
Genome-wide expression profiling in the peripheral blood of patients with fibromyalgia

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ABSTRACT

Objective. *Fibromyalgia (FM) is a common pain disorder characterised by nociceptive dysregulation. The basic biology of FM is poorly understood. Herein we have used agnostic gene expression as a potential probe for informing its underlying biology and the development of a proof-of-concept diagnostic gene expression signature.*

Methods. *We analysed RNA expression in 70 FM patients and 70 healthy controls. The isolated RNA was amplified and hybridised to Affymetrix® Human Gene 1.1 ST Peg arrays. The data was analysed using Partek Genomics Suite v. 6.6.*

Results. *Fibromyalgia patients exhibited a differential expression of 421 genes ($p < 0.001$), several relevant to pathways for pain processing, such as glutamine/glutamate signaling and axonal development. There was also an upregulation of several inflammatory pathways and downregulation of pathways related to hypersensitivity and allergy. Using rigorous diagnostic modeling strategies, we show “locked” gene signatures discovered on Training and Test cohorts, that have a mean Area Under the Curve (AUC) of 0.81 on randomised, independent external data cohorts. Lastly, we identified a subset of 10 probesets that provided a diagnostic sensitivity for FM of 95% and a specificity of 96%. We also show that the signatures for FM were very specific to FM rather than common FM comorbidities.*

Conclusion. *These findings provide new insights relevant to the pathogenesis of FM, and provide several testable hypotheses that warrant further exploration and also establish the foundation for a first blood-based molecular signature in FM that needs to be validated in larger cohorts of patients.*

Introduction

Fibromyalgia (FM) is a common pain disorder affecting some 2-8% of Americans (1-3). The prevalence of FM in Canada and Europe is as high as 4% (4, 5). The 1990 American College of Rheumatology Fibromyalgia Classification Criteria have been a useful guide in identifying a moderately homogeneous group of “FM patients” defined in terms of clinical profiles, aberrant pain physiology and neuroimaging (6). However, there is significant subjectivity in confirming a diagnosis of FM; thus, compromising clinical research where precisely demarcated phenotypes are essential in defining its underlying biology. Attempts to define a characteristic FM genotype have been impeded by the lack of a “gold standard” (7). The same can be said of other common syndromes such as irritable bowel/bladder, chronic fatigue syndrome, headaches, restless leg syndrome (RLS) and most psychiatric diagnoses that rely heavily on clinical judgment.

Like most chronic disorders it is assumed that the aetiology of FM involves both genetic susceptibility as well as environmental triggers. Fibromyalgia aggregates strongly in families, with first-degree relatives bearing an 8.5 fold increased risk of developing the syndrome (8, 9). Additionally patients often have well-documented triggering events that include persistent focal pain disorders (10), childhood abuse (11), injuries (12), depression (13), hypermobility (14), infection with hepatitis C, Sjögren’s syndrome (15), systemic lupus erythematosus (16), HIV (17), early-onset migraine (18) and post-traumatic stress disorder (19).

However, genetic studies in FM exploring single nucleotide polymorphisms (SNPs) variations and/or Genome

Wide Association Studies (GWS) in presumed candidate genes have failed to show any clear associations (9, 20-23). One such SNP study, using a custom chip, with 350 genes known to be involved in the biologic pathways relevant to chronic pain, reported that GABRB3 (Gamma-aminobutyric acid [GABA] A receptor, beta 3), TAAR1 (trace amine associated receptor 1) and GBP1 (guanylate binding protein 1) genes showed significantly different allelic frequencies in FM patients compared to healthy controls (24). A family linkage study genotyped the members of a cohort of 116 FM families (8) showing that the estimated sibling risk ratio was 13.6. There was evidence for linkage at markers D17S2196 and D17S1294 on chromosome 17p11.2-q11.2. Interestingly, this chromosome contains 2 potential candidate genes, the serotonin transporter gene (SLC 6A4) and the transient receptor potential vanilloid channel 2 gene (TRPV2). Although FM has similarities with other rheumatologic diseases, the search for immunological biomarkers for FM has not been very successful. Studies have shown that FM patients present high anti-polymer antibodies as well as other antibodies such as anti-serotonin and anti-phospholipids. The most recent study suggests that anti-polymer antibodies may be a feature of FM patients with the most severe clinical symptoms (25), but none of these markers have demonstrated good diagnostic accuracy (26-31). A systematic meta-analysis of 25 cytokine studies in FM that included 1255 FMS patients and 800 healthy controls revealed that FM patients had higher serum levels of IL-1 receptor antagonist, IL-6, and IL-8 (32).

Over the past few years there have been major advances in genome wide expression studies using gene expression technologies such as high-density Taqman qPCR assays and microarrays, to identify several disease altering genes simultaneously. One study using qPCR for 13 genes compared gene expression at baseline and following moderate exercise in patients with CFS and a subset of 18 patients with FM (33). Two subgroups of patients were identified, one showing increases in mRNA

for sensory and adrenergic receptors; the other group, mostly patients with CFS and orthostatic intolerance, did not show significant post exercise changes. Microarrays are agnostic, and thus can lead to the discovery of candidate genes that had previously escaped attention. A study used Affymetrix GeneChip Human Genome U133 to profile peripheral blood from 9 women with FM (34). The patients were clinically divided into high and low pain and catastrophising groups measured by the Pain Catastrophising Scale (PCS). Differentially expressed genes between high and low PCS scores revealed functional pathways associated with interferon signaling, interferon regulatory activation and dendritic cell maturation that distinguished the two pain groups. The same group recently profiled a subset of only women with FM (n=29) and controls (n=20), and showed upregulation of only 12 genes (>1.8-fold change, $p < .05$) related to immune response and homeostasis (35).

The fact remains that the current standards for diagnosis of FM remain the 1990 criteria for research and the 2010 ACR criteria for clinical practice. We have made efforts to simplify the clinical criteria and the latest 2014 criteria were just published by us, is intended to be an easier to use and more specific diagnostic algorithm (36). Others have shown sub-groups created by using both the 1990 and 2010 ACR criteria is necessary to tailor treatment options based on their diverse clinical profiles (37).

Given the ambiguity regarding the diagnosis of "pure" FM phenotype, it is clear that there is an urgent need to develop objective molecular markers for FM to be used in conjunction with the established clinical instruments.

Herein we report the analysis of mRNA from 70 FM patients and 70 healthy controls to determine if there are significant differences in gene expression. The major aim was to discover gene expression profiles that could shed light on the molecular mechanisms that drive FM and distinguish FM from healthy controls. Second, using rigorous diagnostic modeling strategies, we tested the hypothesis that a blood-based signature could accurately dis-

tinguish FM subjects from healthy controls. If true and successfully validated in another external cohort of sufficient size for statistical power, this would be a first diagnostic gene expression signature for FM.

Materials and methods

Study subjects

This study was approved by the Institutional Review Board at Oregon Health & Science University (IRB#: 7529), and was limited to Caucasian females aged 18 and over. Peripheral blood samples were collected from 70 FM patients and 70 normal healthy control subjects. All subjects were rested for at least half an hour before venipuncture. Fibromyalgia patients had to have been diagnosed by a physician as having FM for at least six months or longer. On entry into the study, an ACR 1990 FM diagnosis was confirmed by a study investigator (RB, KJ). Exclusion criteria for both subjects and FM patients were: chronic inflammatory disorders, chronic peripheral pain disorders, autoimmune disorders, untreated malignancy, the long-term use of corticosteroids, any major organ dysfunction, pregnant or nursing, surgery six weeks prior to blood collection and a Beck depression score ≥ 25 . All subjects continued on current medications (*i.e.*, there was no washout prior to blood draw). Information obtained at study entry included: basic demographics, family history regarding FM and related conditions, current medications, pain at 28 locations, the Revised Fibromyalgia Impact Questionnaire (FIQR) (38), or the Revised Symptom Impact Questionnaire (SIQR -a FM neutral version of the FIQR) in the case of healthy controls (39), and the Beck Depression Inventory (40). The clinical characteristics of the 140 subjects are shown in Table I.

Blood sample collection and microarray analysis

RNA was isolated using The PaxGene RNA isolation kit according to standard protocols. Total RNA was quantified on a Nanodrop spectrophotometer and visualised for quality on an Agilent Bioanalyzer. 200ng of total RNA was amplified and then hybridised to an

Affymetrix® Human Gene 1.1 ST Peg array using standard manufacturer's protocols. Data was analysed using Partek Genomics Suite v. 6.6 using the RMA normalisation protocol. All genes with Log₂ signal intensity less than 4.8 were excluded from the analysis due to their low expression. Differential expression analysis was carried out using a one way ANOVA with a *p*-value <0.005 considered as significant for the biological and molecular function analyses, and a *p*-value <0.001 considered as statistically significant for candidate diagnostic signatures.

Statistical analyses

Class comparison was performed on all study samples using ANOVAs which included a multiple testing correction using False Discovery Rates (FDRs) set at <10% to identify differentially expressed genes. Biological pathway mapping of these genes was done with Ingenuity Pathway Analysis (IPA). Statistical power and sample size calculations were done using the "power analysis" tool implemented in Partek (41, 42). For class prediction and diagnostic accuracies we used the Support Vector Machines algorithm implemented in Partek (*p*<0.001). We also tested these results with corrections for optimism based on the bootstrapping method (43), applied to the model generated using the Logistic Regression algorithm within the Bioconductor package *rms* (43). To calculate the AUCs and generate the Receiver Operating Characteristic (ROC) curves we used the Bioconductor package *ROCR* (44). To detect the lowest fold change of 1.3-fold at a *p*-value <0.005 that we would accept for a biological signal or biomarker gene at a power of 85%, the sample size necessary was 45 samples per group for whole blood. We were more than adequately powered with 70 samples per group.

Results

Clinical characteristics

The FM patients differed significantly from the healthy controls as regards BMI, medication use, medical history of restless leg syndrome, headaches, irritable bowel syndrome (IBS), depression, anxiety, drug use, fatigue and

Table I. Patient characteristics.

	Case	Control	<i>p</i>
Age (mean (SD))	47.4 (12.1)	43.7 (14.2)	0.10
Years since diagnosis with FM	11.5 (9.9) (range 1-47)	N/A	
Years had FM symptoms	17.2 (12.2) (range 1-50)	N/A	
Medical History n (%)			
BMI	32.1 (6.7)	29.0 (7.9)	.01
Beck Depression Inventory	19.1	2.8	<.001
Total FIQ-R score	57.8	5.6	<.001
Restless Legs Syndrome	18 (25.7)	0	<.001
Nocturnal Myoclonus	7 (10.0)	0	.007
Sleep Apnea	13 (18.6)	0	<.001
Migraine	33 (47.1)	9 (12.9)	<.001
Irritable Bowel Syndrome	37 (52.9)	3 (4.3)	<.001
Overactive Bladder	15 (21.4)	1 (1.4)	<.001
Depression	31 (44.3)	1 (1.4)	<.001
Generalised Anxiety Disorder	26 (37.1)	2 (2.9)	<.001
Post Traumatic Stress Disorder	19 (13.6)	1 (1.4)	<.001
Chronic Fatigue Syndrome	13 (18.6)	0	<.001
Multiple Chemical Sensitivity Syndrome	8 (11.4)	0	.004
Medications n (%)			
NSAIDs	9 (12.9)	3 (4.3)	.07
Acetaminophen	31 (44.3)	16 (22.9)	.007
Tricyclic antidepressants	31 (44.3)	6 (8.6)	<.001
SSRIs	14 (20.0)	1 (1.4)	<.001
SNRIs	12 (17.1)	8 (11.4)	.33
Anticonvulsants	20 (28.6)	1 (1.4)	<.001
Opiates	15 (21.4)	1 (1.4)	<.001
Tramadol	27 (38.6)	0	<.001
Cyclobenzaprine	14 (20.0)	2 (2.9)	.001
Sleeping aids	26 (37.1)	1 (1.4)	<.001

BMI: body mass index; NSAIDs: non-steroidal anti-inflammatory drugs; SSRIs: selective serotonin reuptake inhibitors; SNRIs: selective serotonin and norepinephrine reuptake inhibitors.

pain. FM patients also had a significant family history of FM, IBS, headaches and anxiety. As expected, the total FIQ-R scores of the FM patients were significantly higher than the healthy controls (57.8 vs. 5.6, *p*<0.001). Clinical variables are shown in Table I.

Differential gene expression profiles in FM

Since the samples were run in 2 batches, the data were processed using the Partek Batch Remover which employs a mixed model ANOVA to estimate the batch effects and adjust the data to equalise those effects [Partek Genomics Suite 6.6 User Guide]. We then made a class comparison between all FM and control subjects using a 1-way ANOVA model (45) with a *p*-value cut-off of *p*<0.005. This comparison gave us 482 differentially expressed probesets (a collection of probes that define a single molecular species; such as two or more oligonucleotides that hybridised to different regions of mRNA generated

from a single gene) representing 421 known genes. The list of the top 20 differentially expressed genes (14 down-regulated; 6 up-regulated) is shown in Table II and a brief description about the functions of these genes is shown in Supplementary Table I (online).

The most significant canonical pathways were DNA repair, Rac signaling, Integrin signaling and vascular endothelial growth factor (VEGF) signaling. Among the most highly up-regulated genes were OLFM4, SLC14A1, TSPAN7, TSPAN5, SLC6A8 and SLC1A5. Among the down-regulated genes were CPA3, HDC, MS4A2, FCER1A, GATA2, HRH4, IL3RA and ITGB8. There were a total of 26 genes associated with immune/inflammatory processes including the interleukins IL10, IL36 and its receptor, IL25, CCR9 and IL3RA. Figure 1 shows the top significant gene network which illustrates the relationship between the inflammatory cytokines (IL10, IL25 and IL36A) which were up-regulated in FM

Table II. Top 20 differentially expressed genes.

Gene Symbol		p-value	Fold-Change*
PA3	Carboxypeptidase A3 (mast cell)	9.64e-008	-1.72
C1orf150	Chromosome 1 open reading frame 150	4.87e-007	-1.34
MS4A2	Membrane-spanning4-domains, subfamily A	1.51e-006	-1.43
C11orf83	Chromosome 11 open reading frame 83	2.77e-006	-1.11
FCER1A	Fc fragment of IgE, high affinity alpha receptor polypeptide	3.65e-006	-1.41
ITGB8	Integrin, beta 8	4.39e-006	-1.25
HDC	Histidine decarboxylase	9.67e-006	-1.44
GATA2	GATA binding protein 2	1.18e-005	-1.36
APBB2	Amyloid beta (A4) precursor protein-binding	1.18e-005	1.17
FAM46C	Family with sequence similarity 46, member C	2.81e-005	1.25
ENPP3	Ectonucleotide pyrophosphatase	2.91e-005	-1.20
MARCH8	Membrane-associated ring finger (C3HC4) 8	3.28e-005	1.17
AKAP12	A kinase (PRKA) anchor protein 12	3.92e-005	-1.15
TIGD1	Tigger transposable element derived 1	4.30e-005	-1.15
CCDC55	Coiled-coil domain containing 55	4.41e-005	-1.13
MKRN1	Makorin ring finger protein 1	4.63e-005	1.17
RNF11	Ring finger protein 11	7.47e-005	1.17
RGS17	Regulator of G-protein signaling 17	8.77e-005	1.10
TXNDC11	Thioredoxin domain containing 11	9.16e-005	-1.06
CCDC30	Coiled-coil domain containing 30	9.19e-005	-1.12

*All fold changes are Log2 fold changes. Positive number denotes upregulation in FM and negative number denotes downregulation in FM.

whereas the granulocyte specific genes (FCER1A, MS4A2 and CPA3) were down-regulated. (*Red = up-regulated; Green = down-regulated*). There were 14 differentially expressed kinase molecules which include RIOK3, PIK3R2, WNK1, ROCK2, NCK1 and PRKAG2 and 21 differentially expressed transcriptional regulators and transporter molecules. We found that 14 genes were known targets of currently available drugs using the Drug Interaction module in the IPA software (Supplementary Table II - online). These included the histamine receptor gene HRH4I, IL3RA TGA4, and the collagen family genes COL15A1, COL3A1, COL4A6. This approach is a perfect fit for the recent NIH drug repurposing strategy that builds upon existing drug research and development efforts for new candidate therapies based on molecular studies.

Gene expression profiles characteristic of FM

In addition to understanding the biology, we also tested the hypothesis that some of these differently expressed genes could potentially have some value in differentiating FM patients from healthy controls. To discover a robust and unbiased candidate diagnostic signature for all the study subjects we per-

formed a 5-fold cross-validation on the data set by splitting the 140 study samples into an internal training cohort of 112 random samples (~80% of all samples) and an external validation cohort of 28 samples (~20% of all samples). Both cohorts had equal representation of FM and healthy subjects. In practice, each of the external validation groups acted as a test set for the SVM classifier model generated by the list of significant genes ($p < 0.001$) identified by the ANOVA on the corresponding training group. The model was applied first to the training set as an internal validation and to the external cohort with the results of each used to calculate AUC's (Table III) and generate ROC curves (Fig. 2a-b). It is important to note that the internal results are expected to validate the model with high AUC's, but the external cohort was "blinded" to the training of the signature and produced a true and unbiased estimate of the accuracy and the Area Under the Curve (AUC) of the "locked" classifier derived from the training cohort.

The problem with the most common approach in the medical literature, which is the split-sample validation, is that the model is developed in just one portion of the randomly split data and then validated in the remaining por-

tion. Any discrepancy between the predictive accuracies in the development (training) and validation (test) sets is commonly regarded as the evidence of over-fitting or optimism in a given model using any suitable algorithm. Such a split-sample assessment of predictive models has two drawbacks. First, there is a substantial loss of estimation precision from developing the model in only a portion of the data which leads to selection bias, and second, unless sample sizes are extremely large (>200 at least), very little can actually be learned about the model optimism from a single split-sample.

Therefore, in a separate analysis on the entire dataset of 140 samples, we performed a class comparison of the FM vs. healthy controls at a p -value of <0.001 (FDR range 0.002% to ~13%). This analysis yielded 71 differentially expressed probesets. An independent test of the validity of these results uses the Harrell optimism-adjusted method (43) to compensate for the risk of over-fitting a diagnostic classifier in a single-cohort study by calculating the optimism-adjusted measure of discrimination in the form of an AUC. The Harrell method is based on statistical bootstrapping with replacement. Each possible model of a set of genes to classify FM vs. controls is reduced by backward elimination to yield 500 reduced models. Optimism-adjusted measures of discrimination (area under the curve [AUC]) are then derived from the bootstrap model.

Applying this method to the 71 probesets, we found that a subset of 10 candidate genes (CPA3, C11orf83, LOC100131943, RGS17, PARD3B, RNU6-954P-201, TTLL7, C8orf12, KAT2B and RIOK3) represented the "best-fit model" in terms of prediction with an AUC of 0.931 (Fig. 2c-d). This translated to a diagnostic sensitivity for FM of 95% and a specificity of 96%. A multi-dimensional scaling (MDS) plot for this 10 gene set is shown in Figure 3, which shows the clear separation of the FM subjects from the healthy controls.

Influence of comorbid disorders on gene signatures

FM patients characteristically have

Table III. Area Under the Curve (AUCs) for all the internal and external randomised cohorts using the full study dataset.

Dataset	Randomisation					Mean AU \pm SD
	1	2	3	4	5	
Internal Test	0.838	0.873	0.925	0.900	0.900	0.887 \pm 0.03
External Validation	0.796	0.857	0.790	0.933	0.713	0.817 \pm 0.08

associated comorbidities with several common disorders such as irritable bowel syndrome, restless leg syndrome, etc. (Table I). As it is almost impossible to exclude these comorbid disorders during patient recruitment, it is necessary to test if the 71 probeset signature is influenced by these comorbidities and thereby affirm the specificity of the signature to FM. Therefore

we used an Analysis of Covariance (ANCOVA) which blends the ANOVA results with a linear regression analysis of selected clinical variables. We tested continuous variables (weight, and BMI) as well as categorical variables (presence or absence of restless leg syndrome, migraine, irritable bowel syndrome, overactive bladder syndrome, major depression, generalised

anxiety disorder, sleep apnea, post-traumatic stress disorder and chronic fatigue syndrome) with the 71-gene predictive signature. Twenty genes (28%) were associated with BMI, but none of the other variables were associated with the diagnostic signature. From the 10 gene “best-fit” signature, only R1OK3, KAT2B and CPA3 were associated with BMI.

Discussion

After 33 years and close to 5,000 publications, since the term FM was coined in 1990, there is still no generally agreed-upon definition of the FM phenotype or the mechanisms driving its pathogenesis. The current study found

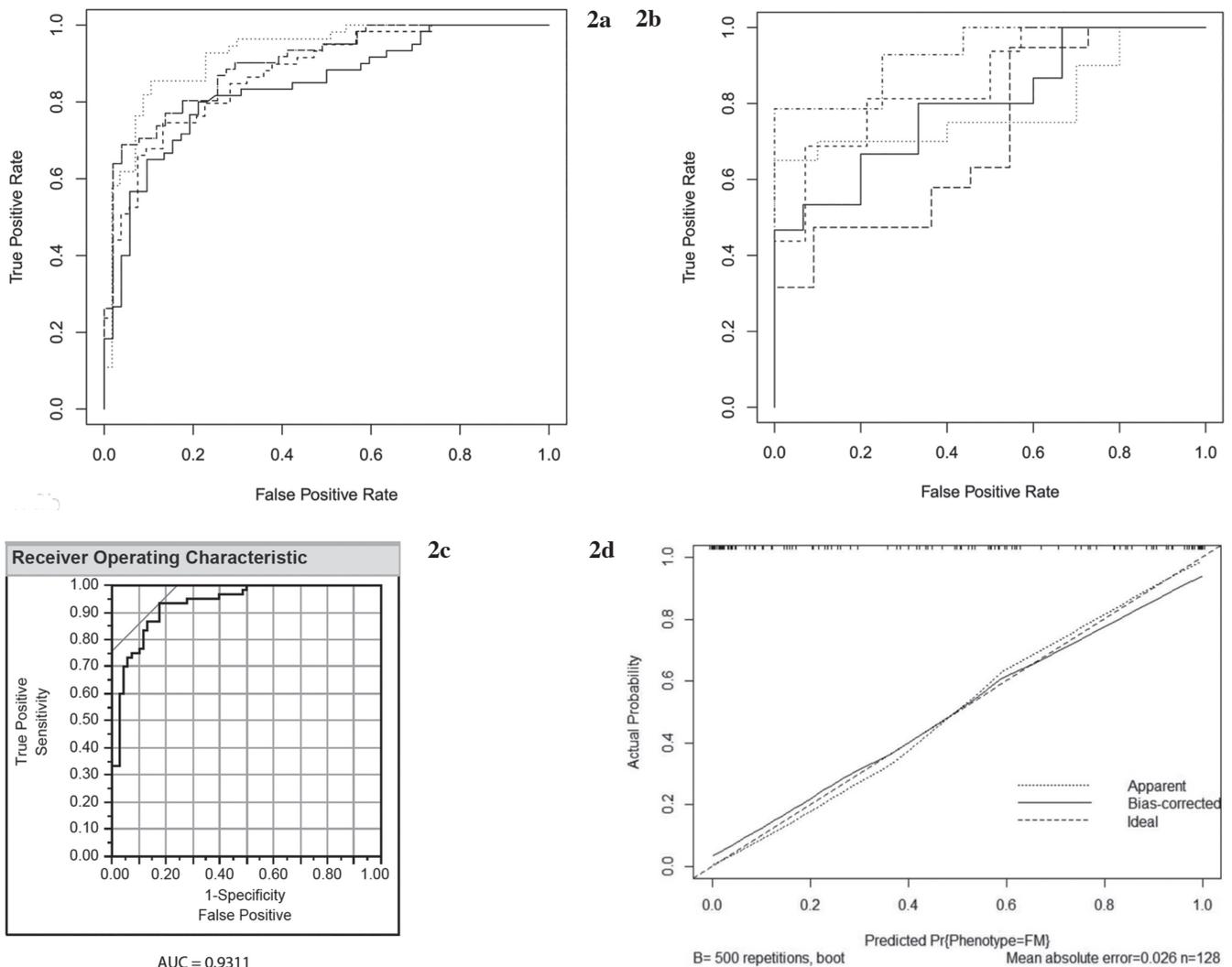
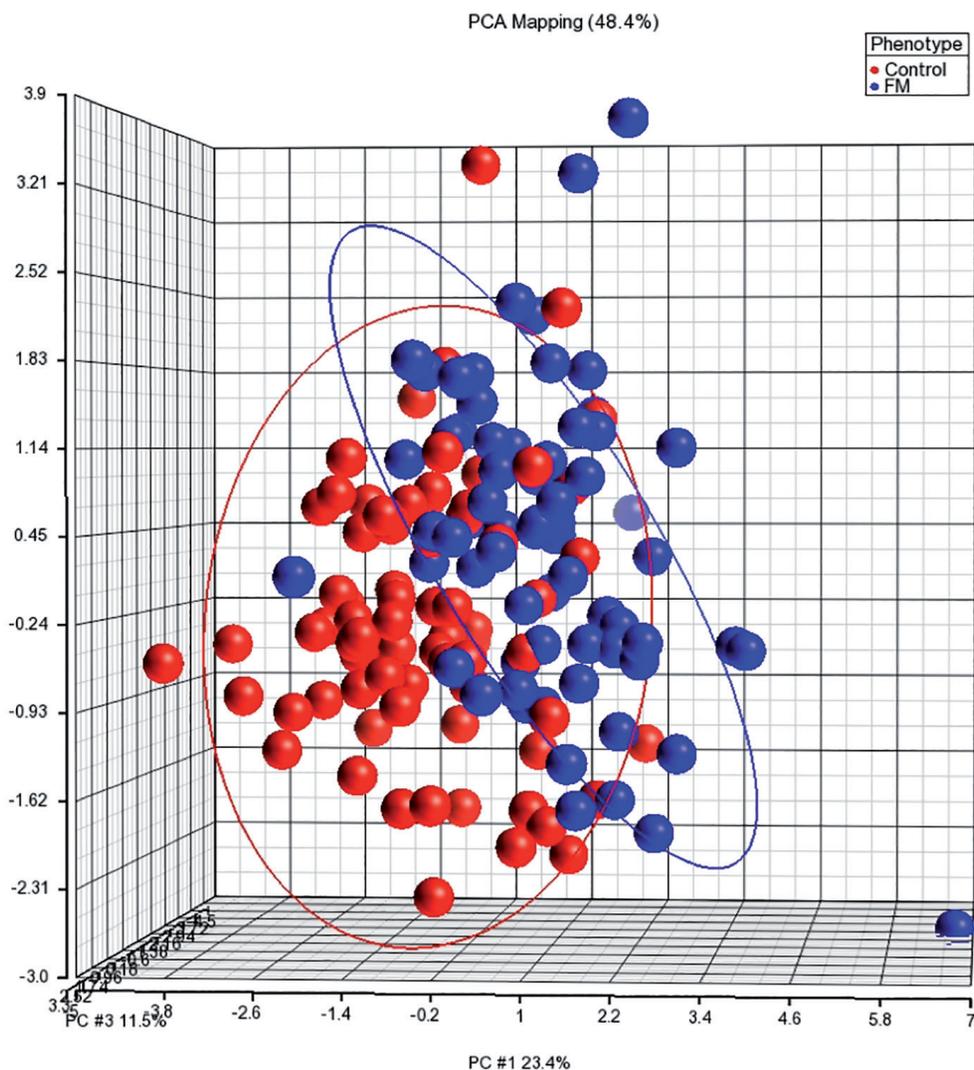


Fig. 2. Receiver Operating Characteristic (ROC) curves for the five randomisations on the Internal (2a) and External cohorts (2b). ROC curves using Logistic Regression models for a subset of 10 candidate genes that represent the “best-fit model” of prediction with an AUC of 0.931 (2c). The bootstrap validation curve nomogram for 500 bootstraps for the 10 genes is shown in 2d. The bias-corrected performance of the classifier (solid line) shows no over fitting when compared to the ideal performance of the classifier (dashed line) demonstrating the robust performance of the 10-gene classifier.

Fig. 3. Multi-dimensional scaling (MDS) plot for the 10 gene set showing the clear separation of the FM subjects from the healthy controls.



482 genes that were differentially expressed between patients and healthy controls. One of the more interesting findings was the differential expression of genes related to hypersensitivity/allergy. For instance a number of genes (CPA3, MS4A2, FCER1A and IL-3RA) are predominantly expressed in granulocytes and dendritic cells and their major function is a granulocyte-mediated inflammatory reaction. CPA3 is a biomarker for local and systemic mast cell degranulation (46). MS4A2 encodes the beta chain of the high affinity IgE receptor. The low affinity receptor of the pro-inflammatory cytokine, IL3, IL3RA is downregulated in FM subjects. These genes are involved in the hypersensitivity reaction and are associated with allergic responses. Fibromyalgia has been sporadically linked to allergic responses

and increased mast cell expression. For example, about 70% of patients with chronic urticaria have concomitant FM and skin biopsies from FM patients have shown IgG deposits and overexpression of mastocytes (47, 48) and FM patients exhibit increased neurogenic inflammation. Furthermore, a study of 25 FM patients revealed that FM skin biopsies had significantly higher values of IgG deposits and collagen III in the dermis and vessel walls, and also a higher number of mast cells (49); while another report also described increased number of mast cells in the papillary dermis of all FM patients compared to healthy controls ($p < 0.001$) (47). Our data illustrates the relationship between FM status and the inflammatory cytokines (IL10, IL25 and IL36A) which are upregulated in FM. IL-10 is known to be anti-inflammatory (50). It

has been shown that IL-10, one with the most potent anti-inflammatory cytokines, regulates the expression of substance P, thus increasing the threshold for pain. IL-25 (51) has been shown to upregulate the expression of pro-inflammatory cytokines especially of the Th2 type (52, 53). IL-10 and IL-25 have been also shown to be key mediators of a Th2 cytokine response, a shift towards which has been linked with chronic fatigue syndrome (CFS) (54). IL36A (IL-1F6) and its antagonist IL-36Ra (IL-1F5) are members of the IL-1 cytokine family. Even though studies have not found any significant differences in IL-1 levels in FM, its receptor has been shown to be upregulated in FM (55).

With respect to neurobiology of FM, there were several solute carrier molecules that were upregulated in the

FM subjects including SLC1A5 and SLC25A22, which are both transporters of the neurotransmitter glutamate in the central nervous system (56, 57). Another potentially relevant gene is GLUL, a glutamine synthetase. GLUL clears L-glutamate, the major neurotransmitter in the central nervous system, from neuronal synapses (58). The metabotropic glutamate receptor (GRM6) was also upregulated in FM subjects; GRM6 is a group III G protein-coupled receptor linked to the inhibition of the cyclic AMP cascade and involved in neuropathic pain signaling in dorsal horn neurons (59).

From our list FM defining genes, some 14 genes are known targets of currently available drugs (Supplementary Table II). These drugs are potential candidates for new therapies in FM patients. Three of the candidate molecules are targets for the treatment of allergies. MS4A2 is a target for omalizumab, a humanised antibody originally used in the control of moderate to severe allergic asthma, not responsive to corticosteroids (60). HRH4 is a target for both Tescmilifene, which inhibits concanavalin-A-induced histamine release in mast cells and acts as a novel antagonist of intracellular histamine and Triprolidene, which is an antihistamine with anticholinergic properties (61). HRH4 has been shown to drive inflammatory responses and the treatment of both mouse and human blood with an H4R antagonist reduced the production of IL-17 when cells were stimulated *in vitro* (62). There were 14 differentially expressed kinase molecules, and 21 transcriptional regulators and 21 transporter molecules. None of these gene targets have been previously recognised in FM and supports the hypothesis that unbiased gene expression profiling may yield novel information. Our hypothesis is that there is an upregulation of immune/inflammatory molecules in the blood of FM patients with a concomitant decrease in pathways related to hypersensitivity and the allergic responses. These findings - in unison with the differential expression of many molecules associated with pain processing, neuro-regulation and

axonal development (most of them downregulated in FM) - suggests that a dysregulation of these pathways is relevant to the pathogenesis of FM.

Lastly we found a panel of 10 genes that differentiated FM from healthy controls with a 95% accuracy. Our findings explicitly employed rigorous statistical tools that compensate for the risk of over-fitting a diagnostic classifier in a single-cohort study. Application of these methods is necessary and important, but not commonly used in similar studies. These findings need to be validated in a large, multicenter, independent cohort of subjects with greater clinical heterogeneity. It is important to consider these performance results for a potential first generation FM molecular diagnostic in context. The first Corus CAD (Coronary Artery Disease) gene expression-based test used RT-qPCR with a 23 gene set signature on over 500 patients with an AUC of 0.72 and a sensitivity of 85% but a specificity of only 43% (63). In contrast, a third generation test for Rheumatoid Arthritis (RA), the Anti-Citrullinated Peptide Antibody (ACPA) showed an AUC of 0.89 in 141 patients who were Rheumatoid Factor positive (RF+) but that dropped to 0.723 in a cohort of 49 RF- patients (64). These metrics dramatically dropped to an AUC of 0.68 in RF- patients with <5 years duration of disease. Testing on different phenotypes of RA patients reveals the importance of testing any molecular diagnostic in real clinical practice and using that experience to inform further refinement.

“Fibromyalgia” is an umbrella term for a heterogeneous collection of phenotypes, sometimes called oligo-phenotypes. For instance, many FM patients have concomitant diagnoses, such as osteoarthritis, depression and obesity. Another group of disorders closely linked to FM, probably through central sensitisation, is irritable bowel, overactive bladder and restless leg syndrome. Excluding all these oligo-phenotypes when designing a clinical study is unrealistic. Moreover, such exclusion would yield data that are not representative of the prevalent FM population. Thus, the “contamination”

of the pure FM phenotype by other disorders implies that gene expression will be similarly contaminated. Exploratory Factor Analysis (EFA) which is based on the premise that a large set of candidate genes can be grouped into a much simpler set of cluster factors (65) revealed that the expression of 34 genes clustered into four meaningful biological factors. Higher expression of these factors was associated with specific aspects of disease in CFS diagnosis and lower depression severity. Bearing these limitations in mind, we found many differentially expressed genes related to the diagnosis of FM. Gene signatures that used these genes show high FM specificity thus confirming that FM specific genes signatures are present in peripheral blood. Another limitation was that we profiled only 70 patients and 70 healthy controls. Moreover, we did not validate the stability of the signature over time, thus our results are a snapshot of the transcriptional profile in peripheral blood. Finally, this work was done at a single center (OHSU) and limited to Caucasian females. A robust biomarker signature must be validated in several centers with an ethnically diverse set of patients before it can be submitted for clinical validity testing.

In conclusion, using an unbiased agnostic approach not shaped by *a priori* biological knowledge, the current study generated two new findings: 1) the discovery of several molecules of potential relevance to the pathophysiology of FM, and 2) the finding of a gene signature that clearly differentiates FM patients from healthy controls.

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Data archiving

All microarray data for this study is available at the NIH Gene Expression Omnibus (GEO) database under the accession number GSE [Series GSE67311](#).

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