



Clinical Validation of a Multi-Biomarker Assay for the Evaluation of Chronic Pain Patients in a Cross-Sectional, Observational Study

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ABSTRACT

Introduction: Chronic pain assessment and post-treatment evaluation continues to be challenging due to a lack of validated, objective tools to measure patient outcomes. Validation of mechanistic pain biomarkers would allow clinicians to objectively identify abnormal biochemistry contributing to painful symptoms.

Methods: We describe the clinical validation of a multi-biomarker assay with algorithmic analysis known as the Foundation Pain Index (FPI) in diverse cohorts of chronic pain patients in a

prospective, cross-sectional, observational validation study. Levels of 11 urinary pain biomarkers were measured and tabulated using a proprietary algorithm to generate FPI scores for chronic pain subjects ($N = 153$) and age- and sex-matched pain-free controls ($N = 334$).

Results: FPI scores were significantly correlated with the 36-Item Short Form Health Survey (SF-36) scores among chronic pain subjects (P value < 0.015) and specific components of SF-36, including emotional well-being, limitations due to emotional problems, and general health (P value < 0.05). Area under ROC analysis (AUROC) revealed FPI to accurately distinguish biomarker profiles between pain-free and chronic pain cohorts (AUROC: 0.7490,

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P value < 0.0001) as well as the SF-36 scores between chronic pain subjects with low vs. high FPI scores (AUROC: 0.7715, P value < 0.01).

Conclusions: Our findings establish the validity and discriminatory power of a novel multi-biomarker test that evaluates the role of biochemistry in chronic pain and correlates with clinical assessments of patients. This test provides novel, reproducible, objective data which may pave the way for non-opioid therapeutic strategies to treat chronic pain.

Keywords: Biomarker; Inflammation; Kynurenine; Micronutrient; Pain

Key Summary Points

Chronic pain assessment and post-treatment evaluation continues to be challenging for medical providers due to a lack of validated, objective tools to measure patient outcomes.

Validation of mechanistic pain biomarkers would allow clinicians to objectively identify abnormal biochemistry contributing to painful symptoms in patients.

The Foundation Pain Index (FPI) is a multi-biomarker assay derived from algorithmic analysis of abnormal urinary metabolites observed in a cross-sectional observational study.

FPI scores strongly segregate biomarker profiles of healthy control subjects and chronic pain patients and correlate with worsening clinical assessments of chronic pain.

This test provides novel, objective data that evaluates the role of biochemistry in chronic pain, which may pave the way for targeted, non-opioid therapeutic strategies.

INTRODUCTION

Pain is a subjective sensation with objective, biochemical underpinnings that continues to challenge healthcare providers and regulatory bodies worldwide. This is mainly due to the risks associated with opioid analgesics commonly used in chronic pain treatment. The relatively high efficacy of opioids for the management of acute pain has been a major driver in the evolution of the current opioid crisis, due mainly to well-documented risks such as tolerance, dependence, and addiction [1]. Unfortunately, a simple solution to the opioid crisis does not exist, as simply removing these addictive analgesics from the clinic has led to a host of additional challenging issues for the healthcare community. The lack of strong evidence for alternative medications to replace opioids in chronic pain adds fuel to this dilemma. Instead, a greater understanding of the biochemical mechanisms that underlie and drive the subjective sensation of chronic pain would allow for novel, personalized, non-opioid therapies that directly modulate pain pathways. Mechanistic pain biomarkers revealing biochemical derangement driving the development or worsening of pain may represent one potential solution to the opioid crisis. Such biomarkers would not only provide physicians with novel, objective data pertaining to the biochemical origin of pain, but could also directly indicate safe, non-opioid therapies aimed at correcting the biochemical dysfunction to alleviate pain and improve function. Biomarker monitoring would also allow for longitudinal evaluation of the efficacy of modulating therapies. This will also provide patients and healthcare providers with objective updates on the biochemical status in a non-invasive manner, which would certainly aid with treatment compliance.

In previous work, we established a high prevalence of abnormal biochemical function in a population of chronic pain patients. We proposed that identifying and correcting such abnormalities would provide a safe and novel treatment modality for the management of pain [2]. As a follow-up to this study, we introduce a

novel, multi-biomarker assay, known as the Foundation Pain Index (FPI), which evaluates diverse biochemical pathways involved in the pathogenesis of chronic pain to provide clinicians with objective and reproducible measures of the biochemical, metabolic, and nutritional drivers of chronic pain. This assay with algorithmic analysis is comprised of 11 well-characterized urinary biomarkers associated with nerve health, chronic inflammation, oxidative stress, and neurotransmitter turnover [3–10]. Clinical interpretations of abnormal biomarker findings in the context of chronic pain are detailed in Table 1.

In the present study, we sought to validate the FPI as an indicator of abnormal biochemical function in a chronic pain population. We also sought to determine the discriminant validity by comparing FPI scores of chronic pain subjects to age- and sex-matched pain-free controls. We established the validity criterion of the FPI by demonstrating a significant correlation between FPI scores and standard clinical assessments of chronic pain (i.e., SF-36 survey). We also discuss in detail the face validity of this innovative test offering. This study provides valuable insight into the validity and utility of the FPI in a clinical setting.

METHODS

Study Subjects

A total of 153 chronic pain patients were enrolled in the ERD2019-02 prospective, multi-center clinical study. This study (ERD2019-02) was reviewed and approved by the Quorum Institutional Review Board (QIRB) and was performed in accordance with the Helsinki Declaration of 1964 and its later amendments. All participants provided informed consent prior to their inclusion of the study. All subjects completed the SF-36 short-form survey and the Hospital Anxiety and Depression Scale (HADS) at the time of sample collection. A single urine sample was obtained from each subject following enrolment and completion of all study questionnaires. Samples were packaged and shipped to Ethos R&D (Newport, KY, USA)

where they were accessioned, prepared, and analyzed according to standard operating procedures. The number of samples provided from pain clinics satisfied criteria of adequate power for data analysis (80%, P value < 0.05).

FPI Scoring Structure

Quantitative results for each of the 11 biomarkers (methylmalonic acid [MMA], homocysteine [HCYS], xanthurenic acid [XAN], 3-hydroxypropylmercapturic acid [3-HPMA], quinolinic acid [QA], kynurenic acid [KA], pyroglutamic acid [PGA], ethylmalonate [EMA], hydroxymethylglutarate [HMG], vanilmandelate [VMA], and 5-hydroxyindoleacetic acid [5-HIAA]) are first normalized using creatinine concentrations and then analyzed via our proprietary algorithm to generate a single numerical score (FPI score) on a scale of 0–100, which describes the severity of abnormal findings. In the pre-validation model, FPI scores were subclassified into tiers that further describe the severity of the abnormal findings based on the severity and number of detected abnormalities (Table 2).

Our working hypothesis is that higher FPI scores, which are represented by a greater number of biochemical abnormalities, are more likely to be contributing to painful symptoms. Patients who exhibit higher FPI scores are therefore more likely to benefit from correction therapy aimed at lowering their baseline FPI score. Conversely, low FPI scores indicate that biochemical function is unremarkable and less likely to be contributing to painful symptomologies. Patients who exhibit low FPI scores at baseline are unlikely to benefit from correction therapy. Low FPI scores indicate that abnormal biochemical function is less likely to be a causative factor in a patient's pain but in no way invalidates a patient's complaint of pain. Such a test could be used in combination with standard clinical assessments and could prove particularly useful in complex cases involving pain of unknown etiology. The assay may also be helpful in cohorts of patients with comorbidities (anxiety, depression, T2D), unresponsive to

Table 1 Component biomarkers of the FPI and clinical interpretation in the context of chronic pain

Biomarker	Clinical interpretation	Relevance to pain and clinical features	Notes	References
Methylmalonic acid	Elevated levels indicate a vitamin B12 deficiency	Vitamin B12 deficiency leads to nerve damage and degeneration of the spinal cord. Peripheral neuropathy is the most common pain presentation	Deficiency can be precipitated by: Medications which lower stomach acidity (such as proton pump inhibitors, H2 receptor antagonists) Metformin Gastric surgery or resection Vegan or vegetarian diets Exposure to nitrous oxide	[3, 11–13]
Xanthurenic acid	Elevated levels indicate a vitamin B6 deficiency	Neuropathy to due to vitamin B6 deficiency starts with numbness, paraesthesias, or burning pain in the feet, which then ascends to affect the legs and hands	Vitamin B6 deficiency can be precipitated by: Use of vitamin B6 antagonists (isoniazid, phenelzine, hydralazine, penicillamine, and carbidopa) Hemodialysis Inflammatory or autoimmune disease	[13–17]
Homocysteine	Elevated levels commonly indicate a B-vitamin (B6/B9/B12) deficiency	Elevated homocysteine results from B-vitamin deficiencies. Elevated homocysteine levels cause inflammation by increasing arachidonic acid and the proinflammatory prostaglandin E2 production	Elevated homocysteine levels result from: B-vitamin (B6/B9/B12) deficiencies Use of diuretic medications Chronic alcohol consumption	[18–20]

Table 1 continued

Biomarker	Clinical interpretation	Relevance to pain and clinical features	Notes	References
3-HPMA	Elevated levels indicate increased exposure to acrolein	Acrolein contributes to inflammatory pain sensitivities through its binding and activation of the transient receptor potential ankyrin 1 receptor (TRPA1) in nerve fibers	Increased acrolein exposure can result from: Chronic tobacco use Foods cooked or fried at very high temperatures Use of certain anti-cancer drugs Spinal cord injury	[21–23]
Pyroglutamate	Elevated levels indicate glutathione depletion	Glutathione depletion renders nerve cells susceptible to oxidative damage which can lead to neuropathic pain	Glutathione depletion can be precipitated by: Chronic use of acetaminophen Poorly controlled diabetes	[24–26]
Ethylmalonate	Elevated levels indicate a carnitine deficiency	Carnitine deficiencies cause muscle aches and fatigue	Carnitine deficiency can be precipitated by: Valproic acid High-fat diets Treatment with acetyl-L-carnitine has been shown to improve nerve conduction and neuropathic pain symptoms	[27, 28]
Hydroxymethylglutarate	Elevated levels indicate a Coenzyme Q10 deficiency	Coenzyme Q10 deficiencies can cause muscle weakness and pain	Coenzyme Q10 deficiency can be precipitated by: Use of statin medications Coenzyme Q10 supplementation ameliorates statin-associated muscle symptoms such as muscle pain and weakness	[29, 30]

Table 1 continued

Biomarker	Clinical interpretation	Relevance to pain and clinical features	Notes	References
5-HIAA	Abnormally low levels indicate decreased synthesis/turnover of serotonin	Abnormally low synthesis/turnover of serotonin can heighten pain sensitivity	Abnormally low synthesis/turnover of serotonin can be precipitated by: Chronic inflammation upregulates indoleamine 2,3-dioxygenase which redirects dietary tryptophan down the kynurenine pathway and away from serotonin synthesis Insufficient intake of high-quality protein Vitamin B6 deficiency	[7, 31, 32]
Vanilmandelate	Abnormally low levels indicate decreased synthesis/turnover of norepinephrine	Abnormally low synthesis/turnover of norepinephrine can heighten pain sensitivity	Abnormally low synthesis/turnover of norepinephrine can be precipitate by: Chronic alcohol use Insufficient intake of high-quality protein Adrenal insufficiency	[33, 34]

Table 1 continued

Biomarker	Clinical interpretation	Relevance to pain and clinical features	Notes	References
Quinolinic acid	Elevated levels indicate cytokine-mediated chronic inflammation	Quinolinic acid is a neuroactive kynurenine pathway (KP) metabolite which serves as a sensitive marker of chronic, systemic inflammation. Upregulation of this pathway has been shown to play a central role in the comorbidity of pain and depression. Quinolinic acid induces its depressive effects through its action on NMDA receptors	Chronic, systemic inflammation can be precipitated by: Autoimmune disease Exposure to LPS from Gram-negative bacteria	[35–39]
Kynurenate	Elevated levels indicate cytokine-mediated chronic inflammation	Kynurenate is a neuroactive kynurenine pathway (KP) metabolites which serves as a sensitive marker of chronic, systemic inflammation. Upregulation of this pathway has been shown to play a central role in the comorbidity of pain and depression	Chronic, systemic inflammation can be precipitated by: Autoimmune disease Exposure to LPS from Gram-negative bacteria	[35–39]

3-HPMA 3-hydroxypropylmercapturic acid, *5-HIAA* 5-hydroxyindoleacetic acid

traditional treatments and poor candidates for opioid therapy.

Inclusion/Exclusion Criteria

Table 3 describes the inclusion/exclusion criteria used for subject enrolment during the ERD2019-02 clinical study.

Pain-Free Control Samples

Control samples from individuals with no history of chronic pain or opioid use, were collected prospectively from Lee Biosolutions (Maryland Heights, MO, USA) and their associated clinical collection sites across the United States. All pain-free control samples were shipped frozen to Ethos R&D (Newport, KY, USA)

Table 2 Tiers used to categorize FPI scores

FPI score	Likelihood of detected abnormalities being pain determinants	Minimum number of expected abnormal biomarkers
0–19	Low	0
20–49	Moderate	1
50–79	Moderately high	2
80–100	High	3

where they were prepared and analyzed according to standard operating procedures.

Analytical Methodologies

Samples were accessioned into the Laboratory Information System (LIS) and frozen at $-20\text{ }^{\circ}\text{C}$ on the date of receipt and remained frozen until the time they were thawed at room temperature and prepared for analysis. Each sample underwent only one freeze/thaw cycle. Samples were prepared on 96-well plates with four-point calibrators, two quality controls, and one negative sample per plate. The urinary biomarkers and creatinine were each analyzed in separate liquid chromatography tandem mass spectrometry (LC–MS/MS) assays. All analytes were quantified using isotopic dilution and analyzed with MassHunter software. Biomarker concentrations were corrected for urinary dilution using creatinine and normalized values reported as $\mu\text{g}/\text{mg}$ creatinine. Samples from healthy and pain subjects were prepared and analyzed in tandem to eliminate any instrument or user bias during LC–MS/MS analysis.

FPI Algorithm and Score Derivation

With the intent to distinguish between chronic pain and pain-free populations, the FPI was developed to represent an algorithm based on binary logistic regression. Individual biomarker results that deviate from previously established normal concentrations collectively contribute to a decision-making process that sorts samples as either normal (pain free) or abnormal (pain).

Table 3 Inclusion/exclusion criteria for the ERD2019-02 study

Inclusion criteria	Exclusion criteria
Men and women between the ages of 21 and 75	Severe or untreated psychiatric disturbance
Long-term use (> 6 months) of an opioid analgesic at a current daily dose of 30 mg morphine equivalents (MME) or greater	Liver and/or kidney disease
Currently under the care of a participating investigator	Pregnancy
Understands and complies with all sample collection procedures	Use of corticosteroid or another immunosuppressive drug during or 1 month prior to sample collection
	Diagnosed with bacterial or viral infection during or 3 months prior to the study
	Being prescribed anti-cytokine therapies
	Use of the following dietary supplements in the previous 3 months: B vitamins (B1, B2, B3, B5, B6, B12); folic acid; magnesium; <i>N</i> -acetyl-cysteine (NAC); ashwagandha; curcumin or turmeric; alpha-lipoic acid; coenzyme Q10; carnitine; tryptophan or other amino acid powder

Various types of supervised machine learning techniques, including principal component analysis, clustering, and linear discriminant

analysis (LDA) were compared among randomly selected pain-free and chronic pain samples. The LDA proved to provide the most robust segregation between groups (P value < 0.001). This model of multiple analysis of variance (MANOVA) with LDA generated weighted coefficients for each biomarker according to the function below:

$$Y = \beta_0 + \beta_1 * E + \beta_2 \times F + \beta_3 \times H + \beta_4 \times I + \beta_5 \times J + \beta_6 \times K \dots$$

Y refers to the model output, E – K ... refer to metabolite concentrations as normalized to creatinine levels ($\mu\text{g}/\text{mg}$) in urine, β_0 is the y-intercept, and β_1 – β_6 ... are coefficients generated by analysis that most distinguishes control (pain free) and experimental (chronic pain) groups for each metabolite.

Biomarkers used in the development of the FPI model were transformed for normal distribution by Box-cox transformations (x^λ , where λ = lambda, R 3.5.1 programming) and verified by normality analysis (D’Agostino and Pearson test, GraphPad Prism 8.3.0).

Statistical Methods

To compare univariate patient characteristics between groups, non-parametric t tests were used to compare means of variables. Multivariate analysis (least squares) were used to determine distinguishable power of the multi-biomarker score. To achieve normal distributions of data prior to MANOVA and correlation analyses, box-cox transformations (λ , lambda) and outlier analyses (determined by $> 2.5 \times$ standard deviation) were applied (Table 4). Biomarkers are expressed as normalized to creatinine concentrations ($\mu\text{g}/\text{mg}$) for each urine sample. Data was analyzed with the level of significance at $\alpha = 0.05$.

Receiver operating characteristics (ROC) and its area under the curve (AUROC) were performed to determine sensitivity and specificity of FPI of pain-free vs. pain subjects and low vs. high FPI among pain patient SF-36 scores.

To account for non-normal distribution of clinical assessments among chronic pain patients, Spearman rank’s coefficient (r) was

Table 4 Lambda (λ) conversion table for biomarkers (x)

X	Lamba (λ)	X	Lamba (λ)
MMA	$1/(\log_{10}(x))$	QA	x^1
HCYS	$x^{-0.25}$	KYNA	$\log_{10}(x)$
XAN	$x^{-0.01}$	HMG	$\log_{10}(x)$
PGA	x^1	EMA	$x^{-0.05}$
VMA	$x^{-0.25}$	3-HPMA	$x^{-0.5}$
5-HIA	$x^{0.8}$		

used. Correlation analysis was performed for group characteristics (age, sex, creatinine levels, biomarker concentrations) between pain-free and chronic pain subjects, as well as among chronic pain patients (biomarker levels, FPI, BMI, daily MME, current VAS, SF-36 score, HADS Anxiety and Depression scores). Components within the SF-36 used for analysis include assessments of physical functioning, limits due to physical health and emotional problems, energy/fatigue, emotional well-being, social functioning, pain, general health, and health change.

RESULTS

Cohort Characteristics

FPI scores were calculated using creatinine-normalized biomarker results from 153 chronic pain patients meeting all inclusion criteria and 334 age- and sex-matched pain-free controls with no history of chronic pain or opioid use. The mean age of chronic pain subjects was 55.5 (55.5 ± 11.4) with a 52% female population. Mean BMI in the chronic pain cohort was 30.9 (30.9 ± 7.57) and 24.8% reported smoking cigarettes. Primary pain diagnoses across the pain cohort included, but were not limited to, low back pain, lumbar radiculopathy, chronic pain syndrome, and cervicgia. The mean daily dose of opioid medication was 65.3 morphine milligram equivalents (MME) (65.3 ± 42.8). At the time of sample collection, the mean self-reported VAS score was 5.92 (5.92 ± 1.9), while

Table 5 Characteristics of patients suffering from chronic pain

Patient characteristics	
Number of subjects	153
Male (%)	48
Female (%)	52
Cigarette smoker (%)	24.8
	Mean ± SD
Age	55.5 ± 11.4
BMI	30.9 ± 7.57
Daily MME	65.3 ± 42.8
Current VAS	5.92 ± 1.9
SF-36 score	1674 ± 667
HADS A score	7.12 ± 4.11
HADS D score	7.43 ± 12.05
SF-36 score assessment (%)	
Physical functioning	45 ± 29
Limits due to physical health	26 ± 33
Limits due to emotional problems	61 ± 45
Energy/fatigue	36 ± 23
Emotional well-being	67 ± 32
Social functioning	54 ± 33
Pain	33 ± 20
General health	47 ± 26
Health change	42 ± 26

Data includes patients' characteristics suffering from chronic pain ($n = 153$) and prescribed long-term opioids (> 6 months prescription) used for multi-biomarker score training

the mean SF-36 score across the pain cohort was 1674 (1674 ± 667). Hospital anxiety and depression surveys revealed a mean anxiety score of 7.12 (7.12 ± 4.11) and a mean depression score of 7.43 (7.43 ± 12.05). Mean component scores from the SF-36 assessment are detailed further in Table 5. Age, sex, and creatinine levels did not correlate with biomarker

concentrations or FPI scores among pain-free and pain subjects. Prescription medications, daily MME, current VAS, and HADS Anxiety and Depression scores did not correlate with biomarker concentrations or FPI scores in subjects with chronic pain.

FPI Performance and Validation

Abnormal biomarker results were detected in a large majority of the chronic pain cohort with significant deviation from normal ranges observed across most individual biomarkers (Table 6).

The majority of pain subjects (86%) exhibited at least one abnormal biomarker (outside of the double-sided, 95% confidence interval reference range). Mean comparisons of individual biomarkers using non-parametric t testing revealed extremely strong discriminatory power ($P < 0.0001$) of five of the 11 biomarkers (methylmalonic acid, xanthurenic acid, pyroglutamate, kynurenic acid, and hydroxymethylglutarate) while an additional three biomarkers (homocysteine, quinolinic acid, and 3-HPMA) exhibited statistically significant discriminatory power ($P < 0.05$) between the pain and pain-free cohorts. Multi-biomarker statistical models (FPI) outperformed individual biomarkers when discriminating between pain-free and chronic pain cohorts (AUROC: 0.7490, $P < 0.0001$) (Fig. 1). Mean FPI scores were evaluated across pain-free and chronic pain cohorts and confirm a greater degree of pain-relevant biochemical perturbations in chronic pain subjects (Fig. 1). Mean FPI scores (0-100 scale) among the chronic pain cohort was 43.9 (43.9 ± 28.0) while the pain-free cohort exhibited a mean FPI score of 20.4 (20.4 ± 20.6) ($P < 0.0001$) (Fig. 2).

Association of FPI Scores with Clinical Assessments of Pain

Criterion validation analysis evaluated the significance of any correlations between FPI scores and validated clinical assessments for chronic pain. FPI scores were significantly associated with overall SF-36 scores ($P = 0.0141$) (Fig. 2), general health ($P = 0.0457$) and even more

Table 6 Biomarker validation across patient and healthy cohorts

	Healthy	Pain			
Number of subjects	334	153			
Male (%)	50	48			
Female (%)	50	52			
	Mean \pm SD	Mean \pm SD	<i>P</i> value	AUROC	<i>P</i> value
Age	54.6 \pm 11.6	54.6 \pm 11.6	0.4273		
Creatinine	125 \pm 76	109 \pm 73	0.0255		
Biomarker ($\mu\text{g}/\text{mg}$ of creatinine)					
Methylmalonic acid	1.06 \pm 0.99	1.49 \pm 2.00	0.0031	0.6191	< 0.0001
Homocysteine	0.32 \pm 0.40	0.36 \pm 0.29	0.3074	0.5717	0.0124
Xanthurenic acid	0.46 \pm 0.31	0.61 \pm 0.41	< 0.0001	0.6381	< 0.0001
Pyroglutamate	31.2 \pm 12.3	44.0 \pm 39.1	< 0.0001	0.6317	< 0.0001
Vanilmandelate	3.25 \pm 1.29	3.42 \pm 2.30	0.2841	0.5560	0.0517
5-HIAA	3.49 \pm 3.83	3.82 \pm 3.55	0.3723	0.5076	0.7930
Quinolinic acid	5.38 \pm 2.34	6.54 \pm 3.59	< 0.0001	0.5735	0.0150
Kynurenic acid	1.64 \pm 0.68	2.26 \pm 1.13	< 0.0001	0.6852	< 0.0001
Hydroxymethylglutarate	3.12 \pm 1.38	4.00 \pm 2.44	< 0.0001	0.6512	< 0.0001
Ethylmalonic acid	3.02 \pm 2.64	3.29 \pm 3.08	0.3266	0.5146	0.6232
3-HPMA	1.44 \pm 2.54	2.19 \pm 3.31	0.0061	0.5825	0.0052
FPI	20.4 \pm 20.6	43.9 \pm 28.0	< 0.0001	0.7490	< 0.0001

Comparison of means (non-parametric *t* test) and ROC curve of discriminating algorithm (healthy vs. pain). Multivariate analysis (least squares) was used to determine distinguishable power of the multi-biomarker score, FPI. To achieve normal distributions of biomarkers, box-cox transformations (λ , lambda) and outlier analyses (determined by $> 2.5 \times \text{SD}$) were applied. Subjects were matched for age and sex to control for collection variation between cohorts. Biomarkers are expressed as normalized to creatinine concentrations ($\mu\text{g}/\text{mg}$) for each urine sample. Data were analyzed with level of significance at $\alpha = 0.05$

AUROC area under receiver operating characteristic curve, 5-HIAA 5-hydroxyindoleacetic acid, 3-HPMA 3-hydroxypropyl mercapturic acid

significantly, with emotional well-being ($P = 0.0044$), and limitations due to emotional problems ($P = 0.0011$) (Table 7).

In the pre-validation model of FPI, scores (0–100 scale) were further categorized into tiers that represent the likelihood of detected abnormalities being pain determinants (Table 2). Validation of scoring tiers was carried

out by examining associations between SF-36 scores and FPI tiers (low, moderate, moderately high, and high). We hypothesized that subjects with severe biochemical disarrangement evident by higher FPI scores would be more impacted, both physically and emotionally by their pain. The SF-36 was selected as the most appropriate, validation clinical assessment to

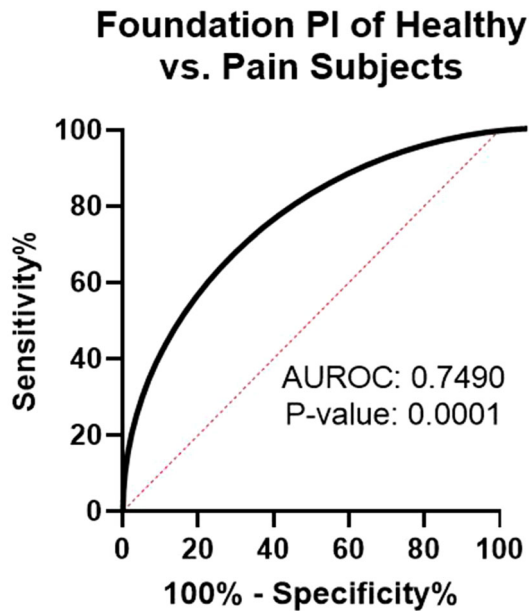


Fig. 1 ROC curve of FPI. Healthy ($n = 334$) and pain ($n = 153$) subjects were matched for sex (female: 50%) and age (avg per group: 55 years old). AUROC area under the receiver operating characteristic

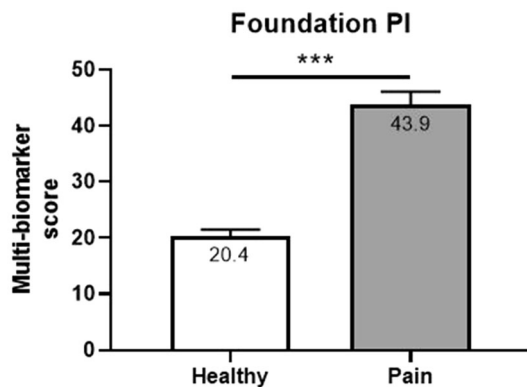


Fig. 2 Comparison of means (non-parametric t test) of FPI. Healthy ($n = 334$) and pain ($n = 153$) subjects were matched for sex (female: 50%) and age (avg per group: 55 years old). *** P value < 0.0001

evaluate both the physical and emotional impact of pain on subjects. Validation analysis was conducted by first comparing SF-36 scores in pain subjects who exhibited LOW FPI scores (< 20) to those who exhibited moderately high and high FPI scores (> 75). Comparison of SF-36 means (non-parametric t test) for the LOW FPI cohort and the high PI cohort reveal a strong

Table 7 Relationship between the FPI score and clinical assessments of chronic pain

	FPI severity (Spearman's r)	P value
Limitations due to emotional problems	0.520	0.0011
Emotional well-being	0.463	0.0044
General health	0.345	0.0457
SF-36 score	0.406	0.0141

Clinical evaluations were compared between pain patients with moderately high to high FPI severity scores (> 75 FPI) and low FPI severity scores (< 20 FPI)

discrimination between SF-36 scores of low and high FPI scores (AUROC: 0.7715; $P = 0.0088$) (Figs. 3 and 4).

Further validation analysis was conducted to evaluate the association between overall SF-36 scores and FPI as a continuous score. Figure 5 illustrates the continuous, significant association ($P = 0.0177$) between FPI scores and overall SF-36 scores across the four tiers of FPI scores (low, moderate, moderately high, and high) (Fig. 5).

Final validation of the scoring tiers was conducted by evaluating both the biomarker and clinical (SF-36) characteristics of subjects

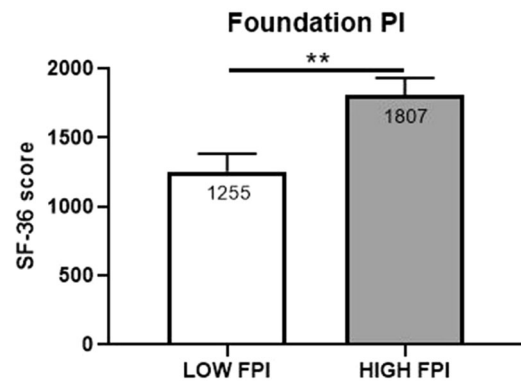


Fig. 3 Comparison of means (non-parametric t test) of FPI severity and SF-36 scores. Clinical evaluations were compared between randomly selected pain patients with moderately high and high FPI severity scores (> 75 FPI; $n = 20$) and low FPI severity scores (< 20 FPI; $n = 20$). ** P value < 0.001

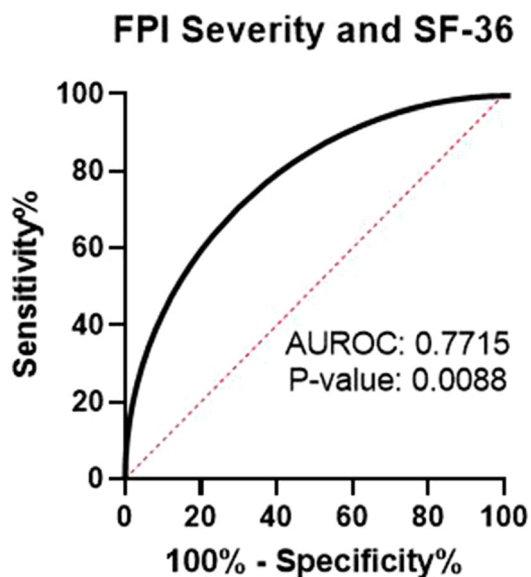


Fig. 4 ROC curve of FPI severity and SF-36 scores. ROC analysis was performed between randomly selected pain patients with moderately high and high FPI severity scores (> 75 FPI; $n = 20$) and low FPI severity scores (< 20 FPI; $n = 20$). *AUROC* area under the receiver operating characteristic curve

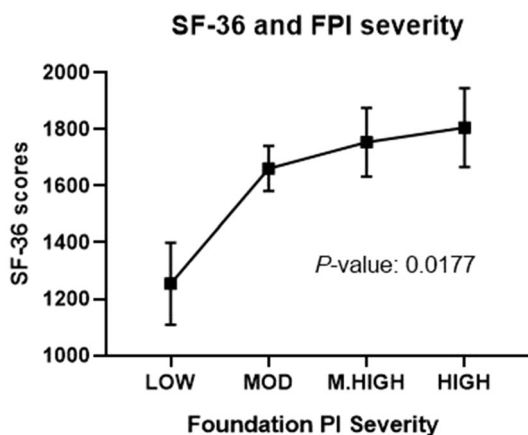


Fig. 5 Association between SF-36 scores and FPI severity among chronic pain patients. Data is represented as mean \pm SEM and was analyzed by linear trend analysis of one-way ANOVA. *MOD* moderate, *M.HIGH* moderately high

within each tier (Table 8). Criteria for validation required a significant (P value < 0.05) difference between the number of abnormal biomarker findings and SF-36 scores across all four

categories with a linear trend confirming that worsening clinical assessments correlate with worsening FPI scores.

DISCUSSION

These results demonstrate the clinical validity and associated correlations between FPI scores and validated clinical assessments of chronic pain (limitations due to emotional problems, emotional well-being, general health, and overall SF-36 scores) in a heterogeneous cohort of chronic pain patients across multiple sites. The FPI score describes the degree and severity of underlying metabolic derangement that may be driving painful symptomology, and therefore, provides novel objective information that will compliment current subjective assessments. In addition to providing mechanistic insight into underlying biochemical derangement in chronic pain, the FPI also directly underscores the potential for novel non-opioid therapies that have been shown to modulate component biomarkers. Novel, biochemical intelligence combined with safe, personalized, non-opioid therapy options will increase the likelihood of successful and prolonged pain control, while reducing healthcare costs and reliance on opioid medications.

During criterion validation analysis, the FPI was strongly associated with limitations due to emotional problems ($P = 0.0011$) and emotional well-being ($P = 0.0044$), both components of the SF-36 survey. The validity and importance of these findings is further supported by examining the component biomarkers and the diverse set of biochemical and metabolic pathways represented by these surrogate markers. Component biomarkers exhibiting the strongest distinguishing power between pain-free and pain cohorts included methylmalonic acid, xanthurenic acid, pyroglutamic acid and the kynurenine pathway metabolites, quinolinic acid, and kynurenic acid. While each of these biomarkers lies along pathways that can directly impact the development, worsening and/or perception of pain, they are also intimately linked to mental health and emotional well-being.

Table 8 Biomarker and clinical characteristics of subjects across all four FPI scoring tiers

Minimum number of expected abnormal biomarkers	Low	Moderate	Moderately high	HIGH	P value	
	0	1	2	3	ANOVA	Linear trend
Average number of abnormal biomarkers	0.24 ± 0.44	1.84 ± 0.74	3.24 ± 0.51	4.72 ± 0.46	< 0.0001	< 0.0001
SF-36 Scores	1255 ± 579	1662 ± 644	1754 ± 555	1806 ± 560	0.0436	0.0177

Grouped, categorical data were analyzed as linear trend analysis of one-way ANOVA

Methylmalonic acid is a sensitive and specific marker of intracellular vitamin B12 status. Elevated levels of this urinary metabolite indicate an increased demand for this critical micronutrient. Vitamin B12 deficiencies are commonly detected in the chronic pain population and can drive painful and neuropsychiatric symptomologies [2, 3, 40–43]. *Xanthurenic acid* is a metabolomic biomarker of vitamin B6 status. Elevated levels of this kynurenine pathway metabolite indicate an increased demand for vitamin B6. Vitamin B6 deficiencies have long been recognized as causes of painful peripheral neuropathies, migraine, chronic pain, depression, and other neuropsychiatric diseases [13, 44, 45]. *Pyroglutamic acid* is a well-characterized metabolomic biomarker of glutathione depletion or a reduced glutathione response capacity. Elevated levels of this urinary marker, indicating a need for glutathione support, is a common finding in chronic pain patients taking daily doses of acetaminophen (APAP). APAP is capable of directly depleting glutathione stores [2, 26]. A reduced glutathione response capacity renders nerve cells susceptible to oxidative damage, which can cause or worsen peripheral neuropathies and drive the development of neuropsychiatric disease [28, 46, 47]. *Quinolinic and kynurenine acid* are neuroactive metabolites of the kynurenine pathway (KP), which can impact the development of pain hypersensitivity and depression through their direct action on NMDA receptors and relationship to serotonin synthesis. The KP is the major pathway responsible for the catabolic degradation of dietary tryptophan. Chronic activation of the KP occurs under conditions of systemic inflammation due to the ability of pro-

inflammatory cytokines to directly upregulate this critical pathway. Under normal conditions, a small percentage of dietary tryptophan is utilized for serotonin synthesis but in the presence of a persistent inflammatory response, tryptophan is preferentially shunted down the KP, at the cost of serotonin synthesis. This phenomenon leads to the accumulation of quinolinic acid (leading to NDMA-mediated excitotoxicity and depression) and decreased serotonin synthesis, which further amplifies heightened pain and depressive symptoms [35, 48–50].

Considering the high prevalence of mental health disorders including anxiety, depression, bipolar disorder, and post-traumatic stress disorder in chronic pain populations, it is timely and significant that we observe and report prevalent perturbations in biochemical pathways capable of afflicting both physical and mental health. Our results provide evidence of criterion validity for the FPI by demonstrating a significant association with the SF-36 and multiple sub-components of this validated assessment for chronic pain. The clinical significance of specific objective markers of pain, which provide mechanistic insight into subjective reports, cannot be overstated, and it has long been recognized that such tools, capable of encompassing self-reported pain scores, would represent a significant advancement for the field of pain management [51]. Figure 5 and Table 8 illustrate the potential clinical significance of the FPI and its associated treatments. To the best of our knowledge, this is the first report of a validated pain biomarker panel that shows association with validated clinical assessments of pain. Strong association between

higher FPI scores and worsening SF-36 responses provides mechanistic insight into the potential role of deranged biochemistry in the worsening and emotional impact of chronic pain. Potentially more exciting than the prospect of objective measures encompassing self-report is the fact that perturbed biochemistry can be corrected with appropriate low-cost therapies and supplementation. If metabolic correction proves capable of improving SF-36 scores, while simultaneously improving FPI scores, it will represent the first laboratory tool of its kind by allowing providers to truly modulate the course of disease.

Face validity of the FPI is supported by the direct relationships between component biomarkers and biochemical pathways known to be involved in the pathogenesis of chronic pain. Many of the metabolic, biochemical, and nutritional abnormalities indicated by the FPI test have been described in the literature as being the underlying causes of pain [13, 22, 25, 27, 52–55]. FPI biomarkers represent and evaluate cytokine-mediated chronic inflammation (kynurenine pathway metabolites), oxidative stress (pyroglutamic acid, ethylmalonate, and hydroxymethylglutarate), micronutrient deficiencies (methylmalonic acid, xanthurenic acid, homocysteine), and neurotransmitter turnover (5-hydroxyindoleacetic acid and vanilmandelic acid), all of which have direct links to the development, worsening, or heightened perception of pain. In addition, many of the non-opioid, biomarker-modulating compounds that would be directly indicated by the FPI test have exhibited significant pain-relieving effects in numerous randomized controlled clinical trials [28, 56–62]. FPI testing will facilitate and simplify patient selection for these important compounds by affording providers the ability to objectively identify patients who exhibit abnormalities and hence require metabolic correction therapies.

Mean comparison testing of FPI scores in pain and pain-free cohorts also provides evidence of discriminant validity by demonstrating significant differences in the mean FPI scores from pain-free subjects, when compared to the chronic pain cohort (P value < 0.0001). Despite strong discriminatory power, this assay

is not intended to diagnose chronic pain. Rather, this innovative assay has been designed and validated to identify patients whose pain complaints may be due, at least in part, to biochemical, metabolic, and nutritional abnormalities. The importance of identifying such patients with a non-invasive, cost-effective test cannot be overstated, as these abnormalities can be treated and corrected with safe, widely available compounds. In addition, patients whose pain is due, at least in part, to underlying biochemical derangement will likely experience no long-term benefit from opioid therapy, as this class of medication is in no way addressing the underlying pathology driving the painful symptoms. Only with objective identification of abnormalities and targeted metabolic correction will these patients experience prolonged pain relief. Therefore, identification of such patients prior to the initiation of opioid therapy represents an immediately available and cost-effective strategy to reduce the opioid burden.

Validation study subjects were recruited from diverse geographic locations and exhibited a wide variety of primary pain complaints including, but not limited to, low back pain, lumbar radiculopathy, chronic pain syndrome, and cervicgia (43%, 31%, 22%, and 14% of pain cohort, respectively). Study subjects were also prescribed a variety of opioid analgesics for pain control. Because of these diverse characteristics, which reflect real-world demographics, the FPI test can be considered a valid objective assessment for chronic pain patients in the clinic. Considering the complexity and biopsychosocial nature of chronic pain, it is not surprising that composite, multi-biomarker statistical models outperformed individual biomarkers during discriminant validation.

Limitations

Chronic pain subjects exhibited abnormal biochemical function across various relevant pathways indicating systemically perturbed biochemistry. While these abnormalities may be responsible for the onset of pain in some patients, other detected abnormalities may reflect the long-term metabolic cost of chronic

pain. Regardless of whether detected abnormalities drove the acute onset of pain or its subsequent chronification, the evidence suggests biologically comprehensive test panels, evaluating multiple pathways are better equipped to characterize pain biochemistry than single marker assays. Furthermore, as biomarkers and FPI scores were assessed by single-point, cross-sectional analysis, longitudinal monitoring through repeat FPI testing in future studies will provide data on the efficacy of modulating therapies, as well as provide patients with objective updates on their biochemical status over time. Such tools will likely improve compliance and motivate patients to adhere to the metabolic correction protocols.

CONCLUSIONS

Objective identification of patients exhibiting high FPI scores will enable providers to initiate novel conversations and implement innovative treatment plans to correct underlying abnormalities and modulate the course of the disease. Ongoing research efforts will seek to determine the impact of FPI testing and subsequent metabolic correction on patients' outcomes in both new and long-term chronic pain patients. Studies will also investigate the impact of FPI testing and metabolic correction following acute injury in order to determine whether the transition from acute to chronic pain can be slowed, or even prevented, by optimizing biochemical function to ensure an appropriate inflammatory response.

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Compliance with Ethics Guidelines. This study (ERD2019-02) was reviewed and approved by the Quorum Institutional Review Board (QIRB) and was performed in accordance with the Helsinki Declaration of 1964 and its later amendments. All participants provided informed consent prior to their inclusion of the study.

Data Availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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