OsteoArthritis and Cartilage (2005) **13,** 762–768

doi:10.1016/j.joca.2005.04.005

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Identification of an urinary metabolite profile associated with osteoarthritis[1]

R. J. A. N. Lamers Ph.D.†, J. H. J. van Nesselrooij Ph.D.†, V. B. Kraus M.D., Ph.D.‡, J. M. Jordan M.D., M.P.H.§, J. B. Renner M.D.§, A. D. Dragomir§, G. Luta M.Sc.§, J. van der Greef Ph.D.† and J. DeGroot Ph.D.‖*

† *TNO Quality of Life & TNO Pharma, PO Box 360, 3700 AJ Zeist, The Netherlands*

‡ *Duke University Medical Center, Department of Medicine, Division of Rheumatology, Allergy and Clinical Immunology, Durham, NC 27710, USA*

§ *Thurston Arthritis Research Center, University of North Carolina, 3310 Doc. J. Thurston, Jr. Building, CB# 7330, Chapel Hill, NC 27599, USA*

‖ *TNO Prevention and Health & TNO Pharma, PO Box 2215, 2301 CE Leiden, The Netherlands*

Summary

Objective: Osteoarthritis (OA) is one of the most common diseases among the elderly. The main characteristic is the progressive destruction of articular cartilage. We lack quantitative and sensitive biomarkers for OA to detect changes in the joints in an early stage of the disease. In this study, we investigated whether a urinary metabolite profile could be found that could serve as a diagnostic biomarker for OA in humans. We also compared the profile we obtained previously in the guinea pig spontaneous OA model.

Methods: Urine samples of 92 participants (47 non-OA controls and 45 individuals with radiographic OA of the knees or hips) were selected from the Johnston County Osteoarthritis Project (North Carolina, USA). Participants ranged in age from 60 to 84 years. Samples were measured by ^{1}H nuclear magnetic resonance spectroscopy (NMR) with subsequent principal component discriminant analysis and partial least squares regression analysis.

Results: Differences were observed between urine NMR spectra of OA cases and controls ($P < 0.001$ for both male and female subjects). A metabolite profile could be determined which was strongly associated with OA. This profile largely resembled the profile previously identified for guinea pigs with OA (~40 out of the ~125 signals of the human profile were present in the guinea pig profile as well). A correlation was found between the metabolite profile and radiographic OA severity ($R^2 = 0.82$ (male); $R^2 = 0.93$ (female)).

Conclusion: This study showed that a urine metabolite profile may serve as a novel discriminating biomarker of OA.

Key words: Osteoarthritis, Biomarker, ^{1}H NMR spectroscopy, Multivariate data analysis.

Introduction

Osteoarthritis (OA), or cartilage degeneration, is the most common form of arthritis[1]. An important feature of the disease is the progressive destruction of articular tissues, resulting in impaired movement, pain and ultimately disability. A variety of systemic and local risk factors have been identified that predispose to the development of OA, including age, gender, bone density, obesity, joint injury and nutritional factors[2]. Despite the growing knowledge on the pathogenesis of OA, its etiology is still not clear and effective disease-modifying treatment is lacking. Diagnosis of OA is currently based on clinical symptoms[3–5] in combination with radiology. Radiological evaluation of joints mainly images bone and is relatively insensitive: a follow-up period of 2 years is often needed to assess disease progression[6]. Magnetic Resonance Imaging has the ability to simultaneously visualize all joint tissues. The technique is currently being optimized but has not yet reached its full potential.

Alternative methods are therefore needed in order to detect osteoarthritic changes in the joints in an early stage of the disease in a quantitative, reliable, and sensitive manner. Biomarkers that monitor molecular events taking place during disease are well suited for this purpose. A good biomarker is disease-specific, reflects actual disease progression, is sensitive to changes due to therapeutic intervention and can predict disease outcome. Currently, no single biomarker exists that meets these requirements[6]. Combining several biomarkers has been shown to improve the discriminatory capability considerably[7]. Recent developments in the field of metabolomics now provide the tools to go one step further: identify profiles of metabolites that

[1]This work was partially funded by a grant from the Dutch Arthritis Association (JDG) and the Association of Schools of Public Health/Centers for Disease Control and Prevention, S1734 (JMJ, JBR, ADD, GL) and the National Institute of Arthritis, Musculoskeletal, and Skin Diseases Multipurpose Arthritis and Musculoskeletal Disease Center 5-P60-AR30701 (JMJ, JBR, ADD, GL).

*Address correspondence and reprint requests to: Dr Jeroen DeGroot,TNO Quality of Life, Business Unit Biomedical Research, Zernikedreef 9, 2333 CK Leiden, The Netherlands. Tel: 31-71-5181384; Fax: 31-71-5181901; E-mail: j.degroot@pg.tno.nl

Received 15 February 2005; revision accepted 14 April 2005.

together serve as a biomarker[8,9]. Biological fluids, such as urine and blood, contain a large number of metabolites that may provide valuable information on the metabolism of an organism, and thus about its health status. Metabolic profiling, also referred to as metabolomics, metabonomics[8], or related terms, is defined as the quantitative and qualitative analysis of the whole complement of small molecules in a sample (cell, tissue, body fluids, etc.). The technology has emerged from approaches to the profiling of body fluid that were developed many decades ago for the study of inborn errors of metabolism and the effects of nutrition. Our previous research has shown that such a metabolomics approach is also feasible for identifying a biomarker profile for OA. We discovered differences between urine samples of 10- and 12-month-old Hartley guinea pigs that spontaneously develop OA using nuclear magnetic resonance spectroscopy (NMR) and multivariate data analysis (MVDA). A metabolite profile was detected which was strongly associated with OA[10]. For initial metabolic profiling it was essential to use samples from a well-defined animal study, rather than using human material, since differences in OA severity, medication, diet and habits create additional variability that would greatly hinder the identification of an OA-specific urinary metabolic profile in humans. However, once identified amidst the numerous other metabolites, the urinary metabolic profile for OA in the guinea pig model may also be quantified in humans. The present study was initiated from this assumption and designed to identify a biomarker profile that could distinguish unaffected from OA affected individuals.

Subjects, materials and methods

STUDY POPULATION AND SAMPLE SELECTION

Urine samples of 92 subjects were obtained from participants in the ongoing Johnston County Osteoarthritis Project (North Carolina, USA), that is described in detail elsewhere[11]. Participants were selected who were not using any medicine for joint complaints (NSAIDs (non-steroidal anti-inflammatory drug) and COX-2 (cyclooxygenase) inhibitors), varied in age between 60 and 84 years, and had a body mass index (BMI) between 21 and 34. Radiographic knee OA was defined from weight bearing bilateral anteroposterior radiographs of the knee, according to the Kellgren–Lawrence (K–L) grading scheme[12]. An OA case was defined as K–L grade ≥ 2 of at least two joints out of the four joints considered (knees and hips); controls were defined as K–L grade 0 in both knees and 0 or 1 in both hips. The selected group of participants consisted of 47 controls (20 males and 27 females) and 45 patients with radiographic knee and/or hip OA (21 males and 24 females). Bone mineral density measurements (hip and spine) were performed with a Lunar DPXIQ dual-energy X-ray absorptiometry machine. Unpaired *t* test (SPSS 11.5) was used to compare controls and cases for adequate matching (Table I).

Table I
The characteristics of the OA and control participants

	Control		*P* value	Case	
	Mean (sd)	Range		Mean (sd)	Range
Female					
Number	27			24	
Age [years]	69.5 (6.0)	[60–84]	0.253	67.7 (4.8)	[61–77]
Height [inches]	63.2 (2.0)	[57.5–66.5]	0.183	62.4 (2.3)	[58.3–65.5]
Weight [lbs]	161 (26)	[109–198]	0.411	156 (25)	[106–204]
BMI	28.4 (4.3)	[20.9–34.0]	0.715	28.0 (3.5)	[21.9–33.9]
BMD left hip	0.86 (0.1)	[0.55–1.16]	**0.049**	0.93 (0.1)	[0.65–1.18]
BMD spine	1.00 (0.2)	[0.49–1.31]	0.112	1.08 (0.2)	[0.73–1.46]
Affected joint [#]	0.0 (0.0)	[0–0]	**<0.001**	2.4 (0.7)	[2–4]
K–L grade right knee	0.0 (0.0)	[0–0]	**<0.001**	1.3 (1.1)	[0–3]
K–L grade left knee	0.0 (0.0)	[0–0]	**<0.001**	1.5 (1.2)	[0–4]
K–L grade right hip	0.9 (0.3)	[0–1]	**<0.001**	1.8 (0.7)	[0–3]
K–L grade left hip	1.0 (0.2)	[0–1]	**<0.001**	1.7 (0.7)	[0–3]
Summed K–L grade	1.9 (0.5)	[0–2]	**<0.001**	6.2 (1.6)	[4–9]
Current NSAID use	none			none	
Current COX-2 inhibitor use	none			none	
Male					
Number	20			21	
Age [years]	66.9 (4.0)	[61–75]	0.512	67.8 (4.8)	[61–76]
Height [inches]	68.4 (3.0)	[61.0–73.5]	0.774	68.1 (2.9)	[63.3–73.5]
Weight [lbs]	177 (17)	[144–212]	0.090	188 (23)	[139–237]
BMI	26.6 (2.2)	[24.0–31.4]	**0.032**	28.4 (3.0)	[23.6–34.0]
BMD left hip	0.98 (0.1)	[0.74–1.31]	0.453	1.01 (0.2)	[0.76–1.52]
BMD spine	1.18 (0.2)	[0.89–1.59]	0.566	1.21 (0.1)	[0.96–1.54]
Affected joint [#]	0.0 (0.0)	[0–0]	**<0.001**	2.2 (0.6)	[2–4]
K–L grade right knee	0.0 (0.0)	[0–0]	**<0.001**	1.5 (1.0)	[0–3]
K–L grade left knee	0.0 (0.0)	[0–0]	**<0.001**	1.3 (1.0)	[0–3]
K–L grade right hip	0.8 (0.4)	[0–1]	**<0.001**	1.7 (0.7)	[0–3]
K–L grade left hip	0.9 (0.3)	[0–1]	**<0.001**	1.6 (0.5)	[1–2]
Summed K–L grade	1.7 (0.6)	[0–2]	**<0.001**	6.1 (1.4)	[4–10]
Current NSAID use	none			none	
Current COX-2 inhibitor use	none			none	

Second morning voided urines were collected and centrifuged at 3000 rpm for 10 min to remove debris, and stored at −80 °C until analyses. The study was approved by the Institutional Review Board of the University of North Carolina School of Medicine and the Centers for Disease Control and Prevention. Written informed consent was obtained from all participants.

NMR ANALYSIS OF URINE SAMPLES

Prior to NMR spectroscopic analysis, 1 mL urine samples were lyophilized and reconstituted in 1 mL sodium phosphate buffer (0.1 mmol/L, pH 6.0, made up with D_2O), to minimize spectral variance arising from differences in urinary pH. Sodium trimethylsilyl-[2,2,3,3,-$^2H_4$]-1-propionate (TMSP; 0.025 mmol/L) was added as an internal standard. NMR measurements were carried out in random order and in triplicate in a fully automated manner on a 600 MHz spectrometer (Avance, Bruker BioSpin GmbH, Rheinstetten, Germany), using a proton NMR set-up operating at a temperature of 300 K. For each sample, 256 free induction decays (FID) were collected. Each FID was induced using a 45° pulse, an acquisition time of 4.10 s and a relaxation delay of 2 s. The FIDs were collected as 64 K data points with a spectral width of 12,000 Hz. The spectra were processed using the standard Bruker software. An exponential window function with a line broadening of 0.5 Hz and a manual baseline correction were applied to all spectra. After referring to the internal NMR reference (TMSP $\delta = 0.0$), line listings were prepared with the standard Bruker NMR software. To obtain these listings all lines in the spectra above a threshold corresponding to about three times the signal-to-noise ratio were collected and converted to a data file suitable for MVDA applications. The NMR data file was imported into Winlin (V2.1, TNO, The Netherlands). Minor variations from comparable signals in different NMR spectra were adjusted and lines were fitted without loss of resolution, after which MVDA was carried out.

MULTIVARIATE DATA ANALYSIS

To correct for urinary dilution NMR data were centered and scaled to unit variance so that small and large peaks contributed similarly to the final study result. Subsequently, Winlin was used to perform principal component discriminant analysis (PCDA) on the data. PCDA is a powerful tool to identify and maximize differences between pre-defined groups in data sets with a large number of variables[13]. In this study, health status (controls vs OA cases) was used as *a priori* knowledge for PCDA discrimination. For PCDA analysis, the respective data set was randomly divided into a training data set and a test data set. PCDA models were built upon the training data set. Next, the test data set was used to test the accuracy of the PCDA model by passing it through this model to obtain the model's prediction of classification of the test data into the clusters from the PCDA training model. Predictions were within the 95% confidence interval.

The resulting discriminants were quantified for each of the urinary NMR spectra. In a two-category discriminant approach, all variance (100%) is explained by the first discriminant. To visualize the differences between NMR spectra of controls and OA cases, the scores of the first discriminant were plotted and the unpaired *t* test was performed to evaluate the statistical significance of the difference between the PCDA scores of the two groups [Excel Office 2003, Microsoft Corporation, USA; Fig. 1(A and C)].

Subsequently, the original NMR spectra were used to calculate which metabolites contributed to the PCDA scores that distinguished controls from OA cases (for male and female cases separately). The combination of these metabolites (visualized via the position in ppm within the NMR spectrum) formed (gender-specific) metabolic profiles for OA. These metabolic profiles provided insight into the type of metabolites responsible for the difference in PCDA scores of the two groups.

Partial least square (PLS) regression analysis was carried out in Matlab (Version 6.5, The MathWorks Inc., Natick, MA, USA) using the PLS toolbox (Version 3.0, Eigenvector Research Inc., Manson, WA, USA) to correlate urine NMR spectra with the sum of knee and hip K–L grades, a measure for OA status. Leave-one-out was used as a method for cross-validation to obtain a goodness of fit (R^2) for the PLS model in its prediction of K–L grades from the urine NMR spectra. The PLS regression vectors, showing NMR signals that were correlated to the K–L grade, were plotted using Excel.

Results

DESCRIPTION OF SAMPLE

The characteristics of the OA and control participants are given in Table I. To minimize variation in urinary metabolites (and thus increase the chance of finding an OA-specific metabolic fingerprint) male and female subjects were analyzed separately and for both genders, cases and controls were matched for age, height and weight. Unpaired *t* test was used to compare controls and cases for adequate matching (Table I). No consistent statistically significant differences were observed except for the presence of OA. The OA patients had a summed K–L grade (knee and hips) ranging from 4 to 10 while the control subjects had a summed K–L grade ranging from 0 to 2.

HUMAN URINARY METABOLITE PROFILE

The underlying hypothesis of the present study is that OA leads to, or is accompanied by, metabolic disturbances that are reflected in an aberrant urinary metabolite composition. NMR with subsequent MVDA revealed such OA-related alterations in the urinary metabolite composition, resulting in a metabolic biomarker fingerprint that distinguished healthy individuals without OA from individuals with OA.

When applying PCDA on the male and female NMR data sets, clear differences for both genders were observed between the NMR spectra of OA cases and controls ($P < 0.001$ for both male and female subjects). Plots of the PCDA scores clearly showed this [Fig. 1(A and C)]. Thus, PCDA resulted in a distinct separation (minimal overlap) between groups based on the metabolite composition and metabolite concentration of urine which is characteristic for each group. In other words, a specific combination of metabolites can almost fully distinguish OA cases from controls.

The profiles of these metabolites for male and female subjects are shown in Fig. 1(B and D). In these 'fingerprints', NMR signals of urinary metabolites were depicted according to their relative abundance in OA vs non-OA subjects. NMR signals of the metabolic profile that displayed an association with OA for both males as well as females and that showed up in the same direction were δ 1.18, 2.02, 2.22, 2.38, 2.58, 2.74, 3.02, 3.14, 3.18, 3.22, 3.26, 3.70, 3.74, 3.78, 3.94 ppm in the positive direction and δ 1.38, 3.58, 3.98, 7.02, 7.06, 7.10, 7.54, 7.58, 7.62, 7.66, 7.78, 7.82 and 7.86 ppm in the

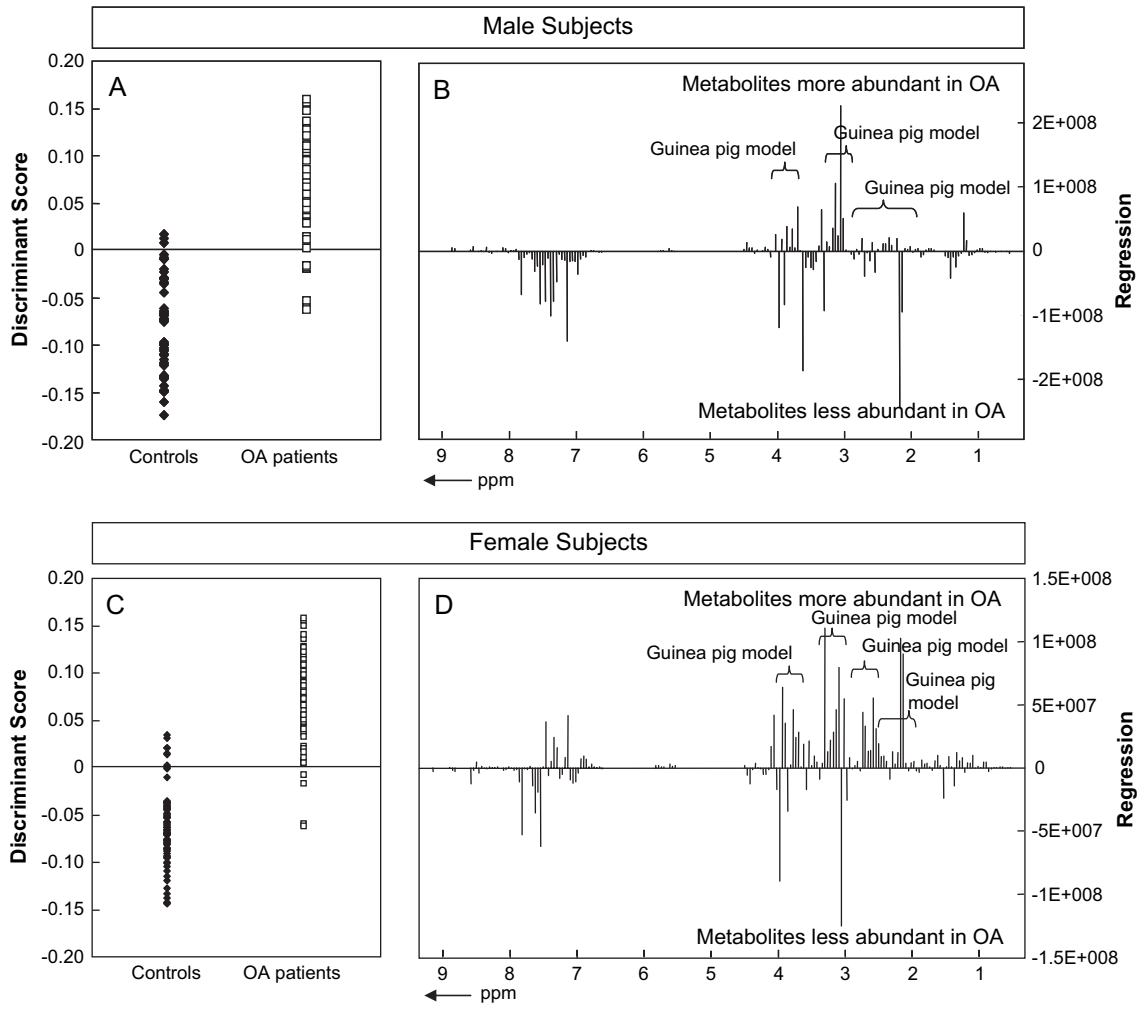

Fig. 1. Plot of the scores of urinary NMR spectra of male (A) and female (C) participants without OA vs participants with OA (the points represent the complete urinary NMR spectra of the subjects). The prevalence of OA is reflected by the urinary composition, as there is a clear difference between the scores of the two groups ($P < 0.001$ for both male and female subjects). In addition, metabolite profiles of male (B) or female (D) participants without OA vs participants with OA. Peaks (representing NMR signals, expressed in ppm) in the positive direction indicate metabolites that are more abundant in urine of participants with OA than in urine of participants without OA. Consequently, metabolites that are less abundant in urine of OA subjects as compared to the controls are presented as peaks in the negative direction. Signals that were also found in the guinea pig OA study are indicated. Please note that some signals are present in the metabolite profiles of male subject but not in female subjects and vice versa. These are therefore not representing OA-specific metabolites, rather they reflect the gender difference.

negative direction. Signals at δ 3.06 ppm in a positive direction and δ 2.14, 2.18, 3.30, 3.62, 7.14, 7.38 ppm in the negative direction varied strongly between males and females.

METABOLITE PROFILE IN RELATION TO SUMMED K–L GRADE

PLS regression was performed to correlate urine NMR spectra with the summed knee and hip K–L grades of subjects. A model was obtained that could predict the K–L grade from the urine NMR spectra for male participants ($R^2 = 0.82$) and female participants ($R^2 = 0.93$) (Fig. 2, left and right panel, respectively), thus showing the sensitivity of the metabolite profile for OA. The NMR patterns (thus metabolite profiles) that were shown by PLS regression to be correlated to the K–L grade, were largely similar to the metabolite profiles obtained by PCDA on controls vs OA cases, respectively (Fig. 3). NMR signals that correlated to OA and that showed similar patterns in men and women were δ 1.18, 2.38, 2.58, 2.74, 3.10, 3.14, 3.18, 3.70, 3.74, 3.78, 3.94 ppm in a positive direction and δ 1.38, 3.58, 3.98, 7.02, 7.10, 7.54, 7.58, 7.62, 7.66, 7.82 and 7.86 ppm in a negative direction.

IDENTITIES OF NMR SIGNALS

Although the NMR signals that make up the fingerprints are not yet characterized (which would require extensive mass spectroscopy), rough identification of the metabolites can be done based on the comparison of their NMR pattern with databases containing NMR signatures of known molecules. According to these databases, the NMR signals at δ 1.18, 2.38, 2.58, 2.74, 3.10, 3.14, 3.18, 3.70, 3.74, 3.78, 3.94 ppm in the positive direction (levels increased with OA) represent, among others, metabolites like hydroxybutyrate, pyruvate, creatine/creatinine and glycerol. Signals at δ 1.38,

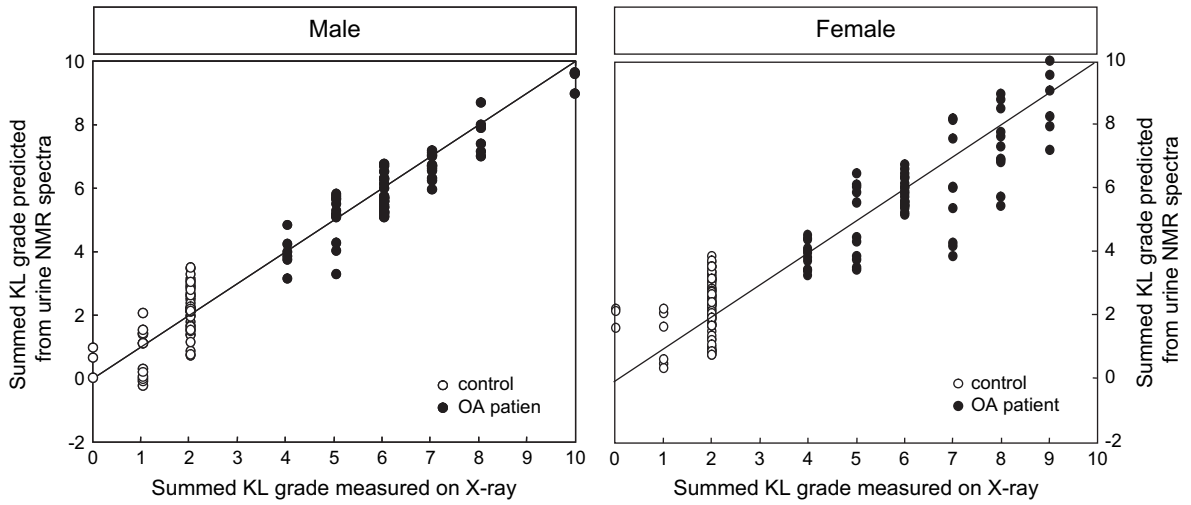

Fig. 2. Plot of measured summed K–L grades vs predicted summed K–L grades from urine NMR profiles for male (A) and female (B) participants, obtained with a PLS model. The goodness of fit (R^2) of the model is 0.93 and 0.82 for male and female subjects, respectively, showing that the correlation between K–L grades and the urine metabolite profile is high.

3.58, 3.98, 7.02, 7.06, 7.10, 7.54, 7.58, 7.62, 7.66, 7.78, 7.82 and 7.86 ppm in a negative direction (levels decreased with OA) represent, among others, compounds like histidine and methylhistidine.

Discussion

A biomarker for OA that is useful for early disease detection, that reflects the course of joint destruction, and that predicts long-term outcome is currently lacking[14]. Such a biomarker could be used to assess disease progression and the effects of therapy and thereby serve as an outcome measure in clinical trials. Ultimately, an ideal OA biomarker would potentially facilitate the development of effective individualized treatment plans and approaches.

Classical biomarker development, based on the detection of known tissue synthesis and resorption markers, has thus far not yielded biomarkers sufficiently specific and/or sensitive enough for the above mentioned applications[6]. The progress in the field of metabolomics and in particular NMR with subsequent MVDA has heralded the advent of a new approach to OA biomarker development. From the overall mixture of metabolites in a biological fluid like urine, a combination of molecules can be identified that together best reflect a disease process[8].

In the present study, we were able to discriminate between subjects who did not have OA and subjects with radiological OA, based on small differences in urinary metabolite composition and metabolite levels as detected by NMR and subsequent MVDA. We identified a urine metabolite profile that was strongly associated with OA and

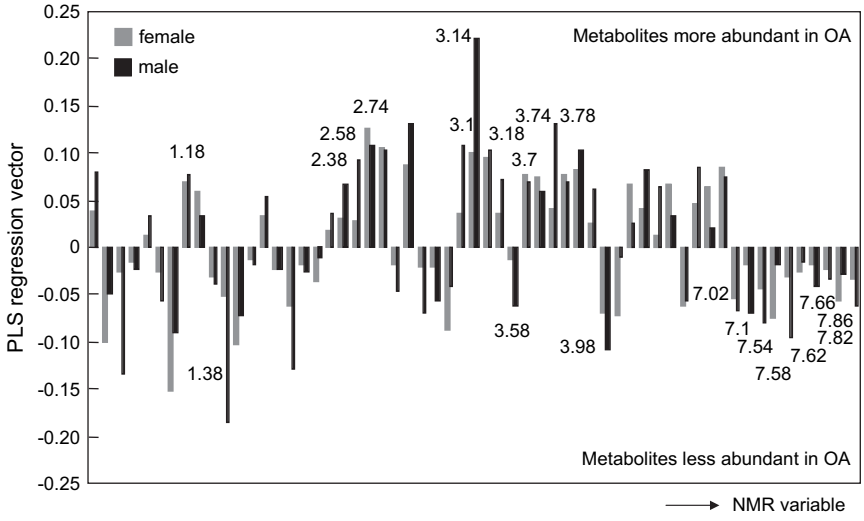

Fig. 3. Plot showing the NMR signals that correlate to the K–L grades according to PLS for both male and female participants. Peaks (representing NMR signals) in the positive direction indicate metabolites that are more abundant in urine of patients with OA than in urine of participants without OA. Consequently, metabolites that are less abundant in urine of participants with OA are presented as peaks in the negative direction. There is a large similarity with the metabolite profiles obtained with PCDA.

which appeared in male as well as in female subjects. A regression model showed that this metabolite profile correlated with the summed K–L scale of radiological OA, for male as well as female subjects. Hence, from the metabolite profile it was possible to discriminate between OA cases and controls and, moreover, to predict the OA state and severity in a sensitive manner. The metabolite profile that we identified in human samples strongly resembled the one we identified previously in samples from the guinea pig model for OA[10]. This further supports the supposition that the metabolite profile could serve as a biomarker for OA.

In addition to its use as a biomarker for OA, the OA-specific metabolic fingerprint also provides information on the cellular processes that occur during the disease and as such, the metabolic profile contributes to our understanding of the pathophysiology of the disease. The presence of hydroxybutyrate, pyruvate, creatine/creatinine and glycerol in the metabolite profile could point to an enhanced use of fat, and hence an altered energy utilization. Since the present study was conducted in urine, which reflects changes in all body tissues, the current data cannot distinguish between general changes in the body metabolism vs local changes in the joint tissues. However, the current data are consistent with studies described in the literature in which the involvement of altered energy metabolism in OA has been proposed based on measurements in normal and osteoarthritic canine synovial fluid[15]. It is possible that some of the metabolites associated with OA are intermediaries in these metabolic pathways.

The metabolite profile for human OA also indicated an alteration in histidine metabolism. Our results demonstrated lower levels of histidine and methylhistidine in association with OA. Again, based on our urinary data, no distinction can be made between a local change in the joint or systemically altered histidine metabolism. However, this finding is consistent with the hypothesis that histidine is metabolised into histamine, itself responsible for stimulating the proliferation of articular chondrocytes into clusters, a characteristic of OA cartilage[16,17]. The synthesis of histamine from histidine is catalyzed by histidine decarboxylase[18,19] and both histamine and histidine decarboxylase have been demonstrated in chondrocytes of OA cartilage[16,17]. These results would suggest that lowered levels of histidine may be caused by over-expression of histidine decarboxylase. However, more studies are needed to confirm this hypothesis.

In conclusion, our study provides evidence of a diagnostic metabolite profile associated with OA that correlates with K–L grades. Our findings are consistent with other studies reporting effects on altered energy and histidine metabolism in association with OA. The metabolite profile may provide a sensitive outcome measurement tool that can be used to evaluate the effects of nutrients and drugs on the incidence and progression of the disease. Results are promising but further research will be necessary to validate this hypothesis. Moreover, this metabolite profile may provide a tool to allow physicians to better quantify the extent of disease. Mass spectroscopy-based identification of the unknown metabolites will be an important next step in promoting an understanding of the disease

Acknowledgments

This study was partially supported by a grant from the Dutch Arthritis Association and the Association of Schools of Public Health/Centers for Disease Control and Prevention, S1734 and the National Institute of Arthritis, Musculoskeletal, and Skin Diseases Multipurpose Arthritis and Musculoskeletal Disease Center 5-P60-AR30701 (JMJ, JBR, ADD, GL). Elly J. Spies-Faber and Gerwin K. Spijksma are gratefully acknowledged for carrying out NMR experiments.

Reference

1. Lawrence RC, Helmick CG, Arnett FC, Deyo RA, Felson DT, Giannini EH, *et al*. Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. Arthritis Rheum 1998; 41(5):778–99.
2. Felson DT. Osteoarthritis: new insights. Part 1: the disease and its risk factors. Ann Intern Med 2000; 133(8):635–46.
3. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, *et al*. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and therapeutic criteria committee of the American Rheumatism Association. Arthritis Rheum 1986;29(8): 1039–49.
4. Altman R, Alarcon G, Appelrouth D, Bloch D, Borenstein D, Brandt K, *et al*. The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hand. Arthritis Rheum 1990; 33(11):1601–10.
5. Altman R, Alarcon G, Appelrouth D, Bloch D, Borenstein D, Brandt K, *et al*. The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hip. Arthritis Rheum 1991; 34(5):505–14.
6. DeGroot J, Bank RA, Tchetverikov I, Verzijl N, Tekoppele JM. Molecular markers for osteoarthritis: the road ahead. Curr Opin Rheumatol 2002;14(5): 585–9.
7. Garnero P, Rousseau JC, Delmas PD. Molecular basis and clinical use of biochemical markers of bone, cartilage, and synovium in joint diseases. Arthritis Rheum 2000;43(5):953–68.
8. Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 1999;29(11):1181–9.
9. van der Greef J, Davidov E, Verheij ER, Vogels JT, van der Heijden R, Adourian AS, *et al*. The role of metabolomics in Systems Biology. In: Harrigan GG, Goodacre R, Eds. Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis. Boston: Kluwer Academic Publishers 2003;170–98.
10. Lamers RJ, DeGroot J, Spies-Faber EJ, Jellema RH, Kraus VB, Verzijl N, *et al*. Identification of disease- and nutrient-related metabolic fingerprints in osteoarthritic guinea pigs. J Nutr 2003;133(6):1776–80.
11. Jordan JM, Linder GF, Renner JB, Fryer JG. The impact of arthritis in rural populations. Arthritis Care Res 1995;8(4):242–50.
12. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis 1957;16:494–502.
13. Hoogerbrugge R, Willig SJ, Kistemaker PG. Discriminant analysis by double stage principal component analysis. Anal Chem 1983;55:1710–2.

14. Otterness IG, Swindell AC, Zimmerer RO, Poole AR, Ionescu M, Weiner E. An analysis of 14 molecular markers for monitoring osteoarthritis: segregation of the markers into clusters and distinguishing osteoarthritis at baseline. Osteoarthritis Cartilage 2000;8(3):180–5.
15. Damyanovich AZ, Staples JR, Chan AD, Marshall KW. Comparative study of normal and osteoarthritic canine synovial fluid using 500 MHz 1H magnetic resonance spectroscopy. J Orthop Res 1999;17(2):223–31.
16. Tetlow LC, Woolley DE. Histamine stimulates matrix metalloproteinase-3 and -13 production by human articular chondrocytes *in vitro*. Ann Rheum Dis 2002; 61(8):737–40.
17. Tetlow LC, Woolley DE. Histamine stimulates the proliferation of human articular chondrocytes *in vitro* and is expressed by chondrocytes in osteoarthritic cartilage. Ann Rheum Dis 2003;62(10):991–4.
18. Salway JG. Metabolism at a glance. 3rd edn. Blackwell Science Ltd, 2003.
19. Michal G. Biochemical Pathways: An Atlas of Biochemistry and Molecular Biology. John Wiley & Sons, Ltd, 1999.