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Serum branched-chain amino acid to histidine ratio: a novel metabolomic biomarker of knee osteoarthritis

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ABSTRACT

Objective There is a pressing need to develop reliable molecular biomarkers in osteoarthritis. The aim of the study was to identify novel serum biomarkers for osteoarthritis using a metabolomics approach.

Methods A two-stage study design was utilised. 123 knee osteoarthritis cases and 299 controls were selected from the TwinsUK cohort as a discovery sample. 76 knee osteoarthritis cases and 100 controls from the Chingford Study were used as replication. Knee osteoarthritis was defined as either radiographic, medically diagnosed or total knee joint replacement due to primary osteoarthritis. All the subjects were unrelated white women. Their serum samples were assessed for targeted metabolite profiling by electrospray ionisation tandem mass spectrometry using the AbsoluteIDQ kit. 163 serum metabolites were assessed and their concentrations obtained. The ratios of metabolite concentrations as proxies for enzymatic reaction rates were calculated and tested for the association with knee osteoarthritis. Significance was assessed after adjustment for multiple testing (Bonferroni method) and potential confounders.

Results In the discovery stage, the authors identified 14 ratios significantly associated with knee osteoarthritis with $p \leq 1.9 \times 10^{-6}$. Two of these 14 ratios were successfully confirmed in the replication stage—the ratios of valine to histidine and leucine to histidine, with $p = 0.002$. The significance remained after adjustment for age and body mass index.

Conclusion This is the first serum-based metabolomic study of osteoarthritis in humans. The branched-chain amino acids to histidine ratio has potential clinical use as an osteoarthritis biomarker and shows the clinical potential of metabolomics.

There are currently no disease-modifying drugs for osteoarthritis and very few in development, due mainly to the costs and problems of radiographic-based clinical trials. There is an urgent need to develop reliable biochemical markers that can be used for characterising the status, prognosis and measures of treatment response in osteoarthritis.¹ The emerging field of metabolomics, in which a large number of small-molecule metabolites from body fluids or tissues are detected quantitatively in a single step, provides immense potential for this purpose.² The method has been applied in the area of cancer, diabetes, inborn errors of metabolism and cardiovascular diseases.²

Using ¹H nuclear magnetic resonance (NMR) spectroscopy, several studies of the animal model of osteoarthritis have demonstrated a clear difference in the biochemical profile of synovial fluids between

normal and osteoarthritis-affected joints,^{3,4} suggesting the technique can be useful in the study of joint metabolism. However, to obtain synovial fluids is invasive and cannot be used in a large-scale study. Lamers *et al*⁵ studied urine samples of osteoarthritic male Hartley guinea pigs and identified a NMR fingerprint that reflected the osteoarthritic changes in guinea pigs. Using the same technique, Lamers *et al*⁶ also studied urine samples of 47 non-osteoarthritis controls and 45 individuals with radiographic osteoarthritis of the knees or hips. They showed that urine NMR spectra can discriminate osteoarthritis cases and controls in both men and women and the metabolic profiles largely resembled the profile identified in the guinea pig model.⁵ They also demonstrated a high correlation between radiographic Kellgren–Lawrence (KL) score and the metabolite profile with $r^2 = 0.82–0.93$. These results provide good evidence of the potential value of a metabolomics approach in osteoarthritis.

All these studies used the NMR spectroscopy technique. Although NMR spectroscopy requires little or no sample preparation and provides reproducible results, it can only detect metabolites with relatively high concentrations and has low sensitivity. Furthermore, difficulty in annotation is a challenge for NMR spectroscopy. On the other hand, the intrinsic high sensitivity (typically pictogram level) of mass spectrometry (MS) detection makes it an important method for measuring metabolites in complex biofluids.² The AbsoluteIDQ kit developed recently by Biocrates Life Sciences AG (Innsbruck, Austria; <http://www.biocrates.com>) in combination with MS allows more than 160 targeted metabolites to be quantified in over four compound classes. The advantage of the kit is that it measures the targeted metabolites in an easy, reliable and robust way, avoiding the uncertainty of non-targeted approaches, and eliminates the effort of cumbersome metabolite identification. The kit has been successfully used in studies of smoking and genetics.^{7,8} Using the same kit, we investigated 163 serum metabolites by targeted metabolomics with the electrospray ionisation–tandem mass spectrometry (MS/MS) technique in order to identify novel metabolic biomarkers for knee osteoarthritis. A two-stage study design was utilised—discovery and replication.

SUBJECTS AND METHODS

Subjects

The discovery sample was derived from the TwinsUK cohort, a group ascertained to study

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the heritability and genetics of age-related diseases (<http://www.twinsUK.ac.uk>). One hundred and twenty-three knee osteoarthritis cases and 299 controls were selected from this source population. Knee osteoarthritis was defined as either radiographic, physician-diagnosed clinical osteoarthritis, or total knee joint replacement due to primary osteoarthritis, whereas the controls were defined as having none of these. All the subjects were unrelated white women.

The replication sample was derived from the Chingford Study, a well-described prospective population-based longitudinal study of osteoarthritis and osteoporosis, comprising 1003 women aged 43 years or above at entry in 1989 derived from the age/sex register of a large general practice in Chingford, North London, who are seen annually and have been described in detail previously.^{9 10} Their knee x-rays were available for years 1, 5, 10 and 15. For the purpose of the current study, we selected 76 cases who had both knees affected by radiographic osteoarthritis defined as a KL score of 2 or greater at both years 10 and 15 and 100 controls who had normal radiographs in both of their knees at years 10 and 15. All the subjects were unrelated white women.

The study was approved by St Thomas' Hospital Research Ethics Committee and all subjects provided informed written consent.

Metabolite measurements

The serum samples collected after an overnight fast of all the study subjects and obtained when their knee x-rays were taken were retrieved from the -80°C freezers and sent to Germany for metabolite measurements.

Liquid handling of serum samples (10 μl) was performed with a Hamilton Star robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and prepared for quantification using the AbsoluteIDQ kit (Biocrates Life Sciences). Sample analyses were performed on the API4000 Q TRAP LC/MS/MS System (Applied Biosystems, Darmstadt, Germany) equipped with a Shimadzu Prominence LC20AD pump and SIL-20AC auto sampler (Shimadzu Deutschland GmbH, Duisburg, Germany). The complete analytical process (eg, the targeted metabolite concentration) was performed using the MetIQ software package, which is an integral part of the AbsoluteIDQ kit. A total of 163 metabolites was measured. The metabolomics dataset contains 14 amino acids, one sugar, 41 acylcarnitines, 15 sphingolipids and 92 glycerophospholipids. Concentrations of all metabolites analysed are reported in μM . A full list of the measure metabolites (with abbreviations) and their biological relevance is presented in table 1.

Reproducibility of the assay was performed in 23 serum samples for all 163 metabolites. The mean of the coefficient of variation (CV) for the 163 metabolites was 0.07 ± 0.05 and 90% of the metabolites had a CV of less than 0.10.

Statistics

The ratios of the pairwise metabolite concentrations ($163\times 162=26\ 406$ ratios) as proxies for enzymatic reaction rates⁷ were calculated. The association between each metabolite/the ratios and knee osteoarthritis was examined using linear regression modelling. The Bonferroni method was used to correct for multiple testing. The significance level was defined as a p value less than 1.9×10^{-6} (correcting 26 569 statistical tests) in the discovery stage and a p value less than 0.003 (correcting 14

Table 1 List of metabolite concentrations determined using the Biocrates AbsoluteIDQ kit

Metabolite class	N	Metabolite name or abbreviation	Biological relevance (selected examples)
Amino acids	14	Arginine, glutamine, glycine, histidine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, (iso)leucine	Amino acid metabolism, urea-cycle, activity of gluconeogenesis and glycolysis, insulin sensitivity, neurotransmitter metabolism, oxidative stress
Sum of hexoses	1	H1	Carbohydrate metabolism
Carnitine	1	C0	Energy metabolism, fatty acid transport and mitochondrial
Acylcarnitines	26	C2, C3, C3:1, C4, C4:1, C5, C5:1, C6 (or C4:1-DC), C6:1, C8, C8:1, C9, C10, C10:1, C10:2, C12, C12:1, C14, C14:1, C14:2, C16, C16:1, C16:2, C18, C18:1, C18:2	fatty acid oxidation, ketosis, oxidative stress, mitochondrial membrane damage
Hydroxy- and dicarboxyacylcarnitines	14	C3-OH, C4-OH (or C3-DC), C5-DC (or C6-OH), C5-OH (or C3-DC-M), C5:1-DC, C5-M-DC, C7-DC, C12-DC, C14:1-OH, C14:2-OH, C16:1-OH, C16:2-OH, C16-OH, C18:1-OH	
Sphingomyelins	10	SM C16:0, SM C16:1, SM C18:0, SM C18:1, SM C20:2, SM C22:3, SM C24:0, SM C24:1, SM C26:0, SM C26:1	Signalling cascades, membrane damage (eg, neurodegeneration)
Hydroxysphingomyelins	5	SM (OH) C14:1, SM (OH) C16:1, SM (OH) C22:1, SM (OH) C22:2, SM (OH) C24:1	
Diacyl-phosphatidylcholines	38	PC aa C24:0/C26:0/C28:1/C30:0/C30:2/C32:0/C32:1/C32:2/C32:3/ C34:1/C34:2/C34:3/C34:4/C36:0/C36:1/C36:2/C36:3/C36:4/C36:5/ C36:6/C38:0/C38:1/C38:3/C38:4/C38:5/C38:6/C40:1/C40:2/C40:3/ C40:4/C40:5/C40:6/C42:0/C42:1/C42:2/C42:4/C42:5/C42:6	Dyslipidaemia, membrane composition and damage, fatty acid profile, activity of desaturases
Acyl-alkyl-phosphatidylcholines	39	PC ae C30:0/C30:1/C30:2/C32:1/C32:2/C34:0/C34:1/C34:2/C34:3/C36:0/ C36:1/C36:2/C36:3/C36:4/C36:5/C38:0/C38:1/C38:2/C38:3/C38:4/ C38:5/C38:6/C40:0/C40:1/C40:2/C40:3/C40:4/C40:5/C40:6/C42:0/ C42:1/C42:2/C42:3/C42:4/C42:5/C44:3/C44:4/C44:5/C44:6	
Lyso-phosphatidylcholines	15	lysoPC a C6:0/C14:0/C16:0/C16:1/C17:0/C18:0/C18:1/C18:2/C20:3/ C20:4/C24:0/C26:0/C26:1/C28:0/C28:1	Degradation of phospholipids, membrane damage, signalling cascades, fatty acid profile
Total	163		

aa, acyl-acyl; ae, acyl-alkyl; a, lyso; Cx:y, where x is the number of carbons in the fatty acid side chain; y is the number of double bonds in the fatty acid side chain; DC, decarboxyl; M, methyl; OH, hydroxyl; PC, phosphatidylcholine; SM, sphingomyelin.

statistical tests) in the replication stage. Confounding effects of age and body mass index (BMI) were also taken into account in the final analysis. All statistical analyses were performed using STATA/SE version 10 for Windows.

RESULTS

One hundred and twenty-three knee osteoarthritis cases and 299 controls in the discovery sample and 76 cases and 100 controls in the replication sample were studied. None of them had renal failure but six people in the discovery and one in the replication sample had type II diabetes. In the discovery sample, the mean age was 56 years in cases and 49 years in controls. The mean BMI (kg/m^2) was 27 in cases and 24 in controls. In the replication sample, the mean age was 66 years in cases and 64 years in controls and the mean BMI was 29 in cases and 25 in controls. Both age and BMI are significantly associated with knee osteoarthritis in both the discovery and replication samples ($p \leq 0.002$) as expected.

Fourteen metabolite concentration ratios were found to be associated with knee osteoarthritis significantly in the discovery sample with p values of less than 1.9×10^{-6} . They are the ratios of valine to tryptophan, histidine, glutamine, arginine, C12-DC and glycine, the ratios of C6 (C4:1-DC) to C3:1, C12-DC and lysoPC a C26:1, the ratio of glycine to ornithine, the ratios of xleucine to tryptophan, histidine, arginine and serine. We replicated the association of these 14 ratios with knee osteoarthritis in the replication sample. The association between the ratios of valine to histidine and xleucine to histidine and knee osteoarthritis was statistically significant after Bonferroni correction for multiple testing in the replication sample (table 2). The effect size in the replication sample was virtually the same as in the discovery sample. The combined analysis of the discovery and replication samples yielded a p value of 1.587×10^{-10} and 8.198×10^{-10} for the two ratios, respectively, after adjustment for cohort effect and the ratios was increased by 0.21–0.24 in the knee osteoarthritis cases compared with the controls (table 2). This association was partly mediated by age and BMI; the effect size was reduced by 42–48% after adjustment for BMI. The results remained virtually unchanged when excluding subjects with diabetes.

In order to examine the association with disease severity, we analysed the association between the two ratios and the KL grades in the replication sample as a proxy and found that the valine to histidine ratio was associated with an increase of 0.09 (SE 0.03) per KL grade ($p \leq 0.001$). The same results were observed for the ratio of xleucine to histidine. In the discovery sample, the difference in the valine to histidine ratio between cases and controls was 0.18 (SE 0.09), 0.26 (SE 0.05) and 0.31 (SE 0.09) for self-reported cases, radiographic cases and joint replacement cases, respectively. This was not the case for the

ratio of xleucine to histidine, for which the effect size was the same for all three case definitions.

In addition, three other ratios showed associations with knee osteoarthritis with $p < 0.01$ in the replication sample (table 2). The effect size was similar except for the ratio of valine to glycine.

Interestingly, all the associated ratios were involved in branched-chain amino acids (BCAA)—namely valine, leucine and isoleucine, which appear to drive the association as the absolute concentration of valine and xleucine (leucine and isoleucine) themselves were significantly increased in knee osteoarthritis cases compared with controls (figure 1), but not for the other four amino acids (all $p > 0.18$).

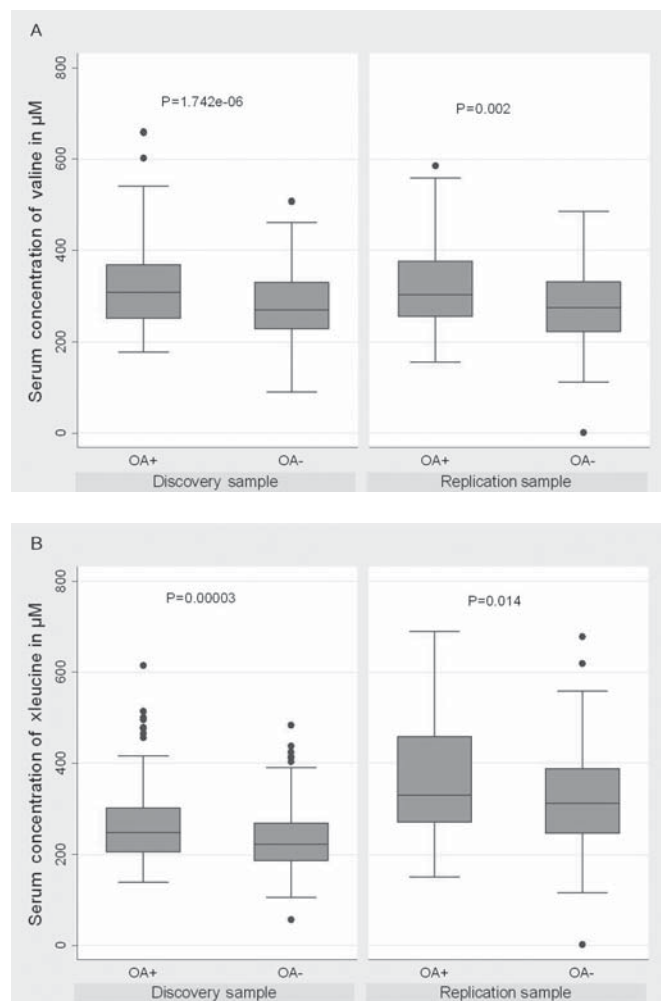


Figure 1 Box plots for the serum concentration of (A) valine and (B) xleucine in the discovery and replication samples. OA, osteoarthritis.

Table 2 Association between knee osteoarthritis and the serum levels of the ratios of metabolites*

Ratios of pair metabolites	Discovery sample (N=422) (123 cases and 299 controls)		Replication sample (N=176) (76 cases and 100 controls)		Combined analysis† (N=598) (199 cases and 399 controls)			
	β (SE)	p Value	β (SE)	p Value	Step 1		Step 2	
					β (SE)	p Value	β (SE)	p Value
Valine/tryptophan	0.32 (0.06)	8.051e-09	0.22 (0.08)	0.009	0.29 (0.05)	1.395e-10	0.18 (0.05)	0.0001
Valine/histidine	0.26 (0.05)	6.801e-08	0.22 (0.07)	0.002	0.24 (0.04)	1.587e-10	0.14 (0.04)	0.001
Xleucine/histidine	0.21 (0.04)	3.179e-07	0.22 (0.07)	0.002	0.21 (0.04)	8.198e-10	0.11 (0.04)	0.005
Valine/arginine	0.25 (0.05)	2.871e-07	0.11 (0.04)	0.004	0.21 (0.04)	2.942e-09	0.17 (0.04)	5.67e-06
Valine/glycine	0.12 (0.02)	7.769e-07	0.08 (0.03)	0.013	0.11 (0.02)	1.349e-08	0.06 (0.02)	0.008

*Linear regression modelling was used and β is expressed as change of a ratio in knee osteoarthritis cases versus controls.

†Combined analysis was adjusted for cohort effect in step 1 and together with age and body mass index in step 2.

DISCUSSION

Using targeted metabolic profiling, we identified that the serum BCAA to histidine ratios were significantly associated with knee osteoarthritis. The strength of the study is that we used a two-stage study design—discovery and independent replication and stringent p values—thus minimising false-positive findings.

The BCAA valine, isoleucine and leucine are structurally similar, having aliphatic side chains that are non-linear. They are essential amino acids making up approximately one-third of skeletal muscle in the human body and play an important role in protein synthesis. Plasma concentrations of the BCAA have been reported to be elevated in human and animal models of obesity,^{11–14} although the exact mechanism for this elevation is yet unclear. Plasma levels of the BCAA have also been reported to increase with increasing age, particularly in women.¹⁵ Consistent with these reports, our data also showed that both age and BMI were significantly associated with the ratio of the BCAA to histidine. Indeed, the association between the ratio of the BCAA to histidine and knee osteoarthritis, although significantly independent, was partly mediated by age and BMI.

Our results are supported by other animal and human data. A recent study of an animal model of osteoarthritis showed an enhancement of the resonance at 0.85 ppm of the ¹H high-resolution magic angle spinning NMR spectra of the osteoarthritis-affected cartilage sample, which could be attributable to the increase in leucine and isoleucine,¹⁶ suggesting the association in the current study could be due to the release of amino acids from joint collagen breakdown. The study of osteoarthritic canine synovial fluid also showed an increased concentration of isoleucine,³ supporting this hypothesis. Leucine has been shown to increase the release of acetoacetate and 3-hydroxybutyrate as a result of the partial oxidation of leucine. An increased concentration of hydroxybutyrate in osteoarthritic synovial fluids and urine sample has been observed,^{3 6 17} also favouring this hypothesis. This may be true given that the BCAA are essential amino acids and cannot be synthesised within the body. An increased level of free BCAA would implicate an increased rate of protein breakdown. Alternatively, BCAA have been shown to increase the production of cytokines including interleukin 1 and 2, tumour necrosis factor and interferon.¹⁸ It could be possible that an increased concentration of the BCAA leads to an increased production of cytokines, which then leads to an increased rate of joint collagen degradation. An increased level of cytokines has been associated with osteoarthritis.^{19 20}

It is also possible that the association between the ratio of BCAA to histidine and knee osteoarthritis could be attributable to a decreased level of histidine. However, the serum concentration of histidine itself is not associated with knee osteoarthritis in the current study. This is consistent with previous reports^{21 22} in which the concentration of histidine in both serum and synovial fluid were equivalent and within the normal range in knee osteoarthritis patients but significantly lower in rheumatoid arthritis patients. However, Lamers *et al*⁶ suggested that osteoarthritis patients had a lower level of histidine and methyl-histidine in urine. The reason for this discrepancy is unclear. The study by Lamers *et al*⁶ used NMR spectroscopy and did not quantify the significance for histidine. In addition, the different biosamples and small numbers used might also be a possible explanation. Histidine is metabolised into histamine, itself responsible for stimulating the proliferation of human articular chondrocytes.²³ The observed association in the current study might thus be interpreted as an indicator of increased cartilage breakdown activity while the synthesis of articular cartilage remains normal.

There are some limitations in the current study. Both our discovery and replication samples consisted of women only; so the results may not be generalisable to men. Given the nature of the cross-sectional study design, the observed association might simply be due to different dietary intakes. A recent study suggested that the ideal most consistent times of day for collecting plasma for global metabolic profiling were fasting morning, postprandial afternoon and nighttime.²⁴ All the metabolites were measured on the serum samples collected after overnight fast in our study in the same way in both cases and controls. Metabolic diseases such as diabetes or renal failure and related medications could potentially confound the results. However, when we excluded the seven subjects with diabetes, the results remained virtually the same. Most importantly, we used a two-stage study design and confirmed the observed association in an independent population-based replication sample, minimising chances of false-positive findings. There was also support for our findings from previous animal data. The diagnoses used were not uniform in the discovery sample—mixing clinical and radiographic (67% were radiographic); however, we found no differences when we analysed separately (data not shown) and any misclassification would have diluted any real results. We did not have the osteoarthritis status for other joints in our discovery sample; however, we did have x-ray data on hands and hips in our replication sample. The main results were not altered after adjustment for hand and hip radiographic osteoarthritis, and the hand and hip osteoarthritis were not themselves significant in the model (data not shown), suggesting that the observed association could be joint specific. Further larger studies are needed to confirm this. In addition, the other three ratios—the ratio of valine to tryptophan, arginine and glycine also showed possible association with knee osteoarthritis. Their non-significance after adjustment for multiple testing is probably due to a relatively small sample size in replication, which could be overcome and reveal more potential metabolic biomarkers for knee osteoarthritis.

In conclusion, this is the first study to find that a serum metabolomic marker is associated with knee osteoarthritis. The ratio of the BCAA to histidine has potential in clinical use as an osteoarthritis biomarker.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the St Thomas' Hospital Research Ethics Committee.

Contributors Study design: GZ, TDS; data collection including phenotypes and blood samples: DJH, TDS, NKA, AJH; metabolite profiling assay: RWS, TI; statistical analysis: GZ, RWS; manuscript preparation: GZ, TDS with critical comments from RWS, TI, DJH, NKA and AJH.

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