulated and disease-associated cytokines, IL-6 and IL-8 (Table 3). It will be interesting to study whether these inflammatory cytokines affect goblet

cells in the intestinal epithelium to promote TFF3 expression and translocation into the circulation.

Unexpectedly, we did not find any significant differences in plasma levels of I-FABP, a marker of enterocyte death and gut permeability, between AH patients, HDC, and HC. Circulatory FABPs are known to have a very short half-life (Grootjans et al., 2010). Previous studies have shown that acute alcohol consumption is associated with elevated levels of circulatory I-FABP levels. Acute alcohol intoxication leads to a significant increase in blood I-FABP levels, which rapidly decreased to normal levels within 4 h (de Jong et al., 2015). In the NIAAA chronic-binge alcohol mouse model of ALD, blood levels of I-FABP were higher in alcohol-fed mice 6 h after the last binge alcohol administration than pair-fed control mice (Samuelson et al., 2017). In addition, individuals with alcohol use disorder (AUD) hospitalized for alcohol withdrawal had significantly higher plasma levels of I-FABP than HC (Donnadieu-Rigole et al., 2018). Available information for 30 out of the 79 AH patients in our study cohort indicated that the median last drinking day before enrollment was 9 days with an IQR of 4–17 days. It is possible that blood levels of I-FABP normalized during this time frame, due to either a rapid turnover in the circulation (Grootjans et al., 2010) or reduced expression (Bottasso Arias et al., 2015). Our results agree with the role of I-FABP as a sensitive marker of acute intestinal damage (Grootjans et al., 2010) and show that REG3α and TFF3 released from injured Paneth cells and goblet cells, respectively, may reflect gut barrier damage in AH patients better than I-FABP. REG3α and TFF3 were both elevated in AH patients and correlated with each other, with disease severity (MELD score, creatinine, and total bilirubin, and with multiple pro-inflammatory cytokines/chemokines/growth factors. However, only REG3α showed a modest correlation with two of the conventional MT markers (sCD14 and LBP), suggesting REG3α might be the better potential marker for gut integrity/MT in AH. Future correlation studies between plasma levels of REG3α/TFF3, *in vivo* gut permeability, and histological evaluations of liver biopsies are needed to address this phenomenon.

Currently, there are no effective medical treatments for AH, leaving only alcohol abstinence as the cornerstone of ALD therapy. While abstinence improves the disease outcome and survival of AH, it does not lead to complete recovery in all patients (O'Shea et al., 2010). Consistent with this idea, liver function of the AH patients significantly improved at the 6 and 12 month follow-ups, but did not fully recover with alcohol cessation (Supplementary Table 1). We have previously showed that circulating levels of several inflammatory markers, including pro-inflammatory cytokines (IL-8 and TNF-α, soluble markers of endothelial cell activation (sCD146, sVCAM-1, and VEGF-A) and soluble immune checkpoints (sCD27, sCD40, sHVEM, and sTIM3) are still higher in abstinent AH patients than HC at 12 months (Li et al., 2017, Xia et al., 2020, Li et al., 2020). Here, we showed that plasma levels of REG3α and TFF3 in abstinent AH patients remained higher, suggesting long-term gut epithelial dysfunction in AH patients. Interestingly, plasma levels of TFF3 at the 12 month follow-up correlated with the 4 soluble immune checkpoints sCD27, sCD40, sHVEM, and sTIM3 in abstinent AH patients ($r = 0.64$, $p = 0.02$; $r = 0.86$, $p = 0.0004$; $r = 0.69$, $p = 0.01$; $r = 0.76$, $p = 0.004$, respectively), suggesting a link between gut barrier dysfunction and immune checkpoint dysregulation in abstinent AH patients.

There are several limitations in our study. Firstly, we did not have *in-vivo* gut permeability data from the AH patients to correlate with the soluble markers of gut epithelial injury. Secondly, we did not have intestinal biopsy tissues to perform histological evaluations of Paneth cell and Goblet cell dysregulation. Thirdly, our conclusion that REG3α plasma levels can predict mortality of AH patients was based on a comparison between 73 survivors and 6 non-survivors. A larger sample size will be required to confirm the mortality prediction with plasma REG3α levels.

In conclusion, we found that AH patients had elevated plasma levels of REG3α and TFF3 and that alcohol abstinence significantly, but not completely, reversed their abnormalities. In addition, several significant correlations were found between the plasma levels of REG3α and TFF3 and disease severity (MELD score), MT, and inflammatory factors in AH patients. Thus, the levels of REG3α and TFF3 may serve as novel biomarkers of MT and gut epithelial damage in AH patients. These two molecules can be further explored as indicators of impairment of specific cell types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AH	alcoholic hepatitis
ALD	alcoholic liver disease
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BT	bacterial translocation
CRP	C-reactive protein
GI	gastrointestinal
GrB	granzyme B
GVDH	graft-versus-host disease
HC	healthy control
HCC	hepatocellular carcinoma

HDC	heavy drinking control
HGF	hepatocyte growth factor
IBD	inflammatory bowel disease
I-FABP	intestinal fatty acid binding protein
IP-10	interferon-inducible protein 10
LBP	lipopolysaccharide-binding protein
LPS	lipopolysaccharides
mCD14	membrane CD14
mCD163	membrane CD163
MCP-1	monocyte chemoattractant protein 1
MELD	model for end stage liver disease
MIP-1β	macrophage inflammatory proteins-1 beta
MT	microbial translocation
NASH	nonalcoholic steatohepatitis
NOD	oligomerization domain
PBMC	peripheral blood mononuclear cell
PIGF-1	placental growth factor
REG3α	regenerating islet-derived protein 3α
sCD14	soluble CD14
sCD163	soluble CD163
SCF	stem cell factor
SDF-1α	stromal cell-derived factor 1α
TFF3	trefoil factor 3
TLR	Toll-like receptors
TREAT	Translational Research and Evolving Alcoholic Hepatitis Treatment
VEGF-A	vascular endothelial growth factor A.

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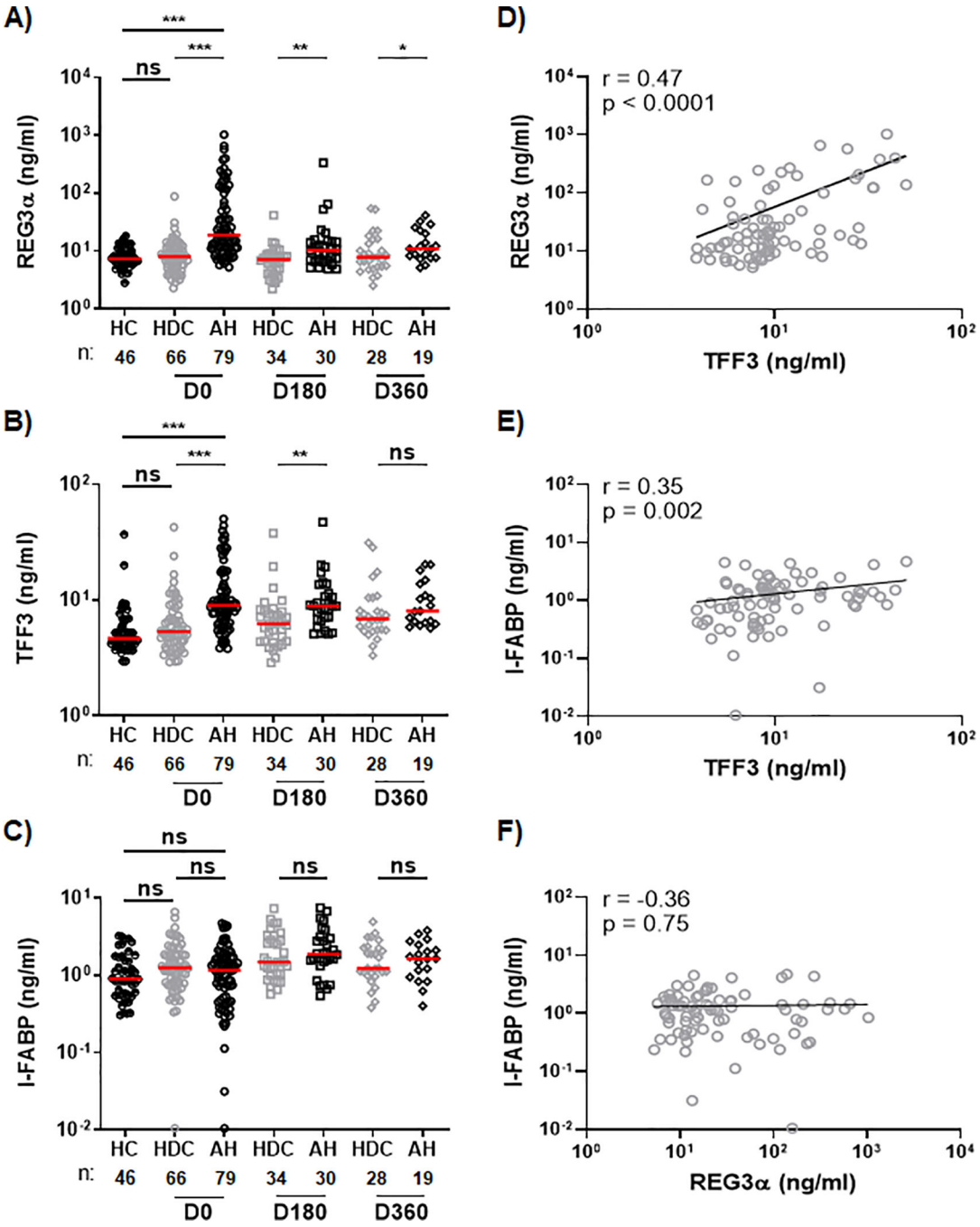

Fig. 1. Cross-sectional analyses of plasma levels of REG3α, TFF3, and I-FABP in AH patients, HDC, and HC.

(**A-C**) Scatter plots showing plasma levels of REG3α, TFF3, and I-FABP in AH patients, HDC, and HC at the baseline and 6 month (D180) and 12 month (D360) follow-ups. Kruskal-Wallis test with Dunn's corrections was performed to compare plasma levels among 3 groups at baseline. Mann Whitney test was used for compare AH versus HDC individuals at 6 and 12 months. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ns, not significant. Horizontal lines represent the median. (**D-F**) Dot plots showing the correlation between plasma levels of

REG3α, TFF3, and I-FABP in AH patients. Spearman's correlation was used to calculate the association. *r*, coefficient. AH, alcoholic hepatitis; HDC, heavy drinking control; HC, healthy control.

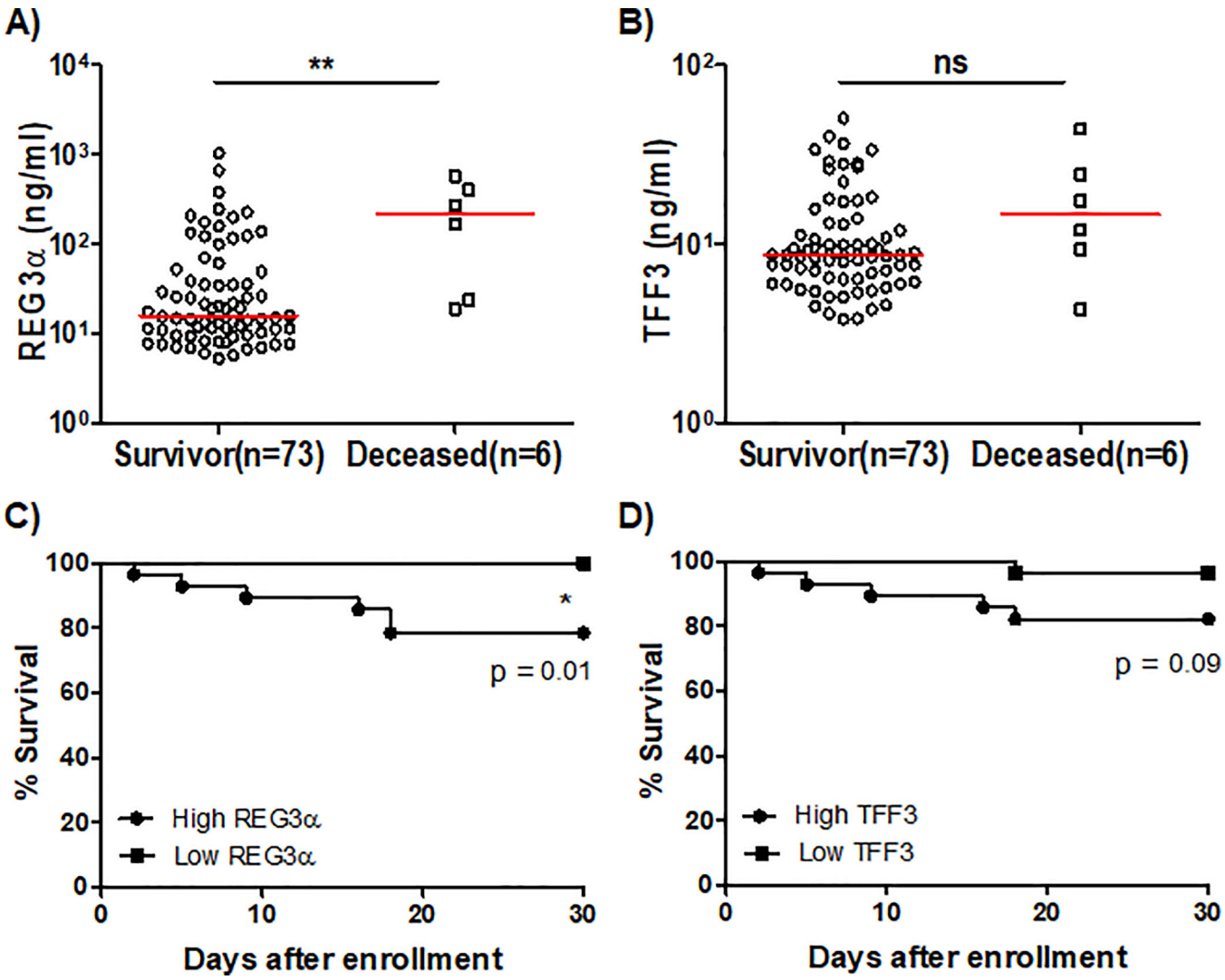

Fig. 2. Higher plasma levels of REG3α in AH patients who died within 30 days after enrollment.

(**A-B**) Scatter plots comparing plasma levels of REG3α (**A**) and TFF3 (**B**) in survivors and deceased AH patients. Mann Whitney test was used for compare the difference. $^{**}p < 0.01$. ns, not significant. Horizontal lines represent the median. (**C-D**) Kaplan-Meier curves showing 30 day survival according to baseline levels of REG3α (**C**) and TFF3 (**D**) in AH patients. The median concentration was used as the cut-off to define patients with low or high concentration. Log-rank test was used for the analysis.$^{*}p < 0.05$. AH, alcoholic hepatitis.

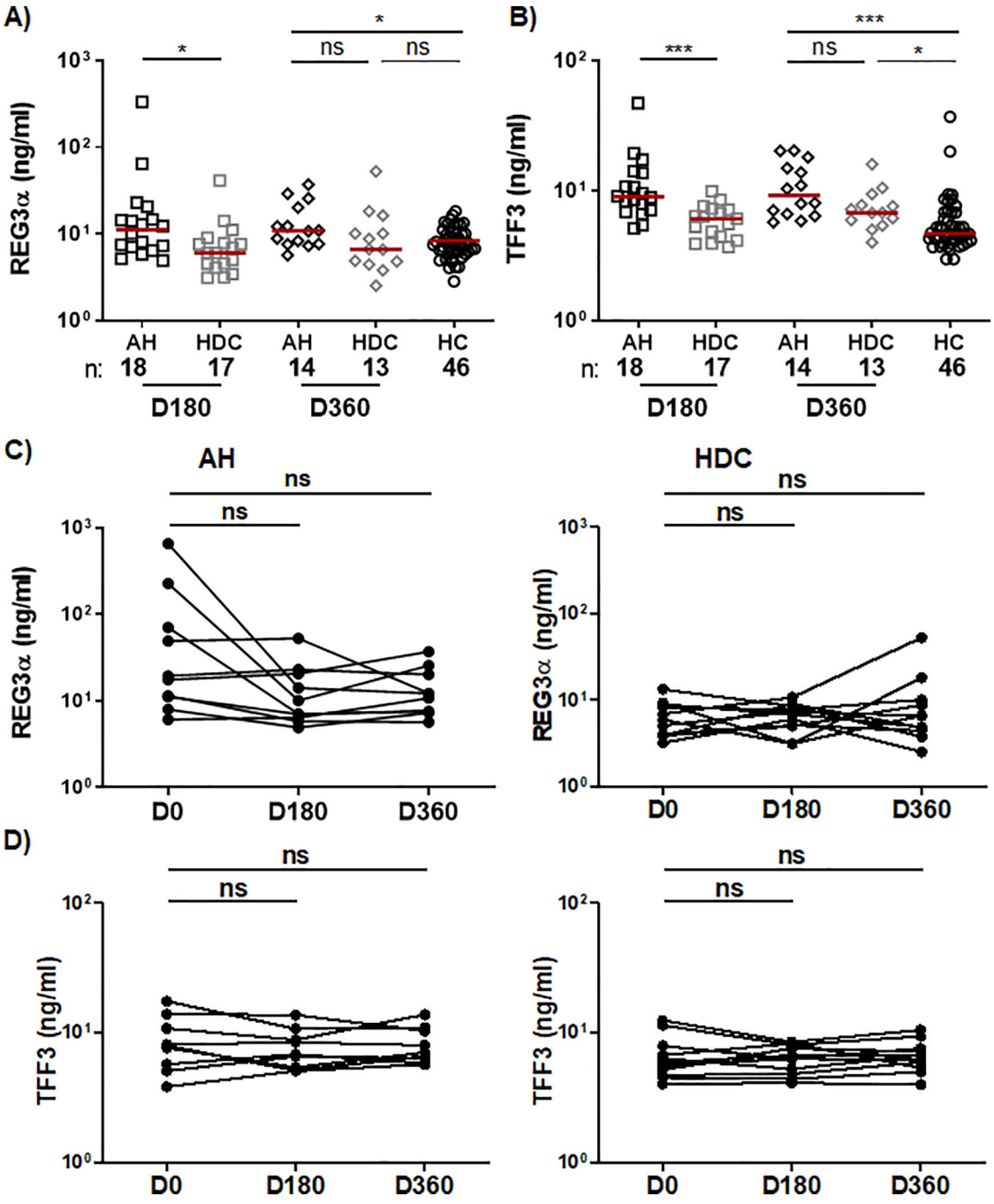

Fig. 3. Plasma levels of REG3α and TFF3 in abstinent subjects at 6 month and 12 month follow-ups.

Scatter plots showing the levels of REG3α (**A**) and TFF3 (**B**) at 6 month (D180) and 12 month (D360) follow-ups in abstinent AH patients and HDC. Mann Whitney test comparing AH vs HDC at 6 and 12 months. The longitudinal graphs showing changes of the plasma levels of REG3α (**C**) and TFF3 (**D**) in AH patients and HDC. Friedman rank sum test with Dunn's corrections was used to compare these the differences between day 0 and 6 or 12

months (n=10 for AH and n=11 for HDC). *$p < 0.05$; ***$p < 0.001$; ns, not significant; horizontal lines represent the median; AH, alcoholic hepatitis; HDC, heavy drinking control.

Table 1.

Characteristics of the study cohort

Variable	Day 0				6 month			12 month		
	HC (n=46)	HDC (n=66)	AH (n=79)	*p*	HDC (n=33)	AH (n=30)	*p*	HDC (n=27)	AH (n=18)	*p*
Age (years)	42 (30–52)	45 (35–53)	44 (35–52)	ns	47 (3655)	43 (33–53)	ns	50 (38–55)	42 (33–53)	ns
Gender (% Male)	59	59	59	ns	62	60	ns	63	67	ns
Drinks/day in last 30 days		8.9 (6.3–16.7)	6.7 (2.5–11.0)	**	0 (0–1.9)	0 (0–0.4)	ns	0.1 (0–3.7)	0 (0–0.03)	ns
Drinking days in last 30 days		28 (23–30)	25 (15–30)	ns	2 (0–16)	0 (0–2)	ns	1 (0–24)	0 (0–1)	ns
MELD score		6 (68)	24 (19–27)	***	7 (68)	10 (8–14)	***	7 (68)	10 (8–13)	***
Creatinine (mg/dL)	0.9 (0.8–1.1)	0.8 (0.7–1.0)	0.8 (0.61.1)	ns	0.9 (0.8–1.1)	0.7 (0.61.0)	*	1 (0.8–1.1)	1 (0.7–1.2)	ns
Total bilirubin (mg/dL)	0.3 (0.3–0.5)	0.6§ (0.4–0.7)	12.9$^{\#\#\#}$ (7.1–19.3)	***	0.4 (0.3–0.6)	1.3 (0.8–3.4)	***	0.4 (0.3–0.7)	0.9 (0.7–1.8)	**
AST (IU/L)	17 (13–20)	24§§ (18–31)	110$^{\#\#\#}$ (88–145)	***	20 (1623)	49 (32–70)	***	21 (17–26)	35 (2657)	**
ALT (IU/L)	11 (7–17)	22§§§ (1631)	46$^{\#\#\#}$ (30–60)	***	15 (11–24)	31 (20–44)	***	18 (13–34)	25 (22–35)	ns
Prothrombin time (INR)		1.0 (0.9–1.0)	1.8 (1.5–2.2)	***	1.0 (1.0–1.0)	1.2 (1.1–1.5)	***	1.0 (0.9–1.1)	1.2 (1.0 −1.5)	*

Note: Data are represented as median and (interquartile ranges). HC, healthy controls; HDC, heavy drinking controls; AH, patients with alcoholic hepatitis; MELD, model for end-stage liver disease; AST, aspartate aminotransferase; ALT, alanine aminotransferase; INR, international normalized ratio. Chi-square test for analysis of categorical variables. Kruskal-Wallis test with Dunn's correction for pairwise comparisons of continuous variables among HC, HDC, and AH patients at enrollment (Day 0). Mann Whitney test comparing AH patients versus HDC at 6 and 12 month follow-ups.

$^{\S}p < 0.05$

$^{\S\S\S}p < 0.001$ for comparison between HDC and HC at Day 0

$^{\#\#\#}p < 0.001$ for comparison between AH patients and HC at Day 0

$^{*}p < 0.05$

$^{**}p < 0.01$

$^{***}p < 0.001$ for comparison between AH patients and HDC; ns, not significant.

Table 2.

Correlations of REG3α, TFF3, I-FABP, and IL-22 with clinical parameters in AH patients

	Variable	REG3α	TFF3	I-FABP	IL-22
Clinical parameters	**MELD**	0.46***	0.55***	0.01	0.27*
	Creatinine	0.54***	0.74***	0.09	0.31*
	Total Bilirubin	0.52***	0.42***	−0.07	0.28*
	INR	−0.05	0.08	0.04	0.01
	AST	0.04	0.1	−0.25*	0.02
	ALT	0.04	−0.11	−0.04	−0.05
	CRP	0.39***	0.26	−0.23	−0.10

Note 1: AH, alcoholic hepatitis; MELD, model for end-stage liver disease; INR, international normalized ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CRP, C-reactive protein. The numbers represent Spearman's coefficients

* $p < 0.05$

*** $p < 0.001$.

Table 3.

Correlations of REG3α, TFF3, I-FABP, and IL-22 with BT markers and inflammatory/growth factors in AH patients

	Variable	REG3α	TFF3	I-FABP	IL-22
BT markers	**sCD163**	0.11	0.24*	0.12	0.004
	sCD14	0.35**	0.17	−0.06	0.18
	LPS	0.03	0.07	0.08	−0.02
	LBP	0.32**	0.02	−0.15	0.10
Inflammatory cytokines/chemokines	**IL-1α**	0.27*	0.26*	−0.11	0.10
	IL-6	0.26*	0.32*	0.01	0.15
	IL-8	0.37**	0.47***	−0.03	0.27*
	IL-10	0.33**	0.27*	0.02	0.01
	IL-21	0.05	0.3*	0.17	0.05
	IP-10	0.26*	0.22	0.002	0.29*
	MCP-1	0.31*	0.18	0.19	−0.02
	MIP-1β	0.39**	0.20	−0.21	0.09
Growth factors	**HGF**	0.33**	0.26*	−0.35**	0.30**
	PIGF-1	0.25	0.27*	0.02	0.03
	SCF	0.18	0.27*	0.03	0.15
	SDF-1α	0.39**	0.28*	−0.02	0.10
	VEGF-A	0.47***	0.42***	−0.03	0.12

Note 3: AH, alcoholic hepatitis; BT, bacterial translocation; LPS, Lipopolysaccharides; LBP, LPS-binding protein. The numbers represent Spearman's coefficients

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$.