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Phenylalanine Is a Novel Marker for Radiographic Knee Osteoarthritis Progression: The MOST Study

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ABSTRACT. ***Objective.*** To identify plasma markers associated with an increased risk of radiographic knee osteoarthritis (OA) progression using a metabolomics approach.

Methods. Study participants were from the Multicenter Osteoarthritis Study (MOST) and were categorized into 2 groups based on the presence of baseline radiographic OA. Subjects in group 1 had unilateral knee OA and subjects in group 2 had bilateral knee OA. Progression was defined as a half-grade or greater worsening in joint space width at 30-month follow-up. For group 1, a participant progressed when their OA knee showed radiographic progression and the contralateral knee developed OA; for group 2, a participant progressed when both knees with OA showed radiographic progression. Metabolomic profiling was performed on plasma samples collected at baseline and logistic regression was performed to test the association between each metabolite and knee OA progression after adjustment for age, sex, BMI, and clinic site. Significance was defined as $P \leq 0.0003$ in the combined analysis.

Results. There were 234 progressors (57 in group 1 and 177 in group 2) and 322 nonprogressors (206 in group 1 and 116 in group 2) included in the analyses. Among 157 metabolites studied, we found that odds of progression were 1.46 times higher per SD increase of phenylalanine level (95% CI 1.20–1.77, $P = 0.0001$) in the combined analysis. Sex-specific analysis showed that an association was seen in women ($P = 0.0002$) but not in men.

Conclusion. Our data suggest that phenylalanine might be a novel plasma marker for higher risk of bilateral radiographic knee OA progression in women.

Key Indexing Terms: knee osteoarthritis, metabolomics, progression, plasma biomarkers, radiograph

Osteoarthritis (OA) is among the most common causes of disability in the older population worldwide[1] and the knee is one of the most common and disabling sites affected[2]. OA development and progression are often insidious, and its evolution can be slow and span many years, leading in some to the need for total joint replacement.

The rate of radiographic knee OA progression has been the subject of a number of studies. The Framingham Osteoarthritis Study, with a mean 8-year follow-up, reported the radiographic progression rate of knee OA [defined as Kellgren-Lawrence (KL) grade 2 disease at baseline showing grade ≥ 3 disease on follow-up] to be 3.0% and 3.9% per year for men and women, respectively[3]. A similar progression rate was reported from the Chingford Study with a more than 14-year follow-up[4]. The low progression rate, in addition to disease heterogeneity, may contribute to the failure of clinical trials to detect any treatment-induced slowing of radiographic progression. In large trials of potential disease-modifying drugs, most patients showed little radiographic progression or had changes within the measurement error of the technique[5,6]. This suggests that to detect the

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effects of treatments in trials within a reasonable time, it might be necessary to select a population that is more likely to progress. Thus, identifying factors that predict rapid progression might increase the success rate of clinical trials and facilitate the development of disease-modifying drugs.

However, the pathogenesis of knee OA progression remains elusive. Recent application of metabolomics to OA research has generated promising results[7,8] and identified several metabolomic markers associated with OA risk[9,10,11,12], but data on the metabolomics of OA progression are still limited[9]. We hypothesized that markers for high risk of knee OA progression can be detected by metabolic alterations and undertook the current study to identify plasma marker(s) associated with radiographic disease progression in patients with radiographic knee OA of KL grade 2 and 3 using a targeted metabolomics approach.

MATERIALS AND METHODS

Study participants were drawn from the Multicenter Osteoarthritis Study (MOST), a longitudinal, prospective, observational study of knee OA in older Americans with OA or at increased risk of developing it[13]. It enrolled 3026 study participants aged 50 to 79 years from 2003 to 2005 and is focused on the baseline and 30-month follow-up examinations, both of which included the acquisition of fixed flexion posteroanterior radiographs. Study participants were recruited and examined at clinical centers at the University of Alabama at Birmingham and the University of Iowa under local Institutional Review Board (IRB; reference numbers FWA00005960 and FWA00003007) approval and with informed consent given prior to inclusion in the study.

Radiographic knee OA progression assessment. Knee OA was defined as KL ≥ 2 and KL < 4 at baseline. Progression was at least a half-grade worsening in joint space width in any compartment at follow-up for baseline OA-affected knees as previously defined[14]. We used this approach to define knee OA progression because it is a comprehensive approach and more sensitive to change than other methods[14]. Participants with unilateral knee OA as group 1 and bilateral knee OA as group 2 at baseline were eligible for the current study. The separation of the 2 groups was arbitrary but represented the reality where either 1 knee or both knees were affected at baseline. Progression was assessed at 30 months follow-up. For group 1, participants with progression had radiographic progression in the knee with baseline OA and the contralateral knee developed OA defined by KL ≥ 2; for group 2, participants progressed when both knees with OA showed progression. We defined control subjects as those in groups 1 and 2 in whom neither knee progressed nor developed OA. We used progression/incidence groups that experienced worsening in both knees (not just 1 knee) as we expected that systemic factors affecting progression would be more likely to be detected in those who had bilateral worsening. Unilateral worsening could arise from an injury to 1 knee. Such study design has been used in genetic case control studies, comparing genetic backgrounds between using 2 extreme groups[15].

Covariates. In addition to information on age, sex, and self-reported race, subjects at baseline had weight and height assessed using previously described methods[16], and BMI was calculated as weight in kilograms divided by squared height in meters.

Metabolic profiling. Baseline overnight fasting plasma samples were retrieved from the MOST biospecimen repository and sent to St. John's, Newfoundland, Canada, for metabolic profiling. Metabolic profiling was performed using the Biocrates AbsoluteIDQ p180 kit, which assesses 186 metabolites including acylcarnitines (n = 40), amino acids (n = 22), biogenic amines (n = 18), hexoses (sum of hexoses; n = 1), and phospho- and sphingolipids (n = 105). The details of the 186 metabolites are listed in Supplementary Table 1 (available with the online version of this article). The profiling was done using an API4000 Qtrap tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies) equipped with Agilent 1100 HPLC system at The Metabolomics Innovation Centre (www.metabolomicscentre.ca). The complete analytical process (e.g., the targeted metabolite concentration) was performed using the MetIQ software package, which is an integral part of the AbsoluteIDQ kit, with concentrations reported in μM. The complete metabolic profiling method using this kit was as described[17].

Statistical analysis. The following quality control (QC) procedures were applied to the metabolomics data. Metabolites were excluded from subsequent analysis if > 10% of the samples had values below the limit of detection (LOD). For those metabolites with < 10% of samples below LOD, subjects with missing values were excluded in the corresponding association tests. Principal component analysis demonstrated that we did not have any batch effect in our experiment; therefore, no correction for batch effects was performed. Among the 186 metabolites, 157 passed the QC procedure and were included in the analysis. The 157 metabolite concentrations were log-transformed to approximate normality and then standardized using a Z-score for use in the subsequent analysis. Z-scores were preferred in the analysis because they were in SD units, making it easier to compare the effect size among metabolites. Z-score was calculated by the following formula:

$$Z = \frac{x - \mu}{\sigma}$$

where x is individual observation; μ is the population mean of the entire study cohort; and σ is the SD of the entire study cohort.

A logistic regression model was utilized to test the association between knee progression and 1 SD difference in each of the metabolites with adjustment for covariates including age, sex, BMI, and clinic site, which was an indicator for clinic centers where the study participants were recruited. Race was not included in the model due to the collinearity with clinic site because all African Americans were from 1 site. The analysis was done in groups 1 and 2 separately, and then a combined analysis of the 2 groups was completed, with adjustment for group as a covariate. The association was considered significant only when the metabolites were associated with knee progression in both groups ($P < 0.05$) and P values were < 0.0003 in the combined analysis. This took into account performing 186 statistical tests using a Bonferroni correction method (0.05/157 = 0.00032). Receiver-operating characteristic (ROC) curve analysis was performed on the identified metabolite to assess its discriminatory ability measured by the area under the curve (AUC).

RESULTS

Among 3026 subjects recruited at baseline from 2003 to 2005, 600 individuals had 1 knee affected by radiographic OA with the contralateral knee unaffected; these persons were eligible for knee OA progression defined in group 1. At 30-month follow-up, 63 progressed. Two hundred twenty did not progress in the affected knee and did not develop incident OA in the nonaffected knee; these were classified as controls. Of these, 57 progression cases and 206 controls had baseline plasma available and were included in the study. In group 2, there were 530 individuals eligible for progression. Of these, 196 had progression in both knees at 30 months, and 177 had baseline plasma available and were included in the study as knee OA progression cases. One hundred twenty-two were classified as controls and, of these, 116 had baseline plasma available and were included in the study.

Baseline age, female sex, race, and clinic sites were not associated with knee OA progression, but baseline BMI was

significantly higher in progressors in group 1, as well as in the combined samples, than nonprogressors (Table 1).

For group 1, six metabolites were associated with knee OA progression at $P < 0.05$. Those metabolites include phenylalanine, arginine, leucine, isoleucine, carnitine, and valerylcarnitine. For group 2, 17 metabolites were associated with knee OA progression including phenylalanine, serine, tryptophan, histidine, lysine, ornithine, asparagine, and a number of phosphatidylcholines with different numbers of carbons and double bonds. The complete results for all the metabolites are provided in Supplementary Table 2 (available with the online version of this article).

Phenylalanine was the only metabolite associated with knee progression in both groups 1 and 2. The plasma concentrations of phenylalanine were statistically significantly different between progressors and nonprogressors in all groups (Table 2). After adjustment for age, sex, BMI, and clinic sites, log-transformed plasma phenylalanine concentration was associated with progression with an OR of 1.66 risk for radiographic knee OA progression in group 1 ($P = 0.004$) and an OR of 1.37 in group 2 ($P = 0.009$). When combining groups 1 and 2, the OR was 1.46 ($P = 0.0001$). Sex-specific analyses showed that higher phenylalanine plasma levels were strongly associated with knee progression in women but not in men (Figure 1). No interaction between sex and phenylalanine for knee OA progression was found when tested.

ROC analysis showed that phenylalanine alone has an AUC of 0.69 and 0.59 for women and men in group 1, respectively, to discriminate knee progression cases from controls. It was 0.59 and 0.52 for women and men in group 2, respectively. No covariates were included in the ROC analysis because they were not associated with knee OA progression in the logistic regression analysis. In the combined analysis, with group included as a covariate as it was significantly associated with knee OA progression in the logistic regression model, the AUC was 0.74 in both women and men. By using R package OptimalCutpoints, we estimated the decision point was 4.2 on the log-transformed unit or 66.7 μmol/L on the original concentration unit.

To further assess how phenylalanine behaves as a marker, we examined the plasma phenylalanine concentration in those who had unilateral, but not bilateral, knee OA worsening, those who were "in-between" the extreme groups of no progression and bilateral progression. Due to limited funding, we only assessed a small number of the eligible subjects to serve the purpose. Specifically, we studied 45 subjects in group 1 whose baseline OA-affected knee had progression, but whose contralateral knee did not develop incident OA at follow-up, and 18 subjects in group 2 who had only 1 knee progressed at the follow-up. We found that the phenylalanine levels in these subjects were higher than the nonprogressors but lower than the progressors, although the difference between the "in-betweeners" was not statistically significantly different from the nonprogressors (Supplementary Table 3, available with the online version of this article).

Patient-reported Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) pain scores were collected previously from the study participants at both baseline and follow-up. We found no correlation between phenylalanine concentration and WOMAC pain scores at either baseline or follow-up (correlation coefficients range 0.004–0.01; P range 0.54–0.88).

DISCUSSION

We have carried out one of the first longitudinal studies examining metabolic risk factors for radiographic knee OA progression. Our results suggest that plasma phenylalanine levels are associated with radiographic knee OA progression, especially in women. To the best of our knowledge, this is the first study that demonstrates that a high plasma phenylalanine level is associated with knee OA progression.

Our data suggest that plasma phenylalanine as a marker behaves well with a linear increase from nonprogressor, in-betweeners, and progressor (Supplementary Table 3, available with the online version of this article). Our failure to find an association with the WOMAC pain score suggests that phenylalanine level is more likely to be associated with structural progression than with clinical symptoms.

The strength of the study is that we used a strict definition of radiographic disease progression in a well-established longitudinal study. We examined both knees with 2 scenarios. One scenario was both knees affected at baseline and the other was 1 knee affected and 1 knee at risk for incident disease. The positive findings for phenylalanine and OA radiographic progression were present in both scenarios. The findings have potential for direct clinical translation because the established method for measuring blood phenylalanine concentration is readily available

Table 1. Descriptive statistics of the study population.

	Group 1		Group 2		Combined	
	Progressors, n = 57)	Nonprogressors, n = 206	Progressors, n = 177	Nonprogressors, n = 116	Progressors, n = 234	Nonprogressors, n = 322
Age, yrs	62.6 ± 7.6	61.8 ± 7.4	63.5 ± 7.6	63.9 ± 8.2	63.3 ± 7.6	62.6 ± 7.8
Sex, % female	56.1	56.8	66.1	71.6	63.7	62.1
BMI, kg/m^2[†]	32.1 ± 5.3*	30.0 ± 5.8	33.1 ± 6.7	32.8 ± 5.9	32.8 ± 6.4*	31.0 ± 6.0
Race, % white	98.3	99.0	98.3	99.1	98.3	99.1
Iowa clinic site, %	54.4	51.5	46.3	56.0	48.3	53.1
Group 2, %[§]	–	–	–	–	75.6*	36.0

* Statistical testing was done with chi-square or t test wherever appropriate. [†] BMI at baseline was significantly higher in cases than controls in group 1 with $P = 0.01$, and in combined analysis with $P = 0.0005$. Group 2 had a significantly higher number of cases in the combined analysis with $P < 0.0001$.

Table 2. Plasma phenylalanine concentrations between knee OA progressors and nonprogressors.

	Progressors	Nonprogressors	*P*
Group 1	72.13 ± 2.08	65.48 ± 0.76	0.0003
Group 2	68.00 ± 0.96	64.28 ± 1.12	0.01
Combined	69.00 ± 0.89	65.04 ± 0.63	0.0002

Values are mean ± SD and *t* test was used in the statistical testing.

at any hospital clinical chemistry lab. In line with this finding, patients with OA should be advised to avoid any food/drinks containing large amounts of aspartame, which can raise blood phenylalanine levels.

Phenylalanine is an essential amino acid that cannot be synthesized within the body but has to be obtained from diet. In addition to serving as a building block for various proteins, phenylalanine can be degraded to tyrosine and other metabolites, which play a significant role in several rare genetic disorders. Alkaptonuria was the first inborn error of metabolism identified to be caused by the aberrant phenylalanine/tyrosine degradation due to mutations in the homogentisate 1,2-dioxygenase (*HGD*) gene, leading to the accumulation of homogentisic acid (HGA) in the connective tissues. People affected by alkaptonuria develop severe OA in their 50s because of the accumulation of HGA in the articular cartilage, leading to stiffness of the cartilage matrix and resulting in the aberrant transmission of loading to underlying subchondral bone[18]. While our study participants were not affected by alkaptonuria, our findings raise the possibility that a high phenylalanine level could result in an overproduction of HGA, some of which may be deposited in articular cartilage before being broken down by *HGD*, thus leading to disease progression. Xu, *et al* previously found that elevated phenylalanine in cartilage was correlated with osteophyte formation in knee OA[19], supporting our hypothesis. Alternatively, type II collagen, which is the major component of the extracellular matrix, is composed mainly of proline, hydroxyproline, and phenylalanine, and breakdown of the cartilage could lead to the release of these amino acids and thus, an elevated phenylalanine level. Plasma proline concentration, but not hydroxyproline, was also higher in the progression cases in group 1 ($P = 0.04$) in the current study (Supplementary Table 2, available with the online version of this article), suggesting this possibility. Further, elevated phenylalanine could lead to adequate availability of tyrosine for the synthesis of receptor tyrosine kinases, most of which could promote cartilage destruction[20,21]. Further studies are needed to reveal the exact mechanism for the observed association.

Previously, an exploratory study of the urinary metabolome with a small sample size reported that baseline urinary glycolate, hippurate, and trigonelline were the most likely metabolites to discriminate knee OA progressors from nonprogressors[22]. The assay we used did not cover these metabolites and we therefore cannot confirm these results. However, these metabolites are common constituents of urine, suggesting that studying the metabolome in different body fluids could provide additional tools for predicting knee OA progression.

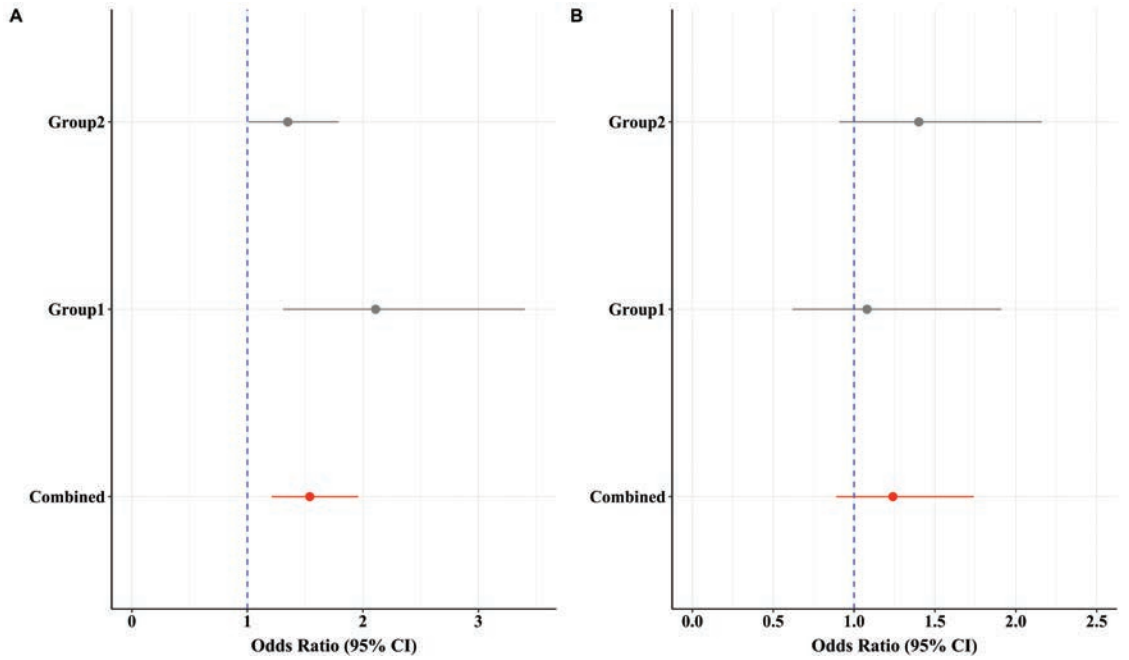

Figure 1. OR for plasma phenylalanine for the risk of radiographic knee OA progression in 30 months for (A) women and (B) men. Group 1 was unilateral knee OA and Group 2 was bilateral knee OA at baseline; the Combined group comprised groups 1 and 2 together. OR were obtained from logistic regression model with adjustment for age, BMI, and clinic sites, and expressed as per SD of the log-transformed plasma phenylalanine concentration. *P* values were 0.002, 0.04, and 0.0002 for group 1, 2, and the combined group in women, respectively. *P* values were 0.30, 0.15, and 0.20 for men, respectively. The group as a covariate was also adjusted in the combined analysis. OA: osteoarthritis.

A number of metabolomic studies have reported several metabolic markers for OA prevalence including arginine deficiency, branched-chain amino acid to histidine ratio, and lysophosphatidylcholine to phosphatidylcholine ratio[8]. We measured these metabolites in the current study but did not find an association between these metabolites and radiographic knee OA progression (Supplementary Table 2, available with the online version of this article), suggesting different metabolic alterations in radiographic knee OA progression.

Low coverage of the metabolome is a limitation of the study. A large number of metabolites in human blood have been detected and we covered only a fraction of them; thus, we might have missed other important metabolites that could be associated with radiographic knee OA progression. The ROC analysis showed that plasma phenylalanine had a moderate discriminatory ability to predict knee OA progression and there may be other metabolites or factors that need to be identified. The strong association was seen in women but not in men. Phenylalanine has been reported to be associated with telomere shortening in men but not in women[23]. Thus, our results may suggest that the association is sex-specific. However, the small sample size of men in the current study may also be a factor, and further studies with sufficient study power are needed to confirm this. Dietary intake could have an influence on plasma phenylalanine levels, and we do not have diet history information on study participants. However, plasma phenylalanine concentration is stable in the fasting state and our measurements were on fasting blood, suggesting it may not an issue. Last, we used a strict case definition of both knees with radiographic changes; thus, our findings may not be generalizable to progression defined as a person with a radiographic change in either knee. However, the in-betweeners presented in Supplementary Table 3 (available with the online version of this article) were in fact patients with progression in only 1 knee. When pooling all the in-betweeners together with all the progressors to create a group of progressors defined by progression in either knee, we found that the newly defined progressors still had a significantly higher phenylalanine level than nonprogressors (68.68 ± 0.76 vs 65.05 ± 0.63, $P = 0.0002$).

In conclusion, our data suggest that phenylalanine might be considered as a novel plasma marker for predicting knee radiographic OA progression, especially in women. These findings provide new insights into the pathogenesis of knee OA progression and have potential clinical applications.

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ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

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