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Original Reports

Serum Metabolome Analysis Identified Amino-Acid Metabolism Associated With Pain in People With Symptomatic Knee Osteoarthritis – A Cross-Sectional Study

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Abstract: **Osteoarthritis (OA) is the most common arthritis affecting synovial joints such as knees and hips of millions of people globally. Usage-related joint pain and reduced function are the most common symptoms experienced by people with OA. To improve pain management, there is a need to identify validated biomarkers predicting therapeutic responses in targeted clinical trials. Our study aimed to identify the metabolic biomarkers for pain and pressure pain detection thresholds (PPTs) in participants with knee pain and symptomatic OA using metabolic phenotyping. Metabolite and cytokine measurements were done on serum samples using LC-MS/MS (liquid gas chromatography integrated magnetic resonance mass spectrometry) and Human Proinflammatory panel 1 kit respectively. Regression analysis was done in a test (n = 75) and replication study (n = 79) to investigate the metabolites associated with current knee pain scores and pressure pain detection thresholds (PPTs). Meta-analysis and correlation were done estimating precision of associated metabolites and identifying relationship between significant metabolites and cytokines respectively. Acyl ornithine, carnosine, cortisol, cortisone, cystine, DOPA, glycolithocholic acid sulphate (GLCAS), phenylethylamine (PEA) and succinic acid were found to be significantly (FDR <.1) associated with pain scores in meta-analysis of both studies. IL-10, IL-13, IL-1β, IL2, IL8 and TNF-α were also found to be associated with the significant metabolites. Significant associations of these metabolites and inflammatory markers with knee pain suggests that targeting relevant pathways of amino acid and cholesterol metabolism may modulate cytokines and these could be targeted as novel therapeutics development to improve knee pain and OA management.**

Perspective: ***Foreseeing the global burden of knee pain in Osteoarthritis (OA) and adverse effects of current pharmacological therapies, this study is envisaged to investigate serum metabolites and***

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The authors have no conflicts of interest to declare.

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molecular pathways involved in knee pain. The replicated metabolites in this study suggests targeting amino-acid pathways for better management of OA knee pain.

Key words: *Osteoarthritis, Knee pain, Serum metabolic profiling, Meta-analysis, Molecular pathways.*

Osteoarthritis (OA) is the most common form of arthritis and overall 44 % of the UK adult population suffers from OA pain.[43] Altered metabolism, inflammation, joint injury and other factors contribute to the development of OA.[9] The most common clinical manifestation leading OA patients to seek medical attention is pain[17] which in 4 of 10 people, is not controlled by simple medication. Certain risk factors such as older age, female gender, and high BMI associate with OA, however, the pathophysiological mechanisms of pain in OA remain unclear. Independent of the degree of peripheral tissue or nerve damage, pain sensitivity is reported to be different between individuals.[27] This is attributed to the fact that pain experience in OA is not only through peripheral nociception but can be modified through central modulation. These subjective pain behavior cause difficulty in assessment of pain and at later stages may contribute to the high prevalence of chronic pain.

Current pharmacological therapies for pain have limited efficacies, restricting functional recovery in OA and encouraging eventual reliance upon opioids which are known to have multiple adverse effects.[37] This is likely to be related to the lack of understanding of the pain pathways and targeted therapeutics against disease progression. There is a growing body of literature supporting evidence of serum metabolites being linked to various types of pain such as chronic back pain, abdominal pain, joint pain and neuropathic pain.[12,16,21] These factors highlight the importance of development of certain tools such as metabolic markers signifying pain and that can be targeted as potential therapeutics. Differences in the serum metabolites in the joint tissues, such as neurotransmitters, amino acids, hormones and various others[41,45,47] potentially stimulate the immune response and thus are likely to be associated with the pathophysiology of OA pain. Metabolic changes mediated through host factors, such as heritability, Single Nucleotide Polymorphisms (SNPs) of genes, and microbial metabolites are also reported to change pain perception.[10,35,36] Based on these evidences, the current study hypothesized that biochemical change in serum metabolites such as amino acids and neurotransmitters is associated with pain in OA via related molecular pathways linked to chronic pain.

The aim of this study was to investigate the relationship of metabolites with both self-reported pain scores and pressure pain threshold traits in people with OA to help identify metabolites associated with pain and pathways related to pain sensitivity in knee pain in OA patients.

Methods

Study Design and Participants Recruitment

In the current study, we compared the relationship of the metabolome with pain traits in 2 independent community-derived cohorts, the Webex (Web-based exercise intervention) cohort (n = 75)[15] which was used as the test study and the KPIC (Knee Pain in the Community) cohort (n = 79)[11] which was used for the replication study. Study flowchart is depicted in Fig 1.

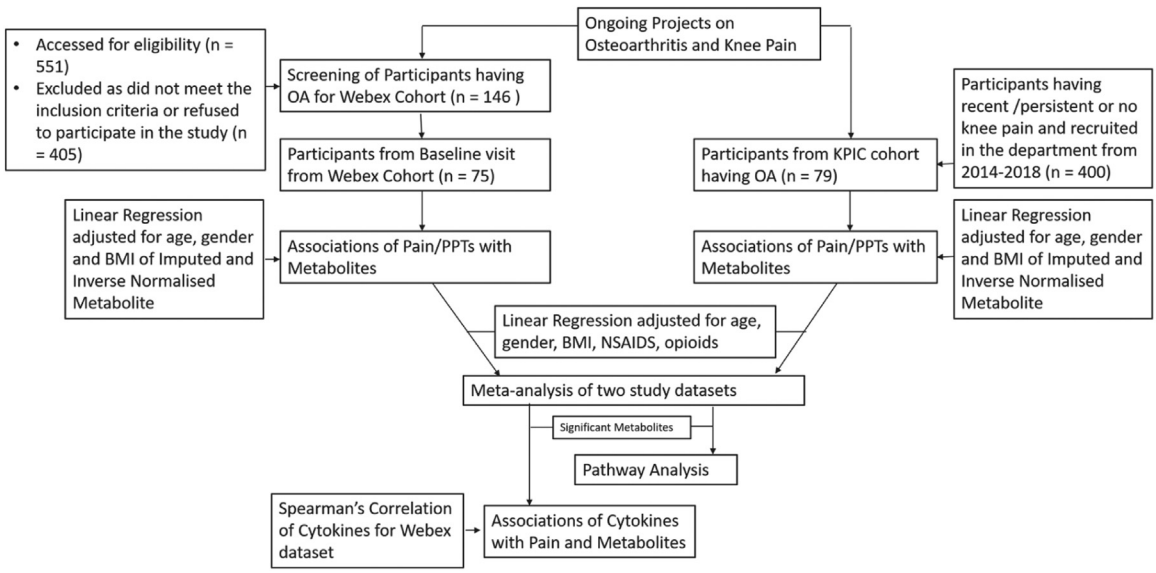

Figure 1. Flow diagram showing study design, conduct and analysis. The Webex study was used as test dataset and KPIC study was used as replication dataset.

Test Study (Webex cohort)

The Webex cohort [15] was set-up to study the effect of an internet-based exercise intervention on the microbiome, metabolome, and on pain and pressure pain detection thresholds at baseline and follow-up at 6 weeks in people with knee pain. Participants aged ≥45 years from the previous/ongoing studies [24] conducted in the Department of Academic Rheumatology at the University of Nottingham who agreed to be contacted in future studies and who had radiographic structural knee OA (Kellgren Lawrence (KL) score ≥1) and current knee pain were recruited for the Webex cohort. All participants were given a detailed explanation of the study design and methods and an informed written consent was obtained prior to the start of the study. Individuals with terminal or mental illness, neurological conditions, sleep apnea, unstable heart condition and soft tissue knee injury within last 3 months were excluded from the study. The study was approved by Research Ethics Committee (REC) (ref: 18/EM/0154), Health Research Authority (HRA) (protocol no: 18021) and the Nottingham University Hospitals NHS Trust Research & Innovation (R&I) department (ref: 18RH004). For the current study, participants from baseline visits (n = 75) were included for analysis as the test dataset.

Replicate Study (KPIC cohort)

The study used to replicate findings for the current study was a dataset from the KPIC cohort [11] which was conducted in the Department of Academic Rheumatology from 2014 to 2018 and recruited participants aged ≥40 years, with or without self-reported knee pain and GP diagnosed OA (if ever or not), both questionnaire-based. The postal questionnaires asked about presence of current knee pain with a validated question: "Have you had knee pain for most days of the past one month?" (Yes/No).[26] Those reporting knee pain were invited for further assessments including standardized knee radiographs, Pressure Pain detection thresholds along with other clinical traits. Individuals suffering from any known terminal illnesses, psychiatric illness, or any other conditions were excluded from the study as this would make them unsuitable to receive a questionnaire. The study was approved by Nottingham Research Ethics Committee 1 (NREC Ref: 14/EM/0015) Participants who reported OA and knee pain (n = 79) of age ≥41 years were included for analysis for the current study.

Clinical Evaluation and Data Collection

Clinical traits data was evaluated based on the self-reported medical history as mentioned in the questionnaire following the inclusion and exclusion criteria as discussed in the protocols published.[11,15] Briefly, pain was measured as current knee pain in both the studies, while sitting at the time of visit and using Numerical Rating Scale (NRS) on the scale of 0 to 10 where 0 is no pain and 10 is the worst pain. Pressure Pain detection Thresholds (PPTs) were measured in triplicate with an algometer and were assessed by applying certain pressure over a region of body and then steadily increasing nonpainful pressure stimulus turning into a painful pressure sensation, determining the pain perception and mechanical pain sensitivity. Our study measured these PPTs using test sites at 1) anterior tibialis which is distal pain-free site, 2) medial joint line which measure pain at the affected site, 3) superolateral patella and 4) superomedial patella, both of which are proximal pain-affected site. The mean of PPT values for each body site were computed from all 3 PPT rounds of measurement for further analysis. Details on use of medications such as NSAIDs and opioids were recorded from the questionnaires.

Sample Acquisition and Profiling

Fasting blood samples were collected from participants at the time of visit and pain assessment. Serum was extracted for metabolomic profiling using an integrated approach of high throughput magnetic resonance mass spectrometry and liquid gas chromatography (LC-MS/MS) using the commercially available Biocrates MxP Quant 500 kit (Biocrates Life Science AG, Innsbruck, Austria).[38] The metabolites concentration was analyzed using Sciex Analyst and data are imported into Biocrates MetIDQ software for further analysis. A total of 630 metabolites were quantified belonging to the class of acylcarnitine, alkaloids, amine oxide, amino acids, bile acids, biogenic amines, carboxylic acids, ceramides, cholesterol esters, cresols, diacylglycerols, dihydroceramide, fatty acids, glycerophospholipids, glycosylceramides, hormones, indole derivatives, nucleobases, sphingolipids, sugars, triglycerols, vitamins and cofactors.

Pro and anti-inflammatory serum cytokines measurement were performed by Affinity Biomarkers, London using the standardized Human Proinflammatory panel 1 assay kit (cat number K151A0H-1), distributed by Meso Scale Discovery and levels of IFNγ, IL10, IL-12P70, IL-13, IL-1β, IL2, IL4, IL6, IL8, TNFα were measured, and concentrations are reported in ng/L.

Metabolite Data Pre-processing

The metabolite data were pre-processed to impute missing data. This was done by replacing missing values with half of the minimum positive value excluding zero. Data were tested for normality and was inverse normalized before downstream statistical analysis.

Statistical Analysis

The current study investigated associations of metabolites (n = 93) belonging to the class of amino acid related, bile acids, biogenic amines, carboxylic acids, cresol, hormones, indole derivative, vitamins and cofactor, nucleobase, amine-oxide and amino acids. Normality tests for clinical traits was done using Kolmogorov-Smirnov tests and significance comparison between the 2 studies was performed with the independent-samples Student t-test, or the Mann–Whitney *U* test as

applicable. To investigate the association of metabolites and pain or PPTs, linear regression analysis was adjusted for age, gender, BMI as confounding factors (model 1). Regression analysis for significant metabolites (n = 26) was then adjusted for age, gender, BMI and intakes of NSAIDs and opioids (model 2).

Model 1: lm (metabolites ~ pain score +age + gender + bmi)

Model 2: lm (metabolites ~ pain score +age + gender + bmi + NSAIDs + opioids)

Meta-analysis of the 2 studies using the fixed random effect model and comparing the regression coefficients from model 2 was done using the REML model using the "metafor" package in R.[31] Pathway analysis was done using the Pathway Analysis tool implemented in the MetaboAnalyst pipeline [29] based on the KEGG metabolic pathways library [18] for humans. The over-representation was tested based on hypergeometric tests which calculate *P*-value as the probability of randomly drawing k or more successes from the population in n total draws. The topology measurement was done using relative-between ness centrality which measures the number of shortest paths going through the node focusing on global network topology.[2] Spearman's correlation was done to analyze the relationship between significant metabolites and cytokines Linear regression and Spearman's correlations were done using "lm. beta" and "Hmisc" package respectively in R. *P*-value < .05 and multiple testing correction using Benjamini-Hochberg (FDR) correction <.1. were chosen as the significance thresholds.

Results

Anthropometric and Clinical Characteristics

Clinical and anthropometric details for the study are shown in Table 1. Overall, the Webex study (n =75) with 53 women (71%) and 22 males (29%) had fasting serum metabolite profiling for all participants except one. The mean age of the participants was 68 years (range 47−84 years). Pain measures on an NRS scale of 0 to10 ranged from 0 to 8 with an average of 3.27.

The KPIC study consisted of 79 participants with 34 men (43%) and 45 women (57%). The average age of the participants (63 years) was matched to a criterion of ±5 years accounting to be in the range of 41 years to 80 years. The pain score in the replication study measured was 2.67 on average.

Metabolome and Pain

Higher levels of acyl ornithine, carnosine, dihydroxyphenylalanine (DOPA), histamine, hypoxanthine nitrotyrosine, phenylethylamine (PEA), spermidine, spermine, taurine, taurolithocholic acid (TLCA), and lower levels of alpha-amino adipic acid (alpha-AAA), anserine, cysteine, cystine, cortisol, cortisone, dehydroepiandrosterone sulphate (DHEAS), glycolithocholic acid sulphate (GLCAS), symmetric dimethylarginine (SDMA), 3-indoleacetic acid were found to be significantly (all FDR <.1) associated with higher current knee pain scores in the test (Webex) dataset. In replicate (KPIC) dataset, higher pain scores were significantly (FDR <.1) associated with higher levels of succinic acid, phenyl ethyl amine and lower levels of, asparagine, beta alanine, spermine, tryptophan and 3-indolepropionic acid (Fig 2, Table 2). Descriptive summary is presented in Supplementary Table 1. PEA was found to be significantly replicated in both study datasets. Overall, a total of 28 and 17 metabolites showed significant associations ($P < .05$) with current knee pain score from regression model 1 in test (Webex) and replicate (KPIC) dataset respectively (Supplementary Fig 1). These results indicate that although most of the metabolites in the replicate study dataset (KPIC) were not significant after multiple correction but showed a similar direction of associations with pain as in the test study dataset (Webex).

Table 1. Anthropometric and Clinical Characteristics of the Participants in Study Datasets

	Webex dataset (N = 75)	*KPIC dataset (N = 79)*	P-*value*
	Mean(SD)	*Mean(SD)*	
Gender	71% F 29% M	57% F 43% M	.14
Age±	68.21 (9.3)	62.75 (8.80)	.0003*
BMI	31.13 (7.08)	29.32 (6.40)	.128
Pain‡	3.27 (2.48)	2.67 (2.80)	.208
Superolateral patella‡	296.05 (144.14)	425.43 (192.05)	5.00E-06*
Superomedial patella‡	321.62 (134.71)	404.88 (176.74)	.004§
Medial joint line	329.87 (172.40)	389.70 (191.71)	.047¶
Tibialis Anterior Muscle‡	351.21 (157.13)	428.22 (191.28)	.004§

Webex dataset is the test study and the KPIC dataset is used as the replicate study. Results are presented as mean (SD) except for gender which is presented as percentages.
**P*-value < .001.
±Age-matched, criteria for age ± 5 years.
‡Independent Student t-test was done for statistical significance inference.
§*P*-value < .005.
¶*P*-value < .05.

Metabolome and Pressure Pain Thresholds (PPTs)

In test (Webex) dataset, altogether 17 metabolites and in replicate (KPIC) dataset altogether 12 metabolites were found to be significantly ($P < .05$) associated with PPTs measured at 4 different regions of the body. However, none of the metabolites were found to be significant (FDR <.1) after multiple testing correction. Nonetheless, the most common metabolite found to be associated with PPTs in the Webex study was SDMA, while cortisone and carnosine were commonly associated with PPTs in the KPIC study (Supplementary Table 2).

Amino-acid Metabolism and Knee Pain

Meta-analysis of standardized linear regression coefficients adjusted for age, gender, BMI, NSAIDs and opioids (model 2) of the 2 cohorts (Webex and KPIC)

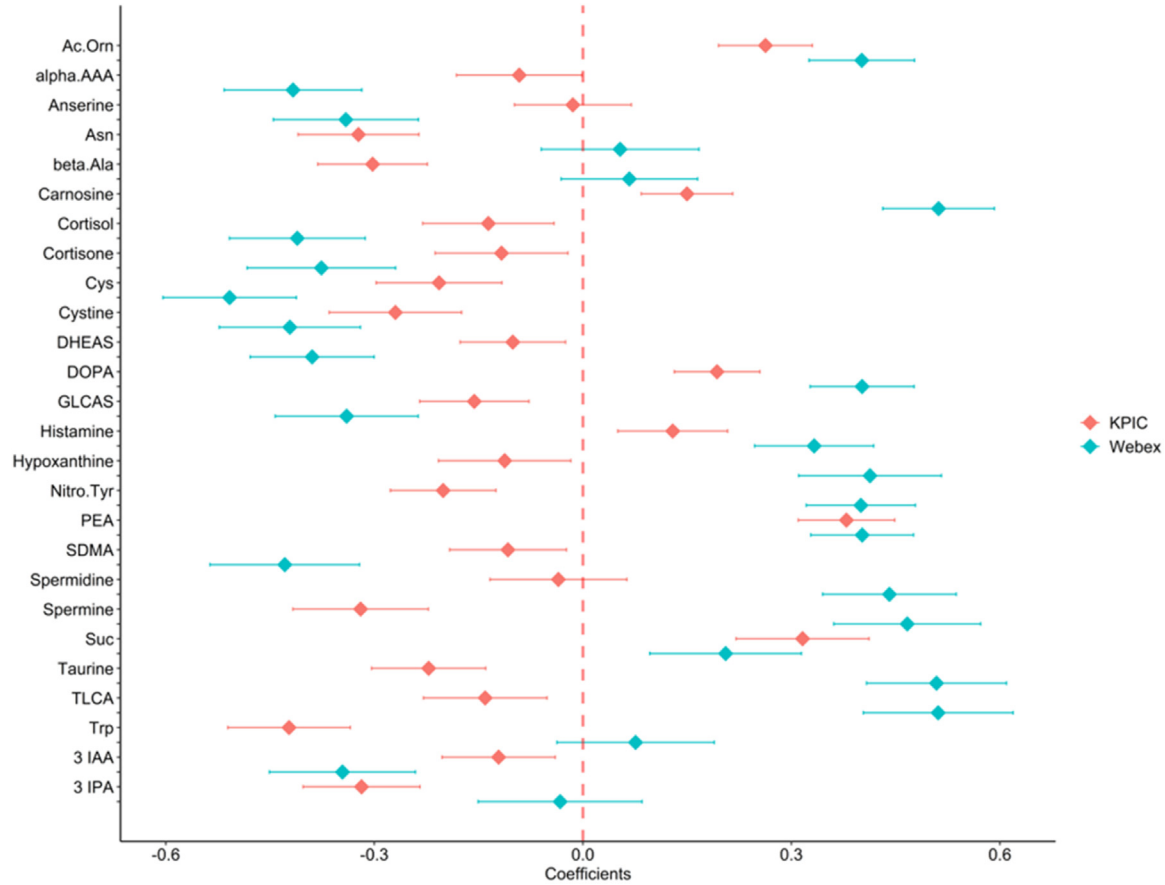

Figure 2. Metabolites associated with Pain scores in Webex and KPIC study datasets. Forest plot depicting the metabolites significantly (FDR <.1) associated with current knee pain in either or both the datasets (Webex as red bars and KPIC as blue bars). Dots represent the coefficients from the regression analysis and horizontal lines represent standard errors.

showed Acyl Ornithine, carnosine, DOPA, PEA and succinic acid to increase significantly with an increase in pain while Cystine, GLCAS, Cortisol and cortisone were significantly decreased with increase in pain. Results are shown in Fig 3A-I and Supplementary Table 3. Pathway analysis showed Phenylalanine metabolism to be most significantly involved ($P < .05$) in current knee pain. Other pathways involved were steroid hormone biosynthesis, arginine biosynthesis, butanoate metabolism, histidine metabolism, citrate cycle, beta-alanine metabolism, propionate metabolism, alanine aspartate and glutamate metabolism, glutathione metabolism, cysteine and methionine metabolism, arginine and proline metabolism, tyrosine metabolism (Fig 4, Supplementary Table 4).

Pain and Pro or Anti-Inflammatory Cytokines in the Test Cohort

The levels of certain pro-inflammatory cytokines such as IL-1β, IL-2, TNFα was found to be decreased while levels of IL-12P70 and IFN-γ was found to be increased with increase in pain. The levels of pleiotropic cytokine, IL-6 and anti-inflammatory cytokines such as IL-10 was found to be decreased while levels of IL-4, IL-8, IL-13 was found to be increased with decrease in pain. However, no cytokine was found to be significantly (FDR <.1) associated with pain scores or PPTs in the test (Webex) study after adjusting for age, gender, BMI, NSAIDs and opioids (Supplementary Table 5).

The association of significant metabolites from the meta-analysis was tested with the cytokine concentrations (Fig 5, Supplementary Table 6). IL-10 and TNFα were significantly correlated with at least 6 metabolites. IL- 13 was found to be significantly associated ($P < .05$) with Acyl ornithine, DOPA, Cortisol and PEA. IL-1β is found to be associated with PEA, and Carnosine. IL-2 is associated significantly with, Cysteine. IL-8 was significantly associated with PEA, Cortisol and Carnosine. IFN-γ, IL-4, IL-6 and IL-12p70 was not found to be having significant associations with any of the significant metabolites.

Discussion and Conclusion

The current study reports reproducible associations of serum metabolites with current knee pain scores and PPTs in individuals with knee OA using the metabolic profiles of 93 metabolites in the 2 independent studies (KPIC and Webex). The metabolites tested belonged to the class of Amino Acid Related, Bile Acids, Biogenic Amines, Carboxylic Acids, Cresol, Hormones, Indole Derivative, Vitamins and Cofactor, Nucleobase, Amine-

Table 2. Metabolites Associated with Pain in Webex and KPIC Study Datasets

	Webex-Pain				*KPIC-Pain*			
Traits	*Beta*	P *Value*	*STD. Error*	*FDR*	*Beta*	P *Value*	*STD. Error*	*FDR*
Asn	.054	.69	.057	.559	-.323	.005†	.043	.039†
Cys	-.508	.00008*	.048	.012†	-.207	.077	.045	.296
Trp	.076	.571	.057	.501	-.423	.0001*	.044	.003†
Ac.Orn ¶	.401	.002†	.038	.034†	.263	.024†	.034	.144
alpha.AAA	-.417	.001†	.05	.032†	-.092	.439	.045	.753
Anserine	-.341	.01†	.052	.09‡	-.014	.906	.042	.856
Carnosine	.512	.00006*	.04	.014†	.15	.211	.033	.546
Cystine ¶	-.422	.001†	.051	.033†	-.27	.021†	.048	.128
DOPA	.402	.002†	.037	.033†	.193	.098	.031	.355
Nitro.Tyr	.4	.002†	.039	.035†	-.201	.095	.038	.346
SDMA	-.429	.001*	.054	.03†	-.108	.352	.042	.692
Taurine	.509	.00008*	.05	.009†	-.222	.062	.041	.261
GLCAS	-.34	.011†	.051	.089‡	-.156	.198	.039	.527
TLCA	.511	.0001*	.054	.011†	-.14	.249	.044	.594
beta.Ala	.067	.584	.049	.506	-.302	.01†	.039	.07‡
Histamine	.333	.009†	.043	.087‡	.129	.292	.039	.65
PEA §	.402	.002†	.037	.036†	.379	.001*	.035	.011†
Spermidine	.441	.001*	.048	.022†	-.035	.771	.049	.823
Spermine	.467	.0003*	.053	.016†	-.32	.006†	.049	.05‡
Suc	.205	.12	.055	.274	.316	.006†	.048	.05†
Cortisol	-.411	.002†	.049	.033†	-.136	.238	.047	.573
Cortisone	-.376	.005†	.053	.054‡	-.117	.34	.048	.693
DHEAS	-.389	.002†	.045	.033†	-.101	.35	.038	.691
3.IAA	-.346	.009†	.052	.087‡	-.121	.268	.041	.61
3.IPA	-.033	.812	.059	.606	-.318	.005†	.042	.042†
Hypoxanthine	.413	.002†	.051	.035†	-.112	.36	.048	.7

Metabolite data was imputed for missing values with half of the minimum positive value excluding zero and was inverse normalised. The table shows beta coefficients, *P*-value, standard error and *P*-values after multiple corrections (FDR) values from the regression model adjusted for age, gender and BMI for the significant metabolites associated with pain. Webex dataset is the test study and the KPIC dataset is used as the replicate study.
**P*-value < .001,
†*P*-value < .05,
‡*P*-value < .1
§indicates metabolites replicated in 2 studies with FDR <.1 and
¶indicated metabolites replicated in 2 studies with *P*-value < .05.

oxide and Amino Acids. Consistent with previous findings, meta-analysis of 2 independent studies demonstrates the association of acyl ornithine, carnosine, cortisol, cortisone, cystine, DOPA, GLCAS, PEA and succinic acid in knee pain in OA patients. Acyl ornithine, a downstream metabolite of arginine catabolism, carnosine, dipeptide of beta-alanine and L-histidine, DOPA, a precursor of dopamine, PEA, a trace amine and succinic acid have been reported to be increased in patients with OA, postural disorders and ligaments rupture, respectively.[1,23,33,34,48] Cystine, a dimer of amino acid cysteine, showed a negative association with pain in the current study and is reported to attenuate synovial fluid thickening and erosion of cartilage in joints with OA.[40] This strengthens the evidence of cystine possessing anti-inflammatory properties and supports their use as a therapeutic target in intervention studies. GLCAS, a sulphate ester with bile acid group and the stress hormone cortisol and carnosine are the intermediates of cholesterol metabolism pathways. There are studies reporting cortisol to heal and improve pain by recovering the tissues, however, studies on cortisol association in OA are scarce. In the current study, the decreased level of cortisol with increase in pain is likely to be attributed to tissue damage progression. However, role of cortisol and its dysfunction could be better understood by evaluating its potential specifically in OA and pain.

Pathway analysis showed amino acids metabolism and cholesterol metabolism pathways to be involved at a higher level of classification. This could be attributed to the fact that the significant metabolites in our study are synthesized from amino acids like tyrosine, glycine, arginine, cystine, histidine, tryptophan, phenylalanine and steroid as another precursor. These results are likely to be related to pain modulation which is primarily mediated through neurotransmitters and amino acids being the main substrate for the production of the neurotransmitters. Similar results were shown in a study,[41] where treatment with amino acid precursors was associated with substantial improvement in chronic back pain, reduction in inflammation, and improvement in back pain correlated with increased amino acid precursors to neurotransmitters in the blood. Meta-analysis with pain and PA results in current study are consistent with the literature and support the heterogeneity of these metabolites to be used as metabolic markers and potential therapeutic

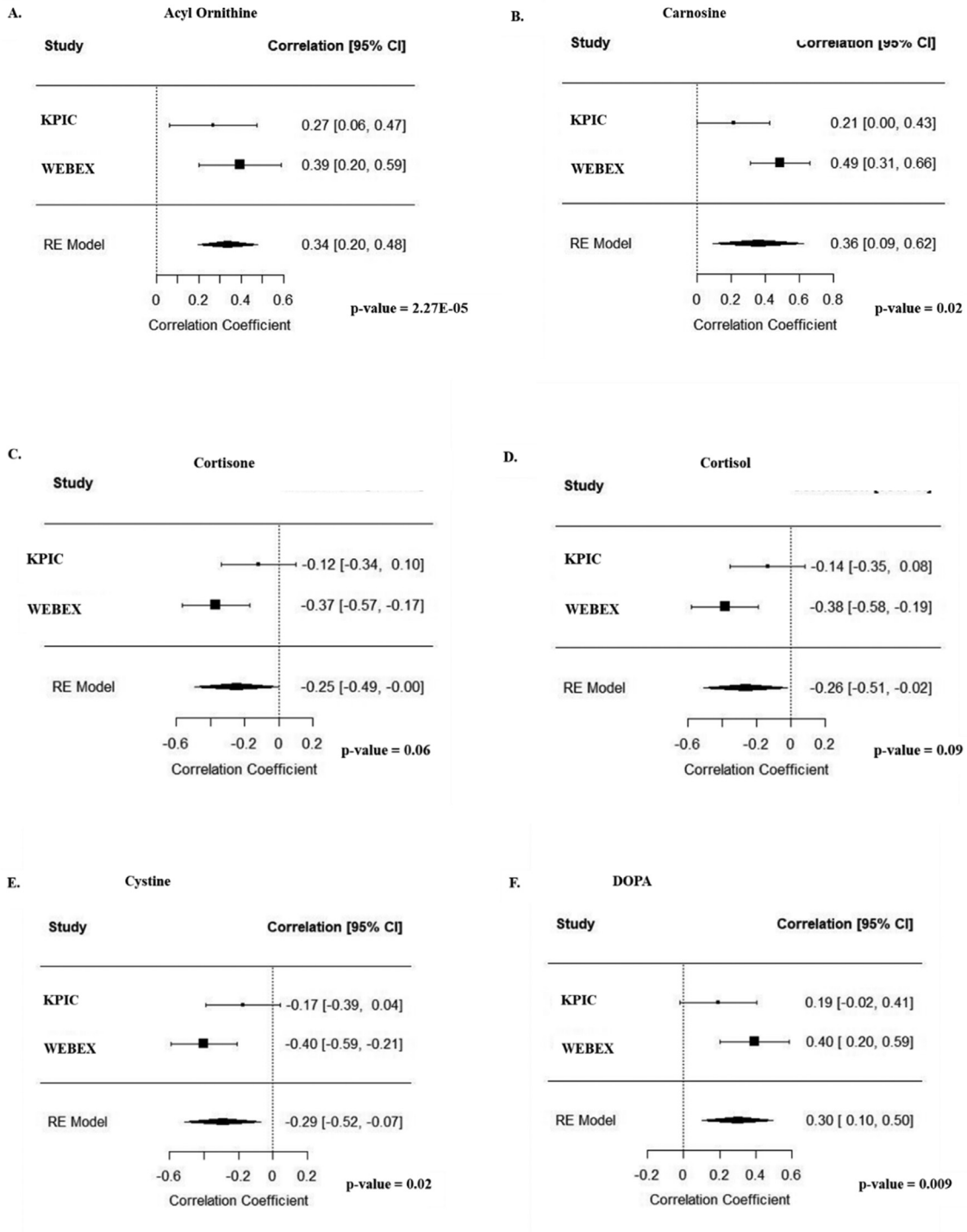

Figure 3. Meta-analysis of test (Webex) and replicate (KPIC) datasets. The forest plots A. Acyl Ornithine, B. Carnosine, C. Cortisone, D. Cortisol, E. Cystine, F. DOPA, G. GLCAS, H. PEA, I. Succinic Acid depict the metabolites significantly associated with pain in replicate (KPIC) and test (Webex) datasets. Values from linear regression adjusted for age, gender, BMI and use of NSAIDs and opioids were inputted for the fixed random effect model-based meta-analysis. *P*-values indicate the statistical significance with FDR <.1.

targets for OA and knee pain. Reproducibility of significant metabolites associated with pain in osteoarthritis in 2 independent studies, supports the validation of these metabolic biomarkers in in-vitro and/or in-vivo studies. It is notable that there are fewer significant metabolites associated with pain in the replicate study. This may partially be explained by the high standard deviation in the metabolite concentration in the study dataset. However, the metabolome data was normalized prior to the statistical inferences. The pain pressure thresholds (PPTs') are a measure of pain perception, however, studies are depicting that the transduction of information signals are processed

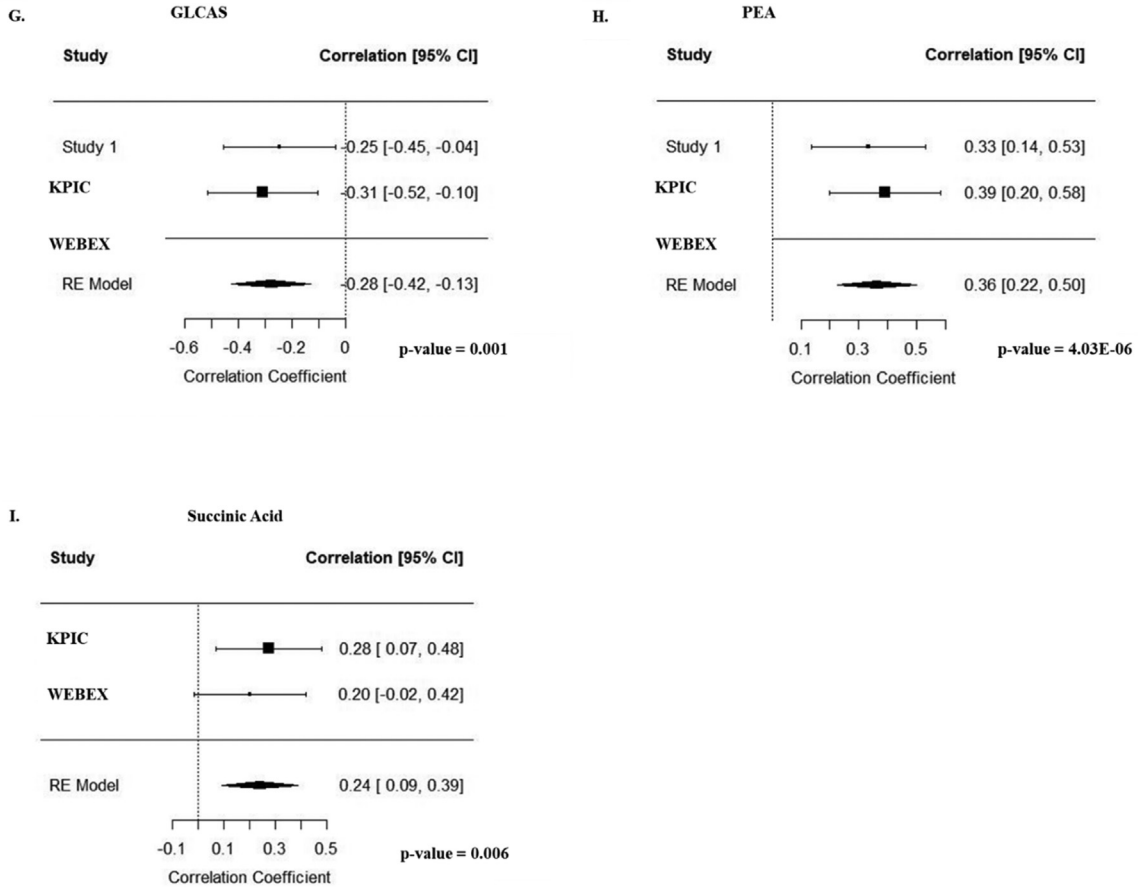

Figure 3. Continued

differently within individuals in the presence or absence of pain or stimuli. The different direction of associations of pain and PPTs with a few metabolites in test and replication study may be due to the significant difference in the PPTs in the 2 study datasets. This also corroborates that there is a lack of true associations because the level of pain experienced amongst individuals varies, and henceforth these results need to be interpreted with caution.

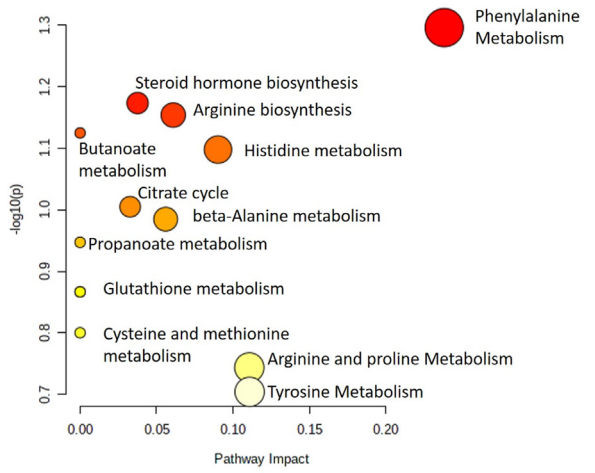

Figure 4. Relevant pathways involved in current knee pain based on the significantly associated metabolites from the meta-analysis. Scatter plot of the pathways enriched due to the significant metabolites. The x-axis and size of the node depict the impact of pathways from topology analysis and the y-axis and color of the node depict the statistical significance in terms of the $\log_{10}$(*P*-values).

Prior studies have noted the importance of cytokines in pain modulation through various mechanisms/neural pathways,[25] increasing pain and thus changes in alteration of sensory neuron excitability. The constant signaling from the inflamed joints may result into the central sensitization[20] which possibly explains the discordance of decrease in certain pro-inflammatory cytokines and increase in a few anti-inflammatory cytokines with pain. This is coherent with other previous studies[19,42] conducted in arthritis patients, reporting difference between the self-reported pain and objective evaluation of inflammation. A review [46] collating evidence from other studies confirms that cytokines are associated with not only inflammatory pain but also with neuropathic pain. This depends upon whether cytokines act upon the receptors in the peripheral or the central sensory system. Although the cytokines were not associated with the pain scores, we observed a number of cytokines like IL-10 and TNFα to be significantly associated with the pain-related metabolites. Similar to other studies,[7,22] the evidence of significant positive correlation of TNF-alpha with cystine and negative association of cortisol with IL-10 in the current study reflects the immune disorder that appears with pain and tissue damage in patients with OA. Synovial fluid from OA

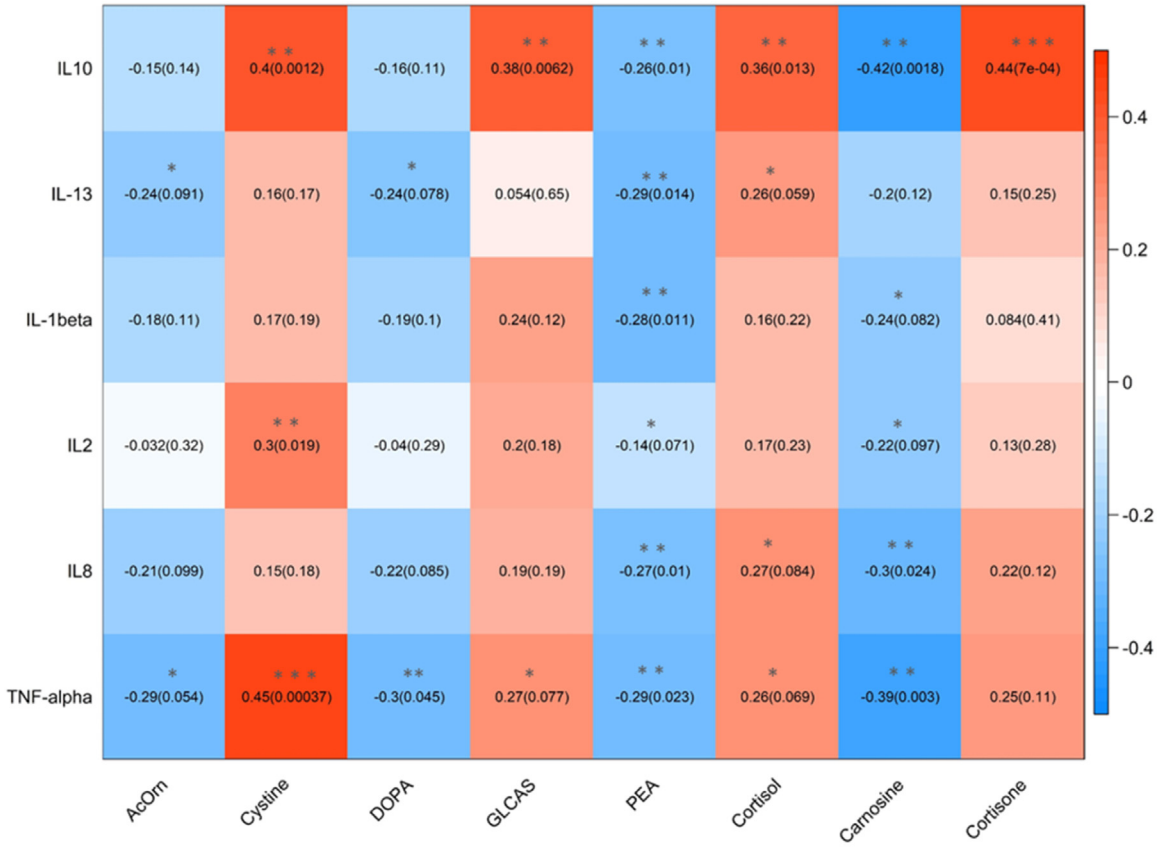

Figure 5. Correlation of replicated metabolites with the cytokines. Heatmap depicting the replicated metabolites in the Webex and KPIC datasets from meta-analysis correlated with the cytokines levels in Webex dataset. Blue to red highlighted values indicates the spearman's coefficients from negative to positive associations. *P*-values in parenthesis indicate the statistical significance of the associations after multiple testing correction (FDR <.1) and are marked with an asterisk.

patients shows increased expression of D1-like dopamine receptors and this increased expression may lead to anti-inflammatory effects.[6] These observations accord with findings from the current study which showed that metabolites are significantly associated with immune-modulatory markers. This supports the hypothesis that pain in OA is not predominantly an inflammatory condition but a degenerative joint disorder with musculoskeletal pain.[3,5,8,39] Inflammation in OA is reported to be of varying degrees and progressing with time making it is different from other inflammatory forms of arthritis such as rheumatoid arthritis, psoriatic arthritis and spondylarthritis.[13]

The use of omics methods in understanding pain and OA is still in its infancy as collated by Teckchandani et al and Gerdle et al [14,44] and there is a scarce metabolomics based research in pain that makes this study as first comprehensive investigation of serum metabolites in knee pain in patients with OA. There are certain limitations to this study. The main limitation is that serum concentration of metabolites may not reflect of the actual concentration of these metabolites at the site of pathology (ie, the knee) or in the central nervous system. However, measuring and testing serum concentration is easily feasible and have also been validated in pain and metabolic syndrome associated with OA reflecting systemic factors influencing pain.[4,28,30,32] The current study did not measure the neurotransmitters such as DOPA or their receptors in brain, and no studies demonstrate the correlation levels in serum vs brain, so it lacks a clearer picture of the metabolism and transportation of the neurotransmitter related metabolites. Also, the bioavailability of cortisol follows certain circadian rhythms of the body, and the current study did not consider the measurement time of these markers in the blood. Furthermore, methods used to determine the presence of OA was not similar in the 2 studies. In the current analysis all the study participants had OA, so this restricts the speculation of these findings to pain experienced in other diseases. The present study is a cross-sectional study so the causal nature of the associations found cannot be determined and prospective studies are required to determine causation.

In conclusion, the current study shows acyl ornithine, carnosine, cortisol, cortisone, cystine, DOPA, GLCAS, PEA and succinic acid are correlated with pain in knee OA and suggest their potential use as metabolic markers in the 2 independent cohorts. Also, these metabolites are associated with immune responses via inflammatory markers such as IL-10, IL-8, TNFα and IL-1β. Although the current study sheds light on the associations between metabolites circulating in serum and pain traits, future work will be required interpreting the WOMAC scores and neurophysiological factors such as Quantitative Sensory Testing (QST) and thus understanding the mechanisms involved in pain modulation. Investigating these modulating factors impacting pain may eventually

translate into metabolic marker development for use in the clinical trials after in vivo/in vitro validations. Given the efficacy of the drugs in pain relief, metabolite therapy holds a promise as potential therapeutics against pain and have anticipated benefits in the treatment and management of OA.

Author Contribution

OM did the analysis and wrote the manuscript. AV and AMV reviewed and edited the manuscript. OM and AMV designed the study. All the authors approved the final version of the manuscript.

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Supplementary data

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