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Inflammatory pattern of the infrapatellar fat pad in dogs with canine cruciate ligament disease

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Abstract

Background: Despite the importance of inflammation during the pathogenesis of cranial cruciate ligament disease (CCLD) in dogs and despite the latest knowledge suggesting a significant role of adipose tissue in osteoarthritis, the infrapatellar fat pad (IFP) was up to now mostly disregarded in veterinary investigations. In the present study, the inflammatory activity of the IFP, the main adipose structure within the stifle joint, was thoroughly investigated to evaluate its potential impact in the pathogenesis of this common disease of our canine companions. Samples of IFP, subcutaneous adipose tissue (ScAT) of the thigh and synovial fluid in both diseased ($n = 36$) and healthy control ($n = 23$) dogs were tested for their immune cell composition but also for interleukins (IL-1 β , IL-6, IL-8, IL-10), degradative enzymes (MMP-1, MMP-3, MMP-13, TIMP-2, iNOS) and adipokines (leptin and adiponectin). Characterization of the immune cell composition was ascertained by fluorescence activated cell sorting. Gene expression and protein release of the inflammatory markers was determined by real RT-qPCR and ELISA.

Results: IFPs of dogs with CCLD had a significantly increased immune cell count with T cells (CD3) as the most abundant immune cells. T cells and macrophages (CD14) were significantly increased compared to healthy controls or corresponding ScAT. In addition, IFPs of dogs with CCLD demonstrated a significant increase on gene as well as protein level of multiple inflammatory indicators (IL-1 β , IL-6, MMP-1, MMP-13) compared to the other tissues. TNF α was only increased on gene expression. Adipokine analysis showed higher secretion of adiponectin and lower leptin secretion in IFP from dogs with CCLD than from controls. In the synovial fluid from dogs with CCLD concentrations of IL-1 β , MMP-1, MMP-13 as well as leptin were significantly increased compared to the synovial fluid from healthy control dogs.

Conclusions: The present study indicates that the IFP is a potential contributory factor in the pathogenesis of CCLD, due to its inflammatory phenotype and the proximity within the stifle joint. To determine the extent of this possible inter-relationship, further studies need to be undertaken.

Keywords: Infrapatellar fat pad, Inflammatory pattern, Cranial cruciate ligament disease, CCLD, Dogs, Osteoarthritis

Background

Cranial cruciate ligament disease (CCLD) is a common disorder and the most common reason for hind limb lameness in canine patients [1, 2]. The majority of veterinary clinicians employ surgical intervention to manage CCLD, yet no such procedures have been shown to prevent further

progression of osteoarthritis (OA) [3, 4]. Consequently, a better understanding of the pathogenesis of osteoarthritic changes seen during CCLD would be advantageous in order to facilitate the development of more effective therapeutic strategies. Besides contributing factors like age, body condition score (BCS), stifle morphology and ligament matrix composition, there is clear evidence that inflammation plays a prominent role in the progression of OA during CCLD [5, 6]. Dogs with CCLD typically have inflammatory changes in the synovial intima, the epiligament and

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the core region of the cruciate ligament [7, 8]. Moreover, various pro-inflammatory and degrading factors known to be involved in the initiation and progression of OA secondary to CCLD are measurable in synovial fluid [9–11]. The ligament, synovium and articular cartilage are regarded as the principal sources of factors secreted into synovial fluid during CCLD. Pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor (TNF)- α have been found to be synthesized by synovium [12, 13]. Matrix metalloproteinases (MMP) from CCL are implicated in the initiation of OA when ligament tissue is exposed to the joint environment [13, 14]. However, the relative contribution of each compartment of the joint to the pathophysiology remains unclear, as is the length of exposure time required in order to initiate the disease.

A characteristic part of the stifle joint, the infrapatellar fat pad (IFP), has mostly so far been disregarded in veterinary medicine. The IFP was considered to have merely biomechanical functions within the knee, serving primarily as a shock absorbing structure and to improve the distribution of lubricant [15]. Due to its intraarticular but extrasynovial localization and the now recognized potential of adipose tissue to act as a source of inflammatory factors, the IFP is progressively gaining attention related to OA, mainly in human medicine [16–19]. New concepts postulate that joint overload is not the sole factor that may result in the development of OA; inflammatory responses within adipose tissue can also play a role. The fact that obese people have an increased risk of developing not only OA of the knee but also of the hand supports this hypothesis. Besides adipocytes, adipose tissue also contains fibroblasts, endothelial cells and immune cells [20–22]. Adipocytes are capable of producing a vast spectrum of inflammatory mediators and potentially contribute to OA [23, 24]. Several studies demonstrated an infiltration of macrophages, lymphocytes and granulocytes in the human IFP during OA [19, 25]. It is postulated that immune cells interact with adipocytes and together are responsible for the release of several cytokines like adipokines or interleukins [26]. Despite increasing effort in research into this field, so far little knowledge is available on how the canine IFP influences both the production and the release of inflammatory mediators.

The aim of our project was to identify the level of inflammatory activity in canine IFP during CCLD. Therefore, a characterization of the immune cell composite, the gene activity on mRNA basis, as well as the final protein production of the important inflammatory mediators in this context was required. To estimate its inflammatory potential those parameters were measured within the IFP, subcutaneous adipose tissue (ScAT) and the synovial fluid of both diseased and healthy control dogs. Our hypothesis was, that the IFP during CCLD shows a significantly increased inflammatory pattern compared to IFP of healthy control dogs and compared to corresponding ScAT.

Methods

Dogs

A total of 59 dogs were enrolled in the present study. Thirty-six client-owned dogs were classified as the CCLD group. The presurgical diagnosis was made based on physical examination; lameness in the affected hind limb that was localized to the stifle joint, detection of cranial drawer, stifle joint effusion; and radiographic evidence of new bone formation in the stifle joint. Study inclusion criteria for the CCLD group were a diagnosis of a complete or partial CCL rupture confirmed by probing and observation during arthroscopy or arthrotomy and unremarkable results of routine hematologic and serum biochemical analyses. Clinical examination of all cases was undertaken and cases presenting any clinical signs indicating illness other than CCLD were excluded, as were those exhibiting traumatic CCL rupture or with a history of performed intra-articular injections. Dogs were admitted for surgical treatment either to the Animal Clinic Thun Süd or the Small Animal Clinic at the Vetsuisse Faculty, University of Bern. A standard anaesthesia protocol was followed in all dogs. This included intramuscular premedication with acepromazine (0.03 mg/kg) and methadone (0.2 mg/kg), induction with propofol (2–8 mg/kg intravenously to effect), and maintenance with isoflurane in an air-oxygen mixture, delivered using a rebreathing system (end-tidal concentration, 1.2–2%). An ultrasound-guided femoral and sciatic nerve block was performed with ropivacaine (0.3 mL/kg 0.5%) for each nerve. A control group consisted of 23 healthy dogs. Inclusion into the control group necessitated normal results for routine hematologic and serum biochemical analyses along with absence of any history of orthopaedic disease, particularly evidence of stifle OA and/or CCLD confirmed during sample collection. Additionally, control dogs had not received any medication for one month prior to sampling. Twenty-one control dogs were euthanized at the end of an unrelated experimental study (BS2197) with an overdose of pentobarbital intravenously after induction of general anaesthesia in accordance with the AVMA guidelines for the euthanasia of animals. Two dogs from the control group were involved in a road traffic accident and were euthanized due to poor prognosis. Various parameters were recorded for each dog including breed, gender, age, and weight. Concerning the BCS, each dog was classified by one individual using a nine-point scale [27] which was sub-grouped into three categories, namely lean to ideal (3–5), overweight (6–7) and obese (8–9). Additionally, the medication history during the last 6 months prior to surgical treatment and the duration of lameness were recorded. Gross pathologic features were assigned to four joint alterations (synovitis, development of osteophytes, meniscus infringement and articular cartilage lesions) as indicated in Table 1. All procedures and the written owner consent form were approved by the commission of animal experimentation of the Canton of Berne and

Table 1 Morphological grading system [60, 61]

| Grade | 0 (normal) | 1 (mild) | 2 (moderate) | 3 (severe) |
|--|--|--|---|---|
| Structure | | | | |
| Synovitis ^a | Thin synovial membrane with thin scattered vessels | Isolated thickening of synovial membrane with granulations and small vessels | Synovial membrane with abundant villous projections and large vessels | Synovial membrane with large villi and abundant vasculature |
| Osteophytes ^b | none | solitary | multiple | confluent |
| Medial meniscus | normal | Fibrillated surface | Undisplaced tear | Displaced tear |
| Articular cartilage lesions ^c | none | Solitary fibrillation | Multiple areas of fibrillation up to solitary erosion | Multiple erosions to the level of subchondral bone |

^aArthroscopic evaluation of the synovial membrane

^bDevelopment of osteophytes along the femoral condyles, the dorsal aspect of the patellar sulcus and within the femoral notch itself, evaluated via arthroscopy

^cArea of femoral trochlea visualized via arthroscopy

Basel, Switzerland (BE116/13; BS2197). Prior to study enrolment, written owner consent was obtained for each dog.

Sample collection

On the day of surgical intervention for CCL rupture, each dog was anaesthetized and stifle joints were aseptically prepared. A synovial fluid sample (0.1 to 0.4 mL) was aseptically collected by percutaneous arthrocentesis. Tissue samples from the thigh ScAT, removed next to the skin incision and the IFP (wet weight, each 1 to 5 g) were obtained from dogs during arthroscopy or arthrotomy. Samples from control dogs were harvested within 30 min after euthanasia. Depending on the conducted experiment, tissue samples were portioned and either immediately processed in cold Dulbecco's modified eagle medium (DMEM, Thermo Fisher Scientific, Zug, Switzerland) for flow cytometry or ELISA procedures, or submerged in RNAlater (Qiagen, Hilden, Germany) for quantitative RT PCR. All samples were stored at -80°C until further analysis unless otherwise stated. Owing to the limited size of the samples, not all experiments could be performed with each sample.

Cell isolation of IFP and ScAT

Freshly obtained adipose tissue samples were dissociated by being cut into small pieces. Stromal vascular cells were isolated from 1 g minced tissues using collagenase (C6885, Sigma-Aldrich, Buchs, Switzerland; 1.3 mg/ mL DMEM/ g adipose tissue) in DMEM on a shaker at 37°C . After the one-hour digestion, the cell suspension was filtered through a 70- μm -mesh (BD Biosciences, Allschwil, Switzerland) in order to remove undigested tissue. The filtered cell suspension was centrifuged at 300 g for 10 min. The floating adipocyte fraction including the supernatant was collected and centrifuged again for 10 min at 300 g. After the second spin, the two pellets were combined and resuspended into 10 mL of 4°C cold FACS buffer (PBS containing 0.5% bovine serum albumin and 2 mM EDTA). Following an additional washing step, cells were counted and then diluted to a final concentration of 1×10^7 cells/ mL. Care was taken

during specimen handling to ensure that cell isolation was as consistent as possible. As much tissue as possible was analyzed to maximize the number of cells isolated; however, in some individual samples, the number of cells isolated imposed limitations on the number of CD makers that could be studied with flow cytometry.

Flow cytometry

An aliquot of 1×10^6 cells was resuspended in 100 μL of FACS buffer before staining with primary antibodies as indicated in Table 2. Before staining with MAC387, samples were permeabilized and fixed using 1% paraformaldehyde for 10 min, followed by 1% Triton for 20 min. All aliquots were incubated with a single antibody against CD3, CD14, CD19, CD21 and MAC387 for 50 min on ice. A second antibody (dilution 1: 800; goat anti-mouse IgG DyLight 649 or 488, AbD Serotec, Germany) was used for CD3 and MAC387 for an additional 30 min. Following staining procedures, cells were washed twice and then resuspended in 0.5 mL FACS buffer. Subsequently, antibody binding was visualized and compensation procedures were performed using a LSRII BD Bioscience flow cytometer. Control gates were set based on staining with isotype controls. Side light-scatter versus forward light-scatter characteristics were used to identify neutrophils, monocytes and lymphocytes. For data acquisition the same instrument settings were used for

Table 2 Primary antibodies for IFP and ScAT staining

| Antibody | Clone | Dilution per 10^6 cells | Supplier |
|----------------------------------|-----------|---------------------------|--------------------|
| Mouse anti-canine CD3 | CA17.2A12 | 1:10 | AbD Serotec |
| Mouse anti-human CD14-Alexa 488 | M5E2 | 1:20 | BD Biosciences |
| Mouse anti-macrophage MAC387 | MAC387 | 1:100 | abcam ^a |
| Mouse anti-CD19-FITC | MB19-1 | 1:50 | abcam ^a |
| Mouse anti-canine CD21-Alexa 647 | CA2-1D6 | 1:10 | AbD Serotec |

^aabcam, Cambridge, UK

each sample and 50,000 events were recorded to generate frequency determination per antibody. Frequency of cell subsets was determined as a percentage of total cells operated by Diva 6 software (BD).

RNA extraction

Total RNA from IFP and ScAT was extracted using a combination of phenol/guanidine hydrochloride reagent, RNeasy purification and on-column DNase treatment (Qiagen) following a protocol for fatty tissue (Qiagen). Briefly, 100 mg of tissue was homogenized in 1 mL TRIzol® (Thermo Fisher Scientific) with a Qiagen TissueLyser for 3 min. After extraction with 200 µL of chloroform and ethanol precipitation, the sample was loaded on the RNeasy spin column. An on-column DNase (Qiagen) treatment was included. Total RNA was finally eluted in 50 µL of RNase-free water and stored at -80 °C until use. Quantity and quality of total RNA were assessed by using an Agilent 2100 bioanalyzer (Santa Clara, USA) for calculation of the concentration, the ratios 18S/28S and RNA integrity number.

cDNA synthesis

Reverse transcription was performed using SuperScript IV reverse transcriptase (Invitrogen, Thermo Fisher Scientific) in accordance to the manufacturer's instructions with few changes. Briefly, 2–5 µg (11 µL) template RNA was preincubated with 50 µM (1 µL) oligo-d(T)20 and 10 mM (1 µL) dNTP mix at 65 °C for 5 min. After incubation on ice, 4 µL 5 x first strand buffer (containing 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 1 µL of 0.1 M DTT, 1.5 µL nuclease-free water and 0.5 µL Superscript IV reverse transcriptase were added to each sample and the combined reaction mixture was incubated at 50 °C for 30 min. Reverse transcriptase activity was terminated by incubation at 65 °C for 15 min, and samples were stored at -80 °C until use.

Quantitative real-time reverse transcriptase PCR (RT-qPCR) assay

IFP and ScAT from both groups were evaluated for gene expression of matrix molecule proteinases (MMP-1, MMP-3, MMP-13), inhibitor TIMP-2, inflammatory indicators (IL-1β, IL-6, IL-8, IL-10, iNOS, TNFα) and a housekeeping gene (β-actin). Canine-specific transcript sequences for selected factors were designed based on gene bank information and synthesized by Microsynth (Balgach, Switzerland) as presented in Table 3. Quantitative real-time reverse transcriptase PCR was performed in duplicates using the 7300 Real Time PCR System (Applied Biosystems) in a 96-well format, with three no template controls used for each assay. The following protocol was executed for each transcript sequence: The reaction volume in each well consisted of 10 µL Power SYBR® green (Applied Biosystems), 0.8 µL of

10 µM forward primer, 0.8 µL of 10 µM reverse primer, 7.4 µL of nuclease-free water and 1 µL of sample cDNA. The standard amplification condition consisted of 1 cycle at 95 °C for 5 min, followed by 42 cycles of 95 °C for 10 s and 60 °C for 30 s and extension at 72 °C for 30 s. Subsequently, a terminal cycle of 95 °C for 15 s, 60 °C for 30 s and finally 95 °C for 15 s was performed. Fluorescence was detected during the extension step of each cycle and during the melt curve analysis for SYBR® green Cyler (Applied Biosystem). Real-time data was then analyzed by using the 7300 System Software (Sequence Detection Software, version 1.3). Data processing was conducted using the 2-ΔΔCT method [28]. The data then was finally presented as the fold change in gene expression normalized to the internal control gene β-actin and relative to the ScAT of healthy controls.

Quantification of selected cytokines, MMPs and adipokines by ELISA

For ELISA, tissue explants (100 mg/ mL) from freshly isolated and washed IFP and ScAT were cultured in DMEM for 48 h at 37 °C and 5% CO₂. To evaluate spontaneous secretory activities of IFP and ScAT, the concentration of selected factors was measured in triplicates in culture supernatants of the tissue explants using commercially available ELISA kits, including canine IL-1β (SEA563Ca, Cloud-Clone Corp, TX, USA), canine IL-6 (SEA079Ca, Cloud-Clone), canine MMP-1 (CSB-E14057c, Cusabio Biotech Co, China), canine MMP-3 (CSB-E13621c, Cusabio), canine MMP-13 (CSB-E14055c, Cusabio), dog adiponectin (CY-8052, CircuLex, Nagano, Japan) and canine leptin (EZCL-31 K, Millipore Corporation, MA, USA). Moreover, the concentration of these factors was also quantified in synovial fluid. The assays were performed according to the manufacturer's protocols. Sample concentrations were calculated based on standard dose-response curves. Results are demonstrated as cytokine content per 100 mg tissue/ mL.

Statistics

All statistical analyses were carried out using MedCalc version 17.8 (MedCalc Software bvba, Ostend, Belgium; <http://www.medcalc.org>; 2017). Summary statistics were performed and normality assessed using the D'Agostino-Pearson test. Data was reported as mean (range) and 95% CI or median and 95% CI for data that was not parametrically distributed. As some data were normally distributed but others were not, nonparametric statistics were used. Fisher's exact test was used to assess differences in age and BCS between the two groups. Differences in cell phenotype, gene expression and protein secretion between the two groups were evaluated for ScAT, IFP and synovial fluid using a Mann-Whitney test. Variations of same parameters within the groups were calculated using a Wilcoxon

Table 3 Oligonucleotide primers used for RT-qPCR

| Target gene | Forward (F) and reverse (R) primer (5'-3') | Amplicon size (bp) | Annealing temperature (°C) |
|----------------|--|--------------------|----------------------------|
| IL-1 β | F-GCCAAGACCTGAACCACAGT | 96 | 60.16 |
| | R-CTGACACGAAATGCCTCAGA | | 59.98 |
| IL-6 | F-ACCGTCTTGTGGAGTTCA | 102 | 60.55 |
| | R-CAGGATCTTGGTACTCATGTGC | | 59.62 |
| IL-8 | F-TTCAGAACTTCGATGCCAGT | 90 | 58.44 |
| | R-GGGCCACTGTCAATCACTCT | | 60.12 |
| IL-10 | F-CAGAGCACCTACCTGAGGA | 97 | 60.40 |
| | R-AAGTCTTCACCCTCCGAAG | | 60.62 |
| TNF α | F-GAGCACTGAAAGCATGATCC | 108 | 58.39 |
| | R-GAGAAGAGGCTGAGGCAGAA | | 59.83 |
| iNOS | F-GTCTGGGAGCCATCATGAAC | 98 | 60.48 |
| | R-GACAAATCAATGGCTTGAGG | | 59.56 |
| MMP-1 | F-TGAATTGGGTCATTCTTTGG | 98 | 59.92 |
| | R-CCTGAGATAGCTGGACATTGC | | 59.85 |
| MMP-3 | F-CCTAGCGCTCTGATGTACCC | 90 | 59.86 |
| | R-GGACTGGATGCCATTACAT | | 60.76 |
| MMP-13 | F-AGTTCGGCCACTCCTTAGGT | 102 | 60.13 |
| | R-CATCGGAAGCATAAAGTGG | | 60.46 |
| TIMP-2 | F-TGTTCAAAGGACCAGACAAGG | 109 | 60.13 |
| | R-TTCTTTCTCCGATGTCCAG | | 60.19 |
| β -actin | F-GGACCTGACCGACTACCTCA | 91 | 60.11 |
| | R-CTTGATGTCACGCACGATTT | | 59.72 |

signed-rank test. Associations between analyzed inflammatory parameters and gross synovitis scores, grade of CCL rupture as well as clustered BCS were evaluated using Kruskal-Wallis tests. A Spearman's rank correlation was used to examine the relationship between the different inflammatory parameters and duration of lameness. All assessments were two-tailed and significance was set at $p < 0.05$.

Results

Dogs

Cranial cruciate ligament diseased group

The CCLD group consisted of 21 females (8 intact, 13 spayed) and 15 males (3 intact, 12 castrated) which represented the following breeds: Labrador Retriever ($n = 10$), Golden Retriever ($n = 5$), Border Collie ($n = 4$), with remaining breeds represented by 3 or fewer dogs. Mean (range) patient age and body weight were 5.9 years (range: 1.2–10.9) and 36.6 kg (range: 14.5–76.0). Relating to the BCS, 19 dogs were in the lean to ideal category, 10 dogs were grouped as overweight, and seven dogs were obese. Within one month prior to surgery, 14 dogs had received daily, 11 dogs intermittent, and 11 dogs no medication (carprofen, firocoxib, meloxicam, robenacoxib, or prednisone), respectively. A partial CCL rupture was diagnosed in 15 dogs (four left, 11 right) and a complete rupture in 21 dogs

with 14 on the left and seven on the right stifle joint. Sixteen dogs (44%) suffered a rupture of the contralateral CCL within a mean time of 13 months (range: 2–48 months) after the diagnosis of unilateral CCL rupture. The mean duration of lameness was 96 days (range: 7–360 days). Based on the grading of the morphological alteration (Table 1) within the joint, five dogs showed mild, 14 dogs moderate and 17 dogs severe synovitis. Dogs with a complete CCL rupture showed statistically significant higher scores of synovitis ($p = 0.038$). Development of osteophytes was observed in 23 dogs, 13 dogs had solitary and 10 dogs multiple osteophytes. Meniscal injury was noted in 16 stifles, whereupon a fibrillated surface was detected in five dogs and 11 dogs had a displaced tear, however, there was no correlation with the degree of synovitis ($r_s = 0.31$; $p = 0.073$; 95% CI -0.03-0.58).

Control

The control group comprised 20 Beagles, two Labrador Retrievers and one Hovawart that fulfilled the inclusion criteria. Of these control dogs, eight were castrated males and 15 spayed females. Mean age and weight were 4.1 years (range: 1.1–10.8 years) and 13.2 kg (range: 7.4–33.2 kg). Based on the BCS, 16 dogs were classified as lean to ideal

and 7 dogs as overweight. In all control dogs, morphological features of joint structures appeared normal.

Inflammatory pattern of IFP, ScAT and synovial fluid Immune composition of the IFP and ScAT

Immune cell composition of IFP and paired ScAT from diseased and control dogs are presented in Fig. 1. Flow cytometry analysis demonstrated a significant difference with more cells/ g tissue present in the IFP of dogs with CCLD than in the paired ScAT. T cells (CD3) were the most abundant immune cells in the IFP from diseased dogs followed by B cells (CD19/21) and macrophages (CD14) in similar percentages. Beside macrophages, neutrophils were numerous recruited in the IFP stained with the antibody MAC387. Moreover, a statistically significant difference was found for T cells ($p < 0.005$) and macrophages ($p < 0.0005$), with elevated levels in the IFP compared to the paired ScAT of dogs with CCLD and to both control adipose tissues from healthy control dogs. In control dogs, the most abundant cells were B cells with no difference between IFP and ScAT. The level of B cells found in ScAT from diseased dogs was significantly lower ($p < 0.0005$) in comparison to that from control dogs. No relationship was seen between number of CD3⁺ or CD14⁺ cells and duration of lameness (CD3, $r_s = 0.07$, $p = 0.7$; 95% CI -0.4-0.5; CD14, $r_s = -0.38$, $p = 0.08$; 95% CI -0.7-0.05), grade of synovitis (CD3, $p = 0.4$; CD14, $p = 0.92$), medication (CD3, $p = 0.37$; CD14, $p = 0.9$) or BCS (CD3, $p = 0.10$; CD14, $p = 0.19$).

Inflammatory gene expression in IFP and ScAT

Gene expressions are presented for IL-1 β , IL-6, IL-8 and IL-10 in Fig. 2; for MMP-1, MMP-3, MMP-13 and TIMP-2 in Fig. 3 and for iNOS and TNF α in Fig. 4. Comparisons were made to internal paired samples of the thigh ScAT

and corresponding tissues of control dogs. The RT-qPCR revealed that genes of various factors, in particular IL-1 β ($p = 0.02$), IL-6 ($p = 0.03$), IL-10 ($p = 0.006$), TNF α ($p = 0.002$), MMP-1 ($p = 0.004$) and also MMP-13 ($p = 0.005$) were more activated in the IFP of diseased dogs compared to the IFP of healthy control dogs. Furthermore, the corresponding ScAT of dogs with CCLD showed an increase in quantity of IL-10 ($p = 0.006$) and TNF α ($p = 0.0005$) when compared to control ScAT. Quantity of MMP-3 gene expression was only higher in ScAT of dogs with CCLD than in ScAT of control dogs. All tissue samples expressed MMP-1, which was the most abundantly expressed factor, particularly in IFP from diseased dogs, with a value more than 1500-fold higher than in ScAT of control dogs and more than 700-fold higher than in IFP of control dogs. Moreover, when comparing adipose tissue sources within groups, we found a statistically significant increase in gene expression of MMP-1 ($p = 0.01$), MMP-3 ($p = 0.03$), and MMP-13 ($p = 0.04$) in IFP compared to paired ScAT (see Fig. 3) within the CCLD group. In contrast, gene expression of iNOS was statistically significantly decreased in both tissues (ScAT, $p = 0.0001$; IFP, $p = 0.008$) of the CCLD group compared to the control group. No significant differences were found within or between groups for gene expression of IL-8 and TIMP-2.

Adipokine, cytokine and MMP secretion

The quantity of selected adipokines, interleukins, and MMPs was measured in the supernatant of IFP- and ScAT-conditioned medium as well as in paired synovial fluid (Figs. 2, 3 and 4). The adipokines adiponectin and leptin were detected in all samples. While no differences of adiponectin secretion were detected within the control group, a statistically significant difference was found in

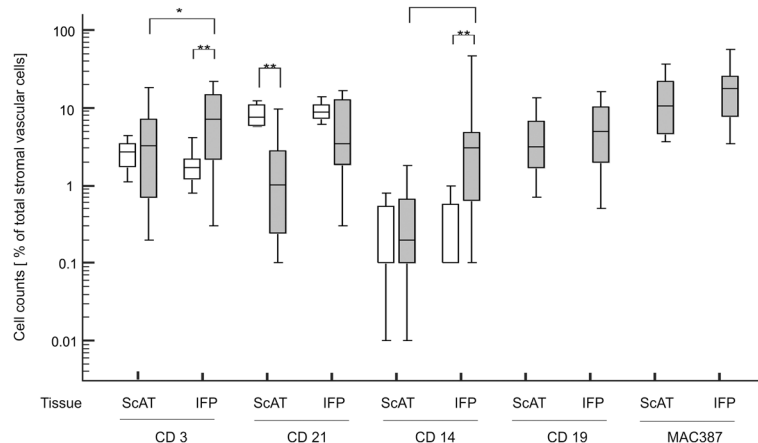
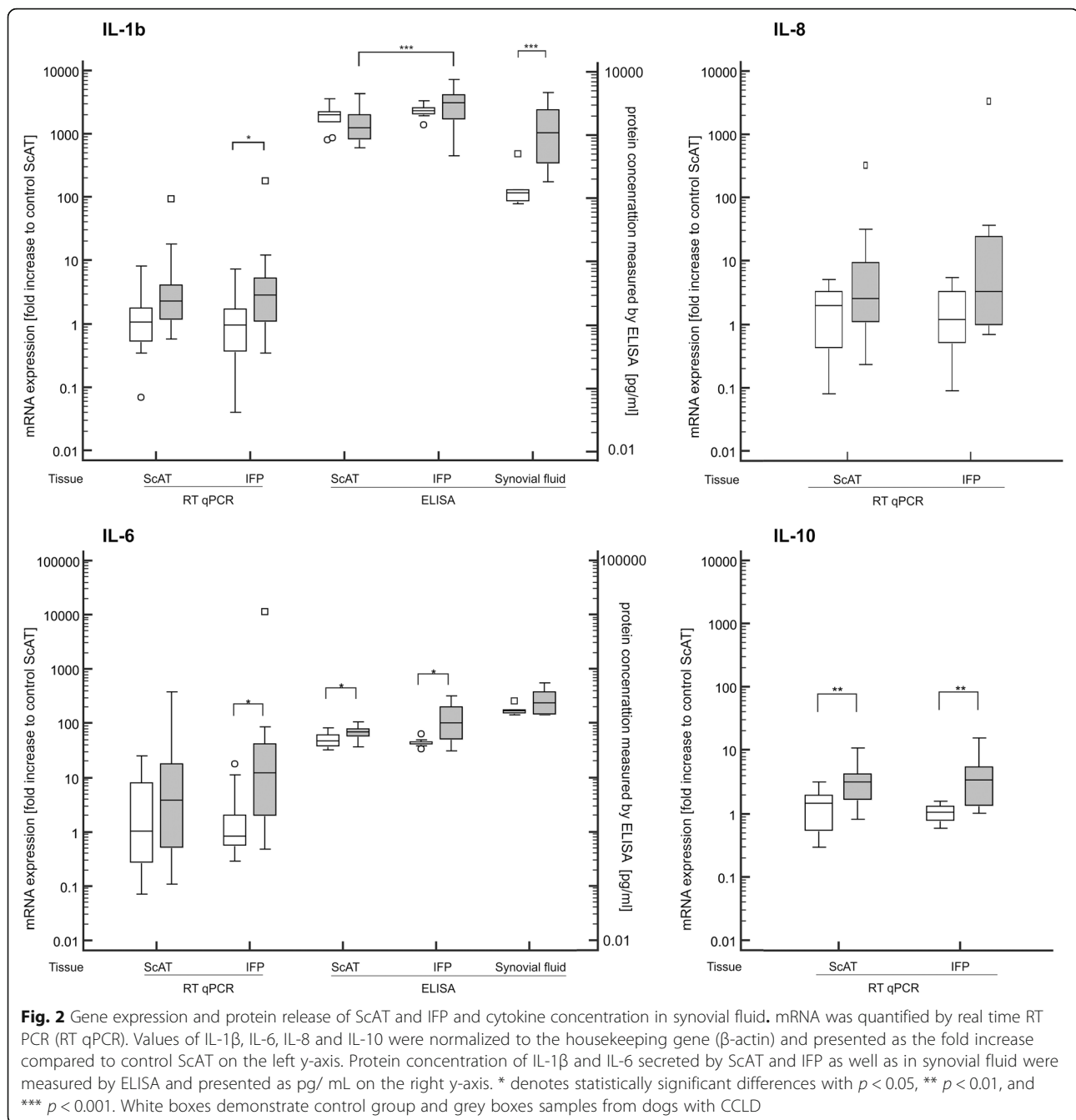


Fig. 1 Immune cell composition of the ScAT and IFP. Cells were isolated from the stromal vascular fraction of ScAT and IFP of control dogs (white boxes) and CCL diseased dogs (grey boxes). Immune cells were characterized by flow cytometry and shown as percentage of total stromal vascular cells. * denotes statistically significant differences with $p < 0.005$, ** $p < 0.0005$

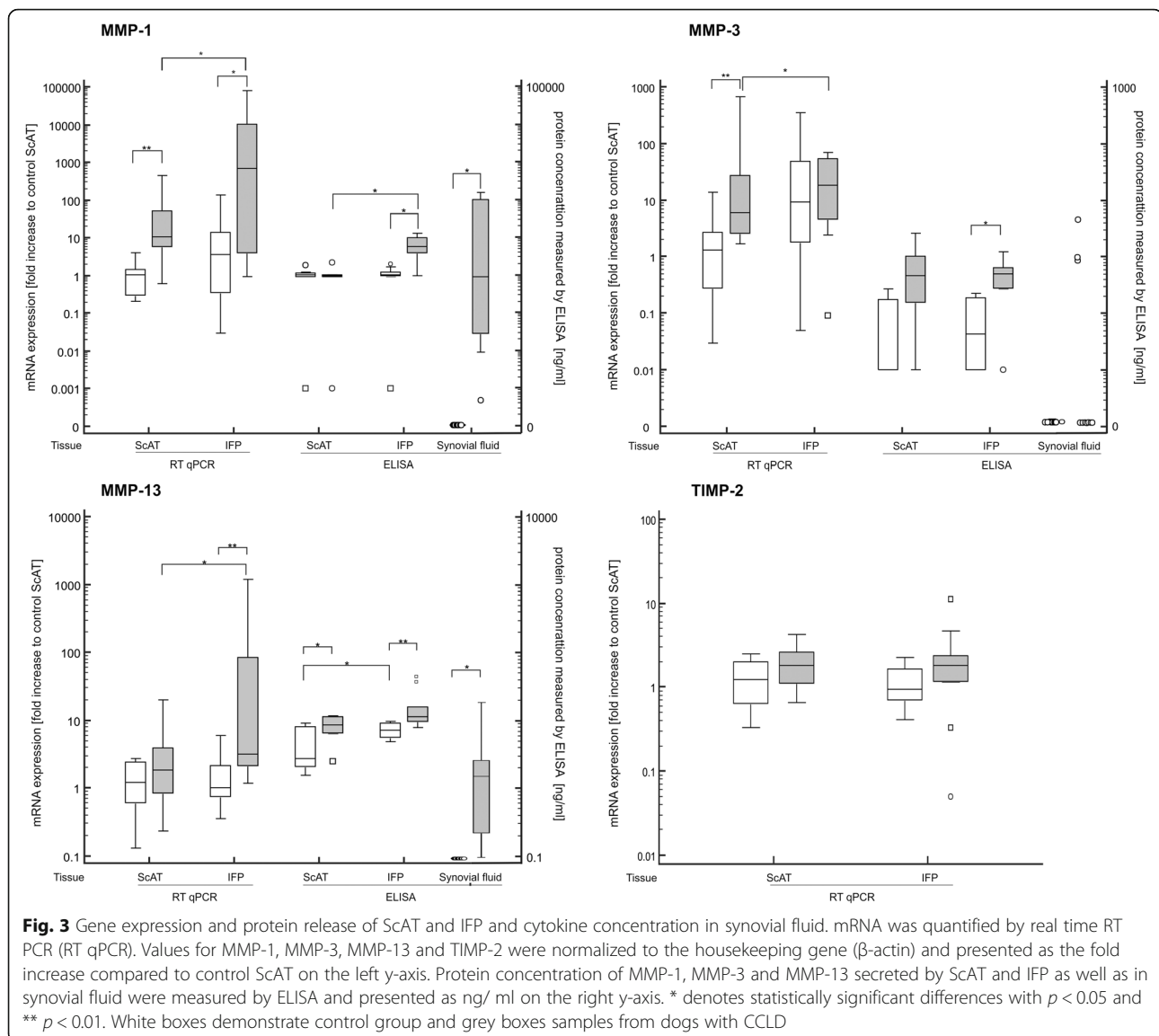


dogs with CCLD. Here, IFP of the involved joints showed higher quantities of adiponectin secreted compared to the paired ScAT ($p = 0.0001$). Interestingly, there was a statistically significant difference in leptin secretion, this being lower in both tissues from diseased dogs compared to controls ($p < 0.003$). Furthermore, a concurrent, statistically significant lower level of secretion in the IFP ($p < 0.01$) was also exhibited in comparison to the ScAT.

Analyzing degrading enzymes, the most obvious differences were detected for MMP-1, similar to the RT-qPCR results. The highest secretion of MMP-1 was detected in

IFP from diseased dogs, this being statistically significantly higher compared to paired ScAT ($p = 0.02$) as well as to IFP and ScAT from control dogs ($p < 0.004$). Fat-conditioned media from CCLD dogs of both, IFP and ScAT, contained statistically significantly higher amounts of MMP-13 compared to healthy control dogs. Regarding MMP-3, the concentration was below the detection limit for all control samples. In diseased dogs, only half of the samples yielded measurable values.

Results of interleukin secretion into fat-conditioned media showed that IL-6 was statistically significantly elevated in IFP



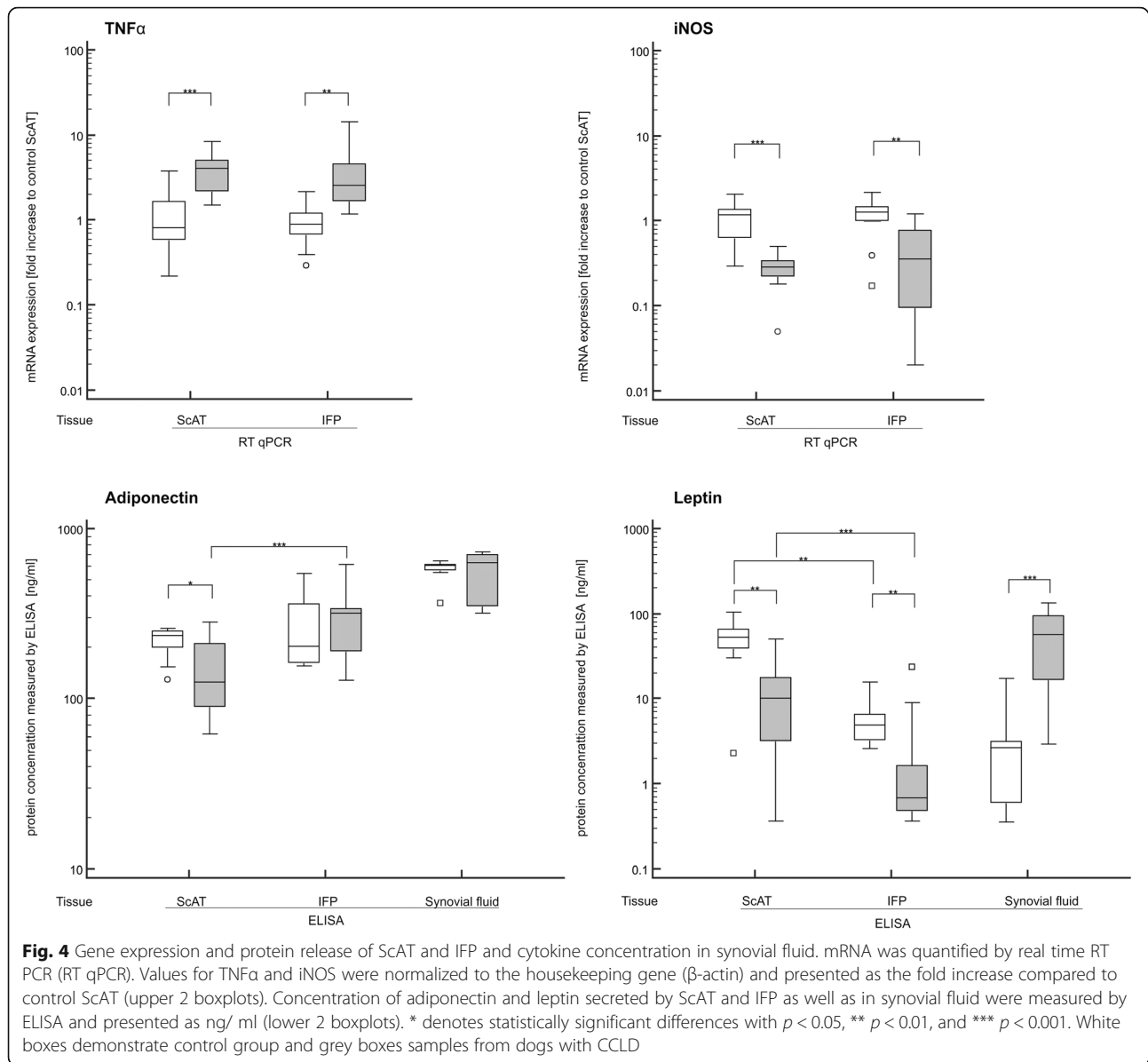
and ScAT from CCLD dogs compared to control dogs. Moreover, there was a statistically significant higher secretion of IL-1 β in the IFP compared to the ScAT ($p = 0.0002$), which was only detected within the CCLD group.

In the synovial fluid from dogs with CCLD, statistically significant higher concentrations of IL-1 β , MMP-1 and MMP-13 as well as leptin were detected compared to the control synovial fluid. The concentration of leptin was 20-fold higher than in control synovial fluid ($p = 0.0002$) and on average 70-fold higher compared to the paired IFP ($p < 0.0001$). In the control synovial fluid, MMP-1 and MMP-13 were below the detection limit, while in synovial fluid of dogs suffering from CCLD, six out of eight dogs showed a statistically significant increase ($p < 0.01$) in these two matrix degrading enzymes. MMP-3 was only detected in three samples obtained from dogs with CCLD.

Statistically significant increases were found with progressive duration of lameness in the levels of IL-1 β ($rs = 0.78$, $p < 0.0001$, 95% CI 0.55–0.9), MMP-1 ($rs = 0.73$, $p < 0.001$, 95% CI 0.38–0.89) and MMP-13 ($rs = 0.84$, $p < 0.0001$, 95% CI 0.61–0.94). No significant relationships were found between clustered BCS, medication or synovitis and the various inflammatory parameters analyzed.

Discussion

Today, it is generally accepted that CCLD exhibits a complexity of biomechanical and biological events. Several studies demonstrated that modulatory changes which take place in the synovial lining, in the ligament as well as in the cartilage are initiated through mutual pro-inflammatory cytokines and matrix degrading enzymes, which are subsequently dispersed by synovial



fluid [5, 29]. The IFP is an additional intra-articular tissue. To date, in veterinary medicine, in comparison to human medicine, there has been significantly less research performed to investigate the role of IFP in progression of joint pathologies.

In the present study, we were able to demonstrate that the IFP exhibits a pro-inflammatory pattern during CCLD. The activated state of the IFP was apparent through an increased gene expression and/or release of pro-inflammatory cytokines like IL-1 β and IL-6, and destructive enzymes like MMP-1 and MMP-13 as well as adipokines such as adiponectin and leptin. Immune cells present in the adipose tissue could be responsible for most of the production and release of inflammatory mediators, except for leptin and adiponectin, which are

mainly secreted by adipocytes [30]. In order to gain insight into the possible cellular origin of the secreted inflammatory mediators, we characterized the cell types present in the IFP and ScAT. Our results revealed substantial differences in the immune cell composition between diseased and control dogs. An altered immune cell composition could subsequently explain the higher secreting activity of inflammatory mediators within the IFP in CCLD. The predominant cell types in the IFP which originated from dogs with CCLD were T cells and macrophages. This did not apply to paired ScAT or the corresponding adipose tissue from control dogs which both showed no increased level of these cell types.

Both cell types can generate an enormous spectrum of cytokines including IL-1 β , IL-6 and TNF α [31–33].

Moreover, macrophages are suggested to be the major regulator of the production of MMP-1 and MMP-3 [34]. The different cell composition could therefore contribute to the higher release of inflammatory mediators by the IFP in CCLD. Studies analyzing the cell composition of IFP from humans with primary OA showed that macrophages followed by T cells were the most abundant immune cells in this tissue [19]. Although T cell counts were lower than in the subcutaneous adipose tissue, they had a predominant inflammatory phenotype. Various human studies have demonstrated that the type and relative abundance of immune cells are variable and depend on both the type of adipose tissue investigated and the adiposity of the individual [35–38]. Human IFP obtained from patients with CCLD had more classical inflammatory macrophages than IFP from patients with primary OA [37]. In contrast, no correlation between the presence of immune cells and BCS has been obtained in our study. However, the number of animals included in our study resulted in small BCS subgroup size with low statistical power.

Immune cell infiltration is postulated to be a major source of pro-inflammatory mediators perpetuating the inflammatory response in adjacent tissue by paracrine and autocrine effects. Studies looking at synovial changes in various stages of canine OA have found a predominance of T- and B-cells and macrophages [39–41].

Infiltrating immune cells are believed to interact with the resident adipocytes through secretion of cytokines, which leads to reciprocal modulation and change of the inflammatory character of the adipose tissue [18, 42]. Our study determined the level of expression of several inflammatory mediators in ScAT and IFP as well as their levels of proteins secreted into fat-conditioned media. One major finding of our study was that IFP obtained from dogs with CCLD demonstrated higher gene expression levels of IL-1 β , IL-6 and TNF α as well as of MMP-1 and MMP-13 compared to both adipose tissues from control dogs. Furthermore, within the CCLD group a significant higher expression of MMP-1, MMP-3 and MMP-13 was detected in the IFP when compared to the paired ScAT. To our knowledge, there is only one publication that investigated the expression of interleukins among other joint tissues in the canine IFP [43]. In contrast to our results, they were not able to show a statistically significant difference in the gene expression of IL-1 β and IL-6 between six IFP samples from dogs with CCLD and five healthy control IFP samples. More data is available for human IFP. Investigations of the IFP in human OA joints revealed a similar expression of inflammatory genes as we identified in the IFP of canine CCLD stifle joints [44, 45]. Moreover, human studies also showed an increase protein secretion for several cytokines [45, 46], similar to the significantly increased

secretory activity of IL-1 β and MMP-1 in the IFP of diseased dogs. The gene expression of iNOS was only significantly downregulated in the IFP of CCLD dogs compared to their healthy control equivalents. Activation of the iNOS system was detected in various joint tissues from dogs suffering from CCLD, suggesting that iNOS is regulated locally and tissue specifically [47].

As the role of adipokines in joint diseases has not been the subject of much investigation in veterinary medicine, we shall address adiponectin and leptin in more detail. A study investigating the inflammatory characteristics of human IFP in knee OA and obesity demonstrated an overproduction of IL-6, which was associated with a decrease of local leptin secretion and an increase in adiponectin secretion [48]. These findings are consistent with our results in dogs with CCLD. In contrast to its protective role against obesity, local adiponectin might act as a pro-inflammatory agent and contribute to matrix degradation in joint tissues [49]. Adiponectin was originally thought to be produced mainly by adipose tissue, meanwhile expression can be induced in synoviocytes as well as in chondrocytes [48, 50]. Because the interaction of articular and periarticular tissues is at least in parts believed to be transmitted through the synovial fluid, we also compared these mediators in the synovial fluid. The concentration of adiponectin in synovial fluid was higher than that secreted by IFP and levels were not different between healthy control and diseased stifle joints, emphasizing that synovial adiponectin is regulated by more than the IFP. Up to date, its role in the pathogenesis of OA is not completely elucidated. There is some evidence that adiponectin affects IL-1 β , IL-6, MMP-1, MMP-3, MMP-13 and NO secretion and suppresses mature macrophage functions such as phagocytosis [51, 52].

Findings regarding leptin are inconsistent in human literature. While some investigators propose a pro-inflammatory role of leptin in the regulation of cartilage metabolism via activation of MMPs [42, 53, 54] others have identified a decrease of locally produced leptin by the IFP [48] in obese OA patients. Concentration of plasma leptin is positively correlated with increased storage of subcutaneous adipose tissue [55]. However, the majority of studies showed that the release of leptin and adiponectin was independent of body mass and gender [56], which is in concordance with our results. Interestingly, we found elevated levels of synovial leptin (in average 70-fold) compared to the IFP production of dogs with CCLD, suggesting that other local tissue must release leptin into the synovial fluid. Recent findings indicate that deteriorated articular cartilage and synoviocytes are another source of adiponectin and leptin within the joint [57].

A limitation of the present study is the fact that we measured the mediators only in IFP, ScAT and synovial fluid. Thus, although we were able to characterize the inflammatory potential of the adipose tissue, it was not

possible to differentiate the origin of mediator production, thereby imposing a restriction on the possibilities for understanding the pathophysiological pathways involved. Additional paired samples of the synovial membrane, ligaments as well as cartilage would have offered a more comprehensive picture and thereby provided assistance in the identification of the pathways. Furthermore, grading of the clinical severity of CCLD and BCS is nonetheless subjective and this may have introduced some insensitive measure bias. We believe that bias and the limited number of samples may be responsible for some of the wide variability associated with gene expression and protein production between the BCS subgroups. The used ELISA for MMP-3 in which measured values were recorded below detection limit could also be considered a limitation. However, various studies were not able to detect MMPs in synovial fluid [14, 58]. One reason could be that MMP-3 strongly expressed in early OA and down regulated in late phase which might establish the observed differences [59]. Therefore, the use of this kit was considered a minor limitation, but may have affected the results associated with MMP-3. In contrast to similar studies in human medicine, the ability in animal studies to obtain control tissue from a healthy control group poses a major advantage.

Conclusions

In conclusion, as reported in similar findings in human medicine, the results of the present study indicate that the IFP has to be considered as a potential factor in the pathogenesis of CCLD. We showed that the IFP might have specific inflammatory phenotypic features independent from the general phenotype found in obesity. Because dogs are often used as models in human medicine, the present and similar studies could be regarded with great interest there to gain further insight into the processes involved. Nevertheless, it would be very interesting to obtain specimens of adjacent joint tissues. Future investigations must be carried out in order to estimate the exact impact of this immunomodulatory tissue within the stifle joint on causing or driving this particular disease.

Abbreviations

BCS: Body Condition Score; CCL: Canine Cruciate Ligament; CCLD: Canine Cruciate Ligament Disease; CD: Cluster of Differentiation; CI: Confidential Interval; DMEM: Dulbecco's Modified Eagle's Medium; DTT: Dithiothreitol; ELISA: Enzyme-linked Immunosorbent Assay; FACS: Fluorescence-activated Cell Sorting; IFP: Infrapatellar Fat Pad; IL: Interleukin; iNOS: inducible Nitric Oxide Synthase; MMP: Matrix Metalloproteinases; mRNA: messenger Ribonucleic Acid; NO: Nitric Oxide; OA: Osteoarthritis; *p*: *P*-value; PBS: Phosphate Buffered Saline; RNA: Ribonucleic Acid; *r*_s: Spearman's Correlation Coefficient; RT-qPCR: Quantitative Real-time Reverse Transcriptase Polymerase Chain Reaction; ScAT: Subcutaneous Adipose Tissue; TIMP: Tissue Inhibitor of Metalloproteinases; TNF: Tumor Necrosis Factor

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MRS merged and interpreted all the data, reviewed the literature and drafted the manuscript. BF carried out the flow cytometry analysis, NK carried out the PCR experiments, UR and DS were the operating surgeons collecting tissue specimens during knee surgery, MD contributed to the design and supervised the execution of all PCR experiments, DES developed the study design and helped to draft the paper, SF designed the study, helped with and supervised the execution of laboratory work, as well as the statistical interpretation of the data and reviewed the final paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were approved by the commission of animal experimentation of the Canton of Berne and Basel, Switzerland (BE116/13; BS2197). Prior to study enrolment, written owner consent was obtained for each dog.

Competing interests

The authors declare that they have no competing interests.

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