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CCL22 is a biomarker of cartilage injury and plays a functional role in chondrocyte apoptosis

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ABSTRACT

Background: Osteoarthritis (OA) is one of the leading causes of disability worldwide. Previous history of knee injury is a significant risk factor for OA. It has been established that low-level chronic inflammation plays a pivotal role in the onset and pathogenesis of OA. The primary aim of this research was to determine if a history of knee joint injury is associated with systemic inflammation. A secondary aim was to determine if systemic inflammation is related to knee pain and joint structure.

Methods: Differences in serum cytokine association networks, knee joint structural changes (MRI), and self-reported pain (i.e., Knee Injury and Osteoarthritis Outcome Score Pain subscale, $KOOS_{PAIN}$ and Intermittent and Constant Osteoarthritis Pain score, ICOAP) between individuals who had sustained a youth (aged 15–26 years) sport-related knee injury 3–10 years previously and age- and sex-matched controls were examined. Proteins of interest were also examined in an OA rat model.

Results: Cytokine association networks were found to differ significantly between study groups, yet no significant associations were found between networks and $KOOS_{PAIN}$ or MRI-defined OA. A group of cytokines (MCP1/CCL2, CCL22 and TNFα) were differentially associated with other cytokines between study groups. In a pre-clinical rat OA model, serum CCL22 levels were associated with pain ($r = 0.255$, $p = 0.045$) and structural changes to the cartilage. CCL22 expression was also observed in human OA cartilage and furthermore, CCL22 induced apoptosis of isolated human chondrocytes.

Discussion: These results suggest that CCL22 may be an early factor in the onset/pathogenic process of cartilage degeneration and/or related to pain OA.

1. Introduction

Knee osteoarthritis (OA) is one of the leading causes of disability worldwide and is universally recognized as a major public health concern [1]. There is increasing evidence that previous history of knee injury is a significant contributor to OA. Prospective studies have reported that knee injury increases the risk of developing radiographic knee OA by ~10 times [2] and it is estimated that 12% of cases of symptomatic OA in the hip, knee, and ankle are due to post-traumatic OA (PTOA) [3]. In fact, more than 50% of individuals with an anterior cruciate ligament (ACL) tear or meniscus injury go on to develop PTOA [4,5]. Furthermore, meta-analysis indicated that even after ACL

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reconstructive surgery, there is still a 3.62-fold risk (range, 2.40–5.47; $P < 0.00001$) of developing PTOA 10 years after such injury [6]. While this information speaks to the increased risk of developing PTOA following knee injury, the time from injury to OA can vary dramatically from years to decades [7]. OA can only be clinically diagnosed at later stages when symptoms (e.g., pain, joint immobility) appear and joint damage/structural changes are severe enough to be detectable radiographically [8]. Because of the lack of effective disease modifying treatments, knee arthroplasty aimed at restoring mobility and quality of life is often the final step for patients with severe OA. Therefore, many researchers in the OA field, strongly believe that OA should be treated/managed at the earliest stages of the disease, when no symptoms have yet appeared and before radiographic evidence of OA is present [9,10]. While this would be ideal, we are yet unable to definitively identify patients at these early stages of the disease, and therefore novel methods for identifying early-stage OA are still required.

Early joint structural changes often include minor soft tissue damage, cartilage defects, meniscal damage, and bone marrow lesions (BMLs); most of these changes cannot be seen on radiographs and are observed using magnetic resonance imaging (MRI) [11]. Sharma et al. reported that these MRI lesions could be representative of early OA as they were associated with the incidence of radiographic OA and subsequent persistent symptoms in participants who initially had Kellgren and Lawrence (K/L) = 0 [12]. A similar study followed 50 individuals with knee scores of K/L = 0 that developed radiographic OA (K/L ≥ 2) 4 years later and found that cartilage T2 values at MRI could predict the development of radiological tibiofemoral OA [13]. However, MRI identified minor tissue damage is also common in the general aged population [14], limiting the specificity of MRI-based prediction of future development of OA.

Intermittent, activity related pain is another sign of early OA [15]. Pain is the hallmark symptom in late stage OA and is the major cause of disability in OA patients [1]. Evidence has shown that there is a poor association between pain and radiographic or MRI findings [16], suggesting that pain is the result of a more complex mechanism than simply early state structural changes. However, animal studies have suggested that OA pain is strongly associated with peripheral and central sensitization throughout the continuum of disease, and this process involves a variety of chemical mediators including inflammatory cytokines [17].

Based on pre-clinical and clinical research over the past decade, OA is no longer considered as simply “wear and tear”. Increasing evidence suggests that low-level chronic inflammation plays a pivotal role in the onset and pathogenesis of OA [18]. Therefore, changes within the expression or activity of effector molecules (e.g. cytokines, growth factors, enzymes) could be prognostic before MRI detectable tissue damage or pain symptoms appear. Disappointingly, so far, no single biochemical marker has proven to be sufficiently robust for the diagnosis of early OA. One possible reason is the complex nature of the pathogenesis of OA, such that a single biochemical marker could be invalidated by various confounding variables including diet, physical activity, and systemic metabolism [8]. To counteract these issues, high-throughput (omic) techniques with advanced, system-level analysis approaches have been employed for the development of OA diagnostics at the advanced/late stage by our group and others. However, these omic based approaches typically require using a broad range investigation of numerous factors [19]. While not yet directly targeted to early OA, such assays consisting of broad-spectrum biomarkers combined with imaging and other techniques could be valuable for asymptomatic early stage OA.

Therefore, the purpose of this research project is to assess the association between systemic cytokines and knee injury history as well as clinical outcome measures indicative of early PTOA (BMI, self-reported pain and MRI-defined OA based upon MOAKS scoring) in a youth knee injury cohort and validate these findings in a pre-clinical rat model of surgical induced OA.

2. Materials and methods

2.1. Human participants

This study included a sub-sample of 145 youth/young adults (15–26 years) from the Alberta Youth Prevention of Early OA (Alberta PrE-OA) cohort for whom baseline serum cytokine and clinical MRI were completed. Specifically, participants included 76 individuals who suffered a youth (under the age of 18 years) sport-related intra-articular knee injury 3–10 year previously and age, sex and sport (at the time of injury) matched uninjured controls. A description of the Alberta PrE-OA cohort, definition of the intra-articular knee injury, and recruitment and injury diagnoses procedures have previously been reported [7]. Briefly, sport-related intra-articular knee injury was defined as a clinical diagnosis of knee ligament, meniscal or other intra-articular tibiofemoral or patellofemoral injury that required both medical consultation and disrupted regular sport participation. Injury diagnoses were based upon diagnostic codes recorded on previous cohort study injury report forms or University Sport Medicine Centre medical records or physician records and confirmed by participants. Uninjured participants reported no previous time-loss knee injury. Exclusion criteria included pregnancy, non-steroidal anti-inflammatory use or cortisone injection within three months prior to testing, a musculoskeletal injury within the previous three months prior to testing that resulted in time loss (i.e., work, school or sport), other arthritides, or any current medical problem that prevented participation in the functional testing aspect of the study (e.g., neurological conditions). Ethics approval was granted from the Conjoint Health Research Ethics Board at the University of Calgary (REB14-2212), Canada and all participants provide signed consent/assent and completed a Physical Activity Readiness Questionnaire (PAR-Q, 2002) prior to testing. All testing was carried out in accordance with the declaration of Helsinki.

2.2. Procedures

After completing a custom designed study questionnaire (i.e., demographics, knee injury, surgery, medical history) the Knee Injury and Osteoarthritis Outcome Score (KOOS) and Intermittent and Constant Osteoarthritis Pain score (ICOAP), participants had their height (cm) and weight (kg) measured before serum samples were collected by a certified phlebotomist using standard venipuncture, with vacuum, non-treated tubes. Serum samples were immediately aliquoted into pyrogen/endotoxin-free polypropylene tubes and stored at −80 °C until required for analysis. All samples were only thawed once (at the time of analysis). Participants were recruited months later (147 days on average) for MRI studies at an offsite facility and reviewed by a musculoskeletal radiologist (JJ) with 13-years of imaging experience, blinded to injury history and intervention, using the MRI OA Knee Score (MOAKS). MRI defined-OA derived from MOAKS scores was based on established criteria [20,21].

2.3. Body mass index

Body mass index (kg/m^2; BMI) was calculated from measurements of participants’ height (to the nearest 0.1 cm; shoes removed) and weight (to the nearest 0.1 kg) assessed using a medical scale and stadiometer (Model 402KL, Pelstar, USA).

2.4. The knee injury and Osteoarthritis outcome score

The KOOS is a self-reported measure designed to evaluate knee related symptoms and function in young active patients with knee injury and OA. It has been validated in different populations varying in age, disease duration and activity levels and it has been shown to have high test-retest reliability [22]. The KOOS consists of 42 items in five subscales: pain ($KOOS_{PAIN}$), other symptoms ($KOOS_{SYMPTOMS}$), function in

daily-living ($KOOS_{ADL}$), function in sport and recreation ($KOOS_{SREC}$), and knee-related quality-of-life ($KOOS_{QOL}$) [23]. Each item was scored on a 5-point Likert scale ranging from 'no problems' to 'extreme problems'. Subscale scores were then summed, and the total sub-scale score transformed to a 0–100 scale (higher scores indicating better outcome). For these analyses, only the scores from the $KOOS_{PAIN}$ subscale was considered.

2.5. Intermittent and constant Osteoarthritis pain questionnaire

The Intermittent and Constant OA Pain Questionnaire is a self-report measure designed to evaluate constant and intermittent pain intensity including frequency and impact on mood, sleep and quality-of-life in persons with hip and knee OA [24,25]. As many patients with knee OA report that their initial presentation of pain was intermittent, often during tasks such as climbing the stairs [15], the ICOAP, which includes a subscale for intermittent symptoms was seen as particularly relevant to the cohort under investigation. This self-report questionnaire consists of 11 items forming two subscales (5 items addressing constant pain and 6 items addressing intermittent pain). The ICOAP has good internal consistency, test retest reliability (ICC = 0.85) and construct validity when compared to KOOS and Western Ontario and McMaster Universities Arthritis Index Scores [25]. Each item was scored on a 5-point Likert scale ranging from no pain to high (disability-severely limiting) pain. Sub-scale scores were then summed, and the total sub-scale score transformed to a 1–100 scale (higher scores indicating poorer outcome).

2.6. Magnetic resonance imaging

Participants underwent bilateral knee MRI studies using typical clinical projections and sequences. Sequences included: Sagittal proton density (PD) TR/TE 1500/10 ms, slice thickness 3.5 mm, field of view (FOV) 150 × 140 mm; Sagittal and coronal PD fat saturated (FS) with TR/TE 2660/28 ms slice thickness 3.5 mm, field of view (FOV) 150 × 140 mm; and 3D gradient echo FIESTA sequence with TR/TE 10.5/4.2 ms, flip angle 55°, slice thickness 1.0 mm and isotropic voxels. All studies were rated by a musculoskeletal fellowship trained radiologist (JLJ) with 13-years of imaging experience, blinded to injury history or surgical intervention using the semi-qualitative MRI OA Knee Score (MOAKS) [20,21]. MRI defined-OA derived from MOAKS scores was based on established criteria [20]. The intra and inter-rater reliability of MOAKS scoring for this sample has been previously reported [26].

2.7. Multiplexed arrays (Human Samples)

Sample analysis was performed by Eve Technologies (Calgary, AB Canada) using the Milliplex MAP Human Cytokine/Chemokine Panel (Millipore), according to the manufacturer's instructions. All samples were assayed at least in duplicate and prepared standards were included in all runs. The following proteins were examined by Luminex in this study for human serum samples: EGF, Eotaxin, FGF2, Flt3L, Fractalkine, GCSF, GMCSF, GROα, IFNα2, IFNγ, IL1α, IL1β, IL1rα, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12 (p40), IL12 (p70), IL13, IL15, IL17A, IL18, IP10, MCP1, MCP3, CCL22, MIP1α, MIP1β, PDGFAA, PDGFAB/BB, RANTES, sCD40L, TGFα, TNFα, TNFβ, VEGFA. The sensitivities of these makers range from 0.1 to 10.1 pg/mL (average 2.359 pg/ml) and the inter-array accuracies range from 3.5% to 18.9% coefficient of variation (average 10.7%).

2.8. Animal model

Sham controls and standardized joint injuries (destabilization of the medial meniscus; DMM) [27] were induced on the left knee joints of rats (n = 18). Three rats were sacrificed at one, two and three, weeks after injury, and nine rats sacrificed at the fourth week. To minimize the individual variance, only the nine rats sacrificed at the 4th week were used for repeat pain and serum cytokine profile studies starting before the injury (day 0). Specifically, before injury and 3, 5, 10, 14, 20, 24 days after injury, serum samples were harvested and processed as described above for cytokine profile analysis.

2.9. Multiplexed arrays (Animal Model)

Milliplex MAP Rat Cytokine/Chemokine Array 23-plex was used and the following cytokines were examined: EPO, IL13, IL10, IL18, IL1α, IL2, MCSF, IL1β, IL4, IFNγ, MIP3α, GMCSF, IL7, TNFα, VEGF, MCP1, IL5, GCSF, RANTES, IL6, GRO, IL17α, IL12p70. Unfortunately, a rat cytokine array matched with the human cytokine array was not available. Therefore, the concentration of CCL22 in rat serum was analyzed independently using sandwich ELISA (LS BIO) following the manufacturers instructions.

2.10. Rat grimace scale (RGS)

Application of the RGS was based on scoring randomised, blinded images of individual rats [28,29]. Briefly, each rat was video-recorded for 15 min at each time point in a plexiglass video chamber (W 14 cm × L 26.5 cm × H 20.5 cm). Recorded video was reviewed by a trained observer (blinded to treatment and time point) and an image captured every 3 min. No images were collected during the first minute of recording to allow the rat to acclimate. Image selection criteria were: absence of movement artifact, a clear view of all relevant facial features (nose, cheek, eyes, ears) and absence of directed behaviours (grooming, rearing, sleeping). Generation of a score with the RGS requires assessment of four "action units": orbital tightening, nose/cheek flattening, ear changes, and whisker change. Each action unit was assigned a score of 0, 1 or 2, and the four scores averaged to generate a single RGS score for each image. A score of "0" reflects an absent action unit, a score of "1" indicates the moderate appearance of an action unit, and a score of "2" indicates the obvious appearance of an action unit (associated with a painful state).

2.11. Histology and immunofluorescent (IF)

Both injured and uninjured (control) rat joints and human cartilage samples were fixed with formalin and embedded in paraffin. Intact knee joints were dissected and fixed in 4% normal buffered formalin (Sigma, St. Louis, MO). Samples were decalcified and embedded in paraffin (VWR, Radnor, PA). Ten μm thick, longitudinal serial sections were stained with Safranin O (Fisher, Waltham, MA) to visualize proteoglycans. In the rat, whole joint sections, medial, lateral and the ACL/PCL insertion sites were graded for signs of OA according to the OARSI Guidelines for rat knee joints [30]. Sections were deparaffinized in CitraSolv (Fisher Scientific; Fairlawn, NJ) and rehydrated through a series of graded ethanol to distilled water steps. Antigen retrieval (10 mM sodium citrate, pH 6.0, brand) and blocking (1:500 dilution; 100 μL rat serum (or goat serum for human samples): 50 mL TRIS-buffered saline, 0.1% Tween 20 (TBST) for 1 h), steps were performed prior to going through sequential wash (TBST) and primary antibody application steps. Primary antibodies directly conjugated to fluorescent probes (Abcam, dylight system) for CCL22 (rat: Cat. No. bs-1761R, Bioss) (human: Cat. No. MAB336, R&D systems); cleaved caspase 3 (rat & human: Cat. No. 9661, Cell Signaling Technology) and the nucleic acid stain DAPI (Sigma) were applied to sections. After antibody staining, sections were mounted using FluorSave reagent (Calbiochem) and coverslipped. A Zeiss Axio Scan.Z1 microscope was used to detect the signal for each antibody.

2.12. Human cartilage biopsies

OA Cartilage biopsies were collected from knee OA patients (n = 8; 3 M/5F, mean age 55.5 ± 7.1 years) undergoing joint replacement. Samples were processes using methods outlined in Histology section. Normal cartilage samples (n = 3; 2 M/1F, mean age 54.2 ± 5.3 years) were obtained from the Southern Alberta Tissue Donation Program. Criteria for control cadaveric donations were an age of 40 years or older, no history of arthritis, joint injury or surgery (including visual inspection of the cartilage surfaces during recovery), no prescription anti-inflammatory medications, no co-morbidities (such as diabetes/cancer), and availability within 4 h of death. Samples were processes using methods outlined in Chondrocyte Isolation and Culture section.

2.13. Chondrocyte Isolation and Culture

Cartilage tissue was cut in pieces of approx. 2 mm^2, then incubated with 1 mg/ml pronase (Roche, Cat. No. 1459643) for 30 min at 37 °C (100 rpm). The cartilage was then incubated with 1 mg/ml collagenase (Serva, Cat. No. 17465) for 24 h at 37 °C (100 rpm). The resultant suspension was filtered (70 μm) and centrifuged. The chondrocytes were resuspended in DMEM/F-12 (Gibco Cat. No. 31330) supplemented with 10% FBS and Anti-Anti. Chondrocytes were incubated with recombinant human CCL22 (Peprotech) or PBS alone and analyzed for Annexin V staining (Thermo Cat. No. BMS500FI) using flow cytometry. Chondrocytes were also stained for CCR4 (Cat. No. 557863, BD Biosciences).

2.14. Data analyses

Statistical analyses were performed using SPSS and R. The participants with missing values were removed from the analyses. The differences of sex, age, BMI, and MRI-defined OA between injured and uninjured group were evaluated using student's *t* test ($\alpha = 0.05$). Univariate logistic regression was used to ascertain the effects of age, sex, BMI, $KOOS_{PAIN}$ and $ICOAP_{TOTAL}$ pain on the likelihood that participants had a previous knee injury. The between-group differences for individual cytokines were accessed by Student's *t* test. The correlations between cytokines and pain scores were determined using Spearman's rank test. Multiple comparisons correction was not applied in this study as experiments were implemented to validate statistic findings.

Cytokine association networks from cytokine profiles of participants with or without a history of injury were constructed independently using the ARACNe algorithm as follows [31]. First, the pairwise mutual information (MI), which was considered as the strength of pairwise cytokine association were estimated for all cytokine pairs. Secondly, by applying the data processing inequality, most indirect associations (lowest strength, or smallest MI value of any 3-cytokine loops) were removed. Finally, networks were constructed with cytokines as nodes and remaining associations as weighted connecting lines. To quantify these perceived differences between networks, the change of centrality (betweenness) of each cytokine was computed. Betweenness has been widely applied in the analysis of biological networks [32] and other types of networks in general [33]. In graph theory, the betweenness of a node measures the number of times it acts as a bridge along the shortest path between all node pairs in the same network. Therefore, in this study, the cytokines with high betweenness can be considered as key connectors within the network, and the change of betweenness of a cytokine between two networks can therefore be considered as an indicator of the topological change of this cytokine. Networks were visualized using Cytoscape 3 with DyNet package [34,35].

Cytokine association networks of the rat at 7-time points (before and 3, 5, 10, 14, 20, 24 days after injury) were created in the same method described above. The connectivity (average node betweenness centrality) of each network of 7 time-points were calculated for the correlation analysis with rat pain score data.

Table 1
Participant characteristics, BMI, $KOOS_{PAIN}$, $ICOAP_{TOTAL}$ and MRI-defined OA by study group.

Outcome		Injured (n = 76)	Uninjured (n = 69)	p-value	95% C.I.	
					Lower	Upper
Sex (%female)		45	43	0.896	0.579	1.869
Age (years; median, range)		23 (15–27)	23 (18–27)	0.246	0.984	1.004
BMI (kg/m^2; median, range)		23.8 (18.6–31.3)	24.9 (19.4–38.9)	**0.003***	1.056	1.294
MRI defined OA (n)		44	19	**< 0.001***	0.202	0.628
Pain	$KOOS_{PAIN}$ (median, range)	94 (58–100)	100 (69–100)	**< 0.001***	0.808	0.939
	$ICOAP_{TOTAL}$ (median, range)	5 (0–43)	2 (0–36)	0.144	0.871	1.020

* $p < 0.05$.

3. Results

3.1. Participant demographics

The demographics of the participants (n = 145) are summarized in Table 1. The median age of participants was 23 years (range 15–27) and 88 were female. The median age of injury was 16 years (range 11–19) and the median time between injury and data/sample collection was 6.9 years (range 4–10). There were no significant differences in sex, age between the injured and uninjured group (Table 1). The injured study group had lower BMI ($p = 0.003$), lower $KOOS_{PAIN}$ ($p < 0.001$) and more MRI-defined OA ($p < 0.001$) than the uninjured group.

3.2. Comparing individual cytokines in cytokine association network for injured vs. uninjured participants

Cytokine association networks were created for both injured and uninjured groups based on the serum levels of 41 distinct analytes. It was found that most cytokines were visually different between two networks (injured vs. uninjured) in terms of their topological patterns (e.g. what "association partners" they connected to) (Fig. 1A). By comparing the betweenness of cytokines in two networks, MCP1/CCL2, CCL22 and TNFα demonstrated the greatest difference in network connectivity between the injured vs. uninjured cytokine networks (Fig. 1A).

3.3. Comparing overall difference between injured vs. uninjured networks

To statistically compare the overall difference between injured and uninjured networks, a permutation test was developed which involved a null hypothesis regarding the average betweenness of cytokines in networks. The null hypothesis stated that if cytokine association networks N_1 and N_2 are randomly organized, then N_1 and N_2 should have the same connectivity (average betweenness of all cytokines). Formally, the null hypothesis H_0 is:

$$H_0: C_\delta = \bar{C}_1 - \bar{C}_2 = 0$$

where $\bar{C}_1$ and $\bar{C}_2$ are the average betweenness centrality of all cytokines in network N_1 and N_2.

The distributions of C_δ were generated empirically through 100,000 permutations (randomly re-assigning group labels and repeated 100,000 times). The two-tailed p-value was computed as

$$p_{two-tailed} = \frac{N_{|C_{\delta obs}| \le C_{\delta sim}}}{N_{perm}}$$

where $N_{|C_{\delta obs}| \le C_{\delta sim}}$ is the number of simulated C_δ that are larger than

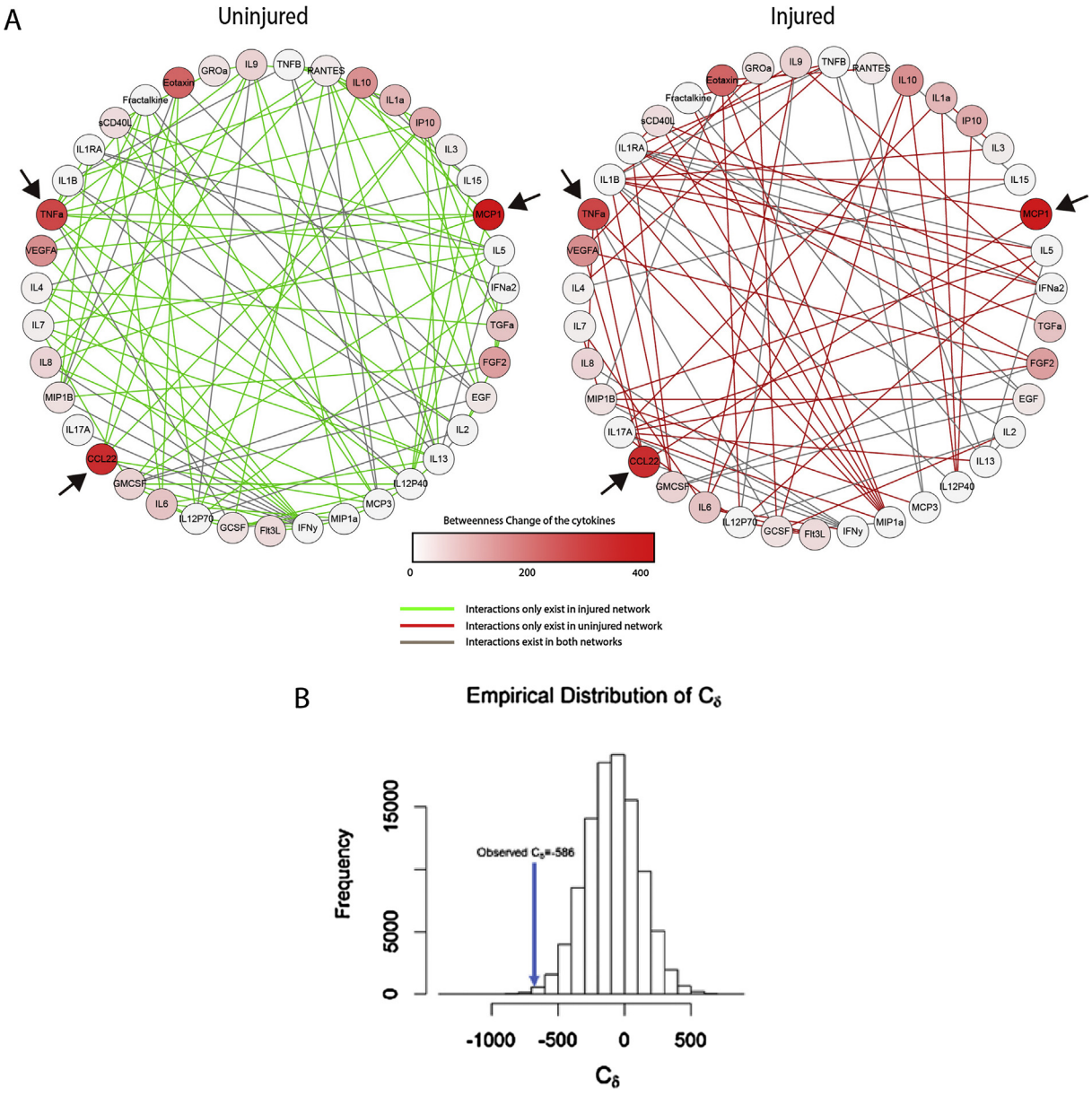

Fig. 1. Networks comparison between injured and uninjured. The injured vs. uninjured cytokine networks were different in terms of the connectivity between cytokines. The connections that only existed in injured (green), uninjured (red) and both (grey) are presented. The absolute betweenness differences between injured vs. uninjured networks per cytokine were calculated and are represented by the shade of red color for each cytokine. The position of MCP1/CCL2, CCL22 and TNFα are marked by arrows (A). The distributions were based on simulated data andN_{perm} = 100,000 permutations (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the absolute value of observed $C_{\delta sim}$. N_{perm} is the number of permutations which is 100,000 in this study.

The connectivity was significantly higher in injured than uninjured network (p = 0.006, Fig. 1B). On average, the injured network consisted of more "key cytokines" that demonstrated bridges/associations to the remainder of cytokines examined. This indicated a non-random or coordinated change in cytokine levels following injury, which suggested that cytokines in the injured group were more associated and co-regulated with each other.

3.4. Cytokine profile comparison using classical statistic approach

While the network analysis was able to identify the difference between injured vs. uninjured cytokine networks as a whole and determine which nodes (individual cytokines) most differed between networks; this analysis was unable to determine if any given cytokine demonstrated a difference in expression between injured vs. uninjured cohorts. Therefore, classical statistic approaches were applied to supplement the findings in the previous network analysis. Multivariate Analysis of Variance (MANOVA) was used to compare cytokine profiles between injured vs. uninjured cohorts. When all analytes were considered together, the two profiles were significantly different (p = 0.001). This was consistent with the analysis using cytokine association networks. However, no significant difference was found within any individual cytokines between two cohorts using Student's *t* test with Bonferroni correction. Moreover, since the injured group tended to demonstrate worse pain scores and MRI-OA scores than the uninjured cohort, the correlations between cytokines and pain and MRI-OA scores were tested, but none were found to be significant. Although not in direct disagreement with the previous finding, this strongly suggests that a factor other than cytokine concentration alone is responsible for the difference observed between networks. It is therefore

very likely that cytokine associations are playing a role in the network analysis, yet this is not being accounted for with univariate statisical methodologies.

3.5. Cytokines correlated with pain in rat OA

Among the most different cytokines, MCP1 and TNFα were found elevated in osteoarthritic joints in previous studies and shown to correlate with the initiation and progression of OA [36]. However, very few studies have reported the correlation between CCL22 and OA. Since it was not possible to determine if CCL22 expression was directly related to the previous joint injury in the human cohort study, a preclinical rat model was used to validate the cytokine association network analysis as well as the role of CCL22 in the initiation/early stages of OA. Standardized joint injuries (destabilization of the medial meniscus, DMM) were induced on the left knee joints of rats (N = 18). Half of the animals were used for repeated serum cytokine analysis, the remainder were sacrificed at different time points after injury for histology analysis of the joint and CCL22 expression/localization.

The expression of twenty-four cytokines/chemokines/growth factors were examined in the serum of rats (pre-injury vs. after DMM) and found to be similar to the analysis conducted in the human cohort study, the connectivity of rat cytokine association network for uninjured (before injury) and injured (days 3–24) were calculated. The network connectivity was increased after the joint injury, which was consistent with the result from the human cohort. Moreover, the connectivity of the network was positively correlated with rat pain ($r = 0.786$, $p = 0.036$) at the different time points examined after DMM injury (Fig. 2). This indicated that in this rat model, the induction of the injury led to an increase in pain, with a corresponding increase in connectivity of in the cytokine association network. Furthermore, CCL22 was also found positively correlated with pain ($r = 0.255$, $p = 0.045$) after joint injury within the rat model while most other cytokines were negatively correlated with pain (Supplementary Table 1).

3.6. CCL22 association with structural changes within the rat joint

Since it was observed that CCL22 was also correlated with MRI structural changes within the human cohort, the correlation between OARSI OA histological scoring and serum CCL22 concentration was assessed in the rat DMM model. Immunofluorescence (IF) was employed to detect the presence and localization of CCL22 in uninjured rat joints in additional to rat injured joints harvested at 1, 2, 3 and 4 weeks after the induction of injury (DMM). The OARSI OA scores and IF findings of all joints are shown in Supplementary Table 2. Serum CCL22 expression levels were correlated with cartilage degeneration width ($r = 0.905$, $p = 0.002$) and surface matrix loss ($r = 0.786$, $p = 0.021$) (Supplementary Table 3). Upon examination of joint tissue with IF, CCL22 was detected in the cartilage or/and synovium in all joints ($n = 12$) with visible damage (total degeneration width > 0 um) (Fig. 3). Within injured joints without cartilage degeneration, osteophytes or synovial inflammation, CCL22 staining was present 3 out of 6 joints and through comparing the histology and IF staining of adjacent slides in these joints without visible damage, CCL22 were found to be only present in the areas of cartilage where noticeable proteoglycan loss was observed (Fig. 3).

3.7. CCL22 association with chondrocyte apoptosis markers within rat cartilage

Since chondrocyte death is a hallmark of OA and it was observed that chondrocytes in areas of cartilage damage stained positive for CCL22 expression (Fig. 3) it was examined if CCL22 was co-localizing with markers of chondrocyte apoptosis. Healthy rat cartilage and areas of cartilage demonstrating increasing levels of damage were doubled stained with CCL22 and the apoptosis marker cleaved caspase 3 (Fig. 4). In healthy cartilage, little to no CCL22 and/or cleaved caspase 3 was detected, however as cartilage damage increased, an increase in CCL22 and cleaved caspase 3 positive cells were observed (Fig. 4). Furthermore, a high degree of co-localization between CCL22 and cleaved caspase 3 was observed in damaged cartilage (Fig. 4).

3.8. CCL22 association with chondrocyte apoptosis within the human cartilage

To validate the expression of CCL22 and it co-localization within apoptotic chondrocytes in human cartilage, biopsies were obtained from patients undergoing joint arthroplasty and stained for CCL22 and cleaved caspase 3 (Fig. 5). CCL22 and cleaved caspase 3 staining was observed in OA cartilage, while CCL22 expression was also observed in the subchondral bone and bone marrow. Furthermore, as observed in damaged rat cartilage, CCL22 positive staining was observed in chondrocytes undergoing apoptosis (e.g. cleaved caspase 3 positive) (Fig. 5).

3.9. CCL22 induces chondrocyte apoptosis

To determine if CCL22 was playing a role in the apoptosis of chondrocytes compared to co-expression in areas of damaged/dying cartilage, human chondrocytes were isolated from cadaveric donors without OA/cartilage pathology and exposed to increasing concentrations of CCL22. Apoptosis was analyzed by Annexin V staining, and the cells were doubled labeled with CCR4, the only known receptor for CCL22. The concertation range of CCL22 employed was determined from previous studies on the concentration range of CCL22 in the synovial fluid of patients with OA (2953 pg/ml ± 3628 pg/ml). The negative control (PBS) demonstrated minimal Annexin V staining (mean = 3.14%) while increasing concentrations of CCL22 increased in the percentage of apoptotic cells (Fig. 6). The most profound effects were observed at 3 ng/ml and 10 ng/ml where 57.23% and 82.21% of chondrocytes stained positive for Annexin V respectively (Fig. 6). It should also be noted that while approx. half of chondrocytes expressed CCR4 in the PBS treated cells, the number of CCR4 positive chondrocytes in the population increased with increasing concentrations of CCL22.

4. Discussion

This study investigated cytokine networks based on the expression of 41 cytokines in individuals that had suffered a sport-related intra-articular knee injury 3–10 years previously in their youth compared to age- sex- and - sport-matched (sport at the time of injury) uninjured controls. Although many of the cytokines have been studied individually in advanced OA cohorts [37], to our knowledge, this is the first study focusing on cytokine networks as a whole in a young cohort without a clinical diagnosis of OA.

Many studies have confirmed OA pain is not always consistent with radiographic or MRI structural changes [16,38,39]. In fact, using the data from this cohort, we have previously reported that we found no evidence of a linear correlation between MRI–defined OA and knee symptoms or function [26]. Although many cytokines have been reported to be responsible for OA pain [40], in this study, we have found no evidence of a correlation between cytokines and $KOOS_{PAIN}$ or $ICOAP_{TOTAL}$ scores. This might reflect that (1) most of the participants in the current study were pre-disease, (2) and/or regardless of disease state were generally asymptomatic, (3) it could be possible that any pain due to the previous injury and/or disease may also have been episodic, (4) and lastly, there may be no (or potentially a weak correlation between pain and structure.

Cytokines are known to be pleiotropic and inter-dependent. As a result, when studying a specific cytokine, ignoring its dependency to/with other cytokines will result in the loss of important information,

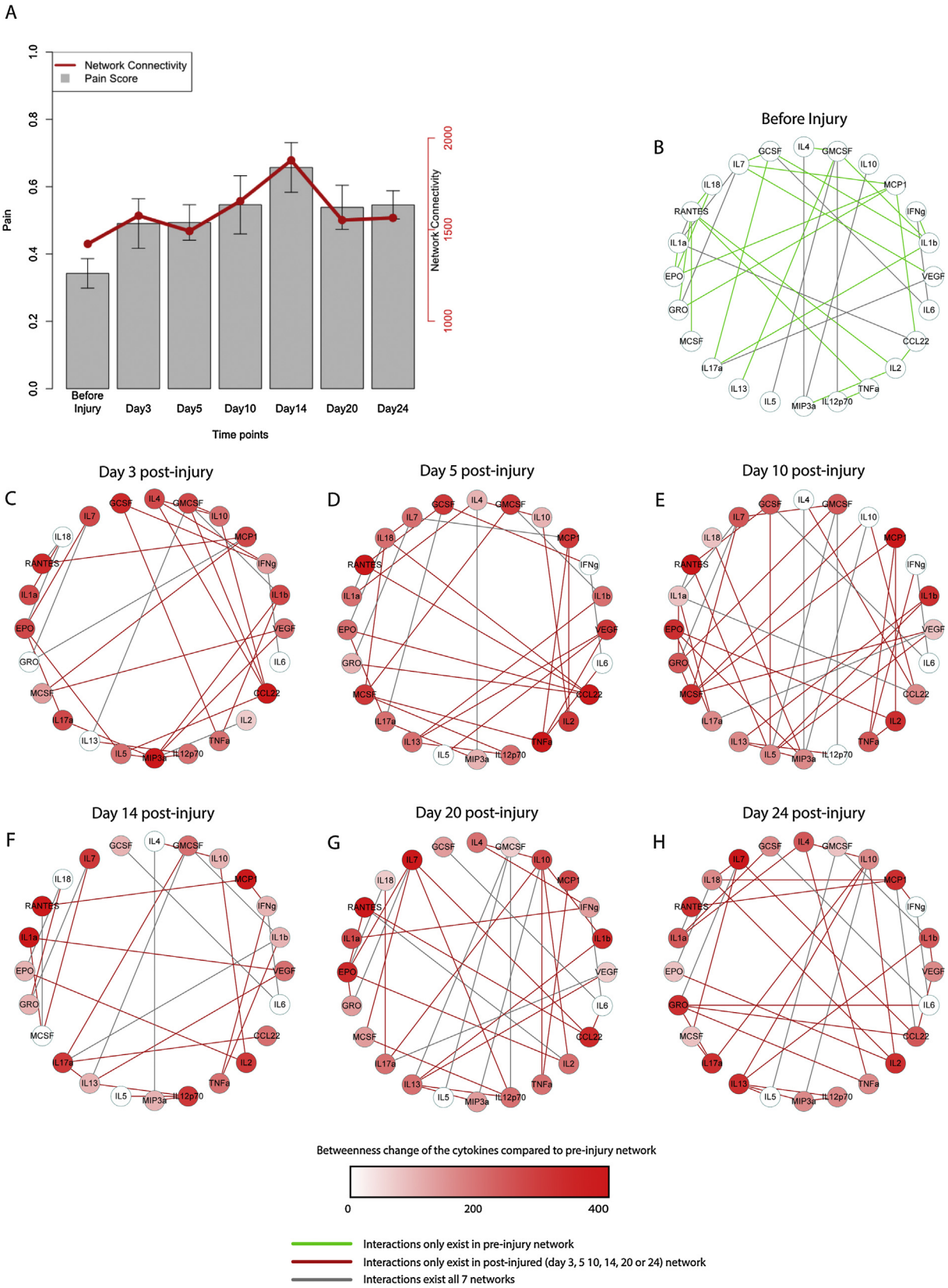

Fig. 2. In a rat DDM model, cytokine associations are correlated with joint pain after injury. The average pain score (Rat Grimace Scale) of rats that underwent DMM surgery (n = 9) at different time points after surgery is shown in the bar graph. The connectivity of the cytokine network for each time point are represented as the red line. The bar graph and line demonstrated a similar relationship at each of the time points examined, and they were found to be statistically correlated (r = 0.786, p = 0.036) (A). Individual cytokine networks pre/post-injury are presented (B-H). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

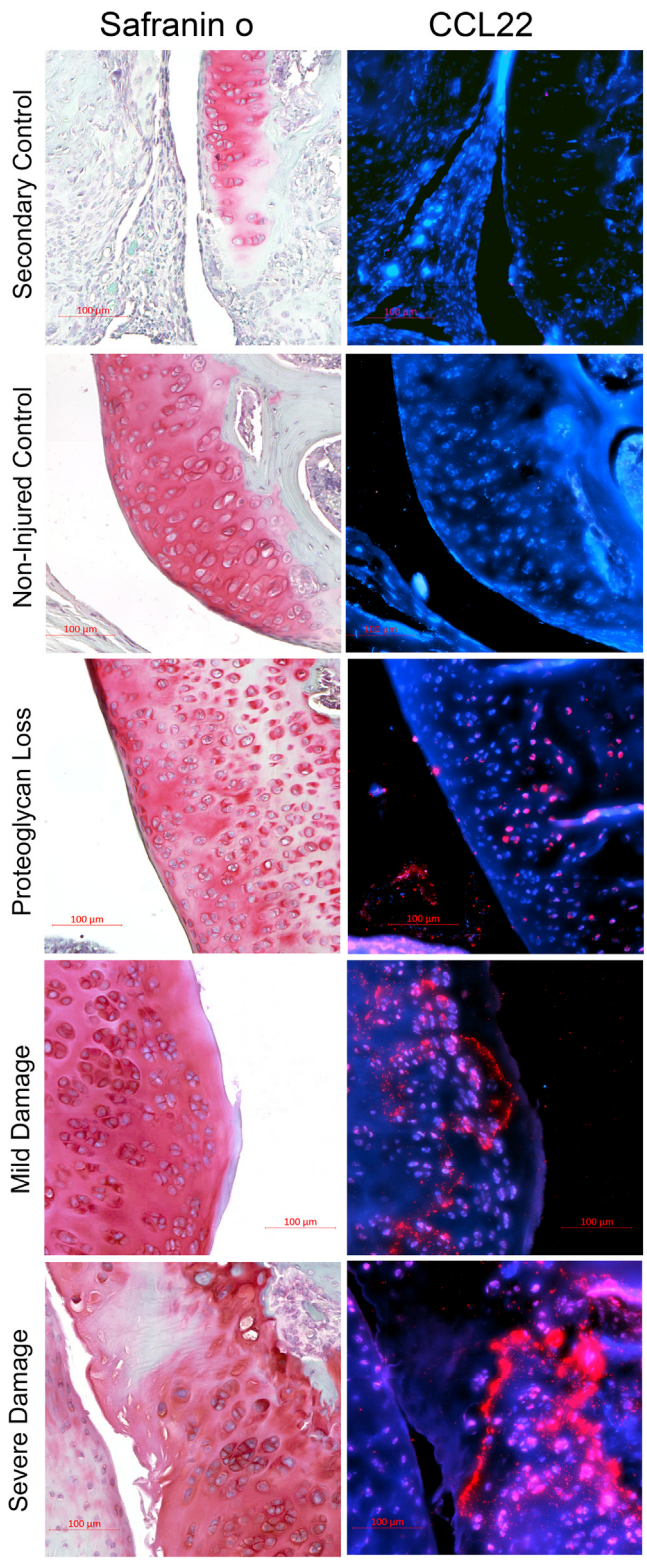

Fig. 3. IF and histology staining of cartilage & synovium. IF and histology slices of serial sections are presented in each row. Safranin O stained sections depict proteoglycan staining (red), while IF staining demonstrates CCL22 staining (red) in relation to DAPI nuclear staining (blue). Scale bars = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

which could lead to an erroneous conclusion. For example, IL6 can be both anti- and pro-inflammatory through different signaling pathways at the same time [41]. Therefore, it is reasonable to look at the relationships between IL6 and its downstream cytokine expressions rather than the level of IL6 alone, to determine which role IL6 is currently playing. In a recent study that analyzed the pairwise correlations between 17 cytokines, Wallner et al. found that drugs modulated cytokine correlations differently instead of causing a general inhibition of the cytokines [42]. This was similar to our finding that injury altered the inflammatory responses that were reflected in overall cytokine associations rather than simply increasing/decreasing the absolute concentration of each cytokine. Because of the complexity (e.g. redundancy, routes of feedback and cross-talk) of cytokine signaling, it is neither appropriate, nor practical, to isolate any single cytokine and its associations from the complete network of cytokines interactions and study them individually. The analysis of biological networks has shown its advantage in understanding complex biological systems as a whole [43–45]. However, even though comparing network structure was involved in most of the studies, very few had statistically evaluated the topological difference between networks. In this study, we developed a permutation test to evaluate the difference of connectivity between networks. The result was consistent with MANOVA applied on original multivariate datasets.

It is known that a history of injury is a significant contributor to knee OA, and chronic inflammation seems to play a pivotal role in the pathogenesis of OA [46]. Does injury alter the local inflammatory environment leading the degeneration of the joint? To address this question, we compared cytokine profiles between participants with and without a history of injury. We found the two groups were significantly different when comparing the cytokine profile by computing the cytokine network connectivity or by using multivariate analysis. The network of injury group had a higher average betweenness, meaning that on average the cytokines of the previously injures participants had a stronger association with each other than seen in the uninjured participants. This suggests that on average the cytokines of the previously injured participants are more highly co-regulated than in the uninjured participants. Although none of the individual cytokines were significantly different when comparing the means between injured vs. uninjured, most cytokines were generally different between the two groups in terms of their “connectivities” in the cytokine association networks. Among the most different cytokines, MCP1 and TNFα were found elevated in osteoarthritic joints by a variety of studies previously and have been shown to be correlated with the initiation and progression of OA [40,47]. But very few studies have reported a correlation between CCL22 and OA. CCL22 has a complex role in inflammation as it is chemokine for both Th2 and Treg cells [48,49]. It has been reported that CCL22 and its receptor CCR4 were found in the synovial membrane of osteoarthritis patients [50] and expressed by subpopulations of sensory neurons [51]. In the rat model of this study, CCL22 was found correlated with pain. Moreover, CCL22 was present not only in severely damaged joints in rats but also in the cartilage before the visible damage occurred. Furthermore, CCL22 expression was observed in chondrocytes undergoing apoptosis (cleaved caspase 3 positive cells), with almost all apoptotic cells being positive for CCL22. This result was validated in human OA cartilage, and we were also able to demonstrate a functional role of CCL22 in the induction of apoptosis of human chondrocytes. While chondrocyte apoptosis is a hallmark of OA [52], to our knowledge CCL22 has not been previously implicated in the induction of apoptosis of chondrocytes and/or any other cell type. However, it has been previously demonstrated the TNFα plays a role in chondrocyte apoptosis [53] and that CCL22 expression is correlated to TNFα activation [54]. Therefore, it may be possible that CCL22 is a mediator in TNFα induced chondrocyte apoptosis, however, as this was not tested in this study, further experiments will be required to examine this directly. Although the observed differences in cytokine profiles between post-injury and uninjured young adults did not associate to significant differences in clinical parameters, this is expected since the patients are young and not yet showing symptomatic OA. This group will be ideal for further longitudinal follow-up.

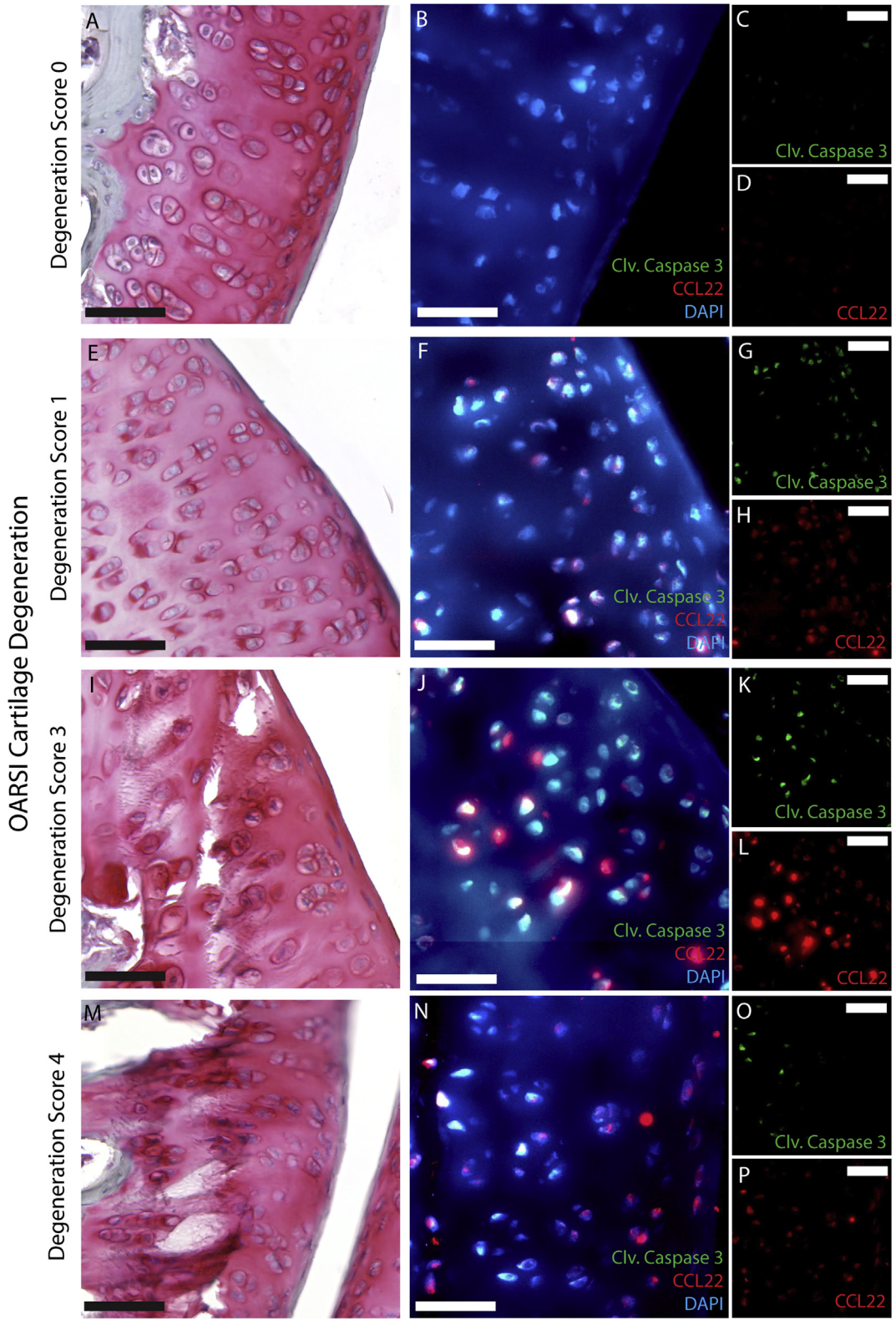

Fig. 4. Co-localization of CCL22 and cleaved Caspase 3 in chondrocytes. IF and histology slices of serial sections are presented in each row. Areas of cartilage were classified by increasing (worsening) OARSI degeneration score based on Safranin O stained sections (A,E,I,M). Serial sections were stained with CCL22 (red) and cleaved (Clv.) caspase 3 (green) (B,F,J,N). As the OARSI degeneration score increased, so did CCL22 and cleaved (Clv.) caspase 3 positive chondrocytes in the cartilage. Furthermore, apoptotic chondrocytes (cleaved caspase 3 positive) also presented with positive CCL22 staining. However, not all CCL22 positive cells were also positive for cleaved caspase. Scale bars = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

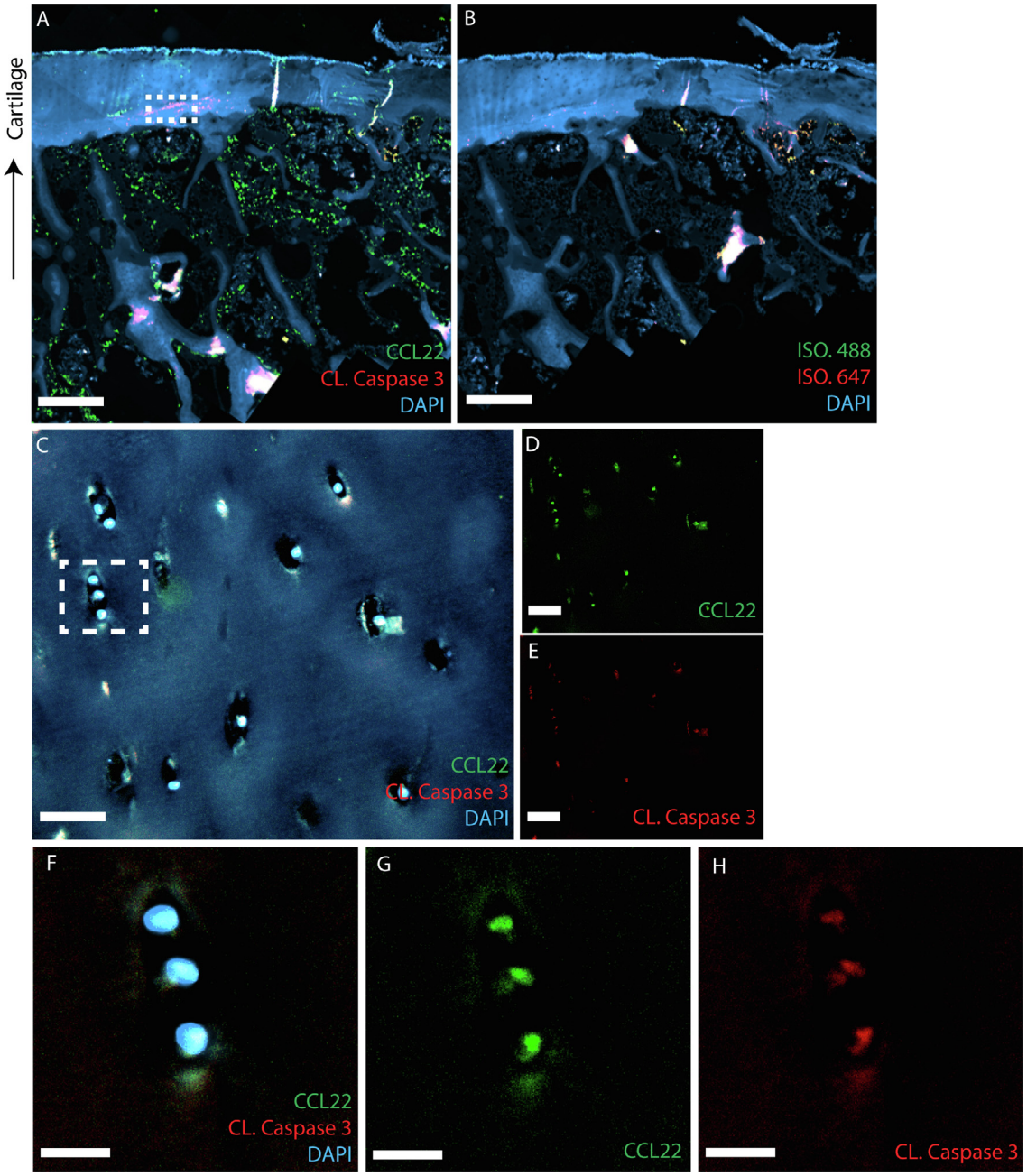

Fig. 5. Co-localization of CCL22 and cleaved Caspase 3 in human cartilage/chondrocytes. Cartilage sections were stained with CCL22 (green) and cleaved (Clv.) caspase 3 (red) (A, C-H) or isotype controls (B). Chondrocytes within OA cartilage demonstrated expression of both CCL22 (C,D) and cleaved caspase 3 (C,E). Furthermore, apoptotic chondrocytes (cleaved caspase 3 positive) also presented with positive CCL22 staining (F-H). Scale bars for A,B = 200 μm; for C-E = 50 μm; for F-H = 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Overall, the findings present in rat models and human OA strongly suggest that CCL22 could play a role in joint pain and initiation of cartilage degeneration.

5. Conclusion

In this study, the associations between cytokines in previously injured and uninjured participants were compared using network analysis. The overall cytokine association networks were significantly different, suggesting that injury had altered the inflammatory environment, which might contribute to joint degeneration. CCL2/MCP1, CCL22 and TNFα were the most different cytokines between two groups. Moreover, no association between cytokine expression knee injury history, self-reported pain symptoms and/or MRI-defined OA was found. In a rat model of OA CCL22 was correlated with pain and structural changes to the cartilage and CCL22 expression was found in damaged cartilage in addition to apoptotic chondrocytes. This result was validated in human cartilage and in isolated human chondrocytes, were CCL22 treatment induced apoptosis in a dose dependent manner. We propose that CCL22 is a novel potential biomarker in the earliest stages of cartilage damage and may also play a functional role in the degeneration of the articular cartilage through induction of chondrocyte apoptosis.

Conflict of interest statement

M. L. Fritzler is the owner of Eve Technologies Corporation, who performed the cytokine assays for this project. All other authors declare

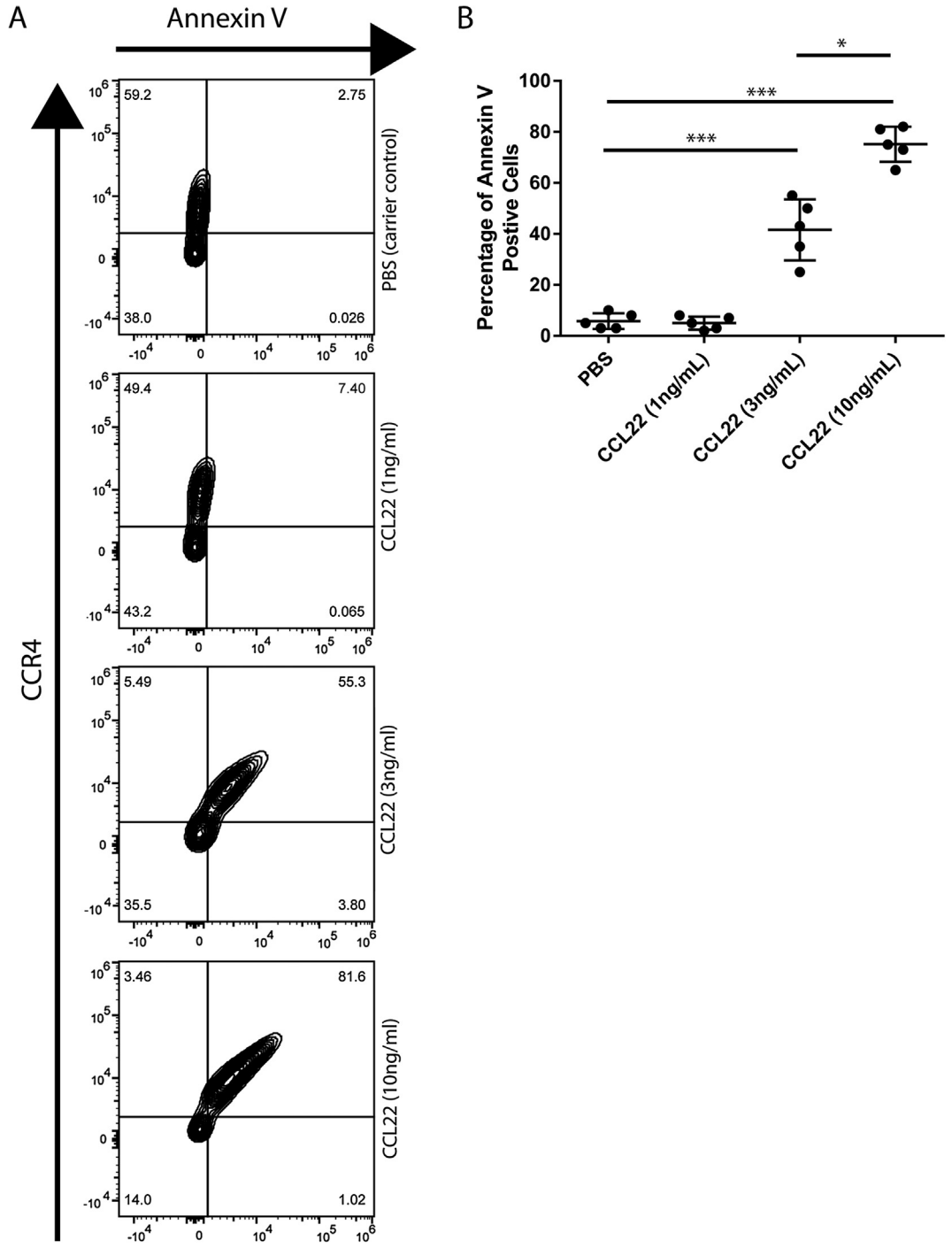

Fig. 6. Apoptosis in isolated human chondrocytes exposed to CCL22. Chondrocytes were isolated from individuals without OA and assayed for apoptosis (Annexin V) and CCR4 (CCL22 receptor) with increasing exposure to CCL22 (A). As CCL22 concentrations exceeded 3 ng/ml, increased levels of Annexin V positive chondrocytes were observed (B). Chondrocytes treated with PBS (carried control) alone and/or 1 ng/ml of CCL22 demonstrated minimal Annexin V staining. *** = $p < 0.001$; * = $p < 0.05$.

that there is no conflict of interest associated with their contribution to this manuscript.

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Author contributions

GR, JLW, CL, DDR, DSJP, PS, MF, MK, APJK, JLJ, CAE, RJK: Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work. Drafting (GR, JLW, CAE, RJK) the work or revising (JLW, CL, DSJP, PS, APJK, JLJ, CAE, RJK) it critically for important intellectual content. RJK: Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2018.11.030.

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