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Evaluation of Serum Cytokines in Cats with and without Degenerative Joint Disease and Associated Pain

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Abstract

Degenerative joint disease is common in cats, with signs of pain frequently found on orthopedic examination and radiographs often showing evidence of disease. However, understanding of the pathophysiology of degenerative joint disease and associated pain remains limited. Several cytokines have been identified as having a role in pain in humans, but this has not been investigated in cats. The present study was performed to use a multiplex platform to evaluate the concentration of 19 cytokines and chemokines in serum samples obtained from cats with and without degenerative joint disease and associated pain. Samples from a total of 186 cats were analyzed, with cats representing a range of severity on radiographic and orthopedic evaluations and categorized by degenerative joint disease scores and pain scores. Results showed that cats with higher radiographic degenerative joint disease scores have higher serum concentrations of IL-4 and IL-8, while cats with higher orthopedic exam pain scores have higher concentrations of IL-8, IL-2, and TNF-α increased concentration of IL-8 in degenerative joint disease and pain may be confounded by the association with age. Discriminant analysis was unable to identify one or more cytokines that distinguish between groups of cats classified based on degenerative joint disease score category or pain score category. Finally, cluster analysis driven by analyte concentrations show separation of groups of cats, but features defining the groups remain unknown. Further studies are warranted to investigate any changes in cytokine concentrations in response to analgesic therapies, and further evaluate the elevations in cytokine concentrations found here, particularly focused on studies of local cytokines present in synovial fluid.

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Keywords

Biomarker; immunoassay; multiplex; arthritis

Introduction

Degenerative joint disease (DJD) may be the most common disease in cats, with a prevalence of up to 92%, and increasing radiographic burden with increasing age (Lascelles, 2010). Radiographic evidence of DJD is frequently accompanied by changes in mobility, activity, and social interactions (Lascelles et al., 2010a; Slingerland et al., 2011; Klinck et al., 2012), yet discrepancies exist between radiographic signs, orthopedic exam findings, and owner-reports of impairment (Bennett and Morton, 2009; Freire et al., 2011). Treatment options for this pervasive disease are lacking, in part due to the difficulty in accurately assessing pain and associated disability in cats. There is a need for objective methods to measure DJD-associated pain in order to advance our understanding of the disease and our ability to develop effective treatments. Accelerometry has been useful as a surrogate measure of mobility and activity in cats (Lascelles et al., 2010b; Benito et al., 2013; Guillot et al., 2013b), and advances in analytical methods for accelerometry data are being developed. However, these monitors remain costly and data extraction and analysis cumbersome, and offer no insight into the pathophysiology of the disease.

Development of biomarkers is a growing initiative in the fields of osteoarthritis (OA) and pain research in human and veterinary medicine (Bauer et al., 2006; Orita et al., 2011b; Kraus et al., 2015). Biomarkers, generally, may comprise any physical, imaging, or biochemical marker of disease or disease symptomology. Ideally, these markers would be sensitive to the presence of disease, indicative of the severity of disease, and responsive to treatments for the disease. In human DJD/OA research, soluble biomarkers of central interest include markers for structural damage to the joint and inflammation, both of which contribute to the clinical manifestation of pain. Inflammatory cytokines, particularly IL-1β, IL-6, and TNF-α, have been implicated in the pathogenesis of pain and joint disease (Zhang J-M, 2007; Miller et al., 2014; Imamura et al., 2015), and elevated concentrations of cytokines have been detected in synovial fluid samples from human DJD/OA patients (Imamura et al., 2014). Several chemokines have also been demonstrated to be associated with chronic pain in both rodent models (Dawes et al., 2013) and human DJD/OA patients (Orita et al., 2011b; Lee et al., 2013).

Despite their promise, the clinical heterogeneity of the disease in humans and the fluctuations in biomarker concentrations associated with intermittent flaring of symptoms have led to the suggestion that combinations of biomarkers, rather than a single universal marker, will be needed in the study of OA (De Ceuninck et al., 2011). This holds true across the category designations biomarkers are suggested to be useful for - Burden of disease, Diagnosis, Prognosis, Efficacy of intervention, and Investigative markers (Bay-Jensen et al., 2016).

In cats, despite the widespread nature of the disease, and the difficulty in measuring the impact of the disease, soluble biomarkers have not been investigated as indicators of DJD

and associated pain. Suggestions from a recent proteomic and genomic study of cats with DJD found that gene expression differences between cats with DJD and an age-matched group of cats without DJD were particularly evident in three main pathways: immune function, apoptosis, and oxidative phosphorylation (Gao et al., 2013). Proteomic analysis of serum found that cats with DJD had an increase in components of the complement system as well as down-regulation of the complement system regulator clusterin (Gao et al., 2013). Up-regulation of the complement system can lead to activation of macrophages and inflammatory cytokine secretion (Haseeb and Haqqi, 2013), and cartilage matrix components can activate complement (Wang et al., 2011). Proteomic analysis of human synovial fluid also found increased expression of complement proteins in DJD/OA patients compared to individuals without OA (Wang et al., 2011). It follows, therefore, that the pattern of cytokine expression may be different in cats with DJD and associated pain compared to cats without DJD, and that this difference could potentially be detected in serum.

The development of multiplex technologies has allowed for the simultaneous quantification of multiple analytes using a very small volume (10–25μL) of sample. Recently, a feline-specific panel has been developed and validated that allows quantification of 19 cytokines and chemokines using multiplex technology[1]. Given the associations between cytokines and painful DJD/OA in synovial fluid samples from humans (Orita et al., 2011b; Dawes et al., 2013), this study was designed to use the feline-specific panel to evaluate concentrations of cytokines and chemokines in well-phenotyped cats with and without DJD. Furthermore, as a preponderance of cats with clinically evident DJD have concurrent chronic kidney disease (CKD, 68% of cats in the study population in one report) (Marino et al., 2014), we sought to evaluate the interactive effect of pain and CKD status on cytokine concentrations. The effects of age and body condition were also investigated as age-associated inflammation is established in people (Greene and Loeser, 2015) and obesity and increased adipokines have been implicated as risk factors for the development of DJDs (Iannone and Lapadula, 2010).

Our central hypotheses were that cytokine/chemokine profiles in cats with DJD would differ from those of normal cats, that these profiles could be used as surrogate measures of pain in cats with DJD, and in cats with DJD and concurrent CKD, and that combinations of cytokines/chemokines would be able to distinguish between cats with painful DJD and normal cats.

Methods and materials

Subjects and samples

Samples used in this study were banked serum samples that had been collected from cats presented to the Comparative Pain Research Laboratory over the period from May 2007 – May 2015 (with approval from the North Carolina State University College of Veterinary Medicine Institutional Animal Care and Use Committee). The cats included in this study represented a mix of cats that presented for several studies, including those that were actively recruited as normal controls (where there was a requirement for no owner-noted

[1]FCYTMAG-20K-PMX Feline Cytokine/Chemokine Magnetic Bead Panel Premixed 19-Plex; EMD Millipore, Billerica, MA USA

mobility impairment) and those that presented for studies of treatments for DJD (Lascelles et al., 2010c; Benito et al., 2013; Gruen et al., 2015). Cats recruited to these DJD studies were required to have owner-noted mobility impairment. All cats had been examined by a veterinarian, and had been evaluated for systemic disease, orthopedic pain, and radiographic evidence of DJD as previously described (Lascelles et al., 2010c; Benito et al., 2013; Gruen et al., 2015). Briefly, all cats received a physical examination, including evaluation of body condition score (BCS) (Laflamme, 1997), followed by an orthopedic examination during which each joint or spinal segment was palpated and gently manipulated, and scored for the presence and severity of pain, crepitus, thickening, and effusion. Pain was scored on the following scale: 0 = no resentment; 1 = mild withdrawal, mild resistance to manipulation; 2 = moderate withdrawal, body tenses, may orient to site, may vocalize or increase vocalization; 3 = orients to site, forcible withdrawal from manipulation, may vocalize, hiss, or bite; and 4 = tries to escape or prevent manipulation, bites or hisses, marked guarding of site. Total pain (TPain) scores were calculated as the sum of the scores for individual joints, with a possible range of 0–80, and were further categorized for some analyses as described in the Statistical Analysis section.

Following the physical and orthopedic examinations, cats were sedated using an individually tailored protocol and orthogonal digital radiographs were made of each joint and spinal segment. Radiographs were evaluated by a single investigator (BDXL) and scored for the presence and severity of DJD using previously published criteria established by our laboratory (Lascelles et al., 2010c). Scores were ascribed according to a 10-point scale where 0 = no evidence of DJD and 10 = ankylosis of the joint. Total radiographic DJD (TDJD) scores were calculated as the sum of the scores for individual joints, with a possible range of 0–200, and were further categorized for some analyses as described in the Statistical Analysis section.

Prior to sedation, all cats had urine samples collected for urinalysis by cystocentesis, and blood samples collected for a complete blood count, serum biochemistry analysis, and for sample archiving. While the timing of the sample collection was not recorded, sample collection typically occurred following the physical examination and prior to the orthopedic examination. However, cases may have occurred where samples were collected following the orthopedic exam as this was not recorded across all studies. For serum samples, whole blood was collected into a sterile 3mL anti-coagulant free plastic tube (red top Vacutainer®) and allowed to clot at room temperature for at least 30 but no more than 60 minutes. Clotted samples were centrifuged at 1163 x *g* for 10 minutes, and serum was removed, aliquoted, and stored in cryovials at −80°C until use. Based on results from the serum biochemistry, urinalysis, and review of radiographs and previous medical records, cats were classified as CKD positive or negative according to the guidelines set forth by the International Renal Interest Society (Lascelles et al., 2010c) and described in Marino et al. (Marino et al., 2014). All examinations and sample collections were performed prior to any treatments being given to the cats. In addition, all cats were required to be either naïve to treatment for pain, or have a 4-week washout period prior to presentation.

Stored serum samples were eligible for inclusion in the study if they had been collected from cats that had been evaluated in the Comparative Pain Research Laboratory who had received

a physical and orthopedic examinations, and radiographic evaluation of each joint and spinal segment. Further inclusion criteria included that the samples had been maintained at −80°C and had not been through more than 1 freeze-thaw cycle prior to testing.

Cytokine assays

Samples were analyzed for concentrations of 19 analytes using commercially available feline-specific multiplex cytokine kits[1]. Given the exploratory nature of the study, kits were chosen that provided an opportunity to evaluate a variety of cytokines and chemokines in a manufacturer validated assay. Kits were used according to manufacturer recommendations, and samples were run in triplicate with all replicates from an individual cat run on the same plate. Quality control samples were run, in duplicate, on each plate.

Plates were analyzed using a dedicated plate reader and software[2,3] and results were analyzed using statistical software packages[4,5]. For each plate, quality control samples, standard curves, and bead counts were assessed according to manufacturer recommendations. Coefficients of variation (CVs) were evaluated for the results from each set of triplicates; CVs less than 20% were considered acceptable (Findlay et al., 2000; Valentin et al., 2011). If a set of replicates had a CV greater than or equal to 20%, individual results were examined. Despite limitations to this approach, if two replicates from a set of three were in close agreement, and the third was markedly different, this third replicate was flagged and the results carried forward were based on the mean of the two remaining replicates. However, if none of the three replicates were in close agreement, results for that set of replicates were not included in any further analyses. Where CVs were acceptable, output results were based on the mean of all three replicates.

Statistical analysis

Descriptive data were tabulated to obtain distributions for age, sex, body condition score, TPain score, TDJD score, and CKD status among the cats. Given that the majority of the cats (85%) were domestic short, medium, and long haired cats (i.e., not pure-bred cats), breed was not included in any of the analyses. TPain scores and TDJD scores were evaluated as both continuous variables and as categorical variables according to the following designations (based on clinically relevant distribution of scores from the prevalence study of randomly selected cats across age groups) (Lascelles et al., 2010c). TPain scores were categorized as 0–2 = negligible/normal (as long as no single joint received a score of 2); 2–4 = low (a score of 2 was placed in this category if a single joint received a score of 2); 5–9 = moderate; ≥10 = high. Given the difficulty in evaluating cats for pain, and unknown significance of low pain scores, analyte concentrations were compared between cats with pain scores in the negligible/normal and low categories using ANOVA. As no significant differences were found between these groups for any analyte, they were pooled into a normal/low group. This was clinically meaningful as cats in this combined category would be considered clinically unaffected by joint pain. TDJD scores were categorized as 0–3 =

[2]MAGPIX; Luminex Corporation, Austin TX USA
[3]xPONENT v.4.2; Luminex Corporation, Austin TX, USA
[4]MILLIPLEX ANALYST v.5.1, EMD Millipore, Billerica, MA USA
[5]SAS v.9.4; Cary, NC USA

negligible/normal; 4–12 = low; 13–24 = moderate; ≥25 = high. Again, given the unknown significance of low DJD scores, analyte concentrations were compared between cats with DJD scores in the negligible/normal and low categories using ANOVA, and were subsequently pooled for further analyses as the normal/low group, with the exception of one analysis (IL-18 analyte concentration as a function of DJD score category and CKD status) where a significant difference between the normal and low groups was found. Cats were also categorized as CKD negative or CKD positive (IRIS Stage 1 – 4, inclusive) as described.

Results where concentrations were below the lower limit of quantification (LLOQ) for a given analyte were imputed as equal to the LLOQ/√2 (Croghan C, 2003), while results that were unable to be used (due to high CVs) were set as missing and designated NA. Analytes with greater than 50% of results below the LLOQ were excluded from the analyses. To address issues of fit, natural log transformation was applied to analyte concentration data for all analyses except correlations.

Correlations between the analyte concentration and TPain and TDJD scores were calculated for each cytokine. The effects of BCS, age, and sex on the natural log transformed analyte concentrations were modeled using ANOVA.

To explore relationships between pain, DJD, CKD, and analyte concentration, several analyses were performed. Natural log transformed analyte concentrations were modeled as functions of:

1. Pain category (3-level), CKD status (Pos/Neg), and the interaction of Pain category and CKD status;
2. Pain category (3-level), DJD category (3-level), and the interaction of Pain category and DJD level; and
3. TPain score, TDJD score, and CKD status (Pos/Neg).

In order to determine if a group of analytes would discriminate between cats with DJD and pain, and those without, discriminant analysis was used in the following comparisons: 1. Cats with normal and low pain/DJD versus cats with moderate and high pain/DJD; 2. Cats with normal and low pain versus those with moderate or high pain in the absence of DJD; and 3. Cats with normal and low DJD versus cats with moderate and high DJD in the absence of pain. Discriminant analysis was performed using step-wise selection to identify analytes for entry into the model. Once analytes were identified, canonical discriminant analysis was run using identified analytes to evaluate model fit, and plots were visually inspected for group separation.

Cluster analysis was performed to allow measured analyte concentrations to delineate groupings of cats, with the intent of identifying unifying features among the clusters. Following clustering, a pseudo-T-squared plot was evaluated to determine the optimal number of clusters. Univariate analyses were used to evaluate the relationship between BCS, age, TPain score, and TDJD score and cluster identification.

For all analyses, significance was set at $p < 0.05$. As these were exploratory analyses, correction for multiple testing was not performed, but comments on the significance given

the number of tests performed is presented in the discussion. All assumptions for each test were evaluated, including assumptions of normality.

Results

Samples from a total of 186 cats were analyzed. Descriptive data for cat age, BCS, sex, and CKD status within each pain and DJD category are presented in Table 1. Pain score data from four cats are missing due to incomplete orthopedic exams (exams unable to be completed due to temperament). Data from these four cats were retained for analyses restricted to DJD scores and DJD score categories.

Assay results for each analyte were tabulated by the number and percent of samples that were above and below the LLOQ, and presented in Table 2. Fifteen of the 19 analytes had results above the LLOQ for at least 50% of the sample sets. Four analytes did not meet this criterion (FAS, GM-CSF, IL-1 β, and PDGF-BB) and were excluded from further analyses. The percentage of samples that were in range and below the LLOQ for each analyte were tabulated by DJD score category and pain score category and are provided as Supplementary Table 1.

Correlations between analyte concentration and TPain and TDJD scores show few significant relationships

Results from correlations between untransformed analyte concentration and TPain and TDJD scores are shown in Table 3. Significant correlations were found between analyte concentration and TPain score for TNF-α and between analyte concentration and TDJD score for IL-4, however correlations for each of these results were considered low (r= 0.24 and r=0.14, respectively).

Body condition score, age, and sex are each significantly associated with individual analytes

Evaluation of the relationship between BCS, age, and sex with natural log transformed analyte concentrations found that overall model results were not significant for the majority of analytes tested, with the exception of IL-13 ($p<0.001$), IL-2 ($p=0.007$), IL-8 ($p=0.006$), and CXCL-1 ($p=0.045$). Specific results showed that BCS was significantly and positively associated with cytokine concentration for IL-13 and IL-2; age was significantly and positively associated with cytokine concentration for IL-8; and sex was significantly associated with cytokine concentration for CXCL-1 (concentrations in females were higher than males) (Table 4). The estimates (the pg/mL increase in cytokine concentration expected for every unit increase in parameter) was low for IL-8, even when considering age is typically measured in years.

Pain score category, but not CKD status, is associated with concentrations of IL-2 and IL-8

Overall model effects for the natural log transformed analyte concentrations and categorical pain score, CKD status, and the interaction between categorical pain score and CKD status showed significant effects for IL-8 ($p<0.0001$) and MCP-1 ($p=0.024$), and near-significance for IL-2 ($p=0.052$). Significant effects were found for pain score category for IL-2 ($p=0.027$)

and IL-8 (p=0.006), while no specific significant effects were found for MCP-1 (all p>0.05). Least-squares means for IL-2 and IL-8 are shown in Table 5. For both IL-2 and IL-8, analyte concentration increased with worsening pain score category. Neither CKD status nor the interaction of pain score category and CKD status had a significant effect on analyte concentration.

DJD score category and the interaction of DJD score category and pain score category are significantly associated with concentrations of IL-4 and IL-8

Overall model effects for transformed analyte concentrations and DJD score category, pain score category, and the interaction of DJD score category and pain score category found significance for IL-4 (p=0.016) and IL-8 (p<0.001). Significant effects were found for DJD category for IL-8 (p=0.038) and for the interaction of DJD category and pain score category for IL-4 (p=0.020) with near significance for DJD category (p=0.056). Tukey-Kramer post-hoc testing showed the same positive relationship for IL-8 concentration reported in the previous model, where concentrations were higher with worsening DJD score category, with a significant difference between the normal/low and the high pain categories. The interaction between DJD score category and pain score category and effect on analyte concentration was less clear for IL-4, with concentrations remaining fairly stable across DJD score categories for the high pain score group, increasing across DJD score categories for the moderate pain score group, and increasing and then decreasing across DJD score categories for the normal/low pain group (Table 6), however the only significant differences were between the analyte concentrations in the high and normal/low DJD category within the moderate pain category.

Increasing TDJD score and TPain score, but not CKD status, are associated with increased concentrations of IL-4, IL-8, and TNF-α

We modeled the natural log of analyte concentrations as a function of TDJD score, TPain score, and CKD status, and found significant overall effects for IL-4 (p=0.043), IL-8 (p<0.0001), and TNF-α (p=0.022). Post-hoc analysis showed that TDJD score was significantly and positively associated with both IL-4 and IL-8 concentrations, while TPain score was significantly and positively associated with both IL-8 and TNF-α concentration (Table 7). No significant effects were found for CKD status and any analyte.

Increased concentration of IL-8 in DJD and pain may be confounded by the association with age

The IL-8 results showed increased serum concentration with increases in DJD score and pain score, and an increased concentration with increased age. We ran two separate models with age as the response variable and pain score category or DJD score category as the explanatory variables; both models were highly significant (p<0.0001) indicating that the relationships between pain score and age, and DJD score and age are too correlated to separate meaningfully. However, using a stepwise selection model allowing for DJD category, CKD status, pain category, age, and all the two-way interactions, only pain category remained in the model suggesting that increased pain score category explains more of the increase in IL-8 concentration than age.

Discriminant analysis was unable to identify one or more cytokines that distinguish between groups of cats classified based on DJD score category or pain score category

Three canonical discriminant models were run to determine if the results from one or more analytes could be used to distinguish between groups of cats based on their DJD score category or pain score category. Canonical discriminant analysis is a method that "finds linear combinations of quantitative variables that provide maximal separation between classes or groups" (Guillot et al., 2013a). Groups of cats undergoing comparison are shown in Table 8.

The first discriminant analysis comparison was between cats with no or low pain and no or low DJD and those with moderate and high pain and DJD. Based on results from step-wise selection, the analytes selected for inclusion in the canonical discriminant analysis model were IL-8 ($p=0.018$) and FLT-3L ($p = 0.024$). The adjusted R^2 of this model was 0.026, and histograms show poor separation of the groups (Figure 1-A). The second comparison was to distinguish between cats with no or low pain (without DJD) from those with moderate or high pain (without DJD). Analytes selected for inclusion in this canonical model were MCP-1 ($p = 0.079$), IL-8 ($p = 0.049$), IFN-γ ($p = 0.072$), and IL-13 ($p = 0.043$). Again, the adjusted R^2 of this model was quite low (0.037) and histograms showed poor separation of the groups (Figure 1-B). The final comparison was between cats with normal and low DJD versus cats with moderate and high DJD in the absence of pain. The analytes selected for inclusion in this model were IL-6 ($p < 0.001$) and IL-8 ($p = 0.003$). The adjusted R^2 of this model was low (0.019) and histograms showed poor separation of the groups (Figure 1-C).

Cluster analysis driven by analyte concentrations show separation of groups of cats, but features defining the groups remain unknown

Cluster analysis was performed using the analyte concentrations to drive the clustering. Observation of the pseudo-T plot indicated that 4, 5, 9, 11, and 15 clusters were appropriate to use. Median values of 5 and 9 clusters were chosen, with 5 clusters ultimately providing reasonable separation of the groups (Figure 2) and number of cats per cluster. Adjusted R^2 for this model with 5 clusters was 0.472, and all analytes except IL-8 ($p=0.302$) and CXCL-1 ($p=0.333$) were significant for the clusters. Univariate and contingency analyses were run to determine the relationship between known variables and cluster identification, with no significant findings for age, BCS, TDJD score, and TPain score (all $p>0.05$).

Discussion

This study showed that a small number of cytokines, IL-4, IL-8, IL-2, and TNF-α are associated with higher radiographic burdens of DJD and higher orthopedic pain scores. Specifically, we showed that cats with higher radiographic DJD scores have higher serum concentrations of IL-4 and IL-8, while cats with higher orthopedic exam pain scores have higher concentrations of IL-8, IL-2, and TNF-α. The association of increased IL-8 concentration with increasing pain score and DJD score was strong, but the confounding increase in IL-8 with increasing age encourages caution regarding conclusions about the importance of this finding. As there were not age matched control samples available for analysis, this finding may be more strongly associated with age than with DJD or pain,

however pain score category appeared to explain more of the increase in IL-8 concentration than age in our model. A recent study has suggested that DJD in cats is associated with changes in genetic and proteomic profiles more extreme than simple aging (Gao et al., 2013).

Based on our findings, we suggest that there are measureable changes in cytokine concentrations in cats with DJD that reflect true associations with DJD burden and pain. As normal ranges for cytokine concentrations in cats have not been determined, we cannot conclude that the increased concentrations found here are greater than normal, but only that they appear increased in cats with DJD and pain relative to normal cats. A recent study by Halpin et al. (Halpin et al., 2016) has supported our findings that several analytes from these kits are below the currently detectable levels in normal cats. Establishment of normal ranges using appropriately phenotyped cats would allow for calculations of positive predictive value based on the findings presented here. Nevertheless, our findings are supported by literature evaluating the associations between cytokines in DJDs in humans (Kapoor et al., 2011; Miller et al., 2014). There is growing interest in the interaction between nociceptors and the immune system, and the function that these interactions play in facilitating pain states (McMahon et al., 2015; Miller et al., 2015). The role of TNF-α in nociceptor sensitization is well supported in the literature (reviewed in (Schaible, 2014)); it has been shown to target nociceptors both directly (via modulation of ion channels) and indirectly (through increased release of downstream cytokines). In addition, TNF-α is able to rapidly increase the firing of A- and C-fibers in the dorsal root ganglion, increasing transmission of input into the spinal cord ((Schafers et al., 2003) and reviewed in (Taves et al., 2013)). Further, one study demonstrated a correlation between serum TNF-α concentration and pain scores in people with knee OA as measured by the Western Ontario and McMaster Universities Osteoarthritis Index (Orita et al., 2011b). There is growing interest and some demonstrated efficacy of targeted therapies against TNF-α in reducing joint pain (Ohtori et al., 2015), though further studies are needed.

TNF-α is commonly mentioned in conjunction with IL-6 and IL-1β, interleukins also capable of sensitizing nociceptors and frequently implicated in the pathogenesis of pain in DJD/OA (Miller et al., 2014). In our study, IL-1β was only detected (above the lower limit of quantification) in 36% of the samples tested, and IL-6 was not associated with any of our measures of DJD or pain. The reason for this discrepancy between our findings and the literature base is unknown. While this could be a species-specific difference, it is more likely that it relates to the use of serum, as most studies of cytokines in DJD/OA have been conducted using *in vitro* preparations or synovial fluid. Still, IL-1β (Imamura et al., 2014) and IL-6 (Imamura et al., 2015) have been detected in serum from patients with OA and our findings deserve further investigation. Il-2 and IL-8 have been less consistently associated with pain, though each has been investigated in experimental or chronic pain conditions. IL-2 has been demonstrated to have both hyperalgesic and analgesic properties in rodent models, dependent on location and route of administration (Song et al., 2002; Cata et al., 2008). In one study, the concentration of IL-8 was found to be higher in plasma samples from OA patients than controls (Mabey et al., 2014), and another study found higher levels of IL-8 mRNA expression from synovial biopsies in OA patients than patients with meniscal tears (Nair et al., 2015), but other studies have failed to show a strong relationship between

IL-8 and pain. It does appear that IL-8 is one of the cytokines downstream of TNF-α that may be produced by chondrocytes (Lotz et al., 1992). In the study reported here, IL-4 concentration was positively associated with radiographic DJD score. To our knowledge, this has not been demonstrated before, and merits further verification and potentially investigation. IL-4 is classically considered an anti-inflammatory cytokine, and may be a marker of chronic inflammation, which could explain the increase seen here in cats with long-standing disease.

Cytokines are ubiquitous as they are involved in cell signaling throughout the body, and may be produced by multiple cell types. The cytokines found to be elevated in this study have many potential sources, including peripheral leukocytes or local sources such as monocytes/macrophages, synovial cells, or chondrocytes in the affected joints. While synovitis in degenerative elbow joints in cats has been shown to be relatively mild (Freire et al., 2014), synovitis in OA in people is also typically scored as mild-moderate, and yet there is recognition of the inflammatory component to the disease (Berenbaum, 2013). Toll-like receptor 4 (TLR4) has been particularly implicated in contributing to inflammation in DJD/OA, with increased TLR4 expression in areas of damaged cartilage in patients with OA (Kim et al., 2006). TLR4 signaling results in an increased inflammatory response and expression of several inflammatory mediators (Gomez et al., 2015). Cytokine production may also occur at the level of the dorsal root ganglion; astrocytes and microglia have been shown to release TNF- α and IL-6 in response to TLR4 activation during inflammation (Taves et al., 2013), and activated microglia in the dorsal root ganglia have been noted in rodent models of DJD/OA (Orita et al., 2011a). Widespread central nervous system glial activation has also been noted in human patients with chronic lower back pain (Loggia et al., 2015). Regardless of source, the increases demonstrated here, detected in the serum, likely indicate that low-level systemic inflammation is present in cats with DJD. The detrimental effects of even mild systemic inflammation on health are the focus of a growing area of research. Low-grade inflammation has been implicated in the development of several chronic disorders and central nervous system diseases including Alzheimer's disease (Le Thuc et al., 2015; Ryan and Nolan, 2015). Other possible sequelae of systemic inflammation include sickness behaviors, insulin resistance, diabetes mellitus, and autoimmune diseases. Cats are susceptible many of these, including an analog cognitive disorder (termed cognitive dysfunction syndrome) and the epidemiological links between these disorders and DJD deserves further research.

Despite the associations discovered, we were unable to determine a cytokine-associated signature of DJD-associated pain in cats. In this study there was no single cytokine or group of cytokines that could reliably distinguish between cats with and without pain and DJD, cats with and without pain in the absence of DJD, or between DJD categories among cats with pain. There are several potential reasons for this, not the least of which is the remarkable complexity with which the balance of cytokines exists in the body, and the multiple sources of these cytokines. A recent study evaluated the ability of serum biomarkers to distinguish between groups of people with rheumatoid arthritis (RA), OA, and those free of disease using artificial neural networking. The resulting decision tree involved 15 nodes, with TNF-α at the top, and failed to group RA, OA, or normal patients together, highlighting the complexity of the interactions (Heard et al., 2014). Other potential explanations overlap

with limitations of the current study, notably that cats were examined and samples were taken at a single time point, while cytokine concentrations may change over time or over the course of the disease. Criteria for staging DJD in cats have not yet been developed, and are hindered by lack of understanding of the critical features of worsening clinical disease, but it is possible that sub-populations of DJD phenotypes in cats exist and are associated with different inflammatory profiles.

The use of serum samples, rather than synovial fluid, has been mentioned previously as another potential limitation in our ability to detect differences between groups of cats. Cytokines and chemokines are typically produced and consumed locally, and detection of increased concentrations in peripheral blood would likely reflect much higher concentrations at the sites of release. An additional limitation in the use of serum is the contribution to cytokine load from comorbid conditions including age (Greene and Loeser, 2015), obesity (Thijssen et al., 2015), and chronic kidney disease (Habenicht et al., 2013). While we evaluated the relationship between measured cytokine concentrations and age, body condition, and the presence or absence of chronic kidney disease and found few positive associations, each likely contributes to the overall circulating concentration of cytokines in these cats. In DJD research, the majority of soluble biomarker work has been performed using synovial fluid samples as these are reflective of the microenvironment within the damaged joint. Unfortunately, synovial fluid is difficult to obtain from cats, even from degenerative joints where a low to moderate amount of effusion is present. The banked serum samples in our lab, from well-phenotyped cats, were a reasonable starting point for this work, as the use of serum is more clinically applicable, and findings from serum could be compared to findings in synovial fluid from a smaller number of cats. Future work using synovial fluid samples from degenerative and normal joints of cats should be performed.

Additional limitations include potential systematic measurement error for TPain scores, as cats were evaluated by one of three separate investigators. Rigorous training of investigators by the lead veterinarian (BDXL) mitigated the impact of this, and all investigators involved in the studies were licensed veterinarians with clinical experience with cats. A further limitation of the TPain score is that it depends on cats' reactions to manipulation rather than spontaneous pain-related behavioral changes. Given the lack of a uniform method to assess these changes, we chose to focus on pain during the orthopedic examination. However, incorporation of these spontaneous measures would be beneficial to understanding clinically-relevant pain in cats. No such limitation is expected regarding DJD scores as a single investigator (BDXL) completed all DJD scoring based on previously published criteria developed by our laboratory.

The noted limitations were motivation for our second approach to cluster analysis, allowing the concentrations of the cytokines to drive the clustering of the cats. This analysis led to better separation of the groups of cats, and the majority of the cytokines used in the analysis were significantly associated with the clustering. However, the common features among cats in a given cluster are yet to be determined. The clustering of the cats does not appear to relate to age, body condition, DJD or pain. Whether these clusters are unrelated to the presence or absence of pain, or represent a limitation of our phenotyping that can be addressed remains to be ascertained.

Another potential limitation of the current study is the age of some of the samples, as these were collected over years of work with cats by our lab. Stability of cytokines in serum has been studied in human samples, but no work has yet been done in cats. Based on the literature using human samples, cytokine concentrations can decrease over years of storage, even when held in −80°C (de Jager et al., 2009; Jackman et al., 2011), and it is reasonable to expect that some degradation could have occurred. As older samples were not systematically different from other, more recently collected samples (i.e., not all normal cat samples were older), the likely impact of sample age would be on decreasing our ability to detect a difference between groups. This must be balanced by a reduction in power if those samples were excluded. Samples included in this study were controlled for freeze-thaw cycle, as concentrations of cytokines have been shown to change (both increase and decrease) in response to repeated freeze-thaws. Again, this has not been systematically evaluated in serum samples from cats, but initial work by the manufacturer of the cytokine panel has shown that this is a potential confounder for cytokines in cats (personal communication) if multiple freeze-thaw cycles occur. Finally, the use of the commercially available panel artificially focused our study to the provided analytes. Other soluble biomarkers of interest, based on findings in other species, include markers of cartilage degradation such as cartilage oligomeric protein or matrix metalloproteinases in serum, or CTX-II in urine (reviewed in (Bay-Jensen et al., 2016)). However, the panel was chosen for discovery work due, in part, to the paucity of feline-specific assays and availability of the technology. Despite its limitations, the 19 analytes provided by the panel include many of those of interest in DJD/OA and pain, and mark the first investigation of these analytes in a population of cats with and without DJD and pain. Further, the multiplex platform allows for screening of multiple analytes using a very small volume of sample, and specific findings from this study can be followed with targeted ELISAs, particularly to verify both the increases found and the absence of increases in IL-1β and IL-6.

In conclusion, we found that there are measurable increases in cytokines that are associated with increased burden of DJD and increased pain in cats relative to cats without DJD and pain. Despite the inability of one or more cytokines to reliably discriminate between groups of cats with and without DJD and pain, this work contributes to the burgeoning field investigating the interactions between the immune system and DJD/OA associated pain. Further work to verify these findings, and extend investigation into the sources of the increased cytokines will help elucidate the mechanisms underlying the associations found here. These findings also support the inclusion of cats in the important translational work on naturally-occurring arthridities, and the need for development of feline-specific assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CV	Coefficient of variation
DJD	Degenerative Joint Disease
LOQ	Limit of Quantification
LLOQ	Lower Limit of Quantification

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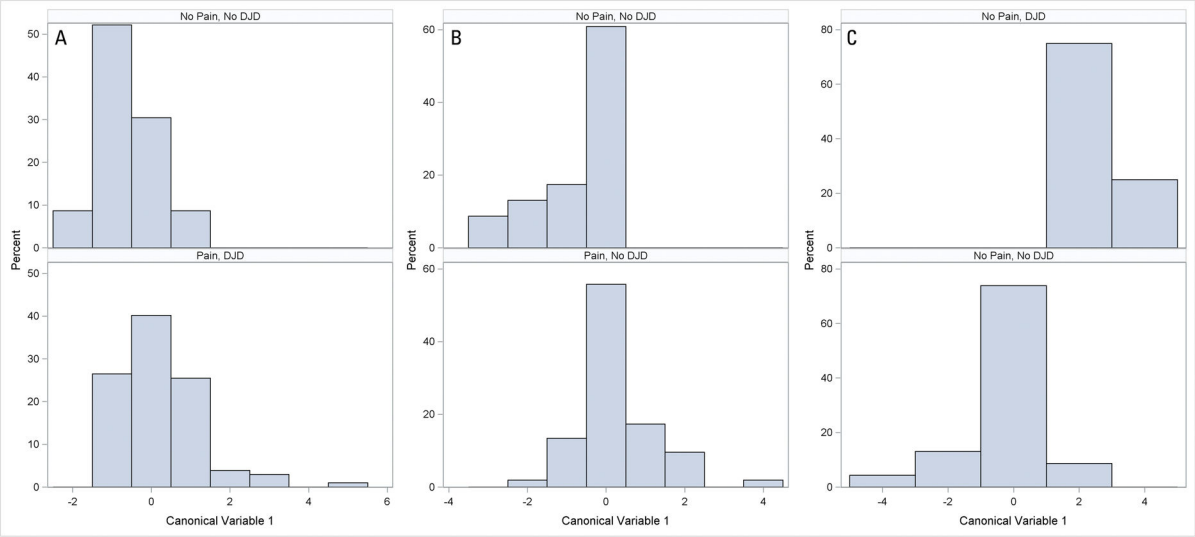

Figure 1.

Histograms showing canonical discriminant analysis results for (A) cats with normal and low pain/DJD (No Pain, No DJD) versus cats with moderate and high pain/DJD (Pain, DJD); (B) cats with normal and low pain in the absence of DJD (No Pain, No DJD) versus those with moderate or high pain in the absence of DJD (Pain, No DJD); and (C) cats with normal and low DJD in the absence of pain (No Pain, No DJD) versus cats with moderate and high DJD in the absence of pain (No Pain, DJD). Poor separation of the groups is seen for all three analyses.

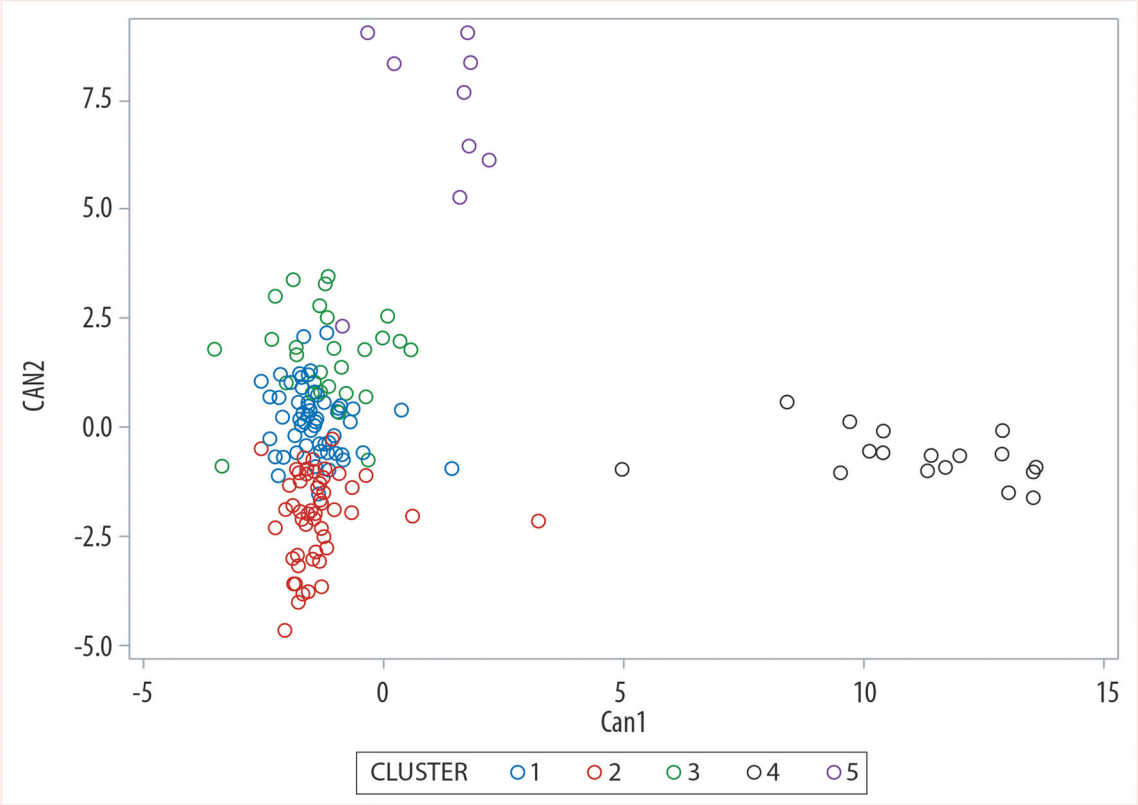

Figure 2.
Results using five cluster showing improved separation of the groups of cats, particularly those in clusters four and five as compared to one, two, and three.

Table 1

Descriptive data for the age, body condition score (BCS), and chronic kidney disease (CKD) status of cats across the pain and DJD categories. Category designations are derived from results of the orthopedic (pain) and radiographic (DJD) evaluations while CKD status is based on International Renal Interest Society guidelines.

DJD Category	Pain Category	N	Age (Median)	Age (Min)	Age (Max)	BCS (Median)	BCS (Min)	BCS (Max)	CKD Status (#Positive)	Sex (Proportion Male)
Normal	Normal	11	4.32	1.41	18.30	5	1	6	0	0.55
	Low	5	3.43	1.04	5.75	5	4	7	2	0.60
	Moderate	2	4.30	2.57	6.03	7.5	7	8	0	1.00
	High	11	9.41	2.07	18.30	6	4	9	3	0.73
Low	Normal	3	5.74	4.92	9.91	4	4	7	1	0.00
	Low	4	10.36	2.05	17.02	5	1	5	3	0.75
	Moderate	5	13.49	10.32	15.55	7	4	7	3	0.80
	High	35	10.76	3.23	16.02	7	4	9	9	0.46
	Missing	1	18.31	18.31	18.31	7	7	7	1	1.00
Moderate	Low	3	11.27	8.31	12.63	7	5	7	0	0.67
	Moderate	5	10.39	5.01	19.27	7	3	8	2	0.60
	High	42	13.02	6.30	18.30	7	3	9	16	0.43
	Missing	2	10.22	9.02	11.41	5	4	6	1	0.00
High	Low	1	18.30	18.30	18.30	3	3	3	0	1.00
	Moderate	7	13.32	10.98	17.01	7	5	7	3	0.29
	High	48	13.48	4.76	19.89	7	1	9	20	0.42
	Missing	1	16.05	16.05	16.05	5	5	5	1	0.00

Table 2

Number of samples that were above (In Range) and below (Low) the LLOQ, and the percentage of samples above the LLOQ for each analyte. NA represents the number of sample sets with missing data. Analytes with >50% of samples above the LLOQ are in bold. None of the samples were above range.

Analyte	Below LOQ or in range?				
	In Range	Low	NA	Total	% In Range
FAS	83	103	0	186	44.6%
FLT-3L	185	0	1	186	**99.5%**
GM-CSF	27	159	0	186	14.5%
IFN-γ	159	26	1	186	**85.5%**
IL-12P40	185	0	1	186	**99.5%**
IL-13	183	3	0	186	**98.4%**
IL-18	165	21	0	186	**88.7%**
IL-1β	67	118	1	186	36.0%
IL-2	105	81	0	186	**56.5%**
IL-4	175	11	0	186	**94.1%**
IL-6	140	45	1	186	**75.3%**
IL-8	162	24	0	186	**87.1%**
CXCL-1	96	90	0	186	**51.6%**
MCP-1	179	6	1	186	**96.2%**
PDGF-BB	37	149	0	186	19.9%
RANTES	186	0	0	186	**100.0%**
SCF	185	0	1	186	**99.5%**
SDF-1	137	49	0	186	**73.7%**
TNF-α	96	90	0	186	**51.6%**

Table 3

Correlation, p-values, and sample sizes between analyte concentrations and TPain scores as well as analyte concentrations and TDJD scores. Significant correlations are in bold.

Analyte	Value	TPain Score	TDJD Score
FLT-3L	Correlation coefficient	0.13	0.049
	P-value	0.08	0.508
	N	181	185
IFN-γ	Correlation coefficient	−0.006	0.006
	P-value	0.934	0.938
	N	181	185
IL-12P40	Correlation coefficient	0.006	−0.088
	P-value	0.941	0.235
	N	181	185
IL-13	Correlation coefficient	0.138	0.098
	P-value	0.064	0.184
	N	182	186
IL-18	Correlation coefficient	0.007	−0.092
	P-value	0.927	0.214
	N	182	186
IL-2	Correlation coefficient	0.119	0.067
	P-value	0.109	0.365
	N	182	186
IL-4	Correlation coefficient	0.048	**0.144**
	P-value	0.516	**0.049**
	N	182	**186**
IL-6	Correlation coefficient	0.103	0.037
	P-value	0.166	0.614
	N	181	185
IL-8	Correlation coefficient	0.093	0.048
	P-value	0.214	0.517
	N	182	186
CXCL-1	Correlation coefficient	0.049	0.029
	P-value	0.515	0.696
	N	182	186
MCP-1	Correlation coefficient	−0.067	−0.075
	P-value	0.373	0.311
	N	181	185
RANTES	Correlation coefficient	0.119	0.097
	P-value	0.11	0.186
	N	182	186

Analyte	Value	TPain Score	TDJD Score
SCF	**Correlation coefficient**	**0.136**	**0.06**
	P-value	0.069	0.419
	N	181	185
SDF-1	Correlation coefficient	0.005	0.03
	P-value	0.951	0.683
	N	182	186
TNF-α	Correlation coefficient	**0.241**	0.113
	P-value	**0.001**	0.123
	N	**182**	186

Table 4

Specific results for analytes with significant overall model effects (IL-13, IL-2, IL-8, and CXCL-1). Shown are the analyte, the parameter with the significant relationship, and the size and direction of the relationship.

	Parameter	Coefficient Estimate	Standard Error	t Value	Pr > |t|
IL-13	BCS	0.162	0.042	3.82	<0.001
IL-2	BCS	0.149	0.057	2.62	0.009
IL-8	Age (days)	0.0002	0.0001	2.94	0.004
CXCL-1	Sex (FS)	0.486	0.207	2.35	0.020

Table 5

Least squares means and standard errors for IL-2 and IL-8 showing the differences between the natural log of analyte concentrations and the levels of pain score category for each of the analytes. Analyte concentrations for both IL-2 and IL-8 were significantly different between the high pain score category and the normal/low category (with higher levels in the high pain category cats), but not significantly different between normal/low and moderate, or between moderate and high.

Analyte	Pain Score Category	Log(Analyte) Least-Squares Mean	Standard Error
IL-2	Normal/Low	1.778	0.320
	Moderate	2.246	0.321
	High	2.659	0.124
IL-8	Normal/Low	2.131	0.276
	Moderate	2.409	0.278
	High	2.973	0.107

Table 6

Results of post-hoc testing (least squares means and standard errors) showing the differences between analyte concentrations between levels of both the pain score category and DJD score category for IL-4. Statistically significant differences were found between moderate pain/normal-low DJD and moderate pain/high DJD, with non-significant differences between the remaining category combinations.

Analyte	Pain Category	DJD Category	Number	Mean	Std Error
IL-4	Normal/Low	Normal/Low	8	3.832	0.277
	Normal/Low	Moderate	9	4.625	0.766
	Normal/Low	High	7	3.336	1.328
	Moderate	**Normal/Low**	**5**	**2.652**	**0.502**
	Moderate	Moderate	6	4.260	0.594
	Moderate	**High**	**4**	**5.330**	**0.502**
	High	Normal/Low	2	4.293	0.196
	High	Moderate	3	4.408	0.205
	High	High	1	4.449	0.192

Table 7

Results of post-hoc testing for analytes with significant overall model effects (IL-4, IL-8, and TNF-α). Shown are the analyte, the parameter(s) with the significant relationship(s), and the size and direction of the relationship(s).

Analyte	Parameter	Coefficient Estimate	Standard Error	t-Value	Pr > |t|
IL-4	TDJD score	0.020	0.008	2.68	0.008
IL-8	TDJD score	0.015	0.007	2.30	0.023
	TPain score	0.049	0.013	3.66	<0.001
TNF-α	TPain score	0.051	0.022	2.33	0.021

Table 8

Distribution of cats into categories for discriminant analyses. Four cats were excluded due to missing pain scores. Shaded blocks show the number of cats in groups that are compared within the series of discriminant analyses.

Table of Pain Category by DJD Category					
Pain Category	**DJD Category**				
	Normal	**Low**	**Moderate**	**High**	**Total**
Normal	11	3	0	0	14
Low	5	4	3	1	13
Moderate	2	5	5	7	18
High	11	35	42	48	137
Total	29	47	50	56	182