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A Microbial Signature of Psychological Distress in Irritable Bowel Syndrome

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ABSTRACT

Objective: Irritable bowel syndrome (IBS) is associated with alterations along the brain-gut-microbiota axis. Previous studies have suggested a parallel segregation of microbial features and psychological burden in IBS. This study aimed at exploring the microbial correlates of psychological distress in patients with IBS.

Methods: Forty-eight patients with IBS (Rome III criteria, M (SD) age = 42 (15) years, 35 female, 25 diarrhea-dominant, 5 constipation-dominant, and 18 alternating-type IBS) were assessed for psychological and clinical variables with validated questionnaires, fecal samples underwent microbial 16S rRNA analyses (regions V1–2). Microbial analyses comprised examination of alpha and beta diversity, correlational analyses of bacterial abundance and comparisons among subgroups defined by thresholds of psychological and IBS symptom variables, and machine learning to identify bacterial patterns corresponding with psychological distress.

Results: Thirty-one patients (65%) showed elevated psychological distress, 22 (31%) anxiety, and 10 depression (21%). Microbial beta diversity was significantly associated with distress and depression ($q = .036$ each, q values are p values false discovery rate-corrected for multiple testing). Depression was negatively associated with *Lachnospiraceae* abundance (Spearman's $\rho = -0.58$, $q = .018$). Patients exceeding thresholds of distress, anxiety, depression, and stress perception showed significantly higher abundances of *Proteobacteria* ($q = .020$ – $.036$). Patients with anxiety were characterized by elevated *Bacteroidaceae* ($q = .036$). A signature of 148 unclassified species accounting for 3.9% of total bacterial abundance co-varied systematically with the presence of psychological distress.

Conclusions: Psychological variables significantly segregated gut microbial features, underscoring the role of brain-gut-microbiota interaction in IBS. A microbial signature corresponding with psychological distress was identified.

Clinical Trial Registration: ClinicalTrials.gov identifier NCT02536131, retrospectively registered.

Key words: anxiety, depression, gut-brain-microbiome axis, irritable bowel syndrome, machine learning, psychological distress.

INTRODUCTION

The understanding of the dynamic interactions between brain and behavior, the gastrointestinal system, and gut microbiota is rapidly evolving (1,2). Psychobiological stress reactions inevitably lead to shifts of homeostasis along the brain-gut-microbiota axis, and the bacterial microbiome is increasingly recognized as a relevant entity within this interplay (3–5). Although coordinated physiological stress responses are necessary for survival, chronic exposure to stress may predispose to dysregulation along the brain-gut-microbiota axis, immune imbalances and to the development of stress-related disorders (6–8). A role of stress in conjunction with microbial factors is also highly presumed in the pathogenesis of irritable bowel syndrome (IBS) (9–11). The underlying interaction between stress and the microbiome seems to be bidirectional: there is evidence both for stress-induced changes in the intestinal microenvironment and the microbiome, and vice versa, effects of microbiome-related factors on psychobiological

stress reactivity. Regarded from a biopsychosocial perspective, environmental factors, behavior and psychological appraisal processes hereby interact with central and autonomous nervous, endocrine, metabolic, and immune functions and the gastrointestinal microbiota via multidirectional pathways (12).

Top-Down Pathways

The release of stress hormones impacts the gut ecosystem by modulating gastrointestinal blood flow, secretion, permeability, and motility, as well as activation of the immune system (13,14). Numerous animal experiments have shown that chronic stress alters the microbiome, with general decreases in diversity and richness or shifts in composition (15–19). Microbial changes may in turn

HADS = Hospital Anxiety and Depression Scale, **IBS** = irritable bowel syndrome, **LefSe** = linear discriminant analysis effect size, **OTU** = operational taxonomic unit (bacteria)

SDC Supplemental Content

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contribute to inflammatory states, altered neurochemistry, visceral pain, and behavioral abnormalities, altogether resembling the picture of IBS. Experimental stress paradigms have therefore been proposed as animal models of IBS (20,21).

Bottom-Up Pathways

Microbiota transplantation from anxious patients with diarrhea-predominant IBS to mice triggered heightened intestinal transit, loss of barrier function, immune activation, and anxiety-like behavior in recipient mice (22). Studies with germ-free animals have demonstrated an important role of microbiota for development and programming of the hypothalamic-pituitary-adrenal axis and behavior (23,24). These mechanisms seem to have pronounced sex-specific aspects (25,26). Also beyond developmentally sensitive temporal windows, gut microbes can affect basal circuits of emotion processing and the autonomous nervous system via ascending vagal pathways from the intestinal lumen (27,28). Interoceptive stimuli can interfere with conscious and subconscious emotional and cognitive processing (29). In this context, the stability of microbial ecosystems can be considered a determinant of gastrointestinal homeostasis with a relevance for appraisals of stress (30). Furthermore, bacterial metabolic pathways affect essential neurotransmitter systems (31,32) and a number of studies point to metabolic and epigenetic effects on the brain with impact on social cognition, reward, and emotion processing (33–35).

Initial evidence demonstrates associations with emotion processing also in humans (36,37), and gut microbial associations with brain morphology and temperamental traits have been identified (38,39). The association between psychopathology and gastrointestinal conditions is widely observed. Microbial shifts were found in depression (40,41), autism (42), as well as inflammatory and functional gastrointestinal diseases (43).

IBS is a brain-gut axis disorder (10,44) associated with high rates of psychopathology (45,46) and altered stress reactivity patterns (47–49). Further mechanisms present in IBS comprise immune activation, heightened gut permeability, alterations in tryptophan and bile metabolism, and pain processing (50–56).

Microbial Characteristics of IBS

A number of studies have attempted to characterize gut microbiomes in IBS compared with healthy controls, which is however complicated by inconsistency and large overlap with healthy microbiomes. Some key findings with a relative concordance among studies include reductions in alpha diversity and richness, depletion of *Bifidobacteria*, elevated abundance of *Proteobacteria*, and elevations in *Ruminococcus* species (10,43,57–60). A heightened *Firmicutes*:*Bacteroidetes* ratio has been observed repeatedly in subgroups of patients with IBS. Several recent studies have reported reductions of bacteria producing methane and/or producing short-chain fatty acids (61–64), but here again, exceptions have been observed (65). One study identified bacteria associated with IBS severity by machine learning techniques, which resulted in a pattern of taxa disseminated over the whole phylogenetic tree (63).

Several observations across studies point to associations between psychological variables and microbiota in IBS. It was repeatedly found that psychological distress segregated patients with IBS in parallel with their microbial composition (66,67) and a recent study reported associations between early life trauma

and microbial features in IBS (68). Moreover, certain bacterial features (e.g., heightened *Proteobacteria* and depleted *Bifidobacteria*) were described in both depression and IBS (40,58,69), and it has been observed that the fecal microbiome of patients with IBS presents strong similarities with that of depressive patients (70).

The aim of this study was, therefore, to explore associations between gut microbial characteristics and psychological distress, anxiety, and depression in patients with IBS.

METHODS

Recruitment

The study was conducted at the University Hospital of Vienna, Outpatient Clinic for Psychosomatics at the Department of Gastroenterology and Hepatology, University Clinic of Internal Medicine III. It included patients with IBS diagnosed according to Rome III criteria, aged between 18 and 89 years, and refractory to other IBS therapies. Exclusion criteria were pregnancy, bowel surgery, mental retardation, insufficient knowledge of German, concomitant severe organic disease or schizophrenia, psychosis, substance-related disorder or panic disorder, and antibiotic treatment within the month before stool collection. Screening for eligibility was performed in routine first interviews at the outpatient clinic under consideration of the hospitals' medical records. The study was conducted between August 2014 and August 2016. Sixty-three patients with IBS were consecutively screened for eligibility, of which five patients declined to participate, five did not meet the inclusion criteria, and 53 were enrolled. Three of these did not provide complete data (questionnaires and stool), in one case microbiome data quality was insufficient, and one was excluded from the analyses because of acute inflammation at the time of sample collection. Complete data sets for analysis were ultimately available from 48 patients (see flowchart in Figure S1, Supplemental Digital Content 1, <http://links.lww.com/PSYMED/A505>). The study protocol was approved by the ethics committee of the Medical University of Vienna (ID: 1502/2014). Informed consent was given by each participant. No financial or other incentives were offered for study participation.

Questionnaires

Anxiety, *depression*, and *psychological distress* were assessed with the Hospital Anxiety and Depression Scale (German version, HADS), (71) a screening instrument for primarily somatically ill patients. Each of the two anxiety and depression scales has seven items, with a four-step response set (scores 0–21 each). Reported internal consistency is Cronbach's α value of .80 (72). Anxiety, depression, and psychological distress (the latter is the sum of anxiety and depression) entered the analyses as continuous scores as well as dichotomized categorizations. Dichotomization was performed for scores higher than 10 to indicate clinically relevant anxiety and depression, respectively. The dichotomous categorization *absence/presence of psychological distress* was the primary variable in this study. It was defined by HADS anxiety or depression scores higher than 10, or a combined score of 16 or higher. This criterion was developed for screening need for mental health support in patients with cancer in a large-scale study and has proven good practicability and validity (73).

Perceived stress was measured with the Perceived Stress Questionnaire, German version, (74) an instrument assessing subjectively experienced stress independent of a specific and objective occasion with 20 items and 4-step response sets. Cronbach's α is .85 or greater for the overall score of the German version (74). Scores were linear transformed to values between 0 and 1, entered the analyses as a continuous variable, and categorized for subgroup analyses based on norm values from healthy adults (mean (SD) stress perception = .33 (0.1)) (74–76). Values greater than one standard deviation above the mean (>0.50, corresponding to the upper 16.7% of the norm) of healthy adults were classified as elevated.

The IBS symptoms *abdominal pain, bloating, diarrhea, and constipation* were assessed by single visual analog scales (0, not at all present, to 100, extremely pronounced) as in a previous study (77).

IBS severity was assessed with the Irritable Bowel Syndrome – Severity Scoring System (78), a questionnaire for clinical assessment of IBS symptom burden and severity. Values range between 0 and 500, with higher values representing higher symptom burden. Values were classified as mild (values ranging 75–175), moderate (175–300), and severe IBS (300–500) as proposed. Sound reproducibility, sensitivity, and specificity are reported for the German version (79), and the scale has been recommended repeatedly for assessment of IBS in methodological reviews (80,81).

Microbiome Analyses

16S Ribosomal RNA Sequencing

Stool samples were collected and frozen by patients, brought to the hospital and deep frozen at -80°C . DNA isolation, library preparation, and sequencing were then performed at the Graz University Center for Medical Research as described in Klymiuk et al. (2016) (82). Frozen stool samples were used for total DNA isolation by combination of mechanical and enzymatic lysis with the MagnaPure LC DNA Isolation Kit III (Bacteria, Fungi; Roche, Mannheim, Germany) according to manufacturer's instructions. Samples were bead beaten for mechanical lysis at 6500 rpm for 30 seconds twice in a MagNA Lyser (Roche). After incubation with lysozyme and Proteinase K, enzymes were deactivated at 95°C for 10 minutes and DNA purification was performed according to kit instructions. PCR amplification was performed with the target specific primers 27f and 357r and 2 μl of total DNA extract was used for a 25 μl of PCR reaction in triplicates containing 1 \times Fast Start High Fidelity Buffer, 1.25 U High Fidelity Enzyme, 200 μM dNTPs, 0.4 μM bar-coded primers, and PCR-grade water (Roche). The final library was quantified using a Quantus Fluorometer (Promega, Mannheim, Germany) and loaded to an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) using a high-sensitivity DNA assay according to manufacturer's instructions for quality control. A 6pM library run was performed on a MiSeqII desktop sequencer (Illumina, Eindhoven, the Netherlands) with 20% PhiX control DNA. The resulting FASTQ files were deposited at <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA386442>. MiSeq paired-end raw sequence forward and reverse reads were subsequently merged using ea-utils v1.1.2 with standard settings, followed by a split library step from QIIME v1.9.1 and removal sequence reads shorter than 200 nucleotides, reads that contained ambiguous bases, or reads with an average quality score of less than 30. Chimeras were removed using USEARCH v6. against 97% clustered SILVA reference database (83). Operational taxonomic units (OTUs) were picked using the QIIME open-reference pipeline to perform clustering steps at 97% sequence similarity, the taxonomy assignment with a UCLUST algorithm, alignment of reference sequences with pyNAST, and generation of a phylogenetic tree with FastTree. An introduction to basic concepts in microbiological ecology relevant to this study is provided in Supplemental Digital Content 2, <http://links.lww.com/PSYMED/A506>.

Diversity and Composition Analyses

Alpha diversity was analyzed at a rarefaction depth of 20,000 sequences using the *observed species*, *Faith's phylogenetic diversity* (PD) and *estimated richness* (Chao1) metrics. Alpha comparisons among subgroups were performed by Mann–Whitney *U* tests and 999 Monte–Carlo permutations.

Beta-diversity analyses were performed using abundance-weighted UniFrac distances as phylogenetic estimates of community similarity. Associations with psychological, clinical, and demographic variables were tested with Adonis (vegan package 2.4-0), a permutational analysis of variance.

Subgroup and Correlation Analyses of Bacterial Abundance

Correlations between OTUs and study variables were calculated using a Spearman correlation after removing OTUs with less than 20% occupancy.

Subgroup analyses of bacterial abundance were performed using Linear discriminant analysis effect size (LEfSe (84)), a method to determine bacteria most likely to explain differences between classes by coupling standard tests for statistical significance with additional tests encoding effect relevance. It identifies bacterial taxa differentially abundant between classes by nonparametric Kruskal–Wallis tests. Significance is subsequently investigated by pairwise Wilcoxon rank-sum tests, and effect sizes are estimated by linear discriminant analyses (85). The lower threshold for reporting logarithmic linear discriminant analyses scores was set at 2.0 (default).

Machine Learning

With regard to the interactional nature of the microbiome and to overcome the boundaries of repeated testing, modeling via machine learning was adopted to complement the analyses. Machine learning was applied after removal of OTUs with less than 10 reads per single sample and feature selection by gradient-boosting classifier (scikit-learn 0.18.1), an algorithm combining weak learner-decision trees and boosting (100 times) to optimize cost function over function space. Each individual decision tree in the gradient-boosting algorithm intrinsically performs feature selection by selecting appropriate split points. This information was used to measure the importance of each feature: the more often a feature was used in the split points of a tree the more important was the feature (weighting). The feature importance was then averaged across all of the decision trees within the model. The sample was randomly split into a training set (32 samples) and a prediction set (16 samples). A model based on the training data was built using the Random Forest Classifier (86,87) with 100 trees and parameter adjusted by GridSearchCV using three-fold cross validation.

Statistical Analyses

Statistical analyses were conducted in QIIME (88), the bioinformatics platform Galaxy (89), R (90), and SPSS 23 (SPSS Inc). Parameter-free tests were chosen throughout the analyses because assumptions of normal distribution were violated in several variables. With regard to the psychological and IBS symptom-related variables, continuous scores were used in the analyses of associations with microbial composition (Adonis tests) and correlation analyses with bacterial abundance. Categorical variables were used in subgroup analyses (alpha diversity, abundance testing with LEfSe) and for the machine learning classifier. *P* values were corrected by Bonferroni's method as default in QIIME or otherwise corrected by Benjamini and Hochberg's method to control for the false discovery rate and given as *q* values. The α level was set at .05 (two-sided) throughout all tests.

RESULTS

Sample Characteristics

Complete data sets, including questionnaires and stool samples, were available from 48 patients with IBS aged 42 (15) years, experiencing IBS for 9 (9) years, and with 35 (73%) female participants. Age ranged between 19 and 70 years and was distributed relatively even with a median of 42 years and two peaks below (at approximately 30 years) and above (at approximately 60 years). With 73% female participants, sex distribution was clearly skewed. Twenty-five patients (52%) experienced IBS with diarrhea (IBS-D), 18 (38%) from IBS with mixed symptoms (IBS-mix), and 5 (10%) from IBS with constipation (IBS-C). A postinfectious onset of IBS (PI-IBS) was known in 9 (19%) of the sample. With a M (SD) severity (Irritable Bowel Syndrome – Severity Scoring System) of 310.3 (77.5), IBS was classified as mild in 3 (6%) patients, moderate in 17 (35%) patients, and severe in 28 (58%) patients. Antidepressants were taken by 9 (19%), Mebeverine by 4 (8%), and proton pump inhibitors by 3 (6%) patients. Further medications each taken by a single patient were the following: cholestyramine,

ursodeoxycholic acid, and domperidone. Two patients reported the intake of a probiotic nutritional supplement.

Psychological Distress

As frequently found in IBS populations and particularly in patients of specialized tertiary centers, there was a high proportion of psychological burden in the sample: 23 (48%) had a psychological disorder in the past or during the study, 21 (44%) were in a psychotherapy during the study, and 38 (79%) reported impairing psychosocial conditions (financial/job strain, history of trauma, family or interpersonal problems). Anxiety was 9.6 (4.13), depression was 7.09 (3.92), and psychological distress was 16.52 (7.42) points high. According to the previously mentioned thresholds (see Methods), 31 patients had elevated psychological distress, 22 had anxiety, and 10 had depression, with 7 experiencing both anxiety and depression (Figure 1A). The distribution of sample characteristics among subgroups with or without clinically relevant psychological distress is shown in Table 1.

Perceived stress and psychological distress were highly correlated (Spearman's $\rho = .83$, $p < .001$). Perceived stress was .537 (.200) in the whole sample, and 34 (71%) patients displayed elevated stress perception. Perceived stress values showed a bimodal distribution (Figure 1B).

Interestingly, no significant correlations between psychological distress and IBS severity or IBS single symptoms (abdominal pain, diarrhea, bloating, constipation) were found; a scatter plot and exact statistics are provided in Supplemental Digital Content 3, <http://links.lww.com/PSYMED/A507>.

Microbial Analyses

The hypervariable regions V1 and V2 of 16S rRNA genes were sequenced from 48 fecal samples and yielded 3067522 sequences and an M (SD) number of MiSeq reads of 63907 (12077) per sample, with a range from 26283 to 84351 sequences. After filtering OTUs with less than 20 total counts and presence in less than 5 samples, the OTU table contained 6.379 OTUs.

Bacterial Diversity and Composition

The bacterial diversity in the fecal samples (alpha diversity) was compared between patients with and without psychological

distress (subgroup analyses based on dichotomized categorization). Diversity in patients with psychological distress was slightly lower than in those without distress, but the difference was not significant (Figure 2, Table 2). Diversity between patients with and without elevated stress, anxiety or depression was also compared, as well as the different IBS subtypes and severity, but there were no significant differences among any of the subgroups (values are summarized in Table S4, Supplemental Digital Content 4, <http://links.lww.com/PSYMED/A508>).

Continuous psychological variables and measures of bacterial composition (weighted beta diversity, which takes into account the abundance of taxa and is therefore sensitive to systematic alterations in large microbial groups) were examined in subsequent analyses. Microbiome composition was significantly associated with psychological distress and depression (Table 3, Adonis testing of abundance-weighted UniFrac distances). Other tested psychological and IBS symptom-related variables showed no association, and the same was found for previously identified microbial covariates age, sex, and antidepressant intake (91–93).

To provide a higher-order perspective on the microbial communities present in the study cohort, the 48 fecal samples were clustered according to their microbiome composition (hierarchical clustering with unweighted pair group method with arithmetic mean of weighted UniFrac distances as shown in Figure 3). This resulted in a two-cluster solution with dissimilarities of 0.39 (0.07) between clusters and 0.27 (0.05) within the clusters.

Distributions of microbial and patient characteristics were tested between the two clusters. *Firmicutes* and *Bacteroidetes* are the two dominant phyla in the human gut, and the *Firmicutes* to *Bacteroidetes* (F:B) ratio thus provides a rough estimate of the composition of a microbial community. It amounted to a median (interquartile range) of 1.92 (1.47–2.51) in the 28 patients of cluster 1 and 12.21 (6.91–28.83) in the 20 patients of cluster 2. The difference was significant at $p < .001$ (Mann–Whitney *U* test). Microbial alpha diversity was almost equal in cluster 1 (*Chao1* = 2232 (409)) and in cluster 2 (2196 (497)), $p = .76$, Mann–Whitney *U* test and Monte–Carlo permutation). Distributions of psychological and IBS symptom-related variables in the two microbial clusters are given in Table 4. Distress, depression, and perceived stress were higher in cluster 1, but significances collapsed

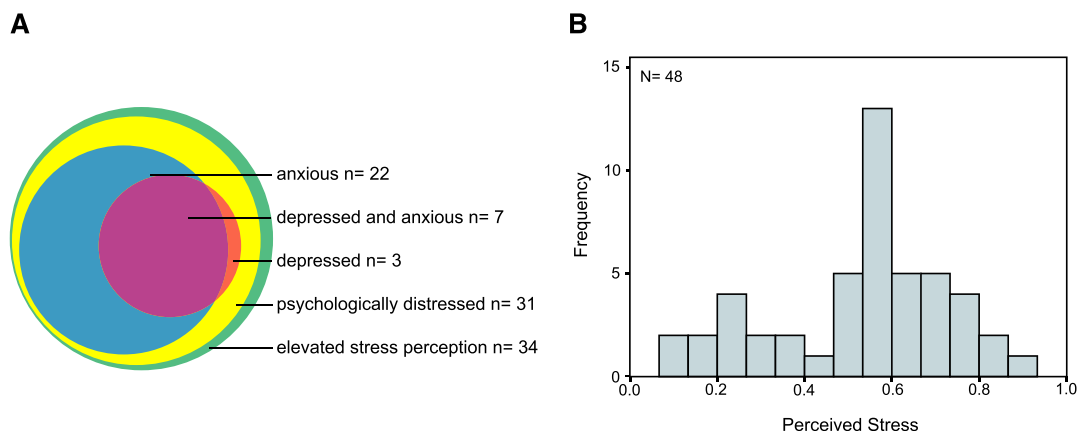


FIGURE 1. Venn diagram of different aspects of psychological burden present in the sample (A). Elevated stress perception was inclusive of psychological distress, psychological distress was inclusive of anxiety and depression, and anxiety and depression overlapped in 7 patients. Histogram of perceived stress values showed a bimodal distribution (B). Color image is available only in online version (www.psychosomaticmedicine.org).

TABLE 1. Sample Characteristics in Patients Without Versus With Psychological Distress

	Not Distressed, <i>n</i> = 17	Distressed, <i>n</i> = 31	<i>p</i>	<i>q</i>
Age, y	33.0 (25.5–54.5)	45.0 (32.0–56.0)	.19	.46
Disease duration, y	7.0 (4.5–12.0)	6.0 (3.0–10.0)	.39	.67
Sex, male/female	6/11	7/24	.34	.65
PI-IBS	3	6	.89	.93
IBS-D	9	16	.73	.82
IBS-C	1	4	.73	.82
IBS-mix	7	11	.73	.82
IBS severity	297 (259–359)	335 (266–371)	.52	.82
Abdominal pain	46.0 (34.5–62.0)	61.5 (45.3–72.0)	.082	.22
Bloating	64.0 (50.5–80.5)	64.0 (57.0–75.0)	.97	.97
Diarrhea	39.0 (4.3–54.3)	53.5 (29.3–66.0)	.054	.18
Constipation	21.0 (2.5–69.0)	20.0 (2.8–52.8)	.69	.82
Debilitating psychosocial conditions	13	25	.73	.82
Taking on psychotherapy	6	15	.382	.84
Diagnosis of psychological disorder	5	18	.057	.18
Perceived stress (PSQ)	0.325 (0.215–0.450)	0.620 (0.570–0.720)		
Psychological distress (HADS)	6.5 (10.0–11.5)	20.0 (18.0–23.0)		
Anxiety	5.0 (3.0–7.0)	12.0 (10.0–14.0)		
Depression	3.0 (2.0–6.0)	8.0 (6.0–11.0)		

IBS = irritable bowel syndrome; PSQ = Perceived Stress Questionnaire; HADS = Hospital Anxiety and Depression Scale.

Median (interquartile range) are shown for metric data, *p* values are two-sided from Mann–Whitney *U* tests, and χ^2 tests for balance of nominal data. *Q* values are *p* values corrected for multiple comparisons by Benjamini and Hochberg's method. Higher values mean higher symptom burden for all questionnaire data.

after false discovery rate correction. IBS symptom-related aspects, such as symptom severity and subtype, and postinfectious onset of IBS were evenly represented in both clusters.

Correlational Analyses

Correlational analyses between relative abundance (species and higher taxonomic levels) and psychological or IBS symptom-related

variables (continuous scores) yielded 11 associations remaining significant after controlling for multiple testing, all with *Firmicutes* members. Anxiety was correlated with the genus *Anaerotruncus* (Spearman's $\rho = .65$, $q = .001$), depression with the family *Lachnospiraceae* ($\rho = -.58$, $q = .018$), as well as seven unclassified species given in Table S5 (Supplemental Digital Content 5, <http://links.lww.com/PSYMED/A509>). IBS severity correlated with the

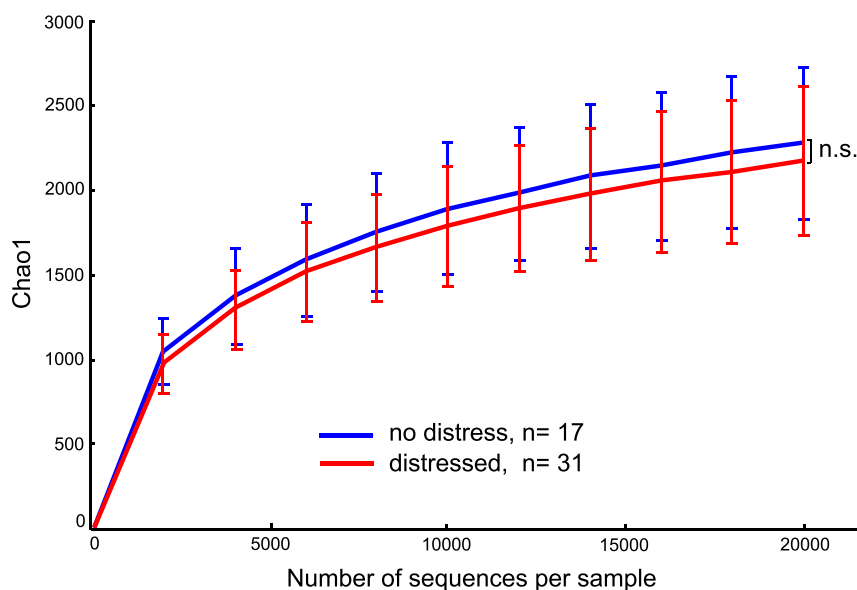


FIGURE 2. Alpha diversity rarefaction curves of estimated bacterial richness (*Chao1*) of patients with versus without psychological distress (HADS). Color image is available only in online version (www.psychosomaticmedicine.org).

TABLE 2. Alpha Diversity in Patients With Versus Without Psychological Distress

	Subgroups Split by Presence of Psychological Distress		<i>p</i>	<i>q</i>
	No Distress, <i>n</i> = 17	Distressed, <i>n</i> = 31		
Observed species	1539 (277)	1443 (251)	.24	.69
Phylogenetic diversity	5046 (795)	4951 (749)	.69	.69
<i>Chao1</i>	2285 (455)	2179 (439)	.46	.69

Observed species, Phylogenetic Diversity, and *Chao1* are metrics depicting different aspects of alpha diversity with M (SD) given. *P* values from Mann–Whitney *U* tests and Monte–Carlo permutation, *q* values (*p* values corrected for multiple comparisons by Benjamini and Hochberg's method).

genera *Ruminococcus* ($\rho = .62$, $q = .005$) and *Coprococcus* ($\rho = .59$, $q = .013$).

Bacterial Abundances Between Subgroups Defined by Psychological Variables

The *Firmicutes* to *Bacteroidetes* ratio was lower, albeit non-significantly, in patients with psychological distress (median (interquartile range) = 2.25 (1.84–5.22) compared with those without psychological distress (median (interquartile range) = 6.56 (2.02–13.66), $p = .091$, Mann–Whitney *U* test. Analyses of relative bacterial abundance between subgroups were performed using LEfSe, a method for detection of significantly elevated bacterial taxa. The presence of psychological distress was associated with four elevated *Proteobacteria* members (phylum to family level) and one *Bacteroidetes* genus (*Barnesiella*). Two *Bacteroidetes* members and the same four *Proteobacteria* members as in psychological distress were increased in elevated perceived stress. Anxiety was associated with five *Bacteroidetes* members and the *Proteobacteria* phylum. The heightened occurrence of *Bacteroides* (genus) and *Bacteroidaceae* (family) in anxious patients showed the highest effect magnitudes among the comparisons by psychological variables. *Proteobacteria* phylum and the *Prevotellaceae* family were elevated in depression. Elevated bacterial taxa from subgroups defined by psychological variables are given in Table 5; all differentially abundant bacteria identified in subgroup analyses are marked in Figure 4.

Bacterial Abundances Between Subgroups Defined by IBS Symptom Variables

Likewise, patient subgroups by IBS symptom-related variables were tested for differences in bacterial abundance. Because the mild IBS group contained only three patients, the mild and moderate IBS groups were combined and compared with severe IBS. The LEfSe analysis yielded three *Bacteroidetes* members (significant from phylum to order level), and three *Firmicutes* members associated with mild/moderate IBS. Postinfectious onset history of IBS was associated with an unclassified *Erysipelotrichaceae* genus. Patients with diarrhea-predominant IBS had higher *Pseudobutyrvibrio* genus, whereas IBS-mix patients had three elevated *Propionibacterium* taxa (members of *Actinobacteria* phylum, significant from order to genus level) (Figure 4, Table 6).

Machine-Learning Model

Machine learning permits modeling of complex and interactional bacterial signatures, and their use in microbiome research has been

encouraged by expert panels. We therefore attempted to complement our analyses by a machine learning approach. A boosting algorithm selected 148 bacteria (altogether a “bacterial signature”) from the whole data set of 48 samples corresponding with presence of psychological distress. With a count of 122,163 sequences, these bacteria covered approximately 3.9% of the total bacterial abundance. The data set was then split in a training ($n = 32$) and a test set ($n = 16$). A Random Forest model was trained to predict the presence or absence of psychological distress in the training set from the relative abundances of the signature bacteria. The bacteria were ranked according to their prediction feature importance (Figure 5; see Methods for detailed information on machine learning and feature ranking). The Random Forest model's prediction accuracy was subsequently demonstrated in the test set ($n = 16$). With an area under the receiver operating characteristic curve of 0.98, the model showed a high prediction accuracy (see Figure S6, Supplemental Digital Content 6, <http://links.lww.com/PSYMED/A510>). However, the conclusiveness of this analysis is tainted by the fact that feature selection was performed on the whole data set including the test set. This was necessary because of the small sample size, as machine learning feature selection techniques require at least ~50 data sets, but is associated with a high likelihood of overfitting (94).

The complete list of signature bacteria, their taxonomic classification, model importance, and representative sequences are given in Table S7 (Supplemental Digital Content 7, <http://links.lww.com/PSYMED/A511>). The top 20 features were all unclassified species, as was confirmed by blast analyses of representative sequences (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Twelve of the top 20 features were members of the *Lachnospiraceae* family, and three were members of the *Ruminococcaceae* family (both members of *Clostridiales* order). These two families were also dominant in the whole set of 148 species, where taken together, they represented

TABLE 3. Associations Between Weighted Composition, Psychological Distress, Anxiety, and Depression

	Pseudo-F	R^2	<i>p</i>	<i>q</i>
Psychological variables				
Distress (HADS)	2.97	0.061	.008**	.036*
Anxiety	2.12	0.044	.029*	.087
Depression	3.09	0.063	.004**	.036*
Perceived stress (PSQ)	1.46	0.031	.13	.24
IBS symptom-related variables				
IBS severity (IBS-SSS)	0.69	0.015	.73	.77
IBS subtype	0.65	0.014	.77	.77
Potential covariates				
Sex	0.83	0.018	.56	.72
Age	0.86	0.018	.53	.72
Antidepressant intake	14.84	0.031	.13	.24

HADS = Hospital Anxiety and Depression Scale; IBS = irritable bowel syndrome; PSQ = Perceived Stress Questionnaire.

Pseudo-F, coefficient of determination R^2 , *p*, and *q* values (*p* values corrected for multiple comparisons by Benjamini and Hochberg's method) from Adonis tests of weighed UniFrac distances.

* $p < .05$.

** $p < .01$.

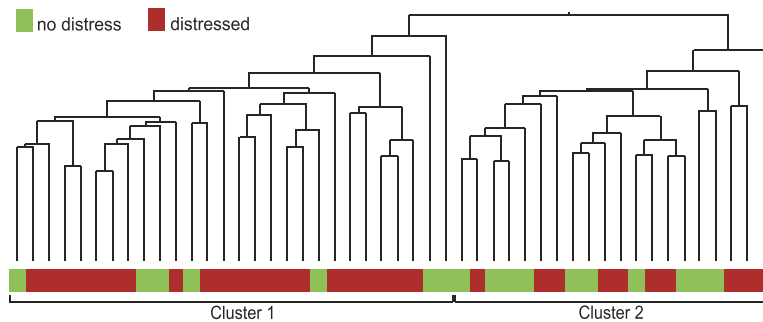


FIGURE 3. Hierarchical dendrogram based on weighted UniFrac distance. Unweighted Pair Group Method with Arithmetic mean clustering revealed 2 clusters with 28 patients in cluster 1 and 20 patients in cluster 2. Patients with psychological distress are indicated in red (dark gray in print grayscale image), those without psychological distress in green (light grey in print). Color image is available only in online version (www.psychosomaticmedicine.org).

66% of the signature OTUs. At the phylum level, most OTUs were members of *Firmicutes* (120 OTUs), followed by *Bacteroidetes* (20 OTUs), whereas *Actinobacteria* counted two OTUs, and *Proteobacteria* one, and five OTUs were unclassified up to phylum level (belonging to unknown bacteria).

DISCUSSION

This study assessed associations between psychological factors and gut microbiota in IBS by using several methods of microbial analysis, including tests of diversity and composition, correlational analyses, subgroup comparisons, and machine learning techniques.

Limitations

Substantial limitations of this study are the lack of a control group and the small sample size. The results must therefore be interpreted with caution, and further studies are required to determine associations between psychological factors and gut microbiota in patients with IBS, and importantly, also in healthy individuals.

Furthermore, the outcomes of this study were highly dependent on self-report assessments with the HADS. The HADS has been subjected to criticism because of psychometric shortcomings (95),

concerning the validity of the subscales anxiety and depression. Our results regarding anxiety and depression should therefore be interpreted carefully. A strength of the HADS however is its familiarity to many clinicians and its wide use in the field (63,66,68), which allows for comparisons across studies.

Another weakness can be seen in the specific OTU clustering technique used in this study, which is implemented in QIIME (88) and has been widely used but was found to inflate OTU counts under certain circumstances (96,97). Methodological advances have recently been made with regard to sequence clustering, and a possible transition from OTUs to higher resolution amplicon sequence variants has been proposed (98–100).

Microbial analyses are also afflicted with general limitations, for example, loss of information in beta-diversity analyses, or possibly misleading results by combination and analysis of bacteria at higher phylogenetic levels without sufficient resolution, the latter also related to the fact that many species are currently not even named. A detailed understanding of microbiome-body interactions cannot be reached until in-depth characterization of these unclassified bacteria has been achieved (e.g., their role in bile acid metabolism or production of neurotransmitter precursors (31,101)).

TABLE 4. Distribution of Main Study Variables Among Microbial Clusters

	Cluster 1, <i>n</i> = 28	Cluster 2, <i>n</i> = 20	<i>p</i>	<i>q</i>
Psychological variables				
Distress (HADS)	19.5 (15.0–23.0)	15.5 (9.3–18.0)	.016	.056
Anxiety	11.0 (7.0–14.0)	9.0 (6.3–11.8)	.063	.11
Depression	8.0 (5.0–11.0)	6.0 (2.3–7.0)	.013*	.056
Perceived stress (PSQ)	0.60 (0.55–0.69)	0.53 (0.27–0.59)	.044*	.10
IBS variables				
Symptom severity	319 (257–375)	316 (282–367)	.98	.98
PI-IBS	5 (18%)	4 (20%)	.85	.98
Subtype IBS-D	13 (46%)	12 (60%)	.49	.68
Subtype IBS-mix	11 (39%)	7 (35%)	.49	.68
Subtype IBS-C	4 (14%)	1 (5%)	.49	.68

HADS = Hospital Anxiety and Depression Scale; IBS = irritable bowel syndrome; PSQ = Perceived Stress Questionnaire.

p values are two-sided from Mann–Whitney *U* tests, and χ^2 tests for the balance of nominal data. Data shown as median (interquartile range) or cases (percent). *q* values are *p* values corrected for multiple comparisons by Benjamini and Hochberg's method.

* *p* < .05.

TABLE 5. Elevated Bacteria in Patient Subgroups Defined by Psychological Variables

Letter in Figure 5	Name	Taxonomic Level	LDA	<i>p</i>	<i>q</i>
Presence of psychological distress					
l	<i>Proteobacteria</i>	Phylum	2.51	.008**	.021*
n	<i>Burkholderiales</i>	Order	2.27	.007**	.020*
m	<i>Betaproteobacteria</i>	Class	2.27	.007**	.020*
o	<i>Alcaligenaceae</i>	Family	2.14	.005**	.020*
f	<i>Barnesiella</i>	Genus	2.02	.022*	.036*
Elevated stress perception					
d	<i>Alistipes</i>	Genus	2.78	.023*	.036*
c	<i>Rikenellaceae</i>	Family	2.78	.024*	.036*
l	<i>Proteobacteria</i>	Phylum	2.50	.007**	.020*
n	<i>Burkholderiales</i>	Order	2.20	.007**	.020*
m	<i>Betaproteobacteria</i>	Class	2.20	.007**	.020*
o	<i>Alcaligenaceae</i>	Genus	2.13	.009**	.021*
Anxiety					
h	<i>Bacteroidaceae</i>	Family	3.01	.022*	.036*
g	<i>Bacteriodes</i>	Genus	3.01	.022*	.036*
d	<i>Alistipes</i>	Genus	2.77	.043*	.043*
c	<i>Rikenellaceae</i>	Family	2.77	.043*	.043*
l	<i>Proteobacteria</i>	Phylum	2.47	.025*	.036*
f	<i>Barnesiella</i>	Genus	2.15	.030*	.040*
Depression					
e	<i>Prevotellaceae</i>	Family	2.92	.028*	.038*
l	<i>Proteobacteria</i>	Phylum	2.16	.020*	.036*

Bacteria with significantly elevated abundance in the respective category/subgroup as identified in LEfSe analyses.

p and *q* values and LDA effect sizes from LEfSe analyses.

* *p* < .05.

** *p* < .01.

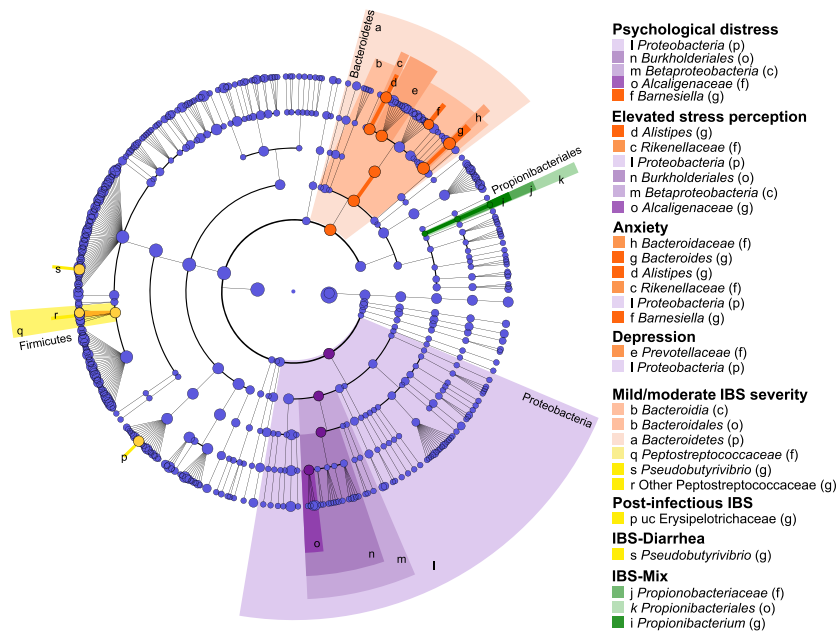


FIGURE 4. Cladogram from LEfSe analyses with differentially abundant taxa. Marked taxa display significant higher relative abundance in patients belonging to the named category. uc = unclassified. Phylogenetic levels are indicated by letters in brackets (*p* = phylum, *c* = class, *o* = order, *f* = family, *g* = genus). See Tables 5 and 6 for significances and effect sizes. Color image is available only in online version (www.psychosomaticmedicine.org).

TABLE 6. Elevated Bacteria in Patient Subgroups Defined by IBS Symptom Variables

			LDA	p	q
Mild/Moderate IBS					
b	<i>Bacteroidia</i>	Class	3.25	.038*	.043*
b	<i>Bacteroidales</i>	Order	3.25	.038*	.043*
a	<i>Bacteroidetes</i>	Phylum	3.25	.038*	.043*
q	<i>Peptostreptococcaceae</i>	Family	2.43	.007**	.020*
s	<i>Pseudobutyrvibrio</i>	Genus	2.36	.003**	.020*
r	Other Peptostreptococcaceae	Genus	2.10	.040*	.043*
Postinfectious IBS					
p	uc Erysipelotrichaceae	Genus	2.74	.043*	.043*
IBS-diarrhea					
s	<i>Pseudobutyrvibrio</i>	Genus	2.73	.019*	.036*
IBS-mixed					
j	<i>Propionibacteriaceae</i>	Family	2.38	.002**	.020*
k	<i>Propionibacteriales</i>	Order	2.19	.002**	.020*
i	<i>Propionibacterium</i>	Genus	2.11	.002**	.020*

IBS = irritable bowel syndrome; uc = unclassified bacterium.

Bacteria with significantly elevated abundance in the respective category/subgroup as identified in LEfSe analyses.

* $p < .05$

** $p < .01$.

Furthermore, it has been argued that functional properties can differ significantly even below the 97% genetic similarity criterion (102). In general, observing alterations of single bacteria can be misleading, because it remains unclear to which extent they are the results of microbe-microbe or microbe-host interactions. Taking into account comprehensive bacterial signatures, as proposed in this study, seem therefore reasonable. However, the machine learning was constrained by the small sample size, the lack of an external validation data set, and possible overfitting. The bacterial signature of

psychological distress can therefore not claim generalization. It can only help orienting research toward relevant bacteria.

Microbial Diversity and Composition

The bacterial diversity and the number of reads per sample in this study were high in comparison with other studies (62,63). Diversity was however not different between the subgroups, which may be due to the small sample size. Because several animal studies have reported decreased alpha diversity after exposure to stress (15,16,103), further investigations are required to assess the association between psychological variables and alpha diversity in humans. Microbiome composition was associated with psychological distress and depression, whereas other potentially confounding variables showed no association. This indicates systematic shifts in certain taxa in parallel with psychological burden. The cohort was separated in two clusters according to microbial composition. Psychological characteristics were equally distributed among these, with a slightly higher presence of psychological distress in the cluster with lower *Firmicutes* to *Bacteroidetes* ratio. This is similar to the results of Jeffery and colleagues (66), where psychological burden was higher in patients belonging to a cluster with microbiomes resembling those of healthy controls. The authors elegantly interpreted this discovery as a more “centrally triggered” IBS. In contrast to their work, our study lacks a control group for comparison with healthy microbiomes.

Irrespective of microbial analyses, the low correlation between IBS symptom burden and psychological distress was a surprising finding of this study. Previous studies reported mixed magnitudes of this relationship (104–106). In our opinion, the findings reflect that IBS is a heterogeneous disorder (43,45) and that association of psychological and IBS symptoms can occur in any configuration.

Differential Abundance of Specific Taxa

Higher abundances of several bacteria belonging to the two major phyla *Proteobacteria* and *Bacteroidetes* were found in patients with psychological distress. Elevated *Proteobacteria* were previously

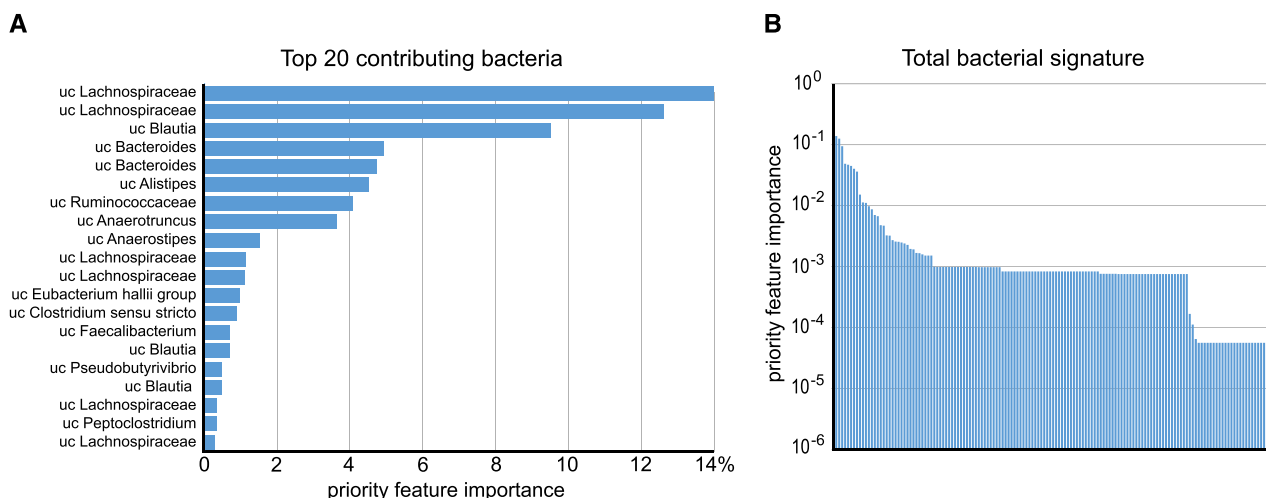


FIGURE 5. Top 20 OTUs with the highest contribution to classifier (A). The lowest known level of taxonomy is given to denote phylogenetic membership, as all top 20 OTUs contributing to the model were unclassified (uc) species. The ranked list of all 148 signature OTUs contributing to classification of psychological distress with logarithmic indication of their contribution to the model (B). Color image is available only in online version (www.psychosomaticmedicine.org).

reported in people with major depressive disorder (40) and as a feature of IBS (58). *Bacteroidetes* members demonstrated a strong presence not only in anxiety (families *Bacteroidaceae* and *Rikenellaceae*) but also in depression (family *Prevotellaceae*). Anxiety correlated positively with the genus *Anaerotruncus*, which was previously found to be increased in animals after prenatal stress (107). A recent study examining IBS and comorbid depression also reported elevated *Bacteroidetes* (70), but studies show mixed results regarding their presence in IBS and in major depression alone (40,62,63,108).

IBS symptom-related variables were also taken into consideration in the microbial subgroup analyses. The severity of IBS was correlated with *Ruminococcus*, which adds to previous findings (58,109). IBS-mix and IBS-C were characterized by elevated *Propionibacterium*. A possible role in slowing down intestinal transit has been previously observed (110) and warrants further investigation. Postinfectious history of IBS onset was associated with a significantly elevated *Erysipelotrichaceae* genus. *Erysipelotrichaceae* were previously found to flourish after treatment with broad-spectrum antibiotics and are rated as highly immunogenic (111). These properties might offer an explanation for subsequent bowel dysfunction after gastrointestinal infections. However, other previously described microbial characteristics of PI-IBS (67,109) were not replicated in our study.

Machine Learning Signature

A comprehensive gut microbial pattern associated with psychological distress was identified with machine learning techniques. Machine learning algorithms are increasingly used to model phenotype-microbe associations (112) or to distinguish between different types of individuals (113,114). Their strength lies in their ability to take into account the complex and interactional nature (115) of the microbiome by simultaneous consideration of several bacterial features and their use in microbiome studies has been encouraged by expert panels (94,116). Machine learning identified associations with psychological distress in a signature of 148 unclassified species, mostly members of the families *Lachnospiraceae* and *Ruminococcaceae*.

CONCLUSIONS

This study assessed relationships between gut microbiota and psychological variables in a sample of patients with IBS. Notably, the study generated further evidence for a relationship between psyche and gut bacteria, underlining the importance of brain-gut alterations and the psychological dimension in IBS. Psychological distress was associated with gut microbiota composition, and a microbial signature corresponding with psychological distress was identified. In-depth characterization of these bacteria might lead to discovery of new biomarkers and therapeutics. The findings further emphasize the relevance of gut bacteria for stress reactivity in humans and for integrated approaches of clinical management of IBS. Future studies will also have to determine, if distress-associated microbial alterations are specific to IBS, a disease picture with both altered microbiome and stress reactivity characteristics, or if similar associations are also present in healthy individuals with varying levels of distress.

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