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Inflammatory, structural, and pain biochemical biomarkers may reflect radiographic disc space narrowing: The Johnston County Osteoarthritis Project

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Abstract

The purpose of this work is to determine the relationship between biomarkers of inflammation, structure, and pain with radiographic disc space narrowing (DSN) in community-based participants. A total of 74 participants (37 cases and 37 controls) enrolled in the Johnston County Osteoarthritis (OA) Project during 2006–2010 were selected. Cases had at least mild radiographic

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DSN and low back pain (LBP). Controls had neither radiographic evidence of DSN nor LBP. Measured analytes from human serum included N-cadherin, Keratin-19, Lumican, CXCL6, RANTES, IL-17, IL-6, BDNF, OPG and NPY. A standard dolorimeter measured pressure-pain threshold. Coefficients of variation (CVs) were used to evaluate inter- and intra-assay reliability. Participants with similar biomarker profiles were grouped together using cluster analysis. Binomial regression models were used to estimate risk ratios (RR) and 95% confidence intervals (CI) in propensity score matched models. Significant associations were found between radiographic DSN and OPG (RR=3.90 95% CI 1.83, 8.31), IL-6 (RR=2.54 95% CI 1.92, 3.36) and NPY (RR=2.06 95% CI 1.62, 2.63). Relative to a cluster with low levels of biomarkers, a cluster representing elevated levels of OPG, RANTES, Lumican, Keratin-19 and NPY (RR=3.04 95% CI 1.22, 7.54) and a cluster representing elevated levels of NPY (RR=2.91 95% CI 1.15, 7.39) were significantly associated with radiographic DSN. Clinical Significance: These findings suggest that individual and combinations of biochemical biomarkers may reflect radiographic DSN. This is just one step towards understanding the relationships between biochemical biomarkers and DSN that may lead to improved intervention delivery.

Keywords

disc space narrowing; biomarkers; epidemiology; phenotype; osteoarthritis

Introduction

Chronic low back pain (cLBP) affects more than 31 million Americans at any given time,[1] has increased threefold in prevalence in 10-years[2], and results in $100–$200 billion per year in expenditures[3]. Due to its association with cLBP,[4–7] a large amount of these expenditures are attributed to intervertebral disc (IVD) degeneration, as identified by disc space narrowing (DSN) on plain film x-ray. A consistent challenge with delivery of interventions has been which specific lumbar spine structures, if any, are the underlying source of cLBP. This challenge has led to the need to develop sub-groups or phenotypes, to decrease the large amount of heterogeneity in the diagnosis of cLBP.

Recently, several biochemical markers have been identified for their potential specificity to the structure of the IVD. N-Cadherin and Keratin-19, biomarkers of IVD structure have been identified as the top biomarker candidates for testing in clinical populations[8] and as a potential key for regenerative medicine efforts[9; 10]. Lumican, a proteoglycan that regulates collagen formation, may represent unique degradation properties of the IVD compared to peripheral joint OA.[11] In addition, recent pilot studies have identified inflammatory biomarkers, osteoprotegerin (OPG)[12], Interleukin-17 (IL-17)[13], RANTES and C-X-C Motif Chemokine Ligand 6 (CXCL6)[14] as significantly associated with lumbar spine IVD degeneration. Interleukin-6 (IL-6), an inflammatory cytokine, and Neuropeptide-Y (NPY), a neuropeptide involved in pain regulation and perception, have been also associated with cLBP.[15] Brain derived neurotrophic factor (BDNF), commonly studied as a pain biomarker[16], has also been found to be derived from IVD cells and may represent structural degradation[17]. Many of these biochemical biomarkers have undergone limited investigation in relation to DSN with human serum and most, to our knowledge, have not been studied in

a community-based sample. Understanding the relationship between these biochemical biomarkers and DSN in a community-based sample can help to uncover the natural history of DSN and intervertebral disc degeneration.

Chronic low back pain is a multidimensional condition with potential contributions from inflammatory, structural, and central pain-based mechanisms. Biochemical biomarkers may provide a means to differentiate groups of individuals on the basis of sources of cLBP such as structural, inflammatory, or central pain-based sources. Our interest was to determine if individual or combinations of biomarkers representing each of these domains were associated with DSN. Therefore, the purpose of this study was to: 1) determine the reliability of biochemical biomarkers with potential specificity to structure of the IVD, inflammation, or pain using human serum from a community-based sample, 2) determine the association between individual biochemical biomarkers and DSN, and 3) determine the association between clusters or combinations of biomarker profiles and DSN. Our hypothesis is that these biomarkers would individually and collectively subgroup individuals with different phenotypes of cLBP based on inflammatory, structural, and/or central pain mechanisms. This study will add to the literature by reporting foundational data to plan future longitudinal studies involving these biomarker-based phenotypes among a community-based sample.

Methods

Data for these analyses come from the Johnston County Osteoarthritis Project (JoCo OA), an ongoing community-based cohort study with the purpose to determine the incidence and progression of radiographic knee, hip and spine OA, the details of which have been described in detail elsewhere.[6; 18] A sample of 74 participants (37 cases and 37 controls) with pair-read lumbar spine radiographs and available serum biospecimens were selected from among 832 JoCo OA participants who enrolled in and completed a study visit between 2006–2010 (Figure 1). The JoCo OA Project recruited both White and African American participants, however our previous work has demonstrated that less than 19% of African Americans have DSN in the cohort.[6] This low overall proportion resulted in a very small number (n=5) available for this pilot analysis. In addition, we have found differences in biochemical biomarker levels by race in previous work.[19] As such only White participants were included in the pilot to decrease the heterogeneity in biochemical marker levels. To be eligible as a case, participants needed to have at least mild radiographic lumbar DSN and the presence of low back symptoms on most days of any one month in the last 6 months. To be eligible as a control, participants needed to have no radiographic evidence of lumbar DSN and report no low back symptoms at baseline and approximately 5 years later at follow-up assessment (2013–2015). Approval for this work was granted by the Duke University Institutional Review Board (Pr00086874).

Radiographic Spine Evaluation

By protocol, women of reproductive age (<50 years of age) were excluded from having lumbar spine radiographs. Lateral lumbar spine films were taken with the participant lying on his or her left side with the central beam centered at the lumbar spine. All lateral lumbar

spine radiographs were graded at each lumbar level by a single bone and joint radiologist without regard to participants' biomarker levels. The Burnett Atlas[20] was used to grade lumbar spine radiographic DSN in a semi-quantitative fashion (0=none, 1=mild, 2=moderate and 3=severe). However, DSN was dichotomized as absent (controls) or present (cases) for these analyses due to sparse data on severity of DSN across strata of cases. For these analyses, the outcome consisted of cases as the index and controls were the referent. We have previously reported the intra-rater radiologist reliability for DSN with a weighted kappa =0.89.[21]

Biochemical Biomarker Reliability and Validity

Details of the participants, collection, and methods of storage of biospecimens have been described elsewhere.[19] Briefly, all participants had blood collected at the clinic visit on the same day that radiographs were taken. Therefore, all samples were collected after completion of morning activity at a time (>1 hour after arising) when these serum markers have attained equilibrium.[22] Serum biomarkers were measured in duplicate for most biochemical biomarkers; however, due to the low volume of serum for this study, IL-6 and NPY were analyzed in singlicate. However, for both assays, the standard curve as well as manufacturer provided controls and a human control serum sample were run in duplicate to assess the mean intra-assay coefficient of variation for each assay (IL-6 – 2.1% and NPY – 0.77%). The manufacturer provided controls for the NPY assay were within the expected range. The reported intra- and inter-assay coefficients of variation for these assays are 3.2% and 5.5% for NPY and 4.0% and 6.4% for IL-6, respectively, demonstrating the reliability of these assays. Inter (within assay) and intra-rater (between assay) reliability measurements were calculated for all duplicate analyzed biochemical biomarkers. Coefficient of variation (CV) was the measure of reliability, with values below 15% representing good reliability. We also calculated the percentage of participants assay measurements that fell within the quantifiable range. The lower limit of quantification (LLOQ) for each assay was reported as the concentration of the lowest standard. MesoScale Discovery is unique in providing detailed information for each VPLEX assay (IL-17, IL-6) regarding the quantitative range. The LLOQ is defined as the lowest concentration at which the CV of the calculated concentration is <20% and the recovery of each analyte is between 80–120% of the known value and is determined for each kit lot. However, it should be noted that in the case of both IL-17 and IL-6, the CVs of all of the standards were <7%, indicating that concentrations above the lowest standard are reliable.

Demographic Characteristics

Demographic data were collected by clinical interview and examination, including age and, race (White or African American), and sex.

Clinical Characteristics

Body mass index (BMI) at the time of interview was calculated from height, measured without shoes, and weight, measured with a balance beam scale. Low back symptoms were collected at clinical interview by asking participants to answer "yes" or "no" to the question "On most days is the past 6 months have you had symptoms of pain, aching or stiffness in your lower back?". Participants underwent weight-bearing postero-anterior knee radiography

of both knees with a Synaflexer™ (CCBR-Synarc, San Francisco, CA) positioning device and bilateral hip radiography with supine anteroposterior pelvis radiographs. All hip and knee radiographs were read for Kellgren-Lawrence[23] score by a single bone and joint radiologist; inter-rater and intra-rater reliability have been reported previously with a weighted kappa of 0.86 and 0.89 for the hip and knee, respectively[24]. Hip and knee OA, for these analyses, were defined as a Kellgren-Lawrence score of 2–4 in at least one extremity.

Pressure-Pain Threshold and Depressive Symptoms

Pressure-pain threshold (PPT) measurements, using a standard mechanical pressure-based dolorimeter, were used to assess each participant's threshold (measured in kilograms) for pressure-pain at the upper trapezius. A single trained research assistant conducted all PPT clinical measurements. The measurement begins with a "practice trial" where a demonstration of the device is conducted with the participant. Measurements were then collected from both the left and right upper trapezius muscle in a systematic fashion consisting of two total measurements. Beginning with the left side, pressure was applied to the trapezius at a rate of 1 kg per second until self-reported pain. If a participant did not report pain at 4 kg, the value was recorded as ">4.0 kg". Trials were continued until two consecutive readings were within +/−0.4 kg for a maximum of four trials. The same procedure was repeated for the right side. Values from the left and right trapezius were averaged to provide a single PPT score. Depressive symptoms were measured with the Centers for Epidemiological Studies Depression (CES-D) Scale.

Analyses

Descriptive statistics in the form of means, medians, standard deviations, ranges or frequencies and percentages, as appropriate, were used to describe demographic, clinical characteristics and biomarker distributions. Concentrations of biochemical biomarkers less than the lower limit of detection (LLOD) were imputed at ½ x the LLOD.[25] Values above the LLOD but below the LLOQ (below the lowest standard) were extrapolated from the standard curve. Independent group t-tests or chi-square tests were used to determine differences in demographics, clinical and biochemical biomarker differences between cases and controls.

Biomarkers differed in their units of measurement and therefore, prior to modeling, all biomarkers were linearly transformed to a 0–1 scale to meet cluster analysis assumptions and allow interpretation across biomarkers with different scales of measurement.[26] Following transformation, one participant continued to have implausible levels (nearly 3,000 times the next highest level) of NPY and was therefore excluded from analyses. Due to differences in demographic and clinical characteristics between cases and controls we used coarsened exact matching[27], as a form of propensity score matching, to balance differences in demographic (age and sex) and clinical characteristics (BMI, knee OA, and hip OA) across cases and controls. This technique reduces imbalance in covariates by temporarily coarsening each variable, exactly matching on these coarsened data. This method has been used with rare exposures to avoid missing data that can occur with 1-to-1 matching.[28] Binomial regression was used to determine the association of each individual biochemical biomarker with radiographic DSN (cases versus controls). Binomial regression was chosen

to focus on risk ratios (RR) and to account for the overestimation of effect that can occur with logistic regression with prevalent outcomes.[29] These models were initially conducted as unadjusted, consisting of only the individual biochemical biomarker as the independent variable and cases with DSN versus controls without DSN as the outcome. Propensity score matched models were then conducted. Finally, the CES-D scale scores were added as a separate variable to the propensity score matched models to further adjust for depressive symptoms. We chose to adjust for CES-D scores since we have found in our previous work that depressive symptoms are associated with PPT and self-reported pain.[30]

We then conducted K-means cluster analysis to group together participants with similar biomarker profiles, ignoring case/control status. K-means cluster analysis was chosen as an exploratory unsupervised learning approach.[31] This approach has been used in other biochemical biomarker studies with smaller sample sizes and number of biomarkers.[26] A 4-cluster solution was chosen to allow for variation across the domains of biomarkers being analyzed (i.e., structural, inflammatory and pain). The number of clusters was motivated by 1) our small sample size which may not support a large number of clusters and 2) previous work with principal component analysis with these data in which the scree plots supported 4 distinct components.[32] Analysis of variance, chi-square tests, and in some cases, Fisher's exact tests, were used to determine differences (ignoring case / control status) for each demographic and clinical measure among clusters. Analysis of variance was used to determine differences of biomarkers across clusters (ignoring case / control status) with post-hoc pairwise mean differences, Tukey-corrected for multiple comparisons, for those with statistically significant overall main effects. Separate binomial regression models were then conducted for the clusters entered as a variable with four categories with indicator variables, with the first cluster as the referent group, and the outcome of cases with DSN versus controls without DSN as the outcome. Our regression models were first conducted as unadjusted bivariate associations between each cluster and case/control status. This approach was then conducted with propensity score models and CES-D score adjustment, as previously described above. As such, our analyses involving the clusters are examining the ratio of 1) the probability of being a case with DSN (versus being a control without DSN) within a particular cluster compared to 2) the probability of being a case with DSN in the referent cluster. Statistical significance was set at $p<0.05$ for all analyses. Risk ratios were the measure of association, and 95% confidence intervals were calculated as a measure of variability. All analyses were conducted in Stata v.15 (College Station, TX).

Results

Description of Reliability and Detection Rates of Biomarkers

Table 1 describes the manufacturer of the assay, biomarker means and distributions, within and between CVs, and the number and proportion of the total samples having concentrations greater than LLOD. The intra-assay CV for all biomarkers assessed ranged from 0.8% to 4.1% and inter-assay CV ranged from 1.3% to 7.7%. The LLOD was not reported by the manufacturer for both N-cadherin and Keratin-19. For these assays, a 10-fold dilution series of human serum samples ranging from 1:1 – 1:10,000 was run to determine the appropriate dilution of sample. For N-cadherin, a 1:20 dilution was used and for Keratin-19, samples

were run undiluted. The values for most biomarkers were above the LLOD. However, N-cadherin and OPG has a small percentage of values below the LLOD, 11% and 1% respectively. Biomarkers with a large proportion of values outside the LLOQ included Keratin-19 (46%), IL-17 (98.6%) and IL-6 (97%) although unlike Keratin-19 and IL-17, 100% of IL-6 values were above the LLOD and 97% of the concentrations were above the lowest standard.

Description of Demographic and Clinical Characteristics for Cases and Controls

Table 2 describes the demographic and clinical characteristics among the cases and controls. Among cases, DSN was mild in 5.4%, moderate in 46.0% and severe in 48.7% of participants. Cases were older (72.9 years [SD=9.7])) compared to controls (64.0 years [SD=6.8])). Cases had similar BMI to controls (30.7 [SD=6.3] vs. 30.3 [SD=5.6], respectively) and a greater proportion of knee (56.8% vs. 32.4%) and hip OA (51.4% vs. 35.1%). Among cases, cLBP was reported mild in n=13 (18%), moderate in n=19 (26.0%) and severe in n=5 (7.0%). Cases had significantly higher ($p<0.01$) OPG (mean=76.6 SD=32.3) than controls (mean=58.0 SD=25.1). Cases had significantly lower ($p=0.01$) PPT values (mean=3.5, SD=1.0) compared to controls (mean=3.9, SD=0.93). No other significant differences were identified among biomarkers.

Individual Biomarker Associations with Disc Space Narrowing

Table 3 presents the regression models between each individual biomarker with cases with DSN compared to controls without DSN as the outcome. Compared to controls, significant associations between inflammatory biochemical biomarkers and cases with radiographic DSN were identified for OPG (RR=4.41 95% CI 2.69, 7.24), IL-6 (RR=2.45 95% CI 1.89, 3.23) and NPY (RR=2.12 95% CI 1.68, 2.69), respectively. After adjusting these models for depressive symptoms, associations remained similar. No significant associations were found for IL-17, CXCL6, RANTES, BDNF or PPT in any of the regression models.

Differences in Demographic, Clinical Characteristics and Biochemical Biomarkers by Clusters

Table 4 describes the distribution of cases and controls, demographics, clinical characteristics, and biomarker distributions by each cluster. No significant differences were found among clusters with respect to cases and controls, demographic, or clinical characteristics. Biomarker levels were lower in the first cluster and therefore this cluster was used as the common referent for the regression analysis. In the second cluster, significantly greater levels of OPG were identified compared to the first, third and fourth clusters ($p<0.0001$ for all pairwise comparisons). Levels of RANTES were significantly greater in the second cluster compared to first and third clusters ($p<0.001$ for both comparisons). Levels of Keratin-19 were significantly greater in the second cluster compared to the first and fourth cluster ($p<0.001$ for both comparisons). Levels of Lumican were significantly greater in the second cluster compared to third and fourth clusters ($p<0.001$ for both comparisons). Levels of NPY were significantly greater in the second cluster compared to the first and third clusters ($p<0.001$ for both comparisons). In the third cluster, significantly higher levels were identified for CXCL6, Keratin-19 and BDNF compared to the first cluster ($p<0.001$). In the fourth cluster, significantly greater levels of NPY were identified compared

to the first cluster ($p<0.001$). No significant differences were found among clusters with respect to PPT. The distributions of each biochemical biomarker by cluster is illustrated in Figure 2.

Associations Between Clusters and Disc Space Narrowing

Table 5 describes the association between each cluster and cases with DSN and controls without DSN. No significant associations were identified in unadjusted analyses. In the propensity score adjusted models matched for age, sex, BMI, knee OA, hip OA, we found significant associations between case status and cluster membership. With cluster 1 as the referent (representing lower levels of biomarkers), a significant association (RR=3.04 95% CI 1.22, 7.54) was found among cases with DSN compared to controls without DSN for the second cluster (representing higher levels of OPG, RANTES, Keratin-19 and Lumican and NPY). A significant association (RR=2.91 95% CI 1.15, 7.38) was also found with cases with DSN compared to controls without DSN in the fourth cluster (higher levels of NPY). Adjusting these models for depressive symptoms slightly attenuated the association for the second cluster (RR=2.76 95% CI 2.76, 7.03) while it slightly increased the association for the fourth cluster (RR=2.97 95% CI 1.17, 7.52).

Discussion

We examined several biochemical biomarkers with potential specificity for inflammation and structure of the IVD as well neuropeptides associated with pain. Most of the biochemical biomarkers from this study demonstrated good reliability and validity. Individually, OPG, IL-6 and NPY were consistently associated with radiographic DSN. Following cluster analysis, we found significantly elevated levels of inflammatory, structural and pain biochemical biomarkers that differed across clusters, suggesting a unique molecular profile for individuals within each cluster. In regression analysis, we identified significant associations between radiographic DSN and one biomarker cluster representing elevated inflammatory, structure and pain biomarkers and as well as a second cluster consistent with an elevated pain biomarker.

In the analysis of biomarker level differences between cases with DSN and controls without DSN, only OPG had significantly higher levels among cases. OPG is a member of the TNF receptor superfamily and has been found to be associated with IVD degeneration in mice[33; 34] and human tissue samples[33]. Our findings of an association between OPG and DSN, indicative of IVD degeneration, are similar but slightly greater than those reported by Xue and colleagues using tissue sample confirmed IVD degeneration.[12] In propensity score matched regression analyses (matched for age, sex, BMI, knee OA, and hip OA) , we observed significant associations of OPG, IL-6 and NPY with DSN. This matching process created balance in potential confounding and selection bias factors between cases and controls that was not present in the analysis of biomarker level differences. IL-6 is a cytokine that has been associated with many different inflammatory diseases and conditions.[35] We found significant associations between IL-6 and DSN. These findings are consistent with the work of Weber and colleagues[36] who identified higher serum levels of IL-6 among participants with MRI confirmed degenerative disc disease compared to controls and those

with disc herniation. However, our IL-6 data should be interpreted with caution since despite 100% of the samples having concentrations greater than the lowest standard, 97% of the samples were below the lower level of quantification for the assay. NPY is a neuropeptide that was also significantly associated with DSN in our analyses. This is consistent with Sowa and colleagues who identified associations with NPY and cLBP among older adults.[15]

Some of the biochemical biomarkers we analyzed as part of the study have had very limited investigation in human serum for lumbar spine disease. Both Keratin-19 and N-Cadherin have been identified as potential candidates for human studies due to consistent findings in human and animal tissue samples with IVD degeneration.[8] The detection rates were good for N-Cadherin but the low percentage of values within the quantifiable range for Keratin-19 may limit this biomarker's reliability. However, we did not find individual statistically significant associations between any of the structural biomarkers (N-cadherin, Keratin-19 or Lumican) in these analyses. Although not statistically significant, the strength of associations for these biomarkers were modest and we have identified them to be correlated to one another in principal components analysis.[32] In the lumbar spine, these biochemical biomarkers are commonly linked to content of the nucleus pulposes[8], which may degrade during the degenerative process in the spine.[37] Advanced degradation of the nucleus pulposes may explain our lack of relationship in these analyses as our older participants had a high proportion of moderate and severe DSN and may not have active nucleus content turnover. Several of the inflammatory and pain / stress biomarkers (CXCL6, RANTES, IL-17 and BDNF) were also not significantly associated with DSN in our regression analyses. One reason for the lack of statistical significance may be due to our small sample size. However, the good detection rates for some of these biomarkers (Lumican, N-Cadherin, CXCL6, RANTES, and BDNF) and the magnitude of associations identified from individual biomarkers by regression analyses support continued examination in a larger sample.

Similar to work on biomarkers of inflammation in the knee[26], we conducted a cluster analysis to determine if participants would have similar profiles of the biomarkers within clusters. To our knowledge, previous lumbar spine studies have not utilized this approach across combinations of biomarkers. A single cluster contained significantly higher levels of inflammatory biomarkers OPG and RANTES, structural biomarkers Keratin-19 and Lumican as well as NPY. Previous studies have identified individual associations between NPY and RANTES with cLBP among older adults[26]. The occurrence of Lumican and Keratin-19 levels within this cluster suggests a phenotype of structural degradation of the IVD. Interestingly, compared across clusters, IL-17, IL-6 and N-Cadherin were not significantly elevated in any cluster. The many samples with low concentrations of IL-17 and IL-6 was surprising considering the consistent identification of these cytokines with IVD cells[38; 39] and LBP[36], respectively in other studies. One explanation for the low concentrations in our study may relate to the older age of our sample and the selection of our participants from a community-based population. The low concentrations of IL-17 and IL-6 may have contributed to the lack of association of these cytokines with DSN in our cluster analysis. BDNF is a neuropeptide that is commonly studied for depression / stress.[40] However, our group has identified BDNF expression by IVD cells[17] and skeletal muscle cells[41]; this may indicate utility for use of this biomarker to reflect structure of the IVD.

Our study has several inherent strengths such as being a community-based sample with a protocol driven approach to radiographic evaluation and serum collection. Our study, however, it is not without limitations. This pilot study was cross-sectional in design and therefore, we are only able to report associations between variables and causality cannot be inferred. This study was also designed to determine the reliability and validity of the selected biochemical biomarkers among a small sample of participants. Therefore, our sample size was not powered to detect significant associations with DSN. We were unable to conduct duplicate analysis for both IL-6 and NPY due to limited serum for this pilot. However, the reliability of these samples compared to the manufacturer provided controls were within expected range. Two of the biomarkers (Keratin-19 and IL-17) examined in this study had a large percentage of sample values outside the quantifiable range indicating that these biomarkers may have limited reliability. By contrast, although below the manufacturer reported lower limit of quantification, all IL-6 values were detectable and 97% had concentrations greater than the lowest standard; these data should nevertheless be interpreted with caution. Our sampling approach included cases with primarily moderate and severe DSN. Our controls were stable with no radiographic DSN at baseline or at follow-up approximately five years later. Therefore, these results may not be generalizable to the entire JoCo OA cohort. The JoCo OA study protocol excluded women of childbearing age from having lumbar spine radiographs to prevent unnecessary radiation exposure and the results may not be generalizable to this subgroup. The phenotype naming was based entirely on biomarker characterization, without consideration of clinical presentation. Future studies incorporating clinical data may further refine these phenotypes names. Furthermore, we found several significant differences demographic and clinical characteristics between baseline cases and controls suggesting potential selection bias and confounding. Our modeling approach suggested that differences in estimates were found when adjusting for these factors with coarsened exact matching. Therefore, we cannot rule out potential bias resulting from those factors not matched upon or controlled for in these analyses.

In summary, significant associations were found between individual biochemical biomarkers, OPG, IL-6 and NPY and cases with DSN compared to controls without DSN. Our exploratory clusters analysis suggests the potential for combinations of biochemical biomarkers to subgroup participants with some clusters having significant associations. However, the findings from this cluster analysis may differ from other analyses depending on several issues including the sample size, statistical approach to determine the number of clusters, and distribution of cases and controls in a sample. Our findings support the notion that biochemical biomarkers may be important towards understanding phenotype development. Combined with other measures such as patient reported outcomes, demographic, clinical characteristics, additional imaging findings and physical performance measures, these findings may assist with deconstructing the discordance between clinical imaging and chronic pain states. A larger, longitudinal study is needed to confirm these results so as to better understand if a temporal relationship exists between biochemical biomarkers and incidence and progression of DSN with and without lower back pain. Given the multidimensional nature of cLBP, different contributions to the underlying cause of pain are possible. We expect these contributions may stem from individual or combinations of inflammatory, structural or pain-based sources. The approach taken here to identify a

biochemical component is an important step towards understanding the relationships between biochemical biomarkers and DSN that may lead to improved intervention delivery.

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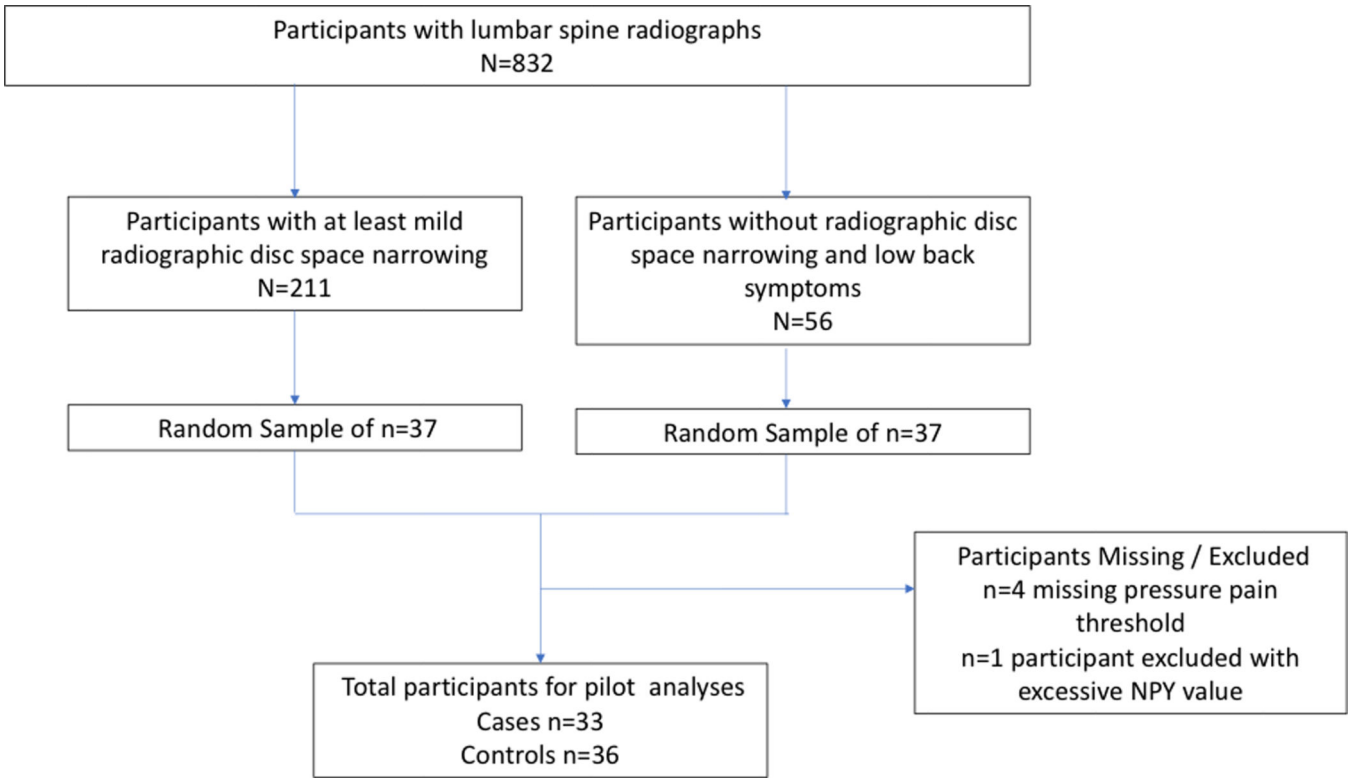
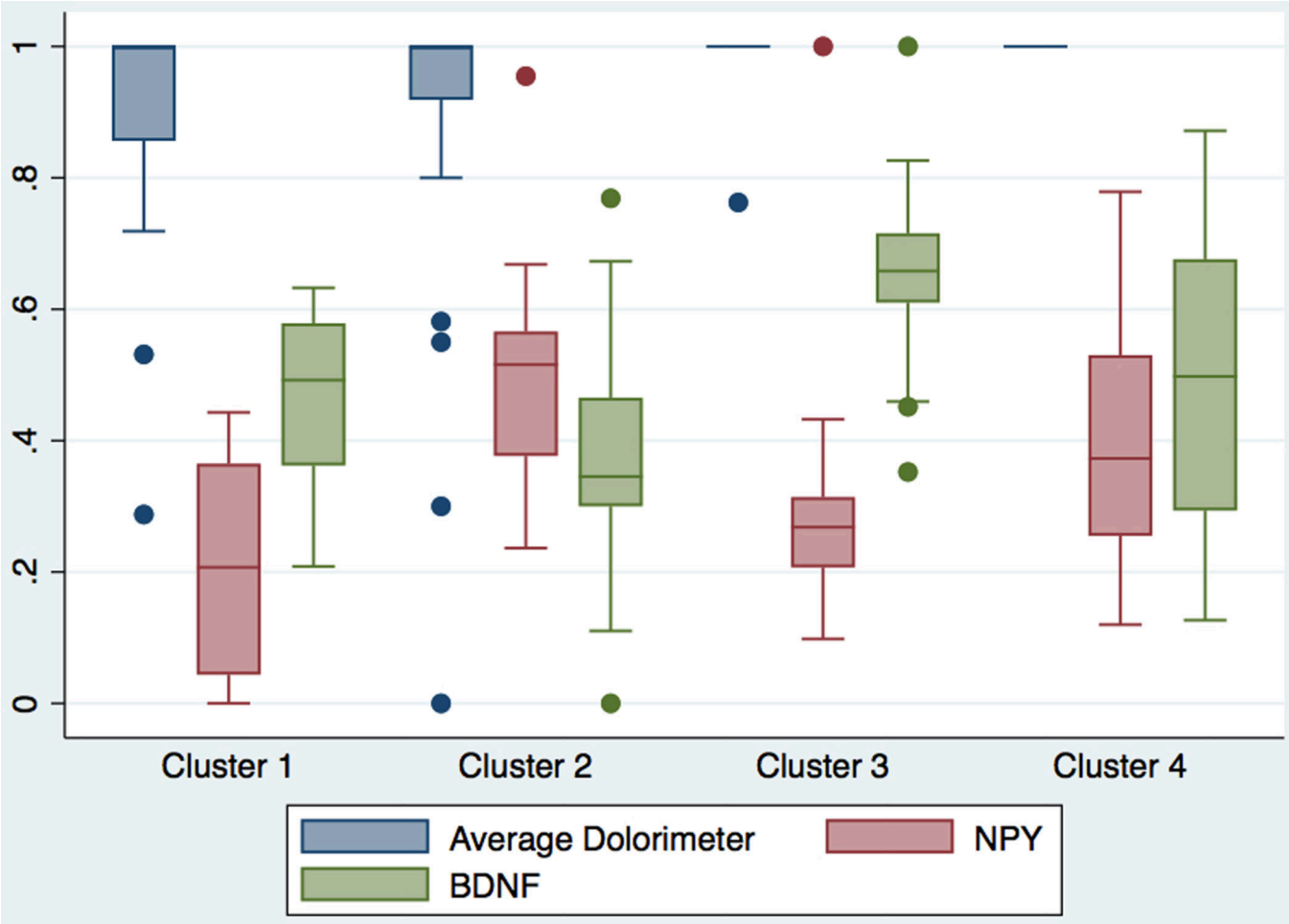
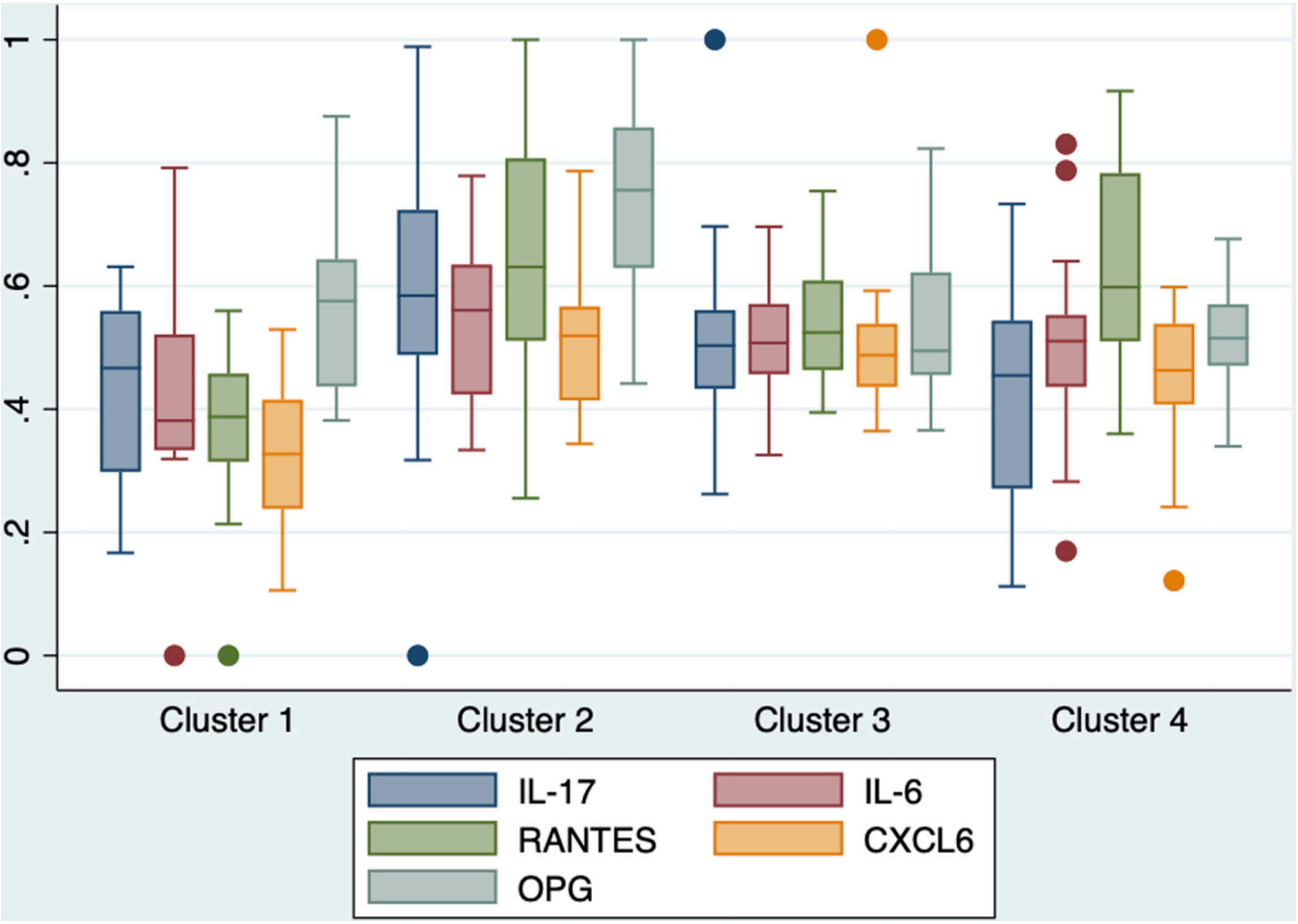


Figure 1.
Selection of cases and control participants from the Johnston County Osteoarthritis Project.





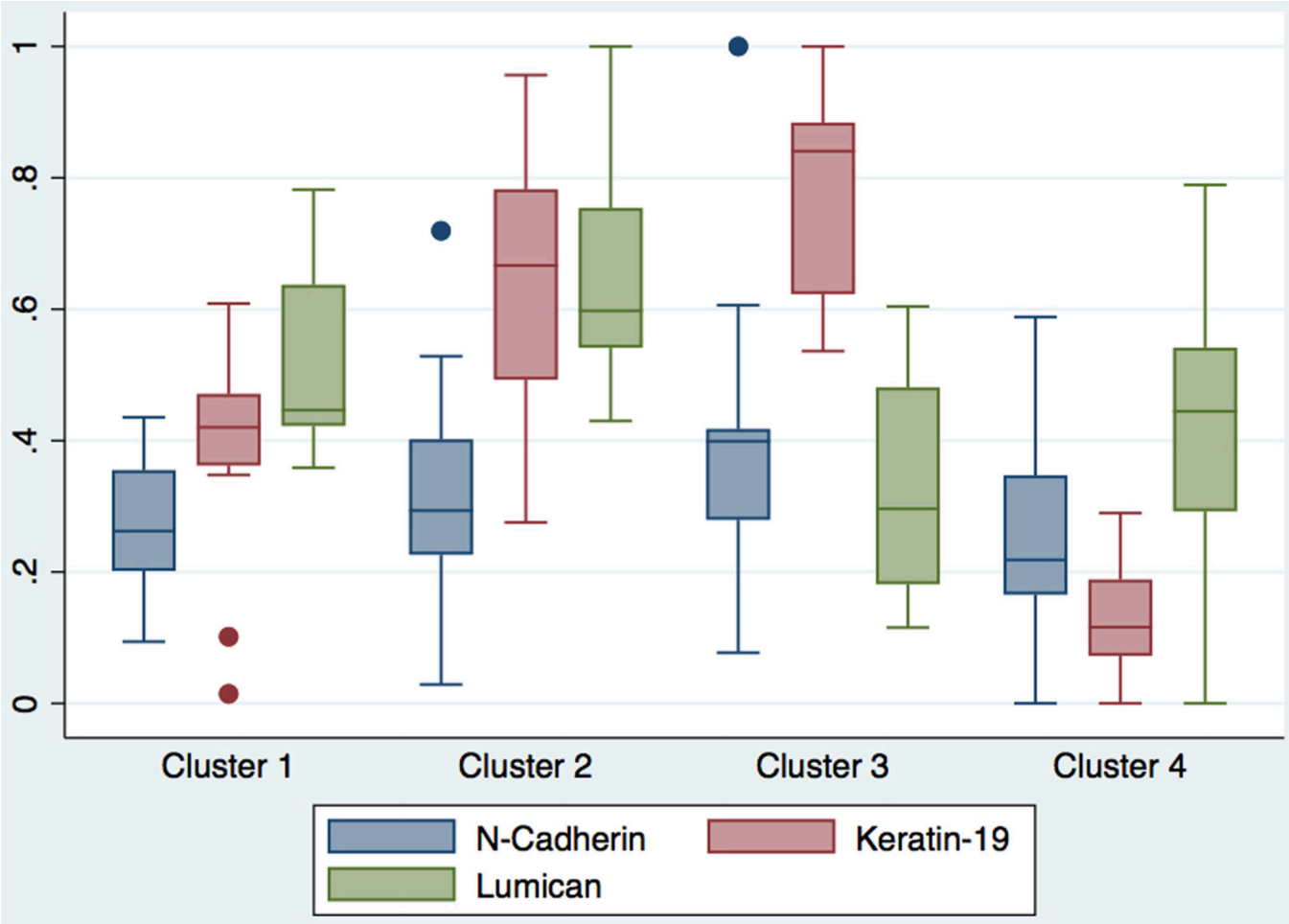


Figure 2.
Distribution of structural (A) inflammatory (B) and pain (C) biochemical biomarkers by clusters

Table 1.

Description of individual biomarkers, reliability and detection rates.

Biomarkers, units / Manufacturer	Mean (SD) (Median; Limits)	Intra-assay CVs	Inter-assay CVs	Quantitative Range of the Assay	LLOD	>LLOD %	Dilution	Within Quantitative Range of the Assay, %
Structure								
N-Cadherin, ng/ml / LifeSpan Bioscience (LS-F4699)	45.5 (7.9) (44.7; 30.7–78.7)	4.1%	4.8%	0.157–10 ng/ml	NS *	NA	20-fold **	100%
Keratin-19, ng/ml / LifeSpan Bioscience (LS-F4498)	0.36 (0.18) (0.33; 0.06–0.87)	3.9%	3.4%	0.31–20 ng/ml	NS *	NA	Undiluted **	46%
Lumican, ng/ml / RayBiotech (ELH-LUM)	104.7 (14.5) (105; 70.3 −139.1)	3.0%	7.1%	0.1–25 ng/ml	0.1 ng/ml	100%	100-fold	100%
Inflammation								
OPG, pg/ml /RayBiotech (ELH-OPG)	67.3 (30.2) (60.5; 0.4–143.6)	3.7%	6.6%	1.2–900 pg/ml	1.0 pg/ml	99%	2-fold	99%
IL-17, pg/ml / MesoScaleDiscovery (K151RFD)	1.1 (1.6) (0.66; 0.1–10.0)	4.0%	1.5%	9.32–3650 pg/ml	0.20 pg/ml	89%	2-fold	1.4%
CXCL6, pg/ml /R&D (DGC00)	175.5 (80.8) (165.1; 50.5–676.6)	4.0%	1.6%	31.3–2000 pg/ml	1.6 pg/ml	100%	Undiluted	100%
RANTES, pg/ml / MesoScaleDiscovery (K151BFC)	102,933.9 (48,498.2) (91,724.8; 12,772–234,641)	2.5%	7.1%	0.61–2500 pg/ml	0.3 pg/ml	100%	50-fold	100%
IL-6, pg/ml / MesoScaleDiscovery (K151QXD)	1.3 (1.0) (1.06; 0.2–7.6)	2.1%	N/A	1.58–488 pg/ml	0.05 pg/ml	100%	2-fold	3.0%
Pain								
BDNF, pg/ml /R&D (DBD00)	29,485.6 (8,573.7) (28,719.0; 10,129–51,571)	3.8%	7.3%	62.5–4000 pg/ml	20 pg/ml	100%	20-fold	100%
NPY, pg/ml /Millipore (EZHNPY-25K)	49.6 (15.1) (48.9; 20.6–360,876)	0.77%	N/A	5–1000 pg/ml	2 pg/ml	100%	Undiluted	100%

SD=standard deviation, OPG=osteoprotegerin, IL-17 = interleukin-17; CXCL6= C-X-C Motif Chemokine Ligand 6; RANTES=C-C Motif Chemokine 5; IL-6=interleukin-6;

*NS=not specified by manufacturer; NA=not applicable BDNF=brain derived neurotrophic factor; NPY=neuropeptide-Y; CV=coefficient of variation; LLOD = lower limit of detection;

**For these assays, a 10-fold dilution series of human serum samples ranging from 1:1 – 1:10,000 was run to determine the appropriate dilution of sample. For N-cadherin, a 1:20 dilution was used and for Keratin-19, samples were run undiluted.

Table 2.

Demographic, clinical, pain sensitivity, radiographic and biomarker levels by case and control status.

Variable	Cases (DSN present) N=37	Controls (DSN absent) N=37	p-value
Mean Age (SD)	72.9 (9.7); Range 55–93	64 (6.8); Range 51–79	**<0.001**
Sex, n (%)			1.00
Female	16 (43.2)	16 (43.2)	
Male	31 (56.7)	31 (56.7)	
Mean BMI (SD)	30.7 (6.3); Limits 19.7 – 47.2	30.3 (5.6); Limits 21.6 – 41.0	0.81
OA, n (%)			
Knee OA	21 (56.8)	12 (32.4)	**0.04**
Hip OA	19 (51.4)	13 (35.1)	0.16
DSN, n (%)			N/A
Mild	2 (5.4)	N/A	
Moderate	17 (46.0)	N/A	
Severe	18 (48.7)	N/A	
Depressive Symptoms, n (%)			0.67
Present	4 (10.8)	2 (5.4)	
Absent	33 (89.2)	35 (94.6)	
Low Back Pain, n (%)			**<0.001**
None	0 (0.0%)	37 (100.0%)	
Mild	13 (35.1%)	0 (0.0%)	
Moderate / Severe	24 (64.9%)	0 (0.0%)	
Biomarkers mean (standard deviation)			
N-Cadherin, ng/ml	45.0 (6.7)	46.1 (9.1)	0.55
Keratin-19, ng/ml	33.6 (21.7)	34.2 (20.3)	0.90
Lumican, ng/ml	106.0 (14.1)	103.4 (14.9)	0.45
OPG, pg/ml	76.6 (32.3)	58.0 (25.1)	**<0.01**
IL-17, pg/ml	0.96 (1.5)	1.30 (1.78)	0.38
CXCL6, pg/ml	162.4 (54.2)	188.5 (100.0)	0.17
RANTES, pg/ml	94,900.7 (41,770.7)	110,967.2 (53,777.8)	0.16
IL-6, pg/ml	1.40 (1.27)	1.17 (0.67)	0.33
BDNF, pg/ml	27, 840.5 (7,953.4)	31,130.6 (8,957.7)	0.10
NPY, pg/ml	51.4 (11.8)	47.8 (17.6)	0.31
Pressure-Pain Threshold, kg	3.5 (1.0)	3.9 (0.23)	**0.01**

OPG=osteoprotegerin, IL-17 = interleukin-17; CXCL6= C-X-C Motif Chemokine Ligand 6; RANTES=C-C Motif Chemokine 5; IL-6=interleukin-6; BDNF=brain derived neurotrophic factor; NPY=neuropeptide-Y; kg=kilogram. Bold items are statistically significant

Table 3.

Associations between individual biomarkers with radiographic disc space narrowing in unadjusted, propensity score matched models and matched and adjusted models.

Biomarker	Unadjusted Risk Ratio (95% CI)	Matched* Risk Ratio (95% CI)	Matched and Adjusted** Risk Ratio (95% CI)
Structural Biomarkers			
N-Cadherin,	0.68 (0.17, 2.75)	1.35 (0.36, 5.10)	1.27 (0.28, 5.91)
Keratin-19	0.95 (0.44, 2.02)	1.74 (0.78, 3.86)	1.65 (0.73, 3.73)
Lumican	1.52 (0.51, 4.57)	1.27 (0.36, 4.47)	1.18 (0.34, 4.07)
Inflammatory Biomarkers			
OPG	**3.43 (1.60, 7.32)**	**4.41 (2.69, 7.24)**	**3.90 (1.83, 8.31)**
IL-17	0.36 (0.04, 3.54)	0.20 (0.02, 2.20)	0.21 (0.02, 2.12)
CXCL6	0.20 (0.02, 2.29)	0.46 (0.03, 6.61)	0.50 (0.04, 6.86)
RANTES	0.48 (0.15, 1.51)	0.70 (0.21, 2.30)	0.71 (0.22, 2.30)
IL-6	**2.32 (1.78, 3.05)**	**2.45 (1.89, 3.23)**	**2.54 (1.92, 3.36)**
Pain Biomarkers			
BDNF	0.45 (0.15, 1.39)	0.62 (0.21, 1.83)	0.68 (0.22, 2.07)
NPY	**2.12 (1.68, 2.68)**	**2.12 (1.68, 2.69)**	**2.06 (1.62, 2.63)**
Pain Measure			
Pressure Pain Threshold	1.53 (0.96, 2.44)	0.99 (0.59, 1.65)	1.00 (0.60, 1.66)

*Matched for age, sex, knee OA, hip OA

**Matched for age, sex, knee OA, hip OA and Adjusted for Depressive Symptoms via CES-D score. Bold items are statistically significant

Table 4.

Descriptive statistics for demographic, clinical characteristics and biochemical biomarkers by clusters.

Variable	Cluster 1 (n=12)	Cluster 2 (n=25)	Cluster 3 (n=13)	Cluster 4 (n=19)	p-value
Case / Control					0.71
Cases	4 (33.3)	13 (52.0)	6 (46.2)	10 (52.6)	
Controls	8 (66.7)	12 (48.0)	7 (53.9)	9 (47.4)	
Mean Age (SD)	66.8 (6.9)	70.0 (11.4)	64.2 (7.1)	68.2 (8.5)	0.31
Sex, n (%)					0.05
Female	8 (27.6)	13 (44.8)	4 (13.8)	4 (13.8)	
Male	4 (10.0)	12 (30.0)	9 (22.5)	15 (37.5)	
Mean BMI (SD)	28.8 (4.9)	31.3 (7.0)	30.6 (4.5)	30.4 (5.9)	0.69
Low Back Pain, n (%)					0.12
None	8 (22.2)	12 (33.3)	7 (19.4)	9 (25.0)	
Mild	0 (0.0)	3 (23.1)	5 (38.5)	5 (38.5)	
Moderate / Severe	4 (20.0)	10 (50.0)	1 (7.7)	5 (25.0)	
Depressive Symptoms, n (%)					0.66
Absent	11 (16.4)	24 (35.8)	13 (19.4)	19 (28.4)	
Present	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	
Biomarker, Mean (SD)					
IL-17	0.64 (0.30)	1.64 (2.0)	1.43 (2.6)	0.64 (0.41)	0.17
OPG	58.8 (24.9)	**88.6 (29.5)**	56.8 (21.0)	51.1 (13.6)	<0.0001
RANTES	57,349.0 (22,093.0)	**121,961.3 (52,406.0)**	89,749.9 (26,379.1)	114,012.4 (43,241.2)	<0.001
IL-6	0.90 (0.75)	1.34 (0.66)	1.08 (0.44)	1.18 (0.85)	0.35
CXCL6	120.3 (36.2)	187.3 (56.5)	**212.5 (142.5)**	164.4 (45.5)	0.02
N-Cadherin	43.8 (5.4)	45.7 (7.3)	49.4 (11.0)	42.9 (7.4)	0.13
Keratin-19	27.7 (11.8)	**44.7 (14.6)**	**54.6 (11.2)**	9.9 (5.8)	<0.0001
Lumican	106.3 (10.1)	**114.8 (9.8)**	92.5 (12.2)	97.4 (14.2)	<0.0001
NPY	36.2 (12.4)	**56.8 (11.8)**	43.6 (16.7)	**49.7 (13.3)**	<0.0001
BDNF	29,287.8 (5,896.0)	**25,857.6 (7,630.3)**	31,326.2 (7,050.3)	29,767.7 (8,596.8)	<0.001
Pressure-pain threshold	3.5 (0.95)	3.5 (1.02)	3.9 (0.26)	4.0 (0.0)	0.05

Bold indicates significantly higher or lower levels

Table 5.

Associations between 4 clusters with radiographic disc space narrowing in unadjusted, propensity score matched models and matched and adjusted models.

Cluster	Unadjusted Risk Ratio (95% CI)	Matched* Risk Ratio (95% CI)	Matched and Adjusted** Risk Ratio (95% CI)
1 – Lower Biomarker Level Group	referent	referent	referent
2 – Inflammation / Pain / Structure	1.56 (0.64, 3.78)	**3.04 (1.22, 7.54)**	**2.76 (1.09, 7.03)**
3 – Inflammation / Structure	1.38 (0.34, 8.68)	2.60 (0.96, 7.04)	2.35 (0.86, 6.41)
4 – Pain	1.58 (0.64, 3.91)	**2.91 (1.15, 7.39)**	**2.97 (1.17, 7.52)**

*Matched for age, sex, knee OA, hip OA

**Matched for age, sex, knee OA, hip OA and Adjusted for Depressive Symptoms with CES-D score. Bold items are statistically significant