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Global Metabolic Profiling of Human Osteoarthritic Synovium

Samuel B. Adams Jr., M.D.[1], **Lori A. Setton, Ph.D.**[1,2], **Elizabeth Kensicki, Ph.D.**[3], **Michael P. Bolognesi, M.D.**[1], **Alison P. Toth, M.D.**[1], and **Dana L. Nettles, Ph.D.**[2]

[1]Department of Orthopaedic Surgery, Duke University Medical Center, Duke University, Durham, NC USA

[2]Department of Biomedical Engineering, Duke University, Durham, NC USA

[3]Metabolon, Inc, Durham, NC USA

Introduction

Osteoarthritis (OA) is a debilitating disease associated with pain and loss of function in numerous diarthrodial joints of the body. Assessments of the severity and/or progression of OA are commonly based on radiographic stages and pain level, which aren't always correlated to severity of disease or joint dysfunction and may be confounded by other factors[1]. There has been recent interest in identifying a biochemical signature of OA[1] that may be detected in serum, urine, and/or synovial fluid that would represent repeatable and predictable biomarkers of OA onset and/or progression. The objective of this study was to use global metabolic profiling to identify a distinct metabolic profile for cultured human synovial tissue from patients with end-stage osteoarthritis (OA) compared to patients with little or no evidence of disease. While metabolic profiles from cultured tissues are not expected to reproduce *in vivo* profiles, it is expected that perturbations in metabolism caused by end-stage disease would result in differences in metabolic profiles *in vitro* compared to tissue with little or no evidence of disease. Because metabolomic perturbations often occur prior to alterations in the genome or proteome, metabolomic analysis possibly provides an earlier window to an altered biochemical profile for OA onset and/or progression, and may

Corresponding Author: Dana L. Nettles, Ph.D, Duke University, Biomedical Engineering, BOX 90281, Durham, NC 27708, Phone: 919-660-8444, Fax: 919-681-8490, dnettles@duke.edu.

Author contributions

SB Adams, Jr. was involved in the conception and design of the study, analysis and interpretation of the data, drafting the article, and critical revision of the article for important intellectual content.

LA Setton was involved in the conception and design of the study, critical revision of the article for important intellectual content, and obtaining of funding.

E Kensicki was involved in analysis and interpretation of data, drafting of the article, critical revision of the article for important intellectual content, statistical expertise, and collection and assembly of data.

MP Bolognesi was involved in provision of the study materials and critical revision of the article for important intellectual content.

AP Toth was involved in provision of the study materials and critical revision of the article for important intellectual content.

DL Nettles was involved in conception and design of the study, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, and takes responsibility for the integrity of the work as a whole, from inception to finished article.

Conflict of interest

E Kensicki is employed by Metabolon, Inc., Durham, NC. All other authors declare no conflict of interest related to the work represented in this study.

provide a unique set of potential drug targets. The synovium was targeted because it has been implicated in OA as a mediator of disease progression; osteoarthritic synovium has been demonstrated to express pro-inflammatory cytokines, such as Tumor Necrosis Factor (TNF), Interleukin-1 beta (IL-1β), and IL-6[2], suggesting that a diseased synovial lining could produce an ideal set of biomarkers for diagnosing OA and/or monitoring disease progression. Media from the culture of synovial explants dissected from diseased human joints (early or end-stage OA) was subjected to global metabolic profiling with a liquid chromatography (LC)/ and gas chromatography (GC)/mass spectrophotometry (MS)-based technology platform. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries developed at Metabolon, Inc (Durham, NC). Global metabolic profiling resulted in the identification of 105 distinct compounds across all sample groups, with 11 compounds showing significantly different relative concentrations between end-stage and no/early disease groups. Metabolites specific to collagen metabolism, branched-chain amino acid metabolism, energy metabolism and tryptophan metabolism were amongst the most significant compounds, suggesting an altered metabolic state with disease progression.

Methods, Results, and Discussion

Synovial tissue samples were collected as discarded tissues (non-human subjects research) from patients undergoing total knee arthroplasty for end-stage OA (“end-stage OA” group, n=11, mean age ± standard deviation: 67.8 ± 7.7) or from patients undergoing ligament or meniscal repair with little or no evidence of OA (“no/early OA” group, n=11, mean age ± standard deviation: 18.9 ± 4.8). Tissue samples were not processed from joints found to have more than one cartilage lesion, greater than Outerbridge grade 2 in the medial or lateral jointspace, or greater than Outerbridge grade 3 changes on the patella. Samples were kept sterile upon collection, dissected to 3 × 3 mm, weighed, and placed into individual culture tubes containing 2 ml of sterile culture media (LG-DMEM (Gibco), 10% FBS (Hyclone), 5 ml Pen-Strep (100 U/100 μg), and 25 mM HEPES buffer). Samples, along with separate control tubes containing media only (n=8), were cultured at 37°C and 5% CO_2 for 48 hours in order to allow metabolites to reach detectable levels. Media was aspirated from vials and frozen at −80°C until analysis. Control media was incubated over the same time course as the other two groups to control for possible changes occurring in media metabolites over the incubation period.

All media (conditioned and control) was analyzed using untargeted, metabolic profiling performed at Metabolon combining three independent platforms: ultrahigh performance liquid chromatography/tandem mass spectrometry (UHLC/MS/MS[2]) optimized for basic species, UHLC/MS/MS[2] optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS) as previously described[3]. In addition, three types of controls were analyzed in concert with the experimental samples: samples generated from pooled experimental samples served as technical replicates throughout the data set, extracted water samples served as process blanks, and a cocktail of standards spiked into every analyzed sample allowed instrument performance monitoring.

Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries developed at Metabolon, Inc. Following log transformation and imputation with minimum observed values for each compound, Welch’s two-sample *t*-tests were used to identify biochemicals that differed significantly between early and late stage disease groups compared to media control, as well as to identify biochemicals that differed between the disease stage groups. An estimate of the false discovery rate (q-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies[4].

One hundred five biochemicals were detected across all conditioned media samples. Of the 11 biochemicals with levels in conditioned media significantly different between the end-stage OA and no/early OA groups, 7 were higher in the end-stage OA group compared to the no/early group (Table 1, $p<0.05$), while 4 were present at lower levels (Table 2, $p<0.05$). In addition, 9 compounds showed trends towards increased (Table 1, $0.05<p<0.1$) or decreased (Table 2, $0.05<p<0.1$) expression with end-stage OA. Among metabolites showing distinct levels in conditioned media of synovium samples were biomarkers of collagen turnover (prolyl-hydroxyproline), cellular energetics (acetylcarnitine, succinate, and glutamine), and branched-chain amino acid (BCAA) catabolism (4-methoxy-2-oxopentanoate and 3-methyl-2-oxobutyrate).

The dipeptide, prolyl-hydroxyproline (PHP), is a compound associated with the breakdown of collagen[5]. Type I and II collagens are compositionally major components of connective tissue, cartilage, and bone, and perturbation of collagen biology has been associated with development of OA[6]. The elevated level of PHP in late disease synovial tissue conditioned media is consistent with an increased capacity for proteolysis of the collagen extracellular matrix with advanced stages of disease, although the specific isoforms involved in this proteolysis cannot be known. A second potential marker of collagen turnover – 4-hydroxyproline – was found to be present in both conditioned media groups when compared to media controls, yet did not show a significant difference between early and late disease groups. Elevated levels of both peptides in urine have been shown to be correlated with bone resorption associated with osteoporosis[7], but have not proven to be significant markers of joint breakdown in rheumatic diseases, which also can perturb collagen metabolism[8]. Differences in PHP levels detected in the present study compared to other studies may reflect an ability to capture less stable metabolites through a 48h period of culture than are present in body fluids.

A second key difference between early/no and late disease conditioned media relates to BCAA catabolism. In general, the cultured synovial tissues acted to increase the amino acid contribution to the media. By contrast, levels observed for tryptophan and the BCAAs, leucine, isoleucine, and valine, in conditioned media samples did not differ from control media, while catabolic products of the BCAAs were evident in both late disease and early/no disease groups. The first-step catabolites of BCAAs (4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate, and 3-methyl-2-oxovalerate) were present in all samples from tissue culture, indicating that their presence in the media is largely via cellular metabolism and excretion. However, a lower level of these metabolites in media from the late-stage OA group suggests an alteration in BCAA metabolism with disease progression. Recently, the ratio of BCAA to the amino acid histidine was suggested as a biomarker for osteoarthritis[9]. In that study, increased serum BCAA correlated with radiographic severity of OA. While we show no differences in the levels of BCAA or histidine between groups in our study, both studies suggest a role for altered BCAA metabolism in osteoarthritis and may prove to be a useful marker of disease progression.

The Tricarboxylic Acid Cycle (TCA) cycle is a key biochemical pathway for the oxidative conversion of carbohydrates for the generation of cellular energy. Carbon contribution to the TCA cycle originates primarily through the metabolism of glucose to pyruvate but also through the anaplerotic contribution of glutamine to the TCA intermediate alpha-ketoglutarate. Indeed, both tissue sample groups significantly depleted the culture media of precursors glucose and pyruvate, and generated elevated media levels of lactate, a product of pyruvate, as well as several TCA intermediates (citrate, cis-aconitate, and malate). However, in late disease culture media, significantly elevated levels for succinate and glutamine suggest that theses cultures display altered energy metabolism compared to early disease cultures. Glutamine is important for oxidative metabolism; thus, elevated glutamine suggests

altered oxidative metabolism in diseased tissue. Moreover, elevated glutamine could also point to a decrease in GAG production, as glutamine is required for the production of GAG[10]. Although not reaching significance, levels for additional TCA intermediates also trend in the direction of reduced oxidative energy metabolism with disease (Table 1). Altered metabolic profiles in urine and synovial fluid from OA patients have also suggested a role for altered energy metabolism in OA[11, 12].

Reduced oxidative metabolism in late disease cultures also may be indicated by the elevated media levels of acetylcarnitine observed for this group (Table 1). Carnitine is a small polar molecule that is required to shuttle long-chain fatty acids across the inner mitochondrial membrane for beta-oxidation and also as a regulator of energy metabolism through control of the Acyl-CoA:CoA ratio. For example, in the mitochondrial matrix, carnitine can facilitate glucose metabolism by accepting the acyl group from acyl-CoAs allowing the key cofactor CoA to participate in additional reactions. Higher acetylcarnitine in the media from late disease tissue culture suggests that acetyl-CoA is not being effectively moved through the TCA cycle. In a recent proteomic study of mitochondria from normal or osteoarthritic chondrocytes, of 22 mitochondrial proteins identified as being altered in OA, 26% were associated with pyruvate metabolism and/or the TCA cycle, also suggesting decreased energy metabolism in diseased cells[13].

In addition to biochemical differences between early and late disease states, 42 biochemicals were significantly elevated or decreased in media from both conditioned media groups compared to the media controls. These compounds provide some information about synovium metabolism, baseline or that associated with pathology, both mild and advanced. Of note, kynurenine, a metabolite of tryptophan that was significantly elevated in both tissue sample groups, is responsive to immune and inflammatory stimulation and is altered in a variety of human disorders and diseases[14]. Elevated tryptophan metabolism and kynurenine levels have also been shown in primary synovial cell cultures in response to elevated interferon-γ, suggesting altered or increased tryptophan metabolism in response to inflammatory cytokines associated with arthritis[15]. In this instance, further understanding of the impact of disease progression on tryptophan/kynurenine metabolism could benefit from further analysis of this pathway in synovial and/or cartilage cells.

This study has two key limitations. First, the distinct age groups represented in the late-stage disease and no/early disease groups are potentially confounding. Metabolism, and notably BCAA metabolites, are known to show age-dependent alterations[3] and further study would be required to unequivocally distinguish disease- from age-related alterations in metabolism. Second, the study is limited by the lack of a true healthy control group. Although the patients in the no/early OA group had substantially less intra-articular pathology, the nature of their injuries does not preclude intra-articular inflammation, as several studies have demonstrated synovial pathology even in early OA[2]. It is likely that a healthy control group would provide for an increased ability to find a greater number of distinctly different metabolites associated with OA onset and progression.

In summary, global metabolic profiling of conditioned media from explanted synovial tissue suggests a distinct metabolic profile for end-stage OA. Metabolites involved with collagen degradation, amino acid/BCAA catabolism, energy metabolism, and lipid and carbohydrate metabolism were found to be significantly different in media from synovial tissue cultures from patients with end-stage OA compared to those of patients with little or no evidence of disease. These metabolites may serve as novel biomarkers for OA diagnosis or to monitor disease progression.

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Table 1

Metabolites Increased in media with End-Stage OA Versus Early/No OA

Metabolite	Pathway	Mean Fold Change	p-Value
	p<0.05		
Pro-hydroxyproline	Collagen degradation	1.60	0.001
Acetylcarnitine	Lipid, carnitine, TCA cycle	1.42	0.002
Myo-inositol	Lipid, inositol	1.30	<0.001
N-acetylornithine	Urea cycle, AA	1.24	0.029
Succinate	TCA cycle	1.24	0.015
Glutamine	AA, TCA cycle	1.19	<0.001
Urea	Urea cycle, AA	1.06	0.03
	0.05<p<0.1		
Beta-alanine	AA, pyrimidine degradation	1.50	0.055
Uracil	Pyrimidine	1.48	0.087
Arabitol	Carbohydrate	1.30	0.095
Catechol sulfate	Benzoate	1.17	0.015
P-cresol sulfate	AA	1.12	0.057

AA = Amino Acid; BCAA = Branched Chain AA; TCA = Tricarboxylic Acid

Table 2

Metabolites Decreased in media with End-Stage OA Versus Early/No OA

Metabolite	Pathway	Mean Fold Change	p-Value
p<0.05			
Gamma-glutamylleucine	Peptide, glutathione	−1.75	<0.001
4-methyl-2-oxopentanoate	BCAA	−1.59	0.045
5-oxoproline	AA, glutathione	−1.30	<0.001
Phenylacetlyglycine	AA	−1.08	0.004
0.05<p<0.1			
3-methyl-2-oxobutyrate	BCAA	−1.64	0.084
Ornithine	Urea cycle, AA	−1.41	0.055
Pyridoxate	Vitamin B6	−1.20	0.092
2-methylbutyroylcarnitine	BCAA	−1.18	0.087

AA = Amino Acid; BCAA = Branched Chain AA; TCA = Tricarboxylic Acid