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Serum Proteomic Biomarkers Diagnostic of Knee Osteoarthritis

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Abstract

Objective: To better understand the pathogenesis of knee osteoarthritis (OA) through identification of serum diagnostics.

Design: We conducted multiple reaction monitoring mass spectrometry analysis of 107 peptides in baseline sera of two cohorts: the Foundation for NIH (n=596 Kellgren-Lawrence (KL) grade 1–3 knee OA participants); and the Johnston County Osteoarthritis Project (n=127 multi-joint controls free of radiographic OA of the hands, hips, knees (bilateral KL=0), and spine). Data were split into (70%) training and (30%) testing sets. Diagnostic peptide and clinical data predictors were selected by random forest (RF); selection was based on association ($p<0.05$) with OA status in multivariable logistic regression models. Model performance was based on area under the curve (AUC) of receiver operating characteristic and precision-recall (PR) curves.

Results: RF selected 23 peptides (19 proteins) and BMI as diagnostic of OA. BMI weakly diagnosed OA (ROC-AUC 0.57, PR-AUC 0.812) and only symptomatic OA cases. ACTG was

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the strongest univariable predictor (ROC-AUC 0.705, PR-AUC 0.897). The final model (8 serum peptides) was highly diagnostic (ROC-AUC 0.833, 95% CI 0.751, 0.905; PR-AUC 0.929, 95% CI 0.876, 0.973) in the testing set and equally diagnostic of non-symptomatic and symptomatic cases (AUCs 0.830–0.835), and not significantly improved with addition of BMI. The STRING database predicted multiple high confidence interactions of the 19 diagnostic OA proteins.

Conclusions: No more than 8 serum protein biomarkers were required to discriminate knee OA from non-OA. These biomarkers lend strong support to the involvement and cross-talk of complement and coagulation pathways in the development of OA.

Keywords

osteoarthritis; diagnostic; serum; knee; coagulation; complement; marker; mass spectrometry; proteomics

Introduction

Currently, OA is diagnosed on the basis of symptoms, physical examination, and radiographic evidence but these methods are reactive, not predictive[1] and lack sensitivity. For example, knee pain has only a 23% sensitivity and 88% specificity for a diagnosis of radiographic OA[2]. Moreover, in what has been termed the “OA iceberg”[3], OA symptoms may manifest prior to imaging abnormalities, thereby being difficult to interpret; for example, based on a systematic literature review, 25–75% of painful knees could not be diagnosed as OA by radiography[4].

Therefore, a search for reliable diagnostic biomarkers of OA is underway. There have been previous efforts to identify diagnostic systemic (serum, plasma, or urine) and synovial fluid biomarkers of OA[1, 5], but even the most prominent candidate, notably urinary C-terminal telopeptide of collagen type II (uCTXII), is still not sufficiently discriminating[6–8]. More recently, many OA diagnostic biomarkers have been proposed based on multi-marker proteomic analysis[9–13]. The goal of our study was to add to this body of work with our targeted mass spectrometry-based multiple reaction monitoring (MRM) analysis of multiple markers in serum using a relatively large sample size compared to most OA diagnostic studies to date. We addressed statistical challenges in the field with the use of precision recall (PR) curves, and random forest (RF)[14] for unbiased variable selection and to impute missing at random (MAR) data.

Methods

FNIH and JoCoOA cohorts

The Foundation for NIH (FNIH) cohort (n=600), a subset of the Osteoarthritis initiative (OAI), is comprised of individuals with Kellgren-Lawrence[15] (KL) grade 1–3 radiographic knee OA at baseline based on scoring using a standardized atlas[16]; the majority (61%) had baseline WOMAC pain score >0. There were sufficient baseline sera to perform proteomic analyses on n=599.

The Johnston County Osteoarthritis Project (JoCoOA) cohort included a subset (n=129) of 'multi-joint controls', all >45 years of age, majority (76%) with baseline WOMAC pain score=0, selected to be: i) free of baseline radiographic OA, based on not meeting designated OA criteria for the hand (KL≥2 in at least 3 hand joints), hip (KL≥2), knee (KL≥1), and lumbar spine (moderate anterior vertebral osteophyte and mild disc space narrowing); ii) the majority (68.2%) having follow-up data confirming lack of radiographic OA development over the subsequent 5–15 years establishing them as non-incident OA controls; iii) having minimal baseline hand and spine symptoms[17, 18]; iv) being free of knee or hip symptoms (pain, aching, or stiffness on most days) at baseline and 5 to 15-year follow-up when available; v) the majority (76%) had baseline WOMAC pain score=0.

Baseline non-depleted sera (tryptic digests of 1/50th volume of neat serum) from a total of 728 individuals, n=599 (FNIH) OA cases and n=129 (JoCoOA) controls, underwent proteomic analysis by mass spectrometry (MS)-based multiple reaction monitoring (MRM) (method previously described[19]). The serum samples were randomly split into twelve sets; sub-aliquots of equal size from each of the samples in Set 1 were used for quality control. The average percent coefficients of variation (%CVs) of the sample pool quality control (SPQC) were 11.4–17.5% for the 12 sets. The average %CVs of the digestion quality control (DQC) samples were 10.2–17.5% for the 12 sets. For more on sample processing, see Zhou et al[19].

Statistical Analysis

The proteomic data were expressed as a ratio of the endogenous to stable isotope labelled (SIL) peptide quantity, a unitless measure. We computed descriptive statistics (mean, median, etc.) of ratios of endogenous peptide to SIL quantities. These ratios were converted to z-scores to compare their values across the multiple biomarkers. The SIL mixture was spiked volumetrically at 20 fmol of SIL per 1 μg of serum assuming a serum protein concentration of 50μg/μL. To evaluate sample quality and outliers, we computed principal components (PC) based on all biomarkers and examined the participant clustering pattern. Two outliers were detected due to their clear deviation from the clusters by comparing the first two PCs, and were removed from further analyses. Three samples (one OA, two controls) with peptide missing rate >15% were excluded. For the remaining samples, missing biomarker data were imputed using the 'missRanger' package by chained random forest implementation[20]; we performed 10 multiple imputations of the missing values for FNIH and JoCoOA together. Following guidance in the literature[21, 22], we included all variables in the imputation scheme that we desired to study: 107 peptides, demographic variables of age, body mass index (BMI), sex, race, and a binary variable indicating OA status.

We annotated each peptide based on their amino acid position and protein name; for example, the two CRAC1 peptides were denoted $CRAC1_{(101–108)}$ and $CRAC1_{(170–178)}$. To select and validate the diagnostic predictors, FNIH and JoCoOA data were split into non-overlapping training (70% of data) and test (30% of data) datasets balanced by age, BMI, sex, and KL grade (controls had knee KL-grade=0 bilaterally). To assess the biomarkers univariately, we performed Wilcoxon-Mann-Whitney (WMW) tests on the training data. The

WMW odds (95%CIs) were calculated for each variable (107 peptides, BMI, and age); the WMW odds can be interpreted as showing the probability that a randomly selected value is higher (WMW-odds > 1) or lower (WMW-odds < 1) in OA than controls. We applied the Benjamini-Yekutieli (BY) adjustment to the p-values to control the false discovery rate (FDR) at level 0.05.

We implemented a three-step process for variable selection. First, the 10 multiple imputed training datasets each underwent 100 repetitions of RF for a total of 1000 repetitions. A variable was considered important if it was selected at least 90% of the time (900 or more times out of 1000 repetitions of selection). Second, the selected peptides were pruned by including only one peptide from each highly correlated peptide cluster (r_s >0.8). Third, the selected independent peptides and clinical variables were used to construct a multivariable logistic regression model for OA status as the outcome. The peptides meeting p<0.05 were considered as the final parsimonious set of biomarkers.

Model performance was evaluated using the area under the curve (AUC) of the receiver operating characteristic (ROC) curve and the precision-recall (PR) curve; the PR curve can be more informative than the ROC curve when there are imbalanced sample sizes such as in this study[23]. The comparator for PR-AUCs was 185/(185+45)=0.80, corresponding to the number of OA cases divided by the total sample size of the test data, in contrast to ROC-AUC for which AUC 0.5 is the reference value. The logistic regression results from the imputed datasets were combined using Rubin's rules[24], then evaluated on the testing data, applying the final coefficients from the training set data. Confidence intervals for the PR-AUC and ROC-AUC were obtained by bootstrapping using the percentile method. For each model, sensitivity and specificity were calculated in the testing set applying the Youden's J statistic calculated in the training set. Sensitivity analyses evaluated performance of the models in the non-symptomatic (baseline WOMAC pain=0) and symptomatic (baseline WOMAC pain>0) FNIH OA participants in the testing set.

All statistical analyses were conducted using R version 4.2.2. The WMW test results were used in Ingenuity Pathway Analysis (IPA, Qiagen) to create network diagrams and identify pathways with which the diagnostic proteins most aligned. STRING (version 11.5) database[25] protein-protein interaction network analyses were conducted to elucidate first order interactions of the identified OA diagnostics. STRING combined interaction scores are reported that reflect direct (physical) and indirect (functional) associations, determined experimentally and by text mining.

Results

Cohort characteristics

Compared to controls (n=127), the knee OA cases (n=596) were slightly older (median age 61 vs. 59 years), with higher BMI (median 30.2 vs. 28.2), and fewer females (59% vs. 63%) (Table 1). The majority of study participants were White (79%, 67%); the FNIH OA cohort included 5 Asian and 11 Other non-White races, while JoCoOA controls were limited to White and Black.

Data Exclusion

Six samples were excluded from analysis based on the following: 1) one OA sample was exhausted in laboratory preparation; 2) two OA cases were designated as outliers based on the clusters of PC1 vs. PC2; and 3) three samples (one OA, two controls) had peptide missing rate >15%. These resulted in 596 FNIH and 127 control study participants in the final analysis dataset. The final missing rate for each peptide ranged from 0.1% to 5.1% and there was one missing BMI value (0.1%).

WMW results and diagnostic variable selection

The WMW analysis revealed 65 peptides with distributional differences between OA and controls ($p<0.05$, Table S1). Of these, 41 peptides remained significant after FDR adjustment (FDR $p<0.05$). The medians of the majority of peptides 101/107 (94%) were lower in OA than control (WMW-odds <1).

Of the 41 peptides with significantly different serum concentrations in cases vs controls, $ACTG_{(96–113)}$ was the top predictor of OA status ($p=1.9\times10^{-13}$) with WMW-odds 3.1, indicating that a randomly chosen OA individual was 3.1 times more likely to have a higher ACTG value than a randomly chosen control individual. We therefore decided to model $ACTG_{(96–113)}$ on its own in addition to the other models described below.

Among all the variables (107 peptides, age, BMI, sex, knee KL grade), random forest (RF) selected 24 variables as important diagnostic indicators at least 90% of the time (Table 2); these consisted of 23 peptides (5 that were higher in OA and 18 lower in OA based on medians corresponding to 19 proteins) (Table S2) and BMI (higher in OA). The assessment of correlation between the 23 selected peptides (Figure S1) identified 17 uncorrelated representative peptides, selected based on their RF variable important scores and WMW results.

OA diagnostic models

For all models (Table 3), PR-AUCs were uniformly higher than standard ROC-AUCs (consistently >0.8). Models with age and sex, with or without BMI, were the weakest predictors (ROC-AUCs =0.57 and 0.593). Neither age nor sex were selected by RF; nor did they add significantly to the prediction provided by BMI alone. For these reasons, we tested the discriminative capacity of the proteomic biomarkers with and without BMI.

All models with peptides performed well as diagnostics of knee OA based on ROC-AUCs or PR-AUCs. $ACTG_{(96–113)}$, the top marker in univariable analyses, alone yielded ROC-AUC and PR-AUC of 0.705 and 0.823, respectively; these AUCs were significantly better than predictions with BMI alone or BMI combined with age and sex. All models with combinatorial peptide biomarkers (23, 17 or 8) performed significantly better as diagnostics of knee OA than $ACTG_{(96–113)}$ alone, with ROC-AUCs ranging from 0.833–0.848, and PR-AUCs ranging from 0.929–0.936, compared with 0.705 and 0.823 for $ACTG_{(96–113)}$ (Table 3). The addition of BMI to peptide only models did not improve the prediction. We also observed that the final parsimonious set of 8 peptides was as good as the models using 23 or 17 peptides (ROC-AUCs: 0.833 vs. 0.848 and 0.841). The forest plot, derived from

multivariable logistic regression of these 8 final significant peptides (Figure 1), show that half were positive predictors of an OA status and half negative (i.e., higher concentrations predicted lower odds of knee OA) predictors.

Sensitivity analyses demonstrated that the proteomic biomarkers were equally effective for diagnosing non-symptomatic and symptomatic OA cases (baseline WOMAC pain=0 and baseline WOMAC pain>0 OA FNIH study participants, respectively), whereas BMI was only diagnostic for symptomatic OA cases (Table 3).

Diagnostic indices of the models

The Youden's index J statistic for the 8 significant peptide model was 0.81 in the training dataset, yielding sensitivity 78% and specificity 78% in the testing dataset (Table 4). Although these results are based on the first of the ten imputed datasets, there were so few missing values that it is expected that the results would be approximately the same across all the 10 multiple imputed datasets. The highest sensitivity (94%) was provided by the combination of the 8 significant peptides plus BMI, but with a sacrifice of specificity (69% vs. 78% for 8 peptides alone). The model of 23 RF peptides yielded the highest overall combination of sensitivity (81%) and specificity (82%).

Network interactions

Five proteins of the complement system, an important part of the innate immune system[26, 27] implicated in the development of OA[28], were identified as OA diagnostics in this study including C1R (*C1R*), IC1 (*Serpin G1*), CFAI (*CFI*), CO5 (*C5*), and MASP1 (*MASP1*) (Figure S2A); all were lower in OA compared with controls consistent with complement consumption in the context of OA inflammation. Three proteins related to coagulation, a pathway linked to the pathogenesis of OA, were also identified as OA diagnostics in this study: KNG1 and FA5 (both lower in OA), and HABP2 (higher in OA, also called factor VII activating protease (FSAP)) (Figure S2B). Results related to KNG1 and FA5 are consistent with coagulation pathway activation and factor consumption in the context of inflammation; results with HABP2 are consistent with its upregulation by inflammatory mediators, such as low molecular hyaluronan fragments, and potential role as an acute phase reactant[25]. With the exception of CRAC1 and CNDP1, STRING analyses revealed a major interaction of all the RF selected proteins (Figure S2C) with interaction scores of the RF selected proteins ranging from 0.403 (CFI / GC interaction) to 0.999 (C1R / SERPING1 interaction), reflecting medium to highest confidence of these interactions (Table S7). STRING analyses also demonstrated medium to high confidence of specific interactions of the complement and coagulation proteins identified as diagnostic of OA with interaction scores ranging from 0.41 to 0.87 (highest for SERPING1 interaction with KNG1) (Table S7). These results provide evidence for crosstalk of several biological pathways in OA, chief among them complement and coagulation cascades.

Discussion

In this study, we utilized MS-based MRM analysis of serum, and a mixture of the regression tree model by random forest for feature selection and logistic regression models for

evaluation of the diagnostic capability of proteomic markers for OA. This culminated in a set of biomarkers that can diagnose both non-symptomatic and symptomatic radiographic OA. Inclusion of BMI in the models had minimal effect on the AUCs. This suggests that these biomarkers would be useful in differentiating the two groups, even in clinical trials with BMI matched cases and controls. Many studies have turned to proteomic strategies to identify novel biomarkers[9] with the plan of future validation. Recently, 677 proteins were identified in the synovial fluid of 10 individuals with OA[29]; interestingly, these included all of the serum proteins that were selected by RF here. Previously in another study, using two-dimensional differential gel electrophoresis (2D-DIGE) and MS, 66 proteins were identified as differentially expressed in healthy (n=10) vs. OA (n=20) synovial fluid[30]. These differentially expressed proteins were associated with three pathways – the acute phase response, the complement pathway, and the coagulation pathway[30] – which were also associated with the differential expression of biomarkers identified here as diagnostic of OA. In cartilage samples obtained at the time of total hip replacement for either OA (n=9) or femoral neck fracture (n=12, control), 7 proteins were identified as upregulated in OA using single reaction monitoring targeted proteomics and MS after controlling the false discovery rate[13]. Another study that targeted 35 peptides by MS-based MRM analysis of 116 sera from 116 individuals (n=39 controls, n=77 OA cases) identified haptoglobin and von Willebrand Factor as upregulated in OA[12]. We add to the prior knowledge by demonstrating diagnostic capabilities of a multi-marker panel of proteomic biomarkers in testing data. Interestingly, many of the protein biomarkers selected here, including ACTG, CRAC1, C1R, CO5, HPT, HEMO, HRG and HABP2, aore also predictive of incident radiographic knee OA[31].

A number of the diagnostic markers identified in this study have putative roles in the pathogenesis of OA including ACTG[32] and CRAC1[19, 33–35], both higher in OA in this and these previous studies. Among these, we previously observed expression of *ACTG* in our existing scRNAseq data, in lesioned and non-lesioned cartilage and synovium[19]. Though there has been some discussion surrounding actins in the OA literature[36], none have yet concerned ACTG that we found to be the most important single predictor of OA. Actin, of which ACTG is a type, is a highly abundant intracellular protein; severe cell injury can cause its release into the systemic circulation[37]. VTDB is an actin-binding protein and acts as an actin-sequestering agent in the extracellular space[37]. VTDB along with gelsolin play a crucial role in the clearance of actin filaments from the circulation[37]; this is consistent with higher ACTG but lower VTDB ($VTDB_{(208–218)}$) that we observed in OA compared to controls. CRAC1 has been studied both as a diagnostic biomarker of OA and as a prognostic biomarker of OA progression. Compared with controls, CRAC1 was upregulated in the synovial fluid[30] and in the secretome of cartilage[38] of individuals with knee OA. Serum CRAC1 has been shown to predict incident radiographic OA[31], radiographic OA progression[19, 39], and knee OA related pain progression[19]. In all cases, CRAC1 was higher in OA progressors and prior to onset of incident radiographic OA compared with controls; this aligns with our finding that CRAC1 was higher in individuals with OA than in controls. Five proteins related to the complement pathways were selected by RF as OA diagnostics in this study: C1R, IC1, CFAI, CO5, and MASP1. Serum concentrations were lower in OA than controls for all five proteins and both C1R and MASP1, members of the final set of 8 diagnostic biomarkers, were associated with lower odds of an OA status. We

previously found that mean serum concentrations of C1R were lower in individuals with knee OA progression compared with non-progressors[19]. Interestingly, C1R and MASP1 are both involved in initiation of the classical- and lectin pathway activation of the complement system, respectively[27]. C1R is a proteolytic subunit of the complement system C1 complex and a component of the C1 complex. MASP-1 is a homologue of C1S and C1r[27]. IC1 (*SERPING1*), an inhibitor of the C1 complex, was also lower in association with OA status. Taken together, the lower concentrations of these peptides are consistent with complement activation and net consumption of complement components in the context of OA, a condition in which it is known that complement can be activated by various extracellular matrix components and their cleavage products, released during OA-associated cartilage degradation[26].

Three proteins related to coagulation, a pathway linked to the pathogenesis of OA, were identified as OA diagnostics in this study: KNG1[30], HPT[10, 12], and FA5. Based on two-dimensional gel electrophoresis, KNG1 was upregulated in knee synovial fluid of individuals with OA versus asymptomatic individuals without radiographic OA[30]. In contrast, lower not higher serum concentrations of KNG1 (both measured peptides) were associated with OA in our study. HPT, which has a systemic anti-inflammatory effect[40] has been shown to be upregulated in synovial fluid in association with meniscal injury[10]. In contrast to a prior study showing a higher serum concentration of $HPT_{(392–401)}$ in association with a 1.22 increased odds of OA compared with control[12], we observed a decreased odds, i.e., that higher HPT was associated with a lower risk of OA. FA5 is both downstream and upstream of thrombin in the coagulation cascade (Figure S2). The peptide we identified as an OA diagnostic, $FA5_{(1506–1517)}$, is part of the activator/connector domain of FA5 that is lost upon proteolytic cleavage by thrombin[41] (thrombin proteolytic activity is associated with OA activity). Thus, a lower serum $FA5_{(1506–1517)}$ concentration would be consistent with an OA status, as observed in this study.

It is typically said that there are no blood tests that can diagnose OA; however, blood tests are often used in clinical settings to facilitate or confirm diagnoses of other arthritides such as rheumatoid arthritis (RA), gout, or lupus. We are now poised, with these strong diagnostic biochemical markers of knee OA, to better understand the pathogenesis of OA and potentially assist in filling the medical need of diagnostics for OA; with as few as 1–8 proteomic markers, a clinical mass spectrometry multiplex laboratory derived diagnostic test for OA would be feasible with minimal serum (a few microliters), and without the need for immunodepletion of high abundance proteins from the clinical sample. Clinical mass spectrometry is currently feasible as demonstrated by its use to diagnose metabolic deficiencies (typically for newborn screening), for toxicology testing, and for quantifying and differentiating between 25-hydroxy vitamins D2 and D3 with high accuracy as these forms cannot be distinguished by immunoassay[42]. Based on the robustness of identification of knee OA using a serum biomarker panel with as few as 1–8 peptides, it would appear that an OA diagnosis by a biomarker is a somewhat easier task than prediction of OA progression[19] or incident OA[31]. This accords with the growing list of biomarkers for achieving a diagnosis of OA. It may be harder however to differentiate OA from other arthritides, chief among them RA, because active OA, like RA, is an inflammatory

arthropathy with inflammatory serum biomarkers able to identify the active (progressing) joint disease[19].

We aimed to optimize several aspects of the design of this study, both technical and statistical. Although proteomics have been utilized by previous studies to identify diagnostic biomarkers of OA, many of these studies relied on older, label-free proteomics[10, 28, 30, 38]. We began with targeted MS-based MRM using SILs which provide a higher accuracy of quantification than label-free methods[43]. It is necessary to examine differences univariately when sample sizes are small, as they were for many of these studies[10, 13, 28, 30, 38]; but with our larger sample size, we were able to use RF to obtain permutation variable importance scores, which are based on prediction, to perform variable selection while taking into account interactions between the variables. Interestingly, our models all performed equally well in terms of ROC-AUC and PR-AUC as the model derived from the top 8 univariate peptides. Due to the unbalanced design of the study, we incorporated PR-AUC in addition to ROC-AUC for the assessment of the prediction models. The ROC curve and its AUC are relatively well known and useful in evaluating predictive models that produce output values over a continuous range[44]. However, ROC curves, and by extension their associated AUC, can be misleading when the sample sizes of cases and controls are different. In this circumstance, the precision recall (PR) curve, can be more informative because it can provide an accurate prediction of future classification performance since it evaluates the fraction of true positives among positive predictions[45]. Additional strengths of this study included a relatively large sample size, and to the best of our knowledge, the most stringent OA controls in the literature to date, derived from the JoCoOA, with low or no burden of multi-joint OA accounting for hand, hip, knee and spine OA. Past studies may have been hampered by unaccounted for occult OA in controls.

There were several limitations of this study. Although another larger cohort was not available for validation of our model, our validation/testing dataset was still substantially larger than the OA sample sets used in discovery by many others[11–13, 30, 38, 46]. Typical for proteomic data, our discovery data also included missing values. However, the SILs enabled us to identify which values were below the limit of quantification and which were MAR so that we could remove peptides accordingly and impute the remaining missing values, assuming that they were MAR. Then, we combined RF, a flexible imputation scheme with multiple imputation, to report appropriate p-values and CIs. Notably, among the peptides that were most highly selected, there were few missing values in the training dataset: three (0.6%) in $C1R_{(229–235)}$, one (0.2%) in $A1BG_{(262–280)}$, one (0.1%) in $CRAC1_{(170–178)}$, and five (1%) in $VTDB_{(95–114)}$ with no missing values in the other peptides. There were also few missing values for the selected peptides in the testing data: one (0.4%) in $A1BG_{(262–280)}$ and five (1.7%) in $VTDB_{(95–114)}$ with no missing values in the other peptides. Combining the results from ten imputed datasets using Rubin's rules relies on a normality assumption. In addition, we can appeal to the central limit theorem to assume normality for the regression coefficients given our sample size. Finally, although based on self-report the majority (98% FNIH and 64% JoCoOA) of sera in both cohorts were obtained after 2–8 hours fasting, we do not know what, if any effect, fasted vs non-fasted state would have on these predictions.

In summary, we identified and validated a model to diagnose radiographic knee OA using serum proteomics. No more than 8 protein serum markers, with or without BMI, were required for discrimination of knee OA from non-OA. Interestingly, many of the proteins indicative of an OA diagnosis have been shown to also identify incident radiographic OA as much as 8 years prior to the onset of radiographic abnormalities. These biomarkers further implicate the complement and coagulation pathways, lending support to the involvement of innate immunity in the development of OA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials availability:

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. All proteomic data for this study are available at ftp://massive.ucsd.edu/ or massive.ucsd.edu.

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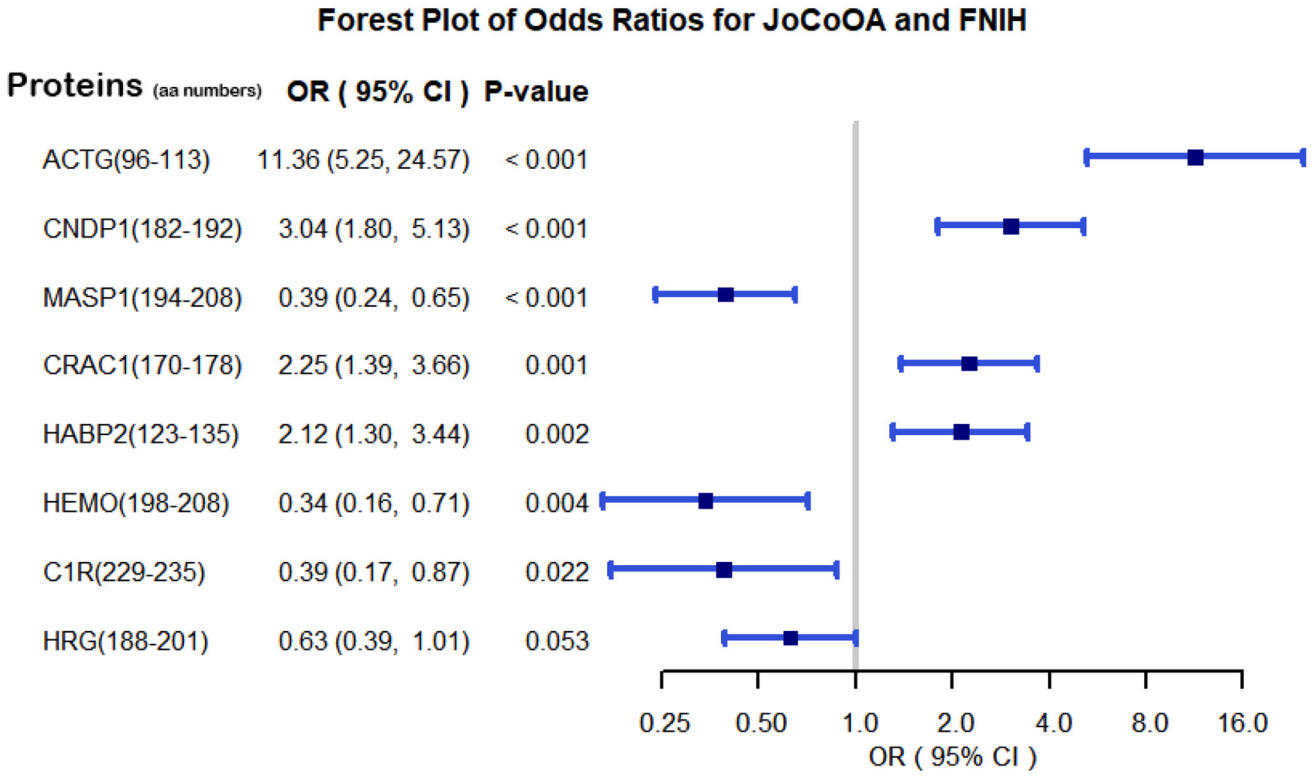

Figure 1. Forest plot of 8 significant OA diagnostic peptides.

Multivariable model results for final 8 peptides selected by random forest as diagnostic predictors of knee OA. (odds of predicting OA, 95% CIs are depicted and p values are shown). aa=amino acid

Table 1:

Summary statistics of the JoCoOA and FNIH cohorts.

Baseline Characteristics	JoCoOA Controls	FNIH OA Cases
Total n	127	596
Female n (%)	80 (63%)	350 (59%)
Age median (years) [Min, Max]	59.0 [45.0, 95.0]	61.0 [45.0, 79.0]
BMI median (kg/m^2) [Min, Max]	28.2 [20.0, 54.4]	30.2 [18.6,46.7]
Race		
Other non-White	0 (0%)	11 (2%)
White	85 (67%)	474 (79%)
Black	42 (33%)	108 (18%)
Asian	0 (0%)	5 (1%)
Knee KL grade		
0	127 (100%)	0 (0%)
1	0 (0%)	75 (12.6%)
2	0 (0%)	303 (50.8%)
3	0 (0%)	218 (36.6%)
WOMAC pain mean (SD)	1 (2.5) *	12.0 (15.5) *

KL: Kellgren Lawrence grade knee OA

WOMAC: Western Ontario and McMaster Universities Arthritis Index pain score normalized on a 0–100 scale

* The majority (76%) of JoCoOA controls had baseline WOMAC pain scores of 0; a total of 231 (39%) of FNIH cases had baseline WOMAC pain scores of 0.

The random parsing led to well-balanced of training and testing sets that were not significantly different from the overall combined cohort shown in the Table (data not shown).

Table 2.

Diagnostic variables (n=24; 23 peptides and BMI[*]) selected by random forest (RF).

Peptide	Protein (Gene) Name	RF importance score	WMW P value	WMW odds (95% CI)	Function	Peptide Sequence	Accession Number
ACTG(96–113)	Actin, cytoplasmic 2 (*ACTG1*)	1	1.85e-13	3.12 (2.28,4.23)	Cell motility	VAPEEHPVLLTEAPLNPK	P63261–1
HEMO(198–201)	Hemopexin (*HPX*)	1	3.02e-10	0.39 (0.29,0.53)	Acute phase protein, transports heme to liver for breakdown	YYCFQGNQFLR	P02790–1
KNG1(317–324)	Kininogen-1 (*KNG1*)	1	3.33e-10	0.39 (0.29,0.53)	Coagulation factor and regulator of Insulin-like Growth Factor	YFIDFVAR	P01042–1
CO5(767–782)	Complement C5 (C5)	1	2.02e-09	0.41 (0.31,0.55)	Complement component	ALEQDLPVNIK	P01031–1
C1R(229–235)	Complement C1r subcomponent (*C1R*)	1	4.28e-09	0.42 (0.31,0.56)	Serine protease, part of the first component of the classical pathway of the complement system	GLTLHLK	P00736–1
A1BG(262–280)	Alpha-1B-glycoprotein (*A1BG*)	1	9.43e-09	0.49 (0.37,0.65)	Novel member of immunoglobulin superfamily	IFFHLNAVALGDGGHYTCR	PO4217–1
CD14(94–111)	CD14 Monocyte differentiation antigen (*CD14*)	1	6.67e-07	0.48 (0.36,0.65)	Coreceptor for bacterial lipopolysaccharide	LTVGAAQVPAQLLVGALR	P08571–1
HPT(392–401)	Haptoglobin (*HP*)	1	3.70e-05	0.55 (0.42,0.73)	Antioxidant, has antibacterial activity, and plays a role in modulating many aspects of the acute phase response	VTSIQDWVQK	P00738–1
HRG(188–201)	Histidine-rich glycoprotein (*HRG*)	1	0.0002	0.59 (0.44,0.78)	Regulates many processes such as immune complex and pathogen clearance, cell chemotaxis, cell adhesion, angiogenesis, coagulation and fibrinolysis	GGEGTGYFVDFSVR	P04196–1
CNDP1(182–192)	Beta-Ala-His dipeptidase (*CNDP1*)	1	0.0009	1.60 (1.21,2.11)	Catalyzes the peptide bond hydrolysis in Xaa-His dipeptides	ALEQDLPVNIK	Q96KN2–1
CRAC1(170–178)	Cartilage acidic protein 1 (*CRTAC1*)	1	0.02	1.39 (1.05,1.82)	**Glycosylated extracellular matrix protein**	GVASLFAGR	Q9NQ79–1
KNG1(479–496)	Kininogen-1 (*KNG1*)	1	0.09	0.79 (0.60,1.04)	See above	LDDDLEHQGGHVLDHGHK	P01042–1

Peptide	Protein (Gene) Name	RF importance score	WMW P value	WMW odds (95% CI)	Function	Peptide Sequence	Accession Number
HPT(216–227)	*HP*	0.999	2.31e-05	0.54 (0.41,0.72)	See above	DIAPTLTLYVGK	P00738–1
HEMO(92–102)	*HPX*	0.999	0.0002	0.58 (0.44,0.77)	See above	NFPSPVDAAFR	P02790–1
IC1(287–298)	Plasma protease C1 inhibitor (*SERPING1*)	0.998	4.99e-06	0.52 (0.39,0.69)	Plays crucial role in regulating important physiological pathways including complement activation, blood coagulation, fibrinolysis and the generation of kinins	LVLLNAIYLSAK	P05155–1
CRAC1(101–108)	Cartilage acidic protein 1 (*CRTAC1*)	0.998	0.10	1.26 (0.96,1.66)	**Glycosylated extracellular matrix protein**	SSPYYALR	Q9NQ79–1
MASP1(194–208)	Mannan-binding lectin serine protease 1 (*MASP1*)	0.983	3.00e-05	0.55 (0.41,0.73)	Functions in lectin pathway of complement, which performs a key role in innate immunity by recognizing pathogens through patterns of sugar moieties and neutralizing them	TGVITSPDFPNPYPK	P48740–1
CFAI(346–358)	Complement factor I (*CFI*)	0.964	3.08e-06	0.51 (0.38,0.68)	Trypsin-like serine protease that plays an essential role in regulating the immune response by controlling all complement pathways	AQLGDLPWQVAIK	P05156–1
VTDB(95–114)	Vitamin D-binding protein (GC)	0.955	0.007	0.68 (0.52,0.90)	Vitamin D transport and storage, scavenging of extracellular G-actin, enhancement of the chemotactic activity of C5 alpha for neutrophils in inflammation and macrophage activation	SCESNSPFPVHPGTAECCTK	P02774–1
FA5(1506–1517)	Coagulation factor V (F5)	0.943	3.54e-06	0.51 (0.38,0.68)	Central regulator of hemostasis.; serves as critical cofactor for the prothrombinase activity of factor Xa that results in activation of prothrombin to thrombin.	EFNPLVIVGLSK	P12259–1
HABP2(123–135)*	Hyaluronan-binding	0.937	0.65	0.94 (0.71,1.23)	Extracellular serine protease that binds	GQCLITQSPPYYR	Q14520–1

Peptide	Protein (Gene) Name	RF importance score	WMW P value	WMW odds (95% CI)	Function	Peptide Sequence	Accession Number
	protein 2 (*HABP2*)				hyaluronic acid, involved in extrinsic pathway of blood coagulation, activates urinary plasminogen activator and coagulation factor VII		
VTDB(208–218)	*GC*	0.934	5.47e-06	0.52 (0.39,0.70)	See above	HLSLLTTLSNR	P02774–1
AMBP(298–309)	Alpha-1-microglobulin (*AMBP*)	0.906	5.06e-06	0.52 (0.39,0.69)	Antioxidant and tissue repair protein	AFIQLWAFDAVK	P02760–1

*Results for BMI: RF importance score 0.995; WMW p value 8.3e-06; WMW odds (95% CI) 1.91 (1.44, 2.54) Amino Acid Number (per Swiss UniProt db searched 4.8.21); Table ordered according to RF score then WMW p value; WMW Odds: >1 indicates higher probability that a randomly chosen OA value will be greater than a randomly chosen control value, <1 indicates higher probability that a randomly chosen OA value will be lesser than a randomly chosen control value. The finally selected 17 uncorrelated peptides are underlined.

The finally selected 8 significant peptides are in bold;

*HABP2 significantly higher in OA than control in multivariable logistic regression (see Figure 1); the remaining proteins yielded similar results by WMW and logistic regression Function data from UniProt (protein UniProt identifiers are provided in Table S1).

Table 3.

Test set results for clinical and proteomic variables as OA diagnostics.

	DISCOVERY ANALYSES (TRAINING SET)		VALIDATION ANALYSES (TESTING SET)		SENSITIVITY ANALYSES FNIH WOMAC pain=0 subset		SENSITIVITY ANALYSES FNIH WOMAC pain >0 subset	
Model	**TRAINING SET ROC-AUCs (95% CIs)**	**TRAINING SET PR-AUCs (95% CIs)**	**TESTING SET ROC-AUCs (95% CIs)**	**TESTING SET PR-AUCs (95% CIs)**	**TESTING SET ROC-AUCs (95% CIs)**	**TESTING SET PR-AUCs (95% CIs)**	**TESTING SET ROC-AUCs (95% CIs)**	**TESTING SET PR-AUCs (95% CIs)**
BMI	0.657 (0.588, 0.721)	0.886 (0.848, 0.926)	0.570 (0.473, 0.665)	0.812 (0.747, 0.882)	0.460 (0.348, 0.58)	0.547 (0.442, 0.678)	0.629 (0.524, 0.73)	0.767 (0.680, 0.864)
BMI, age, sex	0.689 (0.62, 0.753)	0.893 (0.856, 0.932)	0.593 (0.497, 0.687)	0.823 (0.757, 0.893)	0.515 (0.405, 0.629)	0.582 (0.472, 0.725)	0.636 (0.533, 0.732)	0.773 (0.687, 0.868)
ACTG	0.757 (0.697, 0.811)	0.934 (0.906, 0.956)	0.705 (0.612, 0.788)	0.897 (0.844, 0.941)	0.697 (0.591, 0.793)	0.750 (0.633, 0.855)	0.709 (0.617, 0.798)	0.854 (0.780, 0.917)
ACTG + BMI	0.776 (0.719, 0.825)	0.942 (0.92, 0.96)	0.703 (0.608, 0.787)	0.884 (0.828, 0.936)	0.650 (0.537, 0.754)	0.688 (0.563, 0.813)	0.733 (0.635, 0.822)	0.848 (0.766, 0.919)
23 RF peptides	0.929 (0.9, 0.955)	0.984 (0.977, 0.991)	0.848 (0.766, 0.916)	0.936 (0.884, 0.979)	0.837 (0.751, 0.913)	0.834 (0.717, 0.938)	0.854 (0.771, 0.922)	0.908 (0.833, 0.968)
23 RF peptides + BMI	0.938 (0.913, 0.961)	0.987 (0.98, 0.992)	0.853 (0.767, 0.923)	0.929 (0.874, 0.979)	0.832 (0.743, 0.913)	0.809 (0.694, 0.932)	0.864 (0.778, 0.932)	0.903 (0.825, 0.972)
17 uncorrelated peptides	0.919 (0.884, 0.949)	0.981 (0.97, 0.99)	0.841 (0.757, 0.913)	0.932 (0.878, 0.976)	0.834 (0.744, 0.913)	0.826 (0.708, 0.937)	0.846 (0.761, 0.918)	0.901 (0.823, 0.965)
17 uncorrelated peptides + BMI	0.925 (0.89, 0.953)	0.982 (0.972, 0.99)	0.845 (0.758, 0.918)	0.926 (0.871, 0.976)	0.829 (0.74, 0.911)	0.807 (0.691, 0.93)	0.854 (0.766, 0.927)	0.897 (0.818, 0.968)
8 significant peptides	0.907 (0.871, 0.938)	0.978 (0.967, 0.987)	0.833 (0.751, 0.905)	0.929 (0.876, 0.973)	0.830 (0.738, 0.911)	0.819 (0.700, 0.933)	0.835 (0.749, 0.907)	0.898 (0.819, 0.962)
8 significant peptides + BMI	0.916 (0.881, 0.945)	0.981 (0.971, 0.989)	0.842 (0.758, 0.914)	0.925 (0.87, 0.975)	0.824 (0.732, 0.906)	0.802 (0.686, 0.927)	0.853 (0.765, 0.925)	0.897 (0.818, 0.968)

23 peptides and BMI were selected based on random forest (RF)>0.9; 17 uncorrelated peptides were selected based on RF=1, WMW p value and uncorrelated status; 8 significant peptides were selected based on RF=1, WMW p value, uncorrelated status, and association with OA status in multivariable logistic regression; for the list of these sets of peptides see Table 2. 231 (39%) FNIH cases had baseline WOMAC pain scores=0

Table 4.

Youden Indices of biomarker sets for the diagnosis of OA.

Model	Threshold based on optimal Youden's index in training dataset	Sensitivity in testing dataset using training dataset threshold [95% CIs]	Specificity in testing dataset using training dataset threshold [95% CIs]
BMI	0.82	0.52 (0.46,0.59)	0.60 (0.54,0.66)
BMI, age, sex	0.85	0.42 (0.36,0.49)	0.82 (0.77,0.87)
ACTG	0.8	0.71 (0.66,0.77)	0.62 (0.56,0.68)
ACTG + BMI	0.73	0.71 (0.65,0.77)	0.67 (0.61,0.73)
23 RF peptides	0.86	0.81 (0.75,0.86)	0.82 (0.77,0.87)
23 RF peptides + BMI	0.81	0.86 (0.81,0.9)	0.76 (0.7,0.81)
17 uncorrelated peptides	0.83	0.77 (0.72,0.83)	0.84 (0.8,0.89)
17 uncorrelated peptides + BMI	0.79	0.88 (0.83,0.92)	0.73 (0.68,0.79)
8 significant peptides	0.81	0.78 (0.73,0.84)	0.78 (0.72,0.83)
8 significant peptides + BMI	0.9	0.94 (0.91,0.97)	0.69 (0.63,0.75)

For the list of the peptides in each set see Table 2; see Table S3 for the beta estimates for the models of demographics, Table S4 for models of the 23 RF peptides +/− BMI, Table S5 for models of the 17 uncorrelated peptides +/− BMI, and Table S6 for models of the 8 significant peptides +/− BMI