

A biomarker panel and psychological morbidity differentiates the irritable bowel syndrome from health and provides novel pathophysiological leads

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SUMMARY

Backgrounds

The development of a reliable biomarker for irritable bowel syndrome (IBS) remains one of the major aims of research in functional gastrointestinal disorders (FGIDs) and is complicated by the absence of a perfect reference standard. Previous efforts based on genetic and immune markers have showed promise, but have not been robust.

Aim

To evaluate an extensive panel of gene expression and serology markers combined with psychological measures in differentiating IBS from health and between subtypes of IBS.

Methods

Of subjects eligible for analysis ($N = 244$), 168 met criteria for IBS (60 IBS-C, 57 IBS-D and 51 mixed), while 76 were free of any FGID. A total of 34 markers were selected based on pathways implicated in pathophysiology of IBS or whole human genome screening. Psychological measures were recorded that covered anxiety, depression and somatisation. Models differentiating disease and health were based on unconditional logistic regression and performance assessed through area under the receiver-operator characteristic curve (AUC), sensitivity and specificity.

Results

The performance of a combination of 34 markers was good in differentiating IBS from health (AUC = 0.81) and was improved considerably with the addition of four psychological markers (combined AUC = 0.93). Of the 34 markers considered, discrimination was derived largely from a small subset. Good discrimination was also obtained between IBS subtypes with the best being observed for IBS-C vs. IBS-D (AUC = 0.92); however, psychological variables provided almost no incremental discrimination subtypes over biological markers (combined AUC = 0.94).

Conclusions

A combination of gene expression and serological markers in combination with psychological measures shows exciting progress towards a diagnostic test for IBS compared with healthy subjects, and to discriminate IBS-C from IBS-D.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a highly prevalent functional gastrointestinal disorder affecting 10–15% of the population in the western countries,¹ with a higher prevalence in women than men. Patients with IBS are classified into three major groups according to their predominant bowel symptoms: constipation-predominant IBS (IBS-C), diarrhoea-predominant IBS (IBS-D), and IBS with mixed diarrhoea and constipation (IBS-M).²

In current clinical practice, guidelines suggest that the diagnosis of IBS should be based on typical symptoms with judicious exclusion of organic gastrointestinal disorders such as coeliac disease.^{3, 4} Symptom-based criteria such as the Rome criteria for diagnosing IBS have been developed by an international committee of gastroenterologists; however, these are not applied consistently in a clinical practice setting by community gastroenterologists or primary care physicians.⁵ Current clinical practice still leads clinicians to often order a wide variety of tests before making a confident diagnosis of IBS, especially in older patients where the pre-test probability of organic disease (e.g. colon cancer) is much higher.⁶

Most of the tests that clinicians may routinely order, including a complete blood count, serum chemistry, liver enzymes, thyroid function tests and stool sampling, have very low diagnostic values in patients with typical IBS symptoms and no alarm features (such as weight loss, blood in the stool, unexplained iron deficiency anaemia, nocturnal diarrhoea or a family history of inflammatory bowel disease, coeliac sprue or colon cancer).⁷ Notably, such testing can confuse because false-positive results lead to unnecessary diagnostic evaluations, and true-negative results are not necessarily reassuring for doctor or patient. Challenges of diagnosing IBS are further complicated by the fact that IBS patients often present with co-existing functional disorders such as functional dyspepsia, fibromyalgia, chronic pelvic pain or interstitial cystitis. As a result, patients with IBS visit physicians more often, consume more medications and undergo more diagnostic tests than non-IBS patients.^{8, 9}

While the aetiology of this disorder remains obscure, there is a body of evidence suggesting dysregulation of several pathophysiological pathways including serotonin biosynthesis and metabolism,^{10–12} mast cell infiltration and degranulation,^{13–17} visceral hypersensitivity, an exaggerated stress response, immune activation and bacterial infection (post-infectious IBS) or microbiota alterations.^{18–22}

Gene expression profiling in tissue samples taken from patients with IBS has been reported using sigmoid colonic mucosal tissue.²³ Although certain gene expression biomarkers have been recently reported in the literature, these markers were derived from data mining of a published inflammatory bowel disease study.²⁴ However, it is unknown whether there exist 'surrogate' transcriptional biomarkers in peripheral blood cells of patients with IBS.

IBS is widely considered to be a heterogeneous condition possibly resulting in a common constellation of symptoms from multiple distinct pathologies.²⁵ Apart from the biological pathways discussed already, individuals with IBS are also known to suffer elevated levels of mood disorders (anxiety and depression) compared with healthy individuals.^{26, 27} Whether mood disorder lies antecedent to the onset of IBS or results from the symptoms of the disease remain an open question,^{28–30} although the biopsychosocial model³¹ would suggest a bidirectional relationship. There is no strong evidence that we are aware of that IBS subtypes have different mood profiles.

Given the multiple potential pathophysiological aetiologies of this phenotypically heterogeneous disorder, it is unlikely that any single diagnostic test or biomarker will reliably identify subjects with IBS. The poor clinical reliability of using symptom-based criteria alone to diagnose IBS and the poor diagnostic values of the currently available routine diagnostic tests justify the development of a simple but sensitive and specific assay to assist clinicians in making a confident diagnosis of IBS.

A first generation blood-based test for IBS comprising 10 serum biomarkers derived from the literature was reported by Lembo and colleagues³²; the panel of 10 biomarkers had acceptable specificity (88%), but poor sensitivity (50%) in differentiating IBS from health. No further studies using this panel have been reported until now, although it is noted that our study compares IBS with health, having excluded organic disease, which stands in contrast to the combination of functional, organic and healthy subjects that made up the earlier study sample. Further work using the sample reported here and based on discovery methods described in this study employing a pathway-based approach as well as genome-wide gene expression profiling has led to the identification of an additional 24 promising biomarkers. This study reports the diagnostic efficacy of the combined 34 markers, 10 identified in the Lembo paper³² and 24 in this sample.

We aimed to develop statistical models based on the entire panel of 34 biomarkers that would reliably and specifically differentiate IBS from healthy volunteers and differentiate IBS subtypes from each other. As a secondary aim, we sought to determine whether psychological measures added incremental benefit in differentiating IBS subtypes and IBS from health. We hypothesised that psychological factors would improve differentiation of IBS from health, but would provide little or no incremental benefit in differentiating IBS subtypes from each other. This is, to our knowledge, the first prospective study to use gene expression microarrays for identification of differentially expressed genes in peripheral blood samples as potential biomarkers of IBS compared with healthy subjects, and also the first to combine biological markers with well-established psychological markers to differentiate IBS from health.

METHODS

Experimental design

A case-control study design was employed with clearly defined irritable bowel syndrome patients who underwent intensive investigation to ensure that they were functional patients rather than patients suffering from organic gastrointestinal disease as the 'cases'. The controls were similarly well characterised as not suffering from clinically relevant gastrointestinal disorder or other serious disease. This design allows the clearest identification of the biomarker panel's potential in differentiating irritable bowel syndrome from health.

Patients

IBS patients and healthy volunteers were recruited from 12 US tertiary referral centres as well as 23 community gastroenterology clinics. All IBS patients had a physician diagnosis of IBS, met Rome III criteria for IBS and did not have any other gastrointestinal disorders; however, dyspepsia or heartburn was not exclusionary. Patients with extraintestinal functional disorders, organic gastrointestinal disorders, or severe anxiety or depression (HADs score ≥ 18 for either scale) were all excluded. Age- and gender-matched healthy volunteers were Rome III-negative for IBS, did not have chronic gastrointestinal symptoms, any active infections or significant chronic medical conditions. At the time of blood collection enrolled patients were not taking medications that are known to interfere with serotonin metabolism, mast cell degranulation or other inflammatory pathways that were under investigation. Chronic use of nonsteroidal

anti-inflammatory drugs (NSAIDs) was exclusionary with the exception of prophylactic use of low-dose aspirin (< 82 mg). All subjects provided written informed consent for analysis of their blood samples, including separate consents for genetic analyses. The protocol was approved by institutional review boards (IRBs) of the respective academic institutions or by the central IRB, BioMed.

Definition of IBS and IBS subgroups

IBS subjects in this study were required to meet Rome III criteria² and be diagnosed with IBS by experienced gastroenterologists. In addition, subjects were required to experience active IBS symptoms more than twice a week in the month prior to enrolment and be free of comorbidities reported to be highly prevalent in individuals with IBS,³³ including major psychiatric disorders, as well as other nongastrointestinal functional disorders such as fibromyalgia, chronic fatigue and chronic pelvic pain.

Subjects were assigned to the different subgroups of diarrhoea-predominant (IBS-D), constipation-predominant (IBS-C) or mixed IBS (IBS-M) based on predominant bowel habit according to the Rome III subtype table and scored by the Bristol Stool Form Scale, which was asked over a 3-month recall period.

Assessment of IBS severity

Subjects with any degree of IBS severity were enrolled in the study. However, severity was assessed in all IBS subjects via four different measures as there is no consensus definition for categorising IBS patients based on severity. These measures included two validated instruments, the IBS-Severity Scoring System (IBS-SSS)³⁴ and the Functional Bowel Disease Severity Index (FBDSI)³⁵, as well as two self-report scales: a self-report of overall IBS severity (not at all, somewhat, moderately, very or extremely severe in response to, 'rate how severe your IBS is?'); and self-rated pain severity using a 5-point Likert scale (0 = none, 1 = mild, 2 = moderate, 3 = intense and 4 = severe).

Psychological measures

In addition to excluding subjects with a diagnosis of one of the excluded comorbidities, all subjects were administered the Hospital Anxiety and Depression scale or HADs³⁶ to identify and exclude subjects with severe anxiety or depression at screening (anxiety or depression score > 18). Other psychological measures assessed somatisation status using the Patient Health Questionnaire

15 (PHQ-15) and stress status using the perceived stress scale (PSS).³⁷ While no subjects were excluded based on total score on these two scales, the scoring was intended to allow stratification of patients during analyses.

The PHQ-15 assesses the extent to which individuals are bothered by a range of somatic symptoms. Several of these symptoms are gastrointestinal and have been excluded from consideration in this analysis as they may induce a logical and statistical circularity. Specifically, for this analysis, we omitted items 'a: stomach pain', 'd: menstrual cramps', 'l: constipation, loose bowels, diarrhea' and 'm: nausea, gas or indigestion' from the PHQ-15. A total PHQ score was calculated using the remaining items and is referred to as the PHQ non-GI.

Selection of markers

Original 10 biomarker panel. The original panel of 10 biomarkers reported by Lembo *et al.*³² was identified using a seven-step procedure that initially considered more than 60 000 potential biomarkers identified via literature searching and then filtered down to 10 candidate biomarkers through pragmatic considerations around measurement and demonstrated efficacy in differentiating IBS patients from controls.³²

Gene chip human array. Blood samples were collected from eight subjects. Samples were prepared according to the PAXgene RNA preparation protocol. The samples were grouped by class and the group were blinded prior to the screening: group 1 contained three IBS-D; group 2 contained two IBS-C; and group 3 contained three healthy subjects. The screening was performed with Affymetric Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA), an oligonucleotide-probe-based gene array chip containing ~35 000 transcripts, which provides a comprehensive coverage of the whole human genome.

Gene array data analysis. Fluorescence intensities were uploaded to the Array Assist 6.5 and Gene Spring GX10.0 (Agilent Technologies, Santa Clara, CA, USA) software. Data were normalised by quantitative normalisation, and then transferred logarithmically for further analysis to determine changes in a particular gene.

Using a threshold of false discovery rate adjusted P value <0.25 and a fold change >2 , we found 228 differentially expressed genes cumulatively. We then performed a hierarchical clustering analysis to explore whether the gene expression profiles of the DEGs can

separate samples into distinct classes. We used all unmasked probe sets in this analysis.

To select among the 228 differentially expressed genes, a pair-wise t -test was performed between each pair of groups. Fold change, P value and FDR-adjusted P value³⁸ were computed for each probe set on the array in each comparison. Differentially expressed genes (DEGs) were defined as those genes that have a FDR-adjusted P value <0.25 and a fold change >2 . 40 DEGs between IBS-D and healthy volunteers ordered by fold change. To identify genes that can be used for both IBS-C and IBS-D subgroup diagnosis, we further selected 26 genes, which were upregulated in both groups based on >2 fold changes and P values.

Real-time quantitative PCR validation of selected DEGs.

We further validated the 66 selected genes out of 228 by qRT-PCR using samples from 27 healthy volunteers, 19 IBS-C, 22 IBS-D and 17 IBS-M patients. Total RNA was reversed transcribed into cDNA in a 20 μ L reaction using high capacity cDNA reverse transcription kit (Applied Biosystems, Bedford, MA, USA). cDNA was then diluted to 200 ng/ μ L per reaction. Real-time quantitative reverse transcript-polymerase chain reaction (qRT-PCR) was performed in duplicates using two sequence-specific PCR primers and a TaqMan assay-FAM dye-labelled MGB probe to validate the microarray data. Assays were run using $2 \times$ Taqman gene expression master mixes with RNase inhibitor on ABI 7900 Fast thermocycler (Applied Biosystems). FAM dye-labelled β -actin is used as an endogenous control for normalisation and Ct values were obtained for both reference and target gene by auto baseline and auto threshold settings. $\Delta\Delta$ Ct method is used to calculate the % expression.

A panel of 14 genes was subsequently selected based on the microarray/Taqman results confirmation with reference to fold change levels and tested again for confirmation on samples from 97 healthy volunteers, 72 IBS-C, 82 IBS-D and 71 IBS-M patients.

Statistical methods

Identification of biomarkers. As described above, biomarker selection therefore comprised multiple approaches:

(i) Pathway-focused approach targeting pathways implicated in IBS pathophysiology, which resulted in identification of 10 serological markers from pathways involved in pain, serotonin metabolism, mast cell activation and inflammation.³²

(ii) Analysis of differentially expressed genes in IBS and healthy volunteers, which resulted in the identification of 14 differentially expressed genes.

The 24 new markers identified using these approaches were combined with the originally identified 10 markers,³² resulting in a set of 34 markers that were used for further statistical analyses as described below and in Table 1.

Validation of biomarkers

Models have been developed to differentiate IBS from healthy volunteers and to distinguish between IBS subtypes, specifically: (i) IBS from health; (ii) IBS-C from IBS-D; (iii) IBS-C from IBS-M; and iv) IBS-D from IBS-M. All models are based on unconditional logistic regression estimating the probability of a specific disease state (i–iv above) based on a panel of 34 biological markers (biomarkers) all of which are measured on a quantitative scale as described above. For each disease comparison, the diagnostic performance of three models is reported: (i) the full model incorporating all 34 potential biomarkers regardless of statistical significance; (ii) four psychological measures, PHQ (omitting GI items), HAD anxiety and depression and the perceived stress score) in addition to the 34 biomarkers; (iii) backward elimination selection of markers with statistical significance at $P < 0.05$; and (iv) backward elimination selection of markers and the four psychological measures with statistical significance at $P < 0.05$. We regard models (i) and (ii) to be the primary analyses and models (iii) and (iv) to provide an indication of many individual markers and psychological factors are driving the panel's diagnostic performance.

The performance of the panel of 10 markers originally reported by Lembo and colleagues³² is also considered and results are reported in Table 1.

Model performance is reported in terms of overall performance through the AUC with 95% confidence interval and through sensitivity and specificity assessed at a threshold probability identified as the point at which the separate sensitivity and specificity curves cross when both are plotted against diagnostic probability.

Further details of the laboratory methods used to identify the biological markers used in this study are provided in an online supplement (Data S1).

RESULTS

The sample consisted of $n = 294$ individuals of whom $n = 90$ were healthy volunteers (HV) free of functional gastrointestinal disease, while the remaining $n = 204$ met Rome III criteria for irritable bowel syndrome (IBS). A subset of $n = 244$ individuals have data on all 34

biomarkers and this group has been utilised in all statistical analyses reported, while 25 individuals have values recorded for 28 markers and a further 25 individuals have values recorded for only 24 markers. There was no difference in the missing value pattern across IBS and health with 82% of IBS subjects having complete data compared with 84% of the healthy volunteers.

Among the $n = 244$ subjects utilised in this study, the IBS group was divided into 60 IBS-C, 57 IBS-D and 51 mixed IBS (IBS-M), and there were $n = 76$ health volunteers. Study groups did not vary substantially with age or gender (Table 2) except that the IBS-D group was made up of proportionately fewer females than IBS-C, IBS-M and healthy volunteers. IBS subgroups did not differ substantively with respect to any psychological variable (Table 2). IBS subgroups were also not markedly different in average scores on disease severity scales (Table 2). IBS subjects were, however, elevated compared with healthy volunteers in anxiety, depression, somatic symptom reporting and measures of functional bowel symptoms (Table 2).

Performance of the original panel

Lembo *et al.* report an AUC of 0.76 for the discrimination of IBS from health, and the performance of their panel in the current sample was consistent with that with an AUC of 0.74 (95% CI 0.68, 0.81). Performance of this panel in discriminating between subgroups was a little lower than for IBS from health: IBS-C vs. D (0.70, 95% CI 0.61, 0.80), IBS-C vs. M (0.65, 95% CI 0.54, 0.75) and IBS-D vs. M (0.71, 95% CI 0.61, 0.81).

Simple comparisons of IBS and healthy volunteers

Inspection of Table 3 suggests that a small number of biomarkers individually noticeably differentiate the four study groups.

Panel performance in differentiating IBS from health

A model including all biomarkers provides credible differentiation of IBS from health with an AUC of 0.81 (Table 4, Figure 1) and at a threshold probability of 0.60, sensitivity is 0.81 (95% CI: 0.75, 0.87) and specificity is 0.64 (95% CI: 0.54, 0.75). Model selection suggests that a small subset of markers is responsible for the bulk of this performance with a sub-panel of 4 markers (histamine, znf326, rnf26, tTG) yielding an AUC of 0.71 (Table 4).

The addition of four psychological measures to the full panel provided substantial incremental value with an AUC of 0.93 and sensitivity and specificity ≥ 0.80 at a probability threshold of 0.70 (Table 4) and this is

Table 1 | Individual members of the 34 biological markers identified as potentially discriminatory of IBS from health and a brief description of each

	Description
Original Biomarker panel (from Lembo et al. [32])	
Interleukin – 1 β (IL – 1 β)	A pro-inflammatory cytokine that plays a central role in inflammatory diseases such as IBD and is known to be downregulated by glucocorticoids released during stress
Growth-related oncogene – α (GRO – α)	A chemokine associated with chemotactic migration and activation of neutrophils, which may be involved in tissue injury in IBD patients
Brain-derived neurotrophic factor (BDNF)	A nerve growth factor thought to be a regulator of neuronal transmission, which may play an important stimulant role in long-term regulation of gastrointestinal motility
Anti- <i>Saccharomyces cerevisiae</i> antibody (ASCA IgA)	An antibody that may reflect a generalised loss of immunotolerance. High levels of ASCA IgA are frequently found in Crohn's Disease patients
Antibody against CBir1 (Anti-CBir1)	An antibody against bacterial <i>flagellin</i> . Bacterial <i>flagellin</i> is recognised by cells of the gut mucosa, which may then activate innate immunity
Anti-human tissue transglutaminase (tTG)	tTG is a tissue-repair enzyme and the major autoantigen in coeliac disease. Anti-tTG testing can aid in the diagnosis of coeliac disease
Tumour necrosis factor (TNF) – like weak inducer of apoptosis (TWEAK)	A cytokine that controls cellular activities such as proliferation, migration, differentiation, apoptosis and angiogenesis. TWEAK levels are downregulated in autoimmune pathologies
Anti-neutrophil cytoplasmic antibody (ANCA)	Autoantibodies that target antigens present in neutrophils, which have been identified in the serum of 50% to 80% of ulcerative colitis patients
Tissue inhibitor of metalloproteinase – 1 (TIMP-1)	An inhibitor of metalloproteinase (MMPs) that break down extracellular matrix proteins involved in wound healing, angiogenesis and tumour-cell metastasis. In the gut, altered TIMP activity can result in tissue destruction, intestinal barrier function impairment, bacteria influx and excessive immune response
Neutrophil gelatinase-associated lipocalin (NGAL)	Belongs to the lipocalin family of proteins. In the viscera, NGAL is involved in a range of functions including molecular transport and GI mucosal regeneration
New biomarkers (N = 24) (serological markers and gene expression markers)	
Serological Markers (N = 10)	
Histamine	Released by mast cells and involved in allergic reactions. Histamine can cause inflammation and increased permeability of blood vessels. It causes constriction of smooth muscle.
PGE2	Prostaglandin E2 (PGE2) is involved in neuronal function, female reproduction, vascular hypertension, tumorigenesis, kidney function and inflammation
Tryptase	Can be used as an indicator of mast cell activation. Tryptases are mediators of asthma and other allergic reactions, and are also involved in several inflammatory disorders
Serotonin	Serotonin is a neurotransmitter, derived from tryptophan, involved in brain function; Serotonin is primarily found in the gut. Serotonin regulates physiological function such as well-being, appetite, sleep, and pain sensitivity and gut motility.
Substance P	A sensory neuropeptide involved in pain perception. Substance P is also associated with mood disorders, anxiety and stress
IL-12	IL-12 is a heteromeric cytokine involved in naive T cells development. It stimulates the production of INF- γ and TNF- α by T cells. IL-12 also has anti-angiogenic activity
IL-10	IL-10 is an anti-inflammatory cytokine mainly produced by monocytes, and can inhibit the synthesis of IFN- γ , IL-2, TNF α and GM-CSF
IL-6	IL-6 is a pro-inflammatory cytokine. It is an acute phase response cytokine secreted by T cells and macrophages
IL-8	IL-8 is a chemokine and one of the major mediators of the inflammatory response. IL-8 is produced by several cell types and by macrophages. IL-8 is chemoattractant, and is also a potent angiogenic factor

Table 1 (Continued)	
	Description
TNF- α	TNF- α is mainly produced by macrophages and is found in acute and chronic inflammatory conditions
Gene expression markers (<i>N</i> = 14)	
CBFA2T2	Core-binding factor, runt domain, alpha subunit 2; translocated to, 2. Biological role unknown. (May function as a complex with the chimeric protein RUNX1/AML1-CBFA2T1/MTG8, which is produced in acute myeloid leukaemia with the chromosomal translocation t(8,21) potentially repressing AML1-dependent transcription of G-CSF/CSF-3-dependent cell growth) (GeneCards description)
CCDC147	Coiled-coil domain containing 147. Biological role unknown.
HSD17B11	Hydroxysteroid (17-beta) dehydrogenase 11 ¹ . (May participate in androgen metabolism during steroidogenesis) (Gene Cards definition)
LDLR	The low-density lipoprotein receptor (LDLR) gene family consists of cell surface proteins involved in receptor-mediated endocytosis of specific ligands (GeneCards description)
MAP6D1	Encodes a protein highly similar to the mouse MAP6 domain containing 1 protein. May function as a calmodulin-regulated neuronal protein that binds and stabilises microtubules (GeneCards definition)
MICALL1	MICAL-like 1, Cytoskeletal regulator, binds to Rab 13. Participates in the assembly and activity of tight junctions
RAB7L1	Member RAS oncogene family-like 1. Biological role unknown.
RNF26	Ring finger protein 26, contains a C3HC5 type of RING finger, a motif known to be involved in protein-DNA and protein-protein interactions. (GeneCards definition)
RRP7A	Ribosomal RNA processing 7 homolog A (<i>S. cerevisiae</i>). Biological role unknown
SUSD4	Sushi domain containing 4. Biological role unknown
SH3BGR13	Belongs to the SH3BGr = R family, binds to SH3 domain and has SH3/SH2 adaptor activity
VIPR1	Vasoactive Intestinal Peptide Receptor 1, a gut hormone
WEE1	Biological role unknown. May act as negative regulator of entry into mitosis. Activity of WEE1 increases during s and G2 phases and decreases during M Phase
ZNF326	Zinc finger protein 326, Probable transcriptional activator that may play a role in neuronal differentiation. (GeneCards definition)

Table 2 | Demographic psychological characteristics of each disease group and statistical contrasts between *IBS and health and †global test across IBS subtypes

Characteristic	IBS-C (<i>n</i> = 60)	IBS-D (<i>n</i> = 57)	IBS-M (<i>n</i> = 51)	Healthy (<i>n</i> = 76)	<i>P</i> *	<i>P</i> †
Age (mean, s.d.)	38.8 (12.6)	41.1 (13.6)	37.5 (13.3)	38.8 (12.4)	0.9	0.6
Gender (% female)	86	65	85	79	0.9	0.01
Anxiety score (mean, s.d.)	6.52 (3.89)	6.09 (3.42)	6.22 (3.50)	4.12 (2.67)	<0.0001	0.8
Depression score (mean, s.d.)	3.30 (3.98)	3.07 (3.20)	2.41 (2.52)	1.47 (1.85)	0.0002	0.7
Non-GI PHQ score score (mean, s.d.)	5.70 (3.61)	5.81 (3.67)	6.16 (3.32)	1.99 (1.63)	<0.0001	0.5
PSS score score (mean, s.d.)	15.12 (7.80)	12.81 (6.36)	15.00 (7.08)	9.01 (5.80)	<0.0001	0.3
Total IBS-SSS score (mean)	267.20 (91.64)	250.14 (74.13)	266.24 (78.70)	–	–	0.1
Total FBDSI score (mean)	53.37 (51.12)	51.84 (40.76)	67.61 (50.71)	–	–	0.3

* Comparing IBS as one group with health.

† Comparing IBS subtypes.

Table 3 | Statistical description of each of the 34 biological markers in IBS subtypes and healthy volunteers

	Study group												*P
	HV			IBS-C			IBS-D			IBS-M			
	Mean	s.d.	N	Mean	s.d.	N	Mean	s.d.	N	Mean	s.d.	N	
Histamine	181.04	125.57	76	135.33	74.02	60	176.60	119.09	57	126.35	59.91	51	0.02
PGE2	423.35	320.76	76	413.42	452.32	60	507.95	409.98	57	324.62	215.46	51	0.1
Tryptase	9.00	17.08	76	9.72	20.54	60	7.41	6.98	57	7.15	11.98	51	0.6
Serotonin	239.60	105.95	76	247.22	142.80	60	202.12	100.94	57	202.22	97.75	51	0.1
Substance P	515.08	221.24	76	569.01	219.28	60	549.70	201.28	57	573.59	187.46	51	0.1
IL12	8.54	51.94	76	3.35	11.71	60	5.38	23.20	57	0.54	1.51	51	0.3
IL10	5.69	24.11	76	2.25	5.81	60	3.99	15.07	57	1.04	2.50	51	0.5
IL6	0.39	0.33	76	0.36	0.33	60	0.42	0.25	57	0.80	1.98	51	0.02
IL8	5.67	5.33	76	5.95	11.41	60	8.38	11.23	57	5.81	6.63	51	0.3
TNF- α	1.78	1.11	76	1.95	1.37	60	1.82	0.54	57	2.58	4.34	51	0.1
CBFA2T2	0.55	0.34	76	0.58	0.28	60	0.67	0.44	57	0.57	0.35	51	0.5
CCDC147	0.02	0.01	76	0.03	0.02	60	0.03	0.02	57	0.03	0.02	51	0.6
HSD17B11	2.38	1.28	76	2.62	1.35	60	2.66	1.45	57	2.45	1.48	51	0.4
LDLR	0.06	0.03	76	0.06	0.03	60	0.06	0.02	57	0.06	0.04	51	0.8
MAP6D1	0.01	0.00	76	0.01	0.00	60	0.01	0.00	57	0.01	0.00	51	0.5
MICALL1	0.27	0.12	76	0.29	0.10	60	0.25	0.10	57	0.27	0.12	51	0.3
RAB7L1	0.36	0.26	76	0.42	0.20	60	0.36	0.19	57	0.36	0.21	51	0.06
RNF26	0.34	0.17	76	0.37	0.14	60	0.38	0.17	57	0.35	0.14	51	0.1
RRP7A	0.57	0.41	76	0.73	0.60	60	0.61	0.40	57	0.58	0.28	51	0.2
SUSD4	0.09	0.09	76	0.12	0.12	60	0.09	0.07	57	0.11	0.10	51	0.04
SH3BGR13	22.09	8.84	76	23.18	8.07	60	21.99	6.59	57	22.78	7.73	51	0.6
VIPR1	0.36	0.30	76	0.35	0.17	60	0.31	0.14	57	0.36	0.21	51	0.5
WEE1	0.07	0.04	76	0.08	0.05	60	0.06	0.03	57	0.07	0.04	51	0.3
ZNF326	0.31	0.26	76	0.31	0.16	60	0.27	0.12	57	0.28	0.15	51	0.6
ANCA	6.83	5.70	76	8.45	6.49	60	10.26	13.50	57	8.49	7.24	51	0.3
ASCA IgA	7.52	8.59	76	8.58	10.88	60	8.10	12.76	57	7.48	7.72	51	0.6
BDNF	16644.68	4983.28	76	16835.42	5846.83	60	17552.89	5925.02	57	17560.20	5558.95	51	0.9
Anti-CBir1	16.79	20.52	76	13.22	8.75	60	14.35	16.25	57	13.90	11.31	51	>0.9
GRO- α	239.01	208.91	76	413.38	583.09	60	251.01	202.93	57	327.30	288.07	51	0.2
IL1Betan	158.93	194.28	76	171.01	269.50	60	109.88	110.16	57	132.31	135.16	51	0.7
NGAL	139.78	64.82	76	136.61	47.68	60	157.13	46.14	57	151.53	54.93	51	0.01
TMP-1	240.63	45.60	76	238.03	51.55	60	249.45	50.59	57	253.90	69.54	51	0.4
TWEAK	1080.76	443.86	76	1112.27	371.58	60	1221.61	375.27	57	1057.49	401.11	51	0.04
tTG	0.96	4.68	76	0.30	0.27	60	0.29	0.29	57	0.23	0.18	51	0.0002

* P = P value from Kruskal–Wallis test.

reflected in the shape of the ROC curve (Figure 2). Of the four psychological measures, the non-GI PHQ (excluding GI items) OR = 2.41 (95% CI 1.77, 3.27; $P < 0.0005$) and perceived stress OR = 1.12 (95% CI 1.01, 1.23; $P = 0.03$) were most important. The addition of the psychological measures to the sub-panel also improved performance substantially with an AUC of 0.91 and reasonable sensitivity and specificity, although only the PHQ reached statistical significance. Neither age nor gender added to the discriminatory performance of the model once genetic and psychological factors are taken into account.

The four psychological measures, considered independent of the biomarkers, provide useful discrimination between IBS and health with an AUC of 0.86 (95% confidence interval 0.82, 0.91) and sensitivity 0.74 and specificity 0.75 using as a probability threshold where the separate sensitivity and specificity curves cross when plotted against probability of IBS.

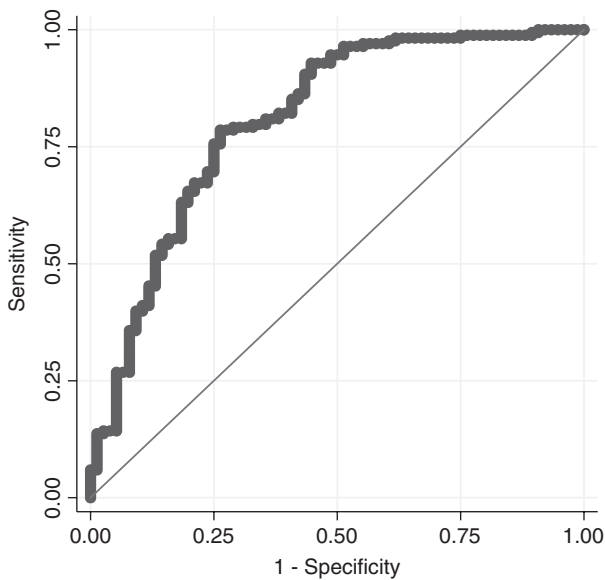
Panel performance in differentiating IBS subtypes

A model including all biomarkers provides good differentiation of IBS-C from IBS-D with an AUC of 0.92 (Table 4) and at a threshold probability of 0.50 achieves

Table 4 | Discriminatory performance of statistical models including biological and psychological markers in differentiating IBS from health and IBS subtypes from each other

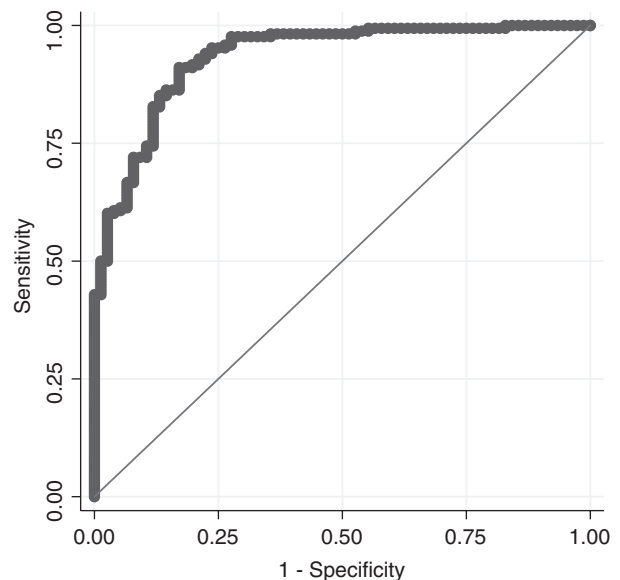
Panel	IBS vs. health	IBS-C vs. IBS-D	IBS-C vs. IBS-M	IBS-D vs. IBS-M
All markers	0.81 (0.75, 0.87) [34] $S_e = 0.81, S_p = 0.64$	0.92 (0.87, 0.97) [34] $S_e = 0.83, S_p = 0.86$	0.85 (0.78, 0.92) [34] $S_e = 0.82, S_p = 0.69$	0.86 (0.79, 0.93) [34] $S_e = 0.84, S_p = 0.67$
Minimum set	0.71 (0.64, 0.78) [4] $S_e = 0.80, S_p = 0.47$ Histamine, znf326, rnf26, tTG	0.75 (0.67, 0.84) [4] $S_e = 0.75, S_p = 0.65$ Histamine, NGALn, micall1, rnf26	0.70 (0.60, 0.79) [4] $S_e = 0.88, S_p = 0.45$ tTG, rab7 l1, IL6, vipr1	0.77 (0.68, 0.86) [5] $S_e = 0.81, S_p = 0.55$ Histamine, vipr1, rnf26, tTG, TWEAK
All markers and psych ²	0.93 (0.90, 0.97) [38] $S_e = 0.85, S_p = 0.88$	0.94 (0.90, 0.98) [38] $S_e = 0.87, S_p = 0.84$	0.88 (0.82, 0.94) [38] $S_e = 0.77, S_p = 0.75$	0.91 (0.86, 0.96) [38] $S_e = 0.79, S_p = 0.84$
Minimum set with psych ²	0.91 (0.87, 0.95) [8] $S_e = 0.82, S_p = 0.83$ tTG, vipr1, znf326, hsd17bl1, wee1, TNFa, PSS, PHQ (non-GI)	0.81 (0.73, 0.88) [8] $S_e = 0.77, S_p = 0.70$ Histamine, rnf26, rrp7a, substance P, NGALn, rab7 l1, PHQ (non-GI), PSS	0.75 (0.66, 0.84) [6] $S_e = 0.72, S_p = 0.65$ IL6, MAP6 dL, vipr1, rab7 l1, PHQ (non-GI), HAD depression	0.80 (0.72, 0.88) [7] $S_e = 0.79, S_p = 0.67$ GROAn, PGE2, TWEAK, RNF26, VIPr1, HAD anxiety, HAD depression

Entries are area followed by 95% confidence interval in parentheses and number of markers included in the model in square parentheses, s_e = sensitivity and s_p = specificity.



The full panel of biomarkers provides adequate overall differentiation of IBS cases from healthy volunteers (AUC = 0.81).

Figure 1 | ROC curve based on full panel for IBS vs. Health. The full panel of biomarkers provides adequate overall differentiation of IBS cases from healthy volunteers (AUC = 0.81).



The full panel of biomarkers in combination with psychological measures provides strong overall differentiation of IBS cases from healthy volunteers (AUC = 0.93).

Figure 2 | ROC curve based on full panel with psychological measures for IBS vs. Health. The full panel of biomarkers in combination with psychological measures provides strong overall differentiation of IBS cases from healthy volunteers (AUC = 0.93).

sensitivity of 0.83 (95% CI: 0.74, 0.93) and specificity of 0.86 (95% CI: 0.77, 0.95). Model selection again suggests that a small subset of markers is responsible for the bulk of this performance with a sub-panel of four markers (histamine, NGAL, micall1, rnf26) yielding an AUC of

0.75 (Table 4). The addition of four psychological measures provided little incremental value to the diagnostic performance, raising the AUC to 0.94, although only one of the measures met the conventional criterion of statis-

tical significance (perceived stress; OR = 1.19, 95% CI 1.01, 1.41; $P = 0.04$).

Adequate differentiation of IBS-C from IBS-M was achieved using all 34 markers (AUC = 0.85, Table 4) and again a sub-panel of four markers (tTG, rab7 11, IL6, vipr1) appears to account for a large proportion of the overall panel's diagnostic performance (Table 4). The additional of psychological measures provided little incremental differentiation.

Adequate differentiation of IBS-D from IBS-M was also achieved using all 34 markers (AUC = 0.86, Table 4) and a sub-panel of five markers (histamine, vipr1, rnf26, tTG, TWEAK) appears to account for a large proportion of the overall panel's diagnostic performance (Table 4). The addition of psychological measures provided little incremental differentiation.

Neither age nor gender add to the discriminatory performance of the model with respect to differentiating any pair of subtypes once genetic and psychological factors are taken into account.

DISCUSSION

This study set out to assess the performance of a set of 34 potential biological markers of irritable bowel syndrome both in terms of differentiating IBS-qualifying individuals from healthy volunteers and in terms of differentiating IBS subtypes from each other. The identification of an array of biological markers that would achieve these differentiations with high sensitivity and specificity would transform IBS from a symptom-based diagnosis of exclusion in clinical practice into a regular medical disease and provide avenues of investigation into possible new pathophysiological mechanisms.

The full set of 34 biomarkers was found to provide encouraging differentiation of IBS from healthy volunteers and with acceptable sensitivity and specificity (Table 4). Furthermore, the addition of four psychological measures covering mood (anxiety and depression), stress and non-GI somatic symptoms yielded excellent overall performance (AUC = 0.93) with both sensitivity and specificity ≥ 0.85 . In the model that included both biological and psychological markers, a small subset of both appears to account for the bulk of the model performance, and therefore there is scope for future research to create a reduced panel with minimal reduction in diagnostic performance. This study was unable to determine whether the incremental value of psychological measures in these models is indicative of a causal association with IBS or not, but does suggest a clinically useful role for psychological factors in the identification of IBS. Interestingly, the performance of

the psychological measures alone in differentiating IBS from health was at least as good and arguably, superior to that of the biological markers alone. Contrasting the discriminatory performance of the biological markers alone (AUC = 0.81), psychological markers alone (AUC = 0.86) and combined biological and psychological markers (AUC = 0.93) raises some intriguing possibilities regarding the pathogenesis of IBS. Based on this observation, it is reasonable to hypothesise that the biological and psychological markers are partly tapping into shared aspects of IBS pathogenesis. On the other hand, the incremental performance of the combined biological and psychological markers suggests that these measures may also be identifying distinct aspects of IBS pathogenesis.

The set of biomarkers studied also showed promise in differentiating IBS subtypes. The data for differentiation of IBS-C from IBS-D were particularly strong with an AUC based on the full panel of biomarkers of 0.92 (Table 4). Performance of this set of biomarkers in differentiating IBS-C from IBS-M (AUC 0.85) and IBS-D from IBS-M (AUC 0.86) was also encouraging. The incremental value of psychological measures in differentiating subtypes appears to be minimal with modest increases in AUC when psychological measures were included (Table 4). This suggests that the influence of psychological factors is limited to differentiating IBS from health and that, conditional on having IBS, psychological factors play little if any role in subtype differentiation.

The subset of markers that were found to provide statistically independent differentiation of IBS subtypes varied considerably between subtype comparisons (Table 4); this provides encouraging although indirect evidence that distinct mechanisms are being identified through the markers selected. For example, four biomarkers provided discrimination of IBS-C from IBS-D: histamine, NGAL, micall1 and rnf26 (see table 4). NGAL belongs to the lipocalin family of proteins; in the viscera, NGAL is involved in a range of functions including molecular transport and mucosal regeneration. Similarly, MICAL-like 1 cytoskeletal regulator binds to Rab 13, and participates in the assembly and activity of tight junctions. Ring finger protein 26 is known to be involved in protein-DNA and protein-protein interactions, which may also impact on intestinal barrier function. Other data suggest that a leaky mucosal barrier may be a key abnormality in IBS.³⁹ Histamine may reflect mast cell dysfunction, also known to be a key pathophysiological marker in IBS.^{40, 41} The highly novel data presented here in turn

suggest that IBS subtypes represent entities that are to some extent biologically distinct.

IBS is likely a heterogeneous disorder making identification of unique biomarkers potentially extremely challenging. To maximise the signal to noise ratio and allow the possible identification of unique biomarkers, the patients enrolled in this study comprised a relatively homogenous IBS population. Specifically, they were diagnosed by experienced gastroenterologists, met established symptom-based criteria (Rome III) for IBS, were experiencing typical IBS symptoms at the time of study enrolment and were free of comorbidities reported to be highly prevalent in IBS patients to avoid identification of confounding markers. These comorbidities included psychiatric disorders such as major depression, anxiety or somatoform disorders, as well as other nongastrointestinal functional disorders such as fibromyalgia, chronic fatigue and chronic pelvic pain. Healthy volunteers enrolled as the control group were adults without any illness, active infection or significant medical condition.

This study adds to the mounting evidence that IBS has an underlying set of biological cause(s).⁴² Strengths of the study include well-characterised cases and controls, and novel application of a biomarker panel. Weaknesses include a relatively small sample size, but significant differences were observed (type II error unlikely). Most importantly, while the set of biomarkers described in this study could distinguish IBS from health, this is insufficient to conclude whether this particular set of markers will be useful for diagnosis – this needs to be confirmed in a study of unselected patients presenting for care (STARD guidelines).⁴³ In particular, further research using a larger sample size and more clinically representative inclusion criteria is needed to establish that the panel is able to differentiate functional gastrointestinal disease from organic gastrointestinal disease.

In conclusion, we have identified potentially novel biomarkers in IBS, although more work is needed to determine the diagnostic utility of the panel. Perhaps most strikingly, a panel of biomarkers alone can discriminate

IBS-C from IBS-D, and psychological measures added little additional information, providing strong novel evidence that may be distinct and measurable disease states that can be objectively identified.

AUTHORSHIP

Guarantor of the article: Michael Jones.

Author contributions: Jones contributed to the design and implementation of statistical analysis, interpretation of results and co-lead preparation of the manuscript. Chey contributed to the design and implementation of the study, and preparation of the manuscript. Singh contributed to the design and implementation of the study, and preparation of the manuscript. Chuang contributed to the design and implementation of the study, and preparation of the manuscript. Shringapure contributed to the design and implementation of the study, and preparation of the manuscript. Hoe contributed to the design and implementation of the study, and preparation of the manuscript. Gong contributed to the design and implementation of the study, and preparation of the manuscript. Talley contributed to the interpretation of results and co-lead preparation of the manuscript. All authors have approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

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