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Detection of osteoarthritis in dogs by metabolic, pro-inflammatory and degenerative synovial fluid biomarkers and traditional radiographic screening: A pilot study

E. de Bakker [a,*], B. Broeckx [b], K. Demeyere [c], V. Stroobants [d], B. Van Ryssen [a], E. Meyer [c]

[a] Department of Small Animal Medical Imaging and Orthopaedics, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
[b] Department of Nutrition, Genetics and Ethology, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
[c] Department of Pharmacology, Toxicology and Biochemistry, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
[d] Department of Virology, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

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ABSTRACT

Secondary osteoarthritis (OA) is a slow progressive, common disorder of synovial joints in dogs. It is characterized by a loss of balance between the synthesis and degeneration of articular cartilage components. Its diagnosis is currently based on the presence of clear radiographic changes, which only occur in the later stages of the disease. Hence, early diagnosis of OA remains a major problem. Therefore, interest in synovial fluid (SF) biomarkers has emerged. Besides pro-inflammatory and degenerative markers, i.e. tumor necrosis factor alpha (TNF-alpha), interleukin-1beta (IL-1beta), tenascin-c (TN-C) and matrix metalloproteinase-2 (MMP-2), metabolic parameters, i.e. pH, glucose and lactate, can potentially be used to detect OA.

The current study demonstrated statistically significant differences in the SF levels of pH, glucose and lactate between OA-affected and normal joints. In addition, the in-house validated immuno-assays for TNF-alpha, IL-1beta, TN-C and MMP-2 allowed to demonstrate also statistically significant differences in the SF concentrations for all these biomarkers - except TNF-alpha - between OA-affected and normal joints. However, no correlation was found between any of these biomarkers and the currently used radiographic scoring system for OA in dogs.

Future research is warranted to explore the potential of these biomarkers in the early detection of OA and in the severity characterization of this disease.

1. Introduction

Osteoarthritis (OA) is characterized by an imbalance between the synthesis and degeneration of articular cartilage components resulting in destruction of joint cartilage, remodeling of the underlying bone, osteophyte formation and variable degrees of synovitis (Fujita et al., 2005). In dogs, OA develops mainly secondary to some specific or predisposing condition, including injury, developmental abnormalities, infectious or immune-mediated arthritis and neoplasia (de Bakker et al., 2017d). The current method for determining OA due to an orthopaedic condition in dogs involves subjective grading schemes based on imaging techniques, mainly radiography and computed tomography (de Bakker et al., 2017d; Fujita et al., 2005). Although a correlation between the pathogenesis of OA and the appearance of specific radiographic features has been demonstrated, the silent onset of OA prevents early diagnosis (de Bakker et al., 2017d). Therefore, substantial interest in synovial fluid (SF) biochemical markers has emerged, not only to objectively assess OA, but also to understand the mechanisms initiating and progressing the disease to enable early diagnosis, predict prognosis and monitor treatment.

As recently reviewed by our group, several biomarkers have been associated with inflammation and OA in canine joints, including pro-inflammatory and degenerative markers (de Bakker et al., 2017d). However, the majority of these biomarker tests have not yet been

Abbreviations: APMA, p-aminophenylmercuric acetate; BW, bodyweight; CV, coefficient of variation; DL, detection limit; ECM, extracellular matrix; H, hour; HA, hyaluronic acid; HIF-1alpha, hypoxia-induced factor – 1alpha; kg, kilogram; L, liter; LOD, limit of detection; LOQ, limit of quantification; m, month; min, minute; mL, milliliter; mmol, millimol; MMP-2, matrix metalloproteinasis 2; MRI, magnetic resonance imaging; µL, microliter; ng, nanogram; OA, osteoarthritis; PBS, phosphate buffered saline; pg, picogram; QL, quantification limit; SD, standard deviation; SF, synovial fluid; TMB, Tetra Methyl Benzidine; TN-C, tenascin-C; w, week.

* Corresponding author.
E-mail address: Evelien.debakker@ugent.be (E. de Bakker).

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validated for canine SF. Another group of biomarkers that can potentially be used to detect OA are metabolic parameters originating from anaerobic glycolysis within the chondrocytes, i.e. pH, glucose and lactate. A few recent studies in dogs and horses have shown that the increased anaerobic metabolism in OA-affected joints resulted in an increased level of lactate and a pH drop (Kraus et al., 2002; Lacitignola et al., 2008). The advantage of these metabolic biomarkers is that their analytical methods are simpler and cheaper compared to the immunoassays for the pro-inflammatory and degenerative biomarkers. Therefore, the first aim of the present pilot study was to compare pH, glucose and lactate values in SF of dogs affected by OA diagnosed on radiography with normal joints of dogs. Since the available commercial immunoassays for the pro-inflammatory biomarkers tumor necrosis factor alpha (TNF-alpha) and interleukin-1beta (IL-1beta), and for the degenerative biomarkers tenascin-c (TN-C) and matrix metalloproteinase-2 (MMP-2) have not been validated for SF, our second aim was the in-house validation of these commercial immunoassays on canine SF. Once validated, as a third aim, the concentrations of TNF-alpha, IL-1beta, TN-C and MMP-2 were compared between affected and normal joints of dogs. The final aim was to investigate whether or not both these groups of biomarkers and the traditional radiographic scoring of OA were correlated.

2. Materials and methods

2.1. Study population

Between August 2014 and February 2017, SF samples of OA affected joints of dogs presented for surgery were obtained as part of the routine diagnostic work-up. Inclusion criteria were the presence of clinical lameness with pain limited to a single joint, confirmation of joint disease based on a thorough orthopaedic examination (e.g. joint distension, decreased range of motion, pain), standard orthogonal craniocaudal and mediolateral radiographic projections and intra-operative assessment. Breed, age, bodyweight (BW), gender and duration of lameness were noted. Whether dogs were on medication (non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and/or nutripharmaceuticals) at the time of sample taking was also noted. Patients receiving medication up to one month before sample taking were considered as no medication.

As a control group, large breed client-owned dogs anaesthetized for an unrelated surgery were included. Inclusion in the control group required the absence of lameness associated with the joint from which the SF sample was obtained and no abnormalities on orthopaedic (including subjective synovial fluid evaluation) and radiographic examination, which was part of the diagnostic work up. Since the synovial fluid volume of normal joints is very limited, the control group was randomly divided in two groups: one group for the metabolic biomarker's validation and the other group for the pro-inflammatory and degenerative biomarkers validation.

All animals were privately owned and presented at the Small Animal Clinic of the Faculty of Veterinary Medicine, Ghent. All dogs went home with the owner after the surgery. The local institutional ethical committee approved the procedure and owners were verbally informed.

2.2. Laboratory methods

2.2.1. Canine SF sampling, processing and preservation

SF was aseptically obtained from the affected or control joint by percutaneous arthrocentesis. For both affected and control joint at least 150 microliter (μL) of SF was required. The acquired SF was spun down (12,000 x g, 1.5 minutes (min)), aliquoted in 0.5 mL eppendorfs and stored at −80 °C until analysis.

2.2.2. Quantification of metabolic biomarkers in canine SF

Prior to analysis, a frozen aliquot of each SF sample was thawed at room temperature and centrifuged (11,500 x g, 10 min at 4 °C). As much as possible, multiple tests were performed on the same day using one aliquot. Due to the high viscosity of SF, accurate pipetting and analysis was challenging. Therefore, the samples were pretreated with bovine testicular hyaluronidase (HA) (Sigma-Aldrich, Saint-Louis, USA), which degrades hyaluronic acid, chondroitin and chondroitin sulphates to tetra- and hexasaccharides. The lyophilized HA was dissolved in phosphate buffered saline (PBS) to a concentration of 10 mg/mL.

2.2.2.1. pH. The pH was measured with a digital meter based on a glass electrode method (LAQUAtwin B-172; HORIBA Scientific, Kyoto, Japan). The devices' sensor was cleaned twice with distilled water and absorbent paper after which a two-points calibration was performed according to the manufacturers' instructions with the included standard solutions at pH 7.00 and 4.00. After each analysis, the sensor was cleaned as described above. For all samples, a volume of 50 μL of SF was applied to the sensor, followed by a sampling sheet B (HORIBA Scientific) to cover the complete surface of the sensor.

2.2.2.2. Glucose and lactate. Hundred μL of SF was applied to a sample cup together with 100 μL of HA and these volumes were mixed by pipetting up and down 10 times. The obtained solutions were applied to the IDEXX Catalyst Dx Chemistry analyzer and glucose and lactate were measured according to the manufacturers' instructions. Measuring range for glucose and lactate was 0.56–38.11 mmol/L and 0.50–12.00 mmol/L, respectively.

2.2.3. In-house validation of biomarker immunoassays for canine SF

2.2.3.1. Sensitivity. Assay sensitivity was calculated based on the mean and standard deviation (SD) of the absorption values of blank samples (assay diluent) to define the lowest concentration of IL-1beta, TN-C and MMP-2 in canine SF that can be reliably distinguished. The limits of detection (LOD) and quantification (LOQ) were defined as the mean blank value (10 repetitive measurements of diluent) + 2.6 x SD and mean blank value + 5 x SD, respectively (Buttner et al., 1979; Maddens et al., 2010).

2.2.3.2. Imprecision. To calculate the reproducibility of the immunoassay, samples were divided in three groups with low, intermediate and high analyte concentrations, covering the analytical range of the test (detailed sub 2.4). To determine the intra-assay coefficient of variation (CV), which indicates the within-day precision of the method, 3 (TNF-alpha), 6 (IL-1beta), 5 (TN-C), and 12 (active MMP-2) selected samples were analyzed in duplicate. First, individual CVs were calculated by dividing the SD of these parallel measurements by their mean and then the mean was determined of these individual CV values to obtain the final intra-assay CV (Jensen et al., 2000). To calculate the inter-assay CV, which indicates the day-to-day repeatability of the immunoassay, measurement of 7 (TNF-alpha), 12 (IL-1beta), 11 (TN-C) and 5 (MMP-2) selected samples was repeated once in time 72 days (TNF-alpha), 41 days (IL-1beta), 27 days (TN-C,) and 140 days (MMP-2) later. For each sample, the CV was calculated and the mean inter-assay CV then determined.

2.2.3.3. Linearity. The precision of the assay results was determined by evaluation of the linearity after dilution. For this purpose, SF samples were serially diluted fivefold with assay diluent.

2.2.4. Quantification of pro-inflammatory and degenerative biomarker concentrations in canine SF

Frozen aliquots of each SF sample were thawed at room temperature and centrifuged (11,500 x g, 10 min at 4 °C). The samples were pretreated with HA, as described sub 2.2. TNF-alpha, IL-1beta and TN-C concentrations were analyzed with commercially available canine and

human sandwich enzyme-linked immunosorbent assays, respectively (Antibodies-online, Aachen, Germany; Cloude Clone Corp, Texas, USA; and Immuno Biological Laboratories, Gunma, Japan). Biologically active and total concentrations of MMP-2 were analyzed with an activity assay (GE Healthcare, UK). For each immunoassay, the absorbance was measured at the indicated wavelength by a plate reader. The concentration of the SF marker in the test sample was interpolated from the standard curve and corrected for the used dilution by using Deltasoft, a microplate analysis software (Deltasoft JV 2.1.2).

2.2.4.1. TNF-alpha. The protocol was carried out according to the manufacturers' instructions. Briefly, precoated wells were filled with 100 µL of 1/2 diluted SF or with the provided standard (range 15.6–1000.0 pg/mL). After a 2 -h (h) incubation at 37 °C, the liquid was removed and 100 µL of detection reagent A was added. Following incubation for 1 h at 37 °C, three washing steps were performed and 100 µL of detection reagent B was added for another 30 min at 37 °C. Five washing steps were performed after removal of the liquid and the bound enzyme was assayed by addition of 90 µL of a chromogenic substrate, Tetra Methyl Benzidine (TMB) in the dark for 10−20 min at 37 °C. After adding 50 µL of stop solution, the absorbance was measured at 450 nm.

2.2.4.2. IL-1beta. The protocol was carried out according to the manufacturers' instructions. Briefly, precoated wells were filled with 100 µL of 1/5 diluted SF or with the provided standard (range 7.8–500.0 pg/mL). After a 2 h incubation at 37 °C, 100 µL of detection reagent A was added to the wells immediately after removing the liquid. Following incubation for 1 h at 37 °C, three washing steps were performed and 100 µL of detection reagent B was added for another 30 min. Five washing steps were performed and the bound enzyme was assayed by addition of 90 µL of TMB in the dark for 20 min at 37 °C. After adding 50 µL of stop solution, the absorbance was measured at 450 nm.

2.2.4.3. TN-C. The protocol was carried out according to the manufacturers' instructions. Briefly, wells precoated with anti-human TN-C mouse IgG antibody were filled with 100 µL of 1/20 diluted SF of the affected dogs or 1/2 diluted SF of the healthy control dogs or with the provided standard (range 0.38–24.00 ng/mL) and were incubated for 1 h at 37 °C. After 8 washing steps, wells were filled with 100 µl of anti-human TN-C mouse IgG antibody conjugated with horseradish peroxidase and incubated for another 30 min at 2−8 °C. Following 9 washing steps, the bound enzyme was assayed by addition of 100 µL of TMB in the dark. After 30 min, 100 µL of 1 N sulfuric acid was added to stop the reaction and the absorbance was measured at 450 nm.

2.2.4.4. MMP-2. The protocol was carried out according to the manufacturers' instructions. Briefly, active and total MMP-2 concentrations were determined on the same plate, in a 1/5 and 1/50 dilution, respectively. Wells precoated with anti-MMP-2 were filled with 100 µL of the appropriate diluted SF or with the provided standard (range 0.19–12.00 ng/mL) and were incubated overnight at 2−8 °C. After four washing steps, 50 µL ready to use p-aminophenylmercuric acetate (APMA) to activate the preform of MMP-2 was added to the wells containing standards (pro MMP-2) and samples where total MMP-2 activity was to be measured. Instead of APMA, 50 µL of assay buffer was added to the wells containing samples, in which active MMP-2 was to be measured. Finally, 50 µL of detection reagent was added to all wells, shaking was performed for 20 s and the absorbance was measured immediately (0 h) at 405 nm, after 3 h (higher endogenous level of MMP-2 : 0.75−12 ng/mL) and after 6 h (lower endogenous level of MMP-2 : 0.13-3 ng/mL) incubation at 37 °C.

2.3. Radiographic scoring of OA

Radiographic synovial effusion was subjectively graded on a scale from 0 to 2 (0: normal; 1: mild; 2: severe), including cranial and caudal joint spaces (Sample et al., 2017).

The radiographic degree of OA was graded according to a previously described scoring system using the Efilm Workstation PACS software (Merge Efilm; Merge eMed; Milwaukee, Wisconsin, USA) (Bennett et al., 2013; D'Anjou et al., 2008; Runge et al., 2008; Wessely et al., 2017). Subchondral sclerosis and osteophytosis at specific locations were scored according to their size using a 0–3 scoring system (0: normal; 1: mild; 2: moderate; 3: severe). All radiographic images were anonymized and randomized for the scoring. EDB and BVR jointly scored the images. Two OA-affected dogs had radiographs from the referring vets, which were excluded for this study as the same standards for scoring could not be applied.

2.4. Statistics

First, a Wilcoxon rank sum test with Bonferroni correction for multiple testing was used to compare age, weight and biomarker concentration between affected and control joints. A comparison was also made between the biomarker concentrations in dogs receiving NSAIDs (yes/no) and joint supplements (yes/no) with a Wilcoxon rank sum test with the Bonferroni correction. Subsequently, all possible pairwise correlations (Spearman correlation coefficient) were assessed between all biomarkers. Finally, a potential correlation (Spearman correlation coefficient) between the metabolic parameters, pro-inflammatory and degenerative biomarkers and radiographic score was evaluated. The significance threshold α was set at ≤ 0.05. All analyses were conducted in R version 3.3.2 ("Sincere Pumpkin Patch").

3. Results

3.1. Patient characteristics

SF samples were collected from a total of 41 dogs: 23 (25 joints) affected with OA due to a stifle problem (cranial cruciate ligament rupture (23 joints) and lateral patellar luxation (2 joints)) in early and late disease stages and 18 normal (21 shoulder joints). Two of the 23 affected dogs developed a bilateral disease during the study period, which resulted in a total of 25 joints. Of the 18 normal dogs, ten were used for the metabolic biomarker validation and the other eight were part of the pro-inflammatory and degenerative biomarker validation. Of the 23 affected dogs, seven were entire males, four castrated males, seven intact females and five spayed females. There were three mixed breeds, two Labrador Retrievers, two Dobermanns, two Bernese Mountain Dogs, two German Shepherd dogs and one individual of the following breeds: Rottweiler, Beagle, Weimaraner, English Cocker Spaniel, Malinois, Vizsla, Pointer, Dog de Bordeaux, Golden Retriever, Akita, Mastino Napolitano, Tamaskan. Mean age of the affected group was 64.8 months (m) (range 3−153 m; standard deviation (SD) 35.5 m), mean bodyweight (BW) was 34.1 kilogram (kg) (range 20−50 kg; SD 8.7 kg) and mean duration of lameness 16.7 weeks (w) (range 0.5 w–104 w; SD 23.6 w). Half of the affected dogs were on medication at the time of sample taking: 11 received NSAIDs, 3 received joint supplements (glycosaminoglycans / chondroitin sulfates) and 1 received a combination of NSAIDs and joint supplements. There was no significant statistical difference in biomarker concentrations between patients with and without either NSAIDs and/or joint supplements (Table 1). Four of the ten control dogs were entire male, one castrated male, four intact female and one spayed female. There were three Labrador Retrievers, and one of each of the following breeds: Malinois, German Shepherd, English Bulldog, Bernese Mountain Dog, Border Collie, American Staffordshire, Pointer. Of the other 8 control dogs, there were two castrated and two intact males, three entire female and one spayed female. There were two Labrador Retrievers and one of each of the following breeds: Malinois, mixed breed, American Staffordshire Terrier, Welsh Springer Spaniel, Beagle and Swiss Shepherd Dog. Mean age of the control group, n = 10

Table 1
Statistical comparison between biomarker concentrations in affected joints with and without NSAIDs and/or joint supplements.

	NSAIDs	Joint supplements
Glucose	p = 1	p = 1
PH	p = 1	p = 1
Lactate	p = 0.676	p = 1
TNF-alpha	p = 1	p = 1
IL-1 beta	p = 1	p = 1
TN-C	p = 1	p = 1
MMP-2 active	p = 1	p = 1
MMP-2 total	p = 1	p = 0.607

p = Bonferroni-corrected p-value; threshold $p < 0.05$.

dogs and n = 8 dogs, was 43 m (range 8–152 m; SD 46.8 m) and 35 m (range 14–97 m; SD 32.7 m) and mean BW was 32.7 kg (range 26.8–41.5 kg; SD 17.5 kg) and 26.8 kg (range 16.0–39.5 kg; SD 8.2 kg), respectively.

There was no statistical difference regarding age and body weight between affected and control dogs (threshold $p < 0.05$; age Bonferroni-corrected p-value = 0.177; body weight Bonferroni-corrected p-value = 1).

3.2. In-house validation of biomarker immunoassays for canine SF

Sensitivity and precision values of the immunoassays for TNF-alpha, IL-1beta, TN-C and MMP-2 for canine SF are shown in Table 2. For IL-1beta and TN-C, the samples were serially diluted to check linearity. The relationship between the measured and the expected concentrations was investigated by regression analysis of the untransformed data. The 95 % confidence interval of the slope of the linear regression equation included 1, indicating a recovery of 100 %. Regarding specificity, these data of serially diluted SF samples fitted a linear model implying that the tested immunoassays for IL-1beta and TN-C are sufficiently flexible to measure a broad range of concentrations and have no matrix interference for canine SF (Fig. 1 left and right). For TNF-alpha, linearity could not be determined since the SF samples could only be diluted 1/2 due to the very low initial concentration. For MMP-2, two dilutions i.e. 1/20 and 1/50 of the same SF sample (n = 9) were measured. As the 1/20 dilution resulted in a higher concentration compared to the 1/50 dilution, suggesting a matrix interference, 1/50 dilutions for all samples were used to generate the research MMP-2 data.

3.3. Comparison of metabolic, pro-inflammatory and degenerative biomarkers in OA affected and normal joints SF

An overview of the results for both biomarker panels is shown in Table 3. For the panel of metabolic biomarkers, the pH in SF of OA-affected joints was significantly higher compared to that of normal joints ($p < 0.01$), with a median of 8.1 (range 7.8–8.9, n = 25) and 7.8 (range 7.6–8.3, n = 10), respectively (Fig. 2a). The glucose concentration was also significantly higher in SF of affected joints compared to that of normal joints ($p < 0.001$), with a median of 5.34 mmol/L (range 3.12–6.82 mmol/L, n = 25) and 3.17 mmol/L (range 2.64–4.28 mmol/L, n = 10), respectively (Fig. 2b). In marked contrast to both these metabolic parameters, the lactate concentration was significantly lower in SF of affected joints compared to normal joints ($p < 0.001$) with a median of 2.02 mmol/L (range 1.18–3.36 mmol/L, n = 25) and 3.47 mmol/L (range 2.52–5.16 mmol/L, n = 10), respectively (Fig. 2c).

For the panel of pro-inflammatory biomarkers, the concentration of TNF-alpha was higher, although not significantly (p = 1) in SF of OA-affected joints compared to normal joints with a median of 187.43 ng/mL (range 61.37–567.24 ng/mL, n = 12) and 135.72 ng/mL (range 29.88–211.96 ng/mL, n = 6), respectively (Fig. 3a). The concentration of IL-1beta was significantly higher in SF of OA-affected joints compared to normal joints ($p < 0.001$) with a median of 116.81 pg/mL (range 4.63–588.67 pg/mL, n = 25), and 18.72 pg/mL (range 1.85–37.97 pg/mL, n = 7 joints) (Fig. 3b), respectively. For the panel of degenerative biomarkers, TN-C was significantly higher in SF of OA-affected joints compared to normal joints (p = 0.01) with a median of 138.15 ng/mL (range 20.34–1073.55 ng/mL, n = 14) and 1.08 ng/mL (range 0.84–2.03 ng/mL, n = 5, respectively (Fig. 3c). The active MMP-2 concentration was significantly higher in affected joints compared to normal joints (p = 0.001) with a median of 26.45 ng/mL (range 16.41–32.30 ng/mL, n = 21) and 13.85 ng/mL (range 8.00–19.71 ng/mL, n = 4), respectively (Fig. 3d). The total MMP-2 concentrations were also significantly higher in SF of affected joints compared to normal joints ($p < 0.01$) with a median of 171.40 ng/mL (range 14.10–283.25 ng/mL, n = 18) and 22.61 ng/mL (range 21.90–23.32 ng/mL, n = 4), respectively (Fig. 3e).

Table 2
Validation results for TNF-alpha, IL-1beta, TN-C and MMP-2 in canine synovial fluid.

		TNF-alpha	IL-1beta	TN-C	MMP-2
Sensitivity	DL	16.61 pg/mL	10.36 pg/mL	0.84 ng/mL	3.59 ng/mL
	QL	18.81 pg/mL	21.40 pg/mL	1.19 ng/mL	9.17 ng/mL
Precision	Intra-assay CV (%)	23.44	3.79	7.11	9.08
	Inter-assay CV (%)	14.45	16.76	9.90	15.07

DL: Detection limit; QL: Quantification limit; CV: coefficient of variation.

3.4. Correlation between pro-inflammatory, degenerative and metabolic biomarkers in canine SF and the radiographic scoring of OA affected joints

No statistically significant correlation was found between each of the metabolic, pro-inflammatory and degenerative biomarkers (Fig. 4). In addition, when comparing each of these biomarkers to the radiographic scoring system, no significant correlation was found either (Fig. 4).

4. Discussion

In general, the measurement of metabolic biomarkers in SF is a relatively easy and cheap way to obtain information on the condition of a joint. Here, we hypothesized that such biomarkers can potentially aid in the detection of canine OA.

Ex vivo and *in vivo* studies on OA-affected joints show an increased lactate production and concomitant pH drop in dogs (de Oliveira El-Warrak et al., 2012d; Proot et al., 2015). Moreover, increased levels of lactate and decreased concentrations of glucose were found in both experimentally and naturally occurring OA in humans (Lee and Urban, 1997; Pfander et al., 2005). This suggests species-independent metabolic profiles on the one hand and similarity between naturally versus experimentally induced OA on the other hand. However, there is a high variability in pH, glucose and lactate concentrations within and between these different studies. Consequently, reference values are generally not available and are more specifically lacking in the dog.

In the current study, statistically significantly lower levels of lactate and higher levels of glucose and pH were found in affected joints compared to normal joints. A first possible explanation for this discrepancy with previous studies is that all SF samples in the present study were enzymatically pretreated with HA, in contrast to the other canine studies. Indeed, a main concern when measuring SF compared to whole blood is the difference in viscosity between both biological fluids. A second possible explanation is the lack of uniformity in analytical methods for these metabolic parameters. Indeed, the pH has been measured using either pH-electrodes or a blood gas analytical device or pH strips, while in the current study an electrode-based digital pH-meter

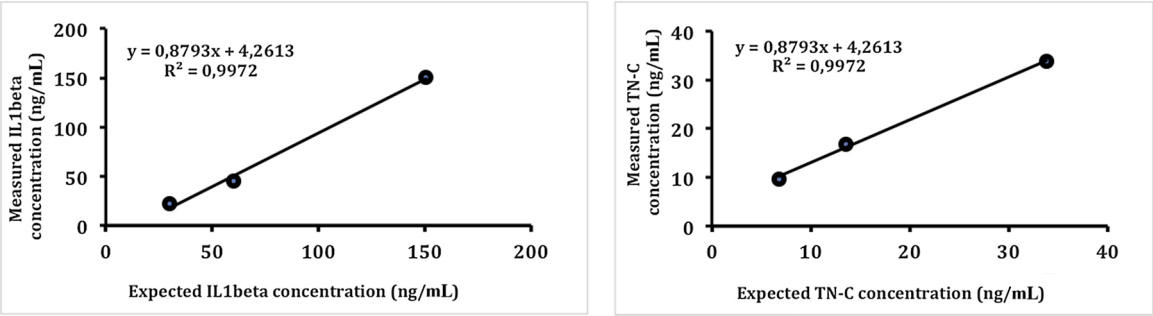

Fig. 1. Relationship between measured and expected concentrations for IL-1beta (left) and TN-C (right) demonstrating a 100 % recovery. R = goodness of fit.

was used although the latter was only validated for pleural effusion or ascites in humans. To quantify glucose and lactate, previous studies used a blood gas analytical device, micro-dialysis and proton nuclear magnetic resonance spectroscopy.

Thirdly, a few samples had several freeze-thaw cycles, which could negatively influence biomarker stability. However, studies on glucose stability in serum and plasma demonstrated only a slight influence of the thaw-freeze process (Cuhadar et al., 2013; Kale et al., 2012; Reynolds et al., 2006).

A fourth influencing factor is the combination of the so-called Warburg phenomenon with synovial effusion. Under normal physiologic hypoxic conditions, the chondrocyte's anaerobic metabolism increases with decreasing pO_2. However, when pO_2 decreases below a threshold value ($pO_2 < 2\%$, *in vitro*), a protective mechanism occurs which decreases cellular metabolism to a minimum (Lee and Urban, 1997; Ruiz-Romero et al., 2009). Chondrocytes in OA-affected joints are exposed to anoxic conditions and pO_2 is negatively correlated to the degree of synovial effusion, which is high in these joints (Pfander et al., 2005). Most likely, chondrocytes in the affected joints of our study, with a distinct synovial effusion, were exposed to such a low pO_2 inhibiting their ATP production and extracellular matrix synthesis. As a result, these cells used less glucose and produced less lactate, explaining the apparently contradictory observation of glucose accumulation in the SF of these joints. In line with this, 70 % of these joints showed a severe distension on radiography.

Complementing the selected metabolic biomarkers, the potential value of selected pro-inflammatory and degenerative biomarkers for canine OA was also evaluated. As recently reviewed by our research group, TNF-alpha, IL-1beta, respectively TN-C and MMP-2, are considered as valuable candidates (de Bakker et al., 2017d). In the present study, the selected commercial immunoassays for TNF-alpha and IL-1beta were not validated for canine SF while both the TN-C and MMP-2 immunoassays were, although their reported validation was rather limited (Boland et al., 2014; Chockalingam et al., 2013). Therefore, we performed an in-house validation for all four biomarkers in a carefully selected pool of normal canine SF spiked with each of the tested analytes, covering the analytical range of each test. Overall, satisfactory validation results were obtained providing the required analytical basis for subsequent application of these four immunoassays in our study. Regarding sensitivity, IL-1beta was found to be the most sensitive parameter being reliably measurable in the pg/mL concentration range, while both TN-C and MMP-2 allowed measurements in the ng/mL concentration range. Regarding precision, intra-assay CV values of less than 10 % were typically obtained, which are considered to be acceptable (Kjelgaard-Hansen et al., 2003). As expected, inter-assay CV values were higher than those for the intra-assay variation, but still acceptable for TN-C and MMP-2. However, a very high inter-assay CV for IL-1beta was obtained, in marked contrast to the excellent intra-assay variation for that biomarker. This unexpected observation can be tentatively explained by repeated freeze-thaw cycle of some of the SF pool aliquots, which might have negatively influenced the stability of this specific biomarker. To avoid this possible freeze-thaw artifact in the subsequent study on OA affected joints versus control joints, we analyzed all study samples within the same day.

Significant differences for IL-1beta, TN-C and MMP-2, but not for TNF-alpha, could be demonstrated between OA-affected and normal joints. This corroborated the increased levels of IL-1beta, TN-C and MMP-2 in dogs with OA located in the hip and stifle joint although these changes occurred concomitant with TNF-alpha in contrast to our findings (Boland et al., 2014; Chockalingam et al., 2013; Fujita et al., 2006, 2005). In humans, IL-1beta and TNF-alpha stimulate the production of cartilage degradative enzymes and inhibit synthesis of cartilage matrix, collagen and proteoglycan (Fujita et al., 2006). In dogs, it is known that both IL-1beta and TNF-alpha are synthesized by the synovium and potentiate the upregulation of MMP-2, which in turn participates in the macrophage-mediated joint destruction (Boland et al., 2014). The presence of these cytokines together with the decreasing pO_2 in OA affected joints stimulates the stabilization of hypoxia-induced factor (HIF)-1alpha in the chondrocyte increasing the expression of hypoxia-responsive glycolysis genes (Pfander et al., 2005). In addition, pro-inflammatory cytokines and HIF-1alpha stimulate MMP-2 leading to OA progression (Pfander and Gelse, 2007). A study on human TN-C in OA-affected joints demonstrated a correlation between TN-C levels and proteoglycan loss, most likely due to a Toll-like receptor 4-dependent TN-C induction of matrix degradation (Patel et al., 2011). TN-C regulates the expression of matrix metalloproteases, including MMP-2, but is also inducible in chondrocytes by the inflammatory cytokines, IL-1beta and TNF-alpha (Nakoshi et al., 2010; Rettig et al., 1994). Higher but no significant differences were seen for TNF-alpha in OA-affected compared to normal joints, this could be due to the small sizes of both groups, in combination with the heterogenous profile of the dogs included as stated below in the limitations of the present pilot study. Analytical factors might also play a role as previous studies measured TNF-alpha with a cytotoxicity bioassay while here a validated canine immuno-assay was used.

Although an effect on the metabolic, pro-inflammatory as well as degenerative biomarker profiles between control versus OA affected joints was observed, the present study was unable to detect significant correlations between these three groups of biomarkers in OA-affected joints. Likely, other mediators are influencing these proteins as part of the complex molecular network responsible for their regulation by paracrine and autocrine secretion mechanisms orchestrated between several joint cells during the pathophysiology of OA (de Bakker et al., 2017d; Fujita et al., 2006). The highest correlation that the present study could demonstrate was a negative one between TN-C and the active form of MMP-2 (Fig. 4). Increased production of extracellular matrix molecules (ECM), including TN-C during the early stages of OA, has been described (Aigner et al., 2001; Loening et al., 2000). As protease-mediated cartilage destruction continues, fragments of ECM proteins are generated, which accumulate during disease progression (Sofat et al., 2012). These specific ECM proteins become endogenous catabolic factors potentiating further joint damage in OA. Of relevance

Table 3
Overview of the metabolic, pro-inflammatory and degenerative biomarker results for affected and control joints as well as radiographic scoring for the affected joints.

Affected joint	METABOLIC PROFILE					PRO-INFLAMMATORY AND DEGENERATIVE BIOMARKERS			
	pH	glucose (mmol/L)	lactate (mmol/L)	TNF-alpha (pg/mL)	IL-1beta (pg/mL)	TN-C (ng/mL)	MMP-2 (ng/mL)		radiographic score (0–10)
							active	total	
1	8.0	5.50	2.12	81.7	164.21	–	29.30	209.10	4
2	8.6	6.00	1.36	108.45	82.05	139.78	29.00	146.65	4
3	8.1	4.02	1.64	61.37	89.46	–	26.65	181.40	4
4	8.1	5.94	2.16	–	41.16	–	18.55	–	4
5	8.2	5.38	1.78	120.35	159.97	–	26.45	69.45	5
6	8.1	3.90	1.94	–	159.08	–	32.30	–	4
7	8.2	4.48	1.50	69.08	588.67	142.69	20.81	14.10	8
8	8.3	6.82	3.36	359.01	88.46	75.58	27.85	141.70	8
9	8.9	5.34	1.18	–	105.12	–	24.50	169.70	4
10	8.1	4.08	2.08	–	144.98	–	28.35	173.10	–
11	8.9	4.24	2.98	254.5	108.41	20.34	29.85	136.15	6
12	8.2	5.16	2.72	–	98.17	–	27.30	163.85	1
13	8.1	5.54	1.86	–	100.20	101.38	30.50	149.70	5
14	8.0	5.70	2.10	447.32	279.34	89.94	32.20	173.70	1
15	8.1	6.70	2.08	576.24	123.03	–	–	–	9
16	8.2	3.12	2.02	–	185.41	754.10	21.65	283.25	7
17	7.9	5.74	2.60	–	237.71	95.40	17.47	208.75	5
18	8.3	3.88	1.86	–	41.12	136.51	19.65	242.15	4
19	7.8	4.90	2.56	359.53	105.78	961.00	18.95	211.30	6
20	7.9	5.04	2.68	535.8	196.92	1073.55	17.22	–	5
21	8.0	4.60	2.06	82.86	116.81	–	16.41	104.80	8
22	7.9	3.72	1.66	–	4.63	20.34	–	–	3
23	8.2	5.40	1.98	–	172.00	183.80	–	–	–
24	7.8	6.08	1.70	–	146.92	337.99	17.15	181.75	3
25	7.8	6.26	1.60	–	4.63	–	–	–	3
median	**8.1**	**5.34**	**2.02**	**187.43**	**116.81**	**138.15**	**26.45**	**171.40**	**4**
range	**7.8 – 8.9**	**3.12 – 6.82**	**1.18 – 3.36**	**61.37 – 567.24**	**4.63 – 588.67**	**20.34 – 1073.55**	**16.41 – 32.30**	**14.10 – 283.25**	**1 – 9**

Control joint	METABOLIC PROFILE			PRO-INFLAMMATORY AND DEGENERATIVE BIOMARKERS				
	pH	glucose (mmol/L)	lactate (mmol/L)	TNF-alpha (pg/mL)	IL-1β (pg/mL)	TN-C (ng/mL)	MMP-2 (ng/mL)	
							active	total
1	7.6	3.72	2.72					
2	8.3	2.64	3.10					
3	7.6	2.96	3.84					
4	7.5	3.40	2.74					
5	7.6	3.34	2.98					
6	7.7	2.78	5.16					
7	7.8	4.28	2.52					
8	7.8	3.36	5.00					
9	8.1	2.78	4.82					
10	8.0	3.00	4.06					
11				84.56		2.03		
12				186.88	35.00			
13				211.96		1.92		
14				29.88	16.45	0.84		
15				72.44	63.97			
16				203.31	40.09	0.84		
17					1.85	0.84	7.99	23.32
18					4.63		3.59	15.49
19					22.85			
20							14.69	19.71
21							14.55	23.44
Median	**7.8**	**3.17**	**3.47**	**135.72**	**18.72**	**1.08**	**13.85**	**22.61**
Range	**7.6—8.3**	**2.64 – 4.28**	**2.52 – 5.16**	**29.88 – 211.96**	**1.85 – 37.97**	**0.84 – 2.03**	**8.00 – 19.71**	**21.90 – 23.32**

Two of the OA-affected dogs had radiographs from the referring vet, which were excluded for analysis in this study.

for the observed negative correlation in the current pilot study, when TN-C is cleaved, these TN-C fragments obtain novel proteolytic activity absent in the full-length molecule, and mediate catabolic pathways in articular cartilage, e.g. activation of MMP-2 (Fichter et al., 2006; Sofat et al., 2012).

Radiography remains the first step in the diagnostic work-up of a joint problem (Bogaerts et al., 2018; Piermattei et al., 2006). This is mainly due to the wide availability of this imaging technique and its relatively low cost. The most important early radiographic sign of OA affected canine joints is synovial effusion, although not always radiographically clearly visible (Sample et al., 2017; Wall et al., 2015). Radiographic detection of articular osteophytes and sclerosis is limited by factors such as morphologic distortion, geometric magnification, and superimposition of overlying structures (D'Anjou et al., 2008). This can explain why the present pilot study failed to demonstrate a significant correlation between radiographic severity of OA and levels of biomarkers, since early signs might not have been recognized on radiographs. Although a published standardized OA scoring system was used,

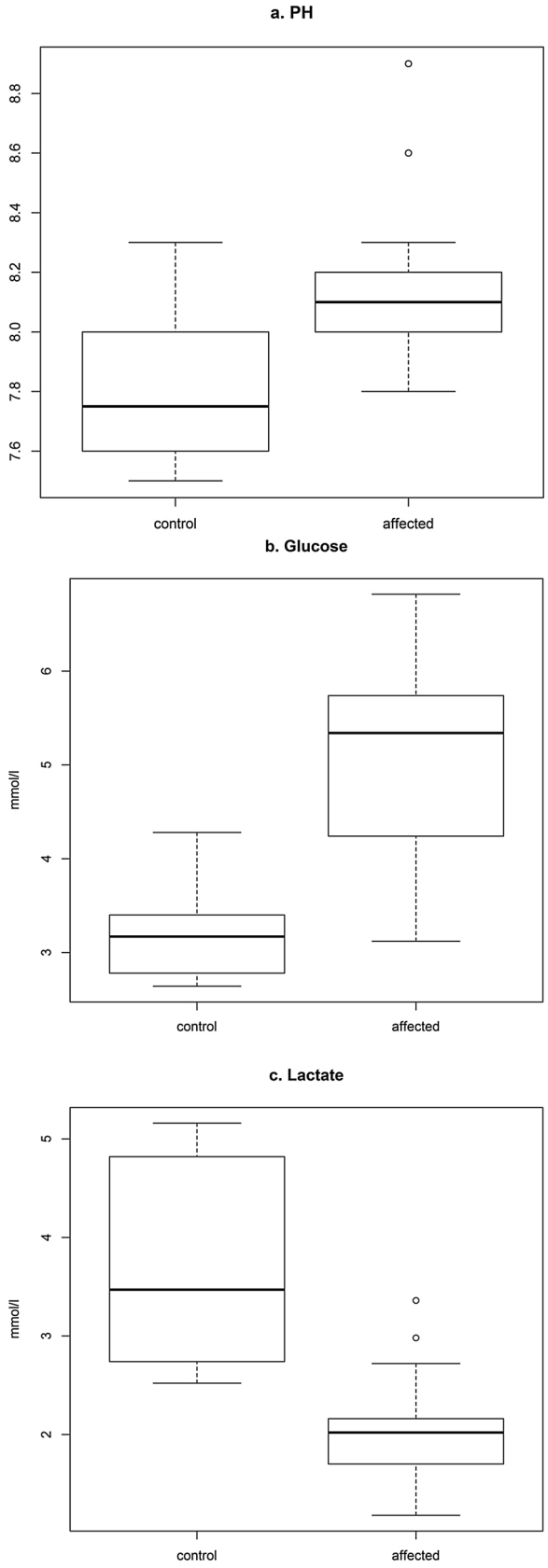

Fig. 2. Comparison between the metabolic parameters pH (a), glucose concentration (b) and lactate concentration (c) in synovial fluid of affected joints ("affected") and normal joints ("control"). Graph bars represent the median ± interquartile range; whiskers represent the values with Q1 – 1.5 interquartile distance (IQR) < x < Q1 of Q3 < x < Q3 + 1,5 IQR.

it cannot be excluded that this method might still be a contributing factor to the absence of a significant correlation between the biomarkers and radiographic scores. The use of more advanced imaging techniques, including magnetic resonance imaging (MRI), could provide greater anatomic detail of cartilage and non-cartilaginous structures, including bone (D'Anjou et al., 2008). More specifically, MRI has been recognized as the only diagnostic tool that allows evaluating the joint as a' whole organ' (D'Anjou et al., 2008). Therefore, changes associated with OA detected by MRI may correlate better with biomarker activities. The tomographic nature of MRI is an important feature that facilitates the detection of osteophytes. A human study demonstrated the presence of osteophytes on MRI in 72 % of men and 67 % of women with normal knee radiographs (Taouli et al., 2002). Also, a canine study on stifle joints with experimental OA demonstrated that MRI is more sensitive for detection of osteophytes compared to radiography (D'Anjou et al., 2008). However, important limitations of the use of MRI in veterinary medicine are the high cost and the need for general anesthesia.

This prospective pilot study had several limitations, the main one being the heterogenicity and limited sample size of both the patient and control groups. Indeed, no restrictions regarding demographic features (age, breed, sex) were imposed for the inclusion criteria. In addition, the composition of the studied population might not be representative for the total dog population. The results could be exposed to a selection bias since the included dogs were presented to a referral center.

Future research should focus on the influence of factors such as homogenous classification of affected and control dogs, specific joints and stage of the disease. Therefore, a large multicenter study, including both first line and referral centers, involving affected and control joints, should be designed. Both groups of dogs should be homogenous regarding age, breed, bodyweight and gender. The number of affected and corresponding control joints will be determined based on feasibility (statistical analysis). The staging of disease severity (and OA staging) will be performed with radiography and more advanced imaging techniques (MRI). Since an optimal panel of biomarkers remains to be found, the final goal of this future study would be to develop an ideal synovial fluid biomarker panel to identify joints affected with early OA (Allen et al., 2019; Fujita et al., 2005). Other potential metabolic biomarkers could be added to the current panel after their evaluation in future research. One such marker is urea, which reflects passive transport and has been reported as a local parameter to correct for synovial fluid dilution in both canine and human OA patients (Budsberg et al., 2006; Kraus et al., 2002). We here additionally suggest that a non-hypothesis-based metabolomics, sometimes called metabonomics, approach to identify novel metabolic biomarkers, as only recently described for human OA could be performed at first for canine OA (Huang et al., 2020; Makarczyk et al., 2021; Southan et al., 2020).

5. Conclusion

Our pilot study demonstrated statistically significant differences in the SF concentrations of the metabolic parameters pH, glucose and lactate between OA- affected and normal joints. The validation of the selected pro-inflammatory and degenerative biomarkers was satisfactory for TNF-alpha, IL-1beta, TN-C and MMP-2 and allowed to demonstrate statistically significant differences for all biomarkers except TNF-alpha between OA-affected and normal joints. No correlation was found between these three groups of biomarkers and the currently used radiographic scoring system for OA in dogs. Hence, future research is warranted to explore the potential of these biomarkers in the earlier detection of OA and in the severity characterization of this disease in which MRI could be included as a superior imaging technique compared to radiography.

Ethics approval and consent to participate

The chairperson of our institutional ethics committee evaluated and

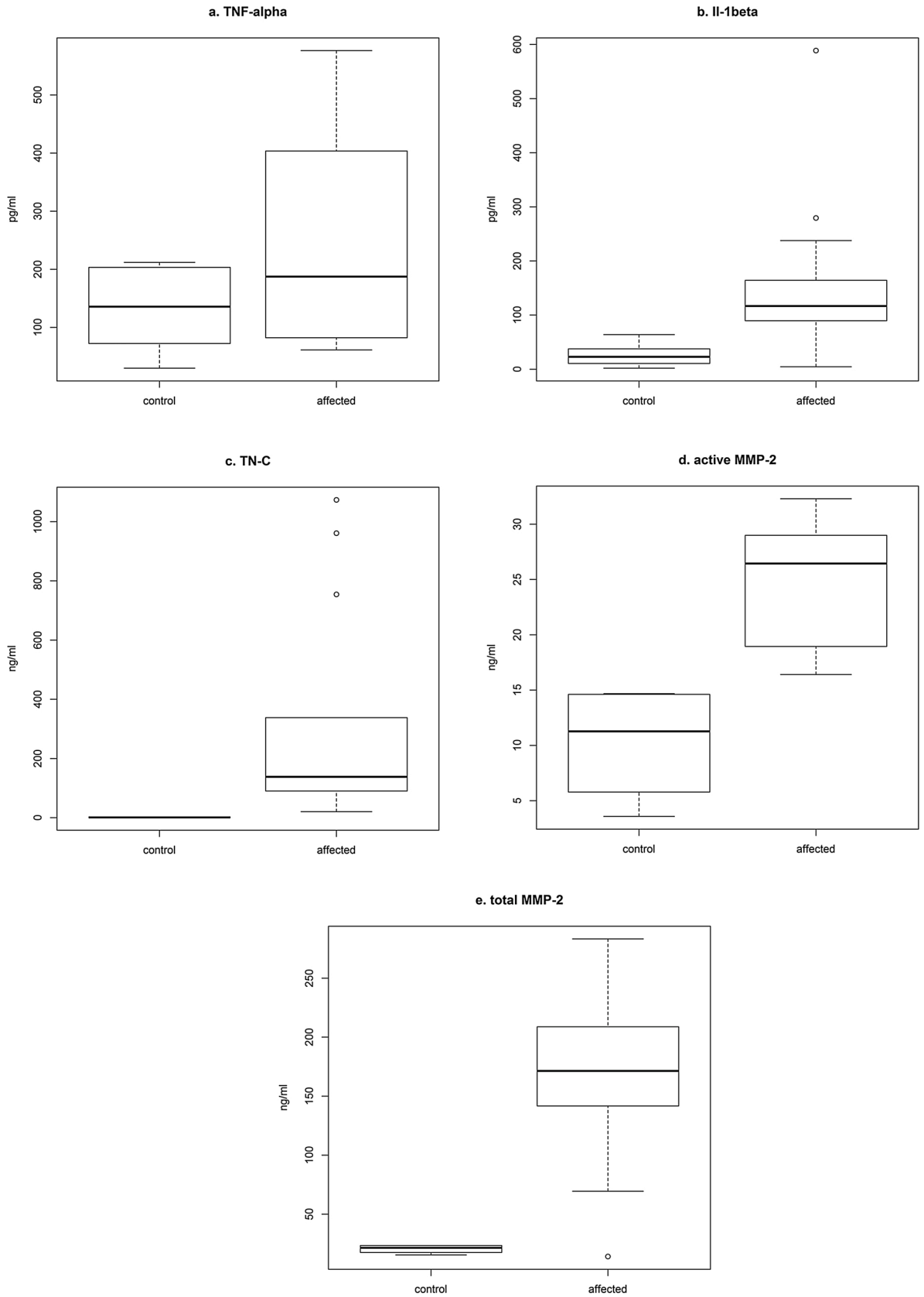

Fig. 3. Comparison between TNF-alpha (a), IL-1beta (b), TN-C (c) and active (d) and total (e) MMP-2 in synovial fluid of affected joints ("affected") and normal joints ("control"). Graph bars represent the median ± interquartile range; whiskers represent the values with Q1 – 1.5 interquartile distance (IQR) < x < Q1 of Q3 < x < Q3 + 1,5 IQR.

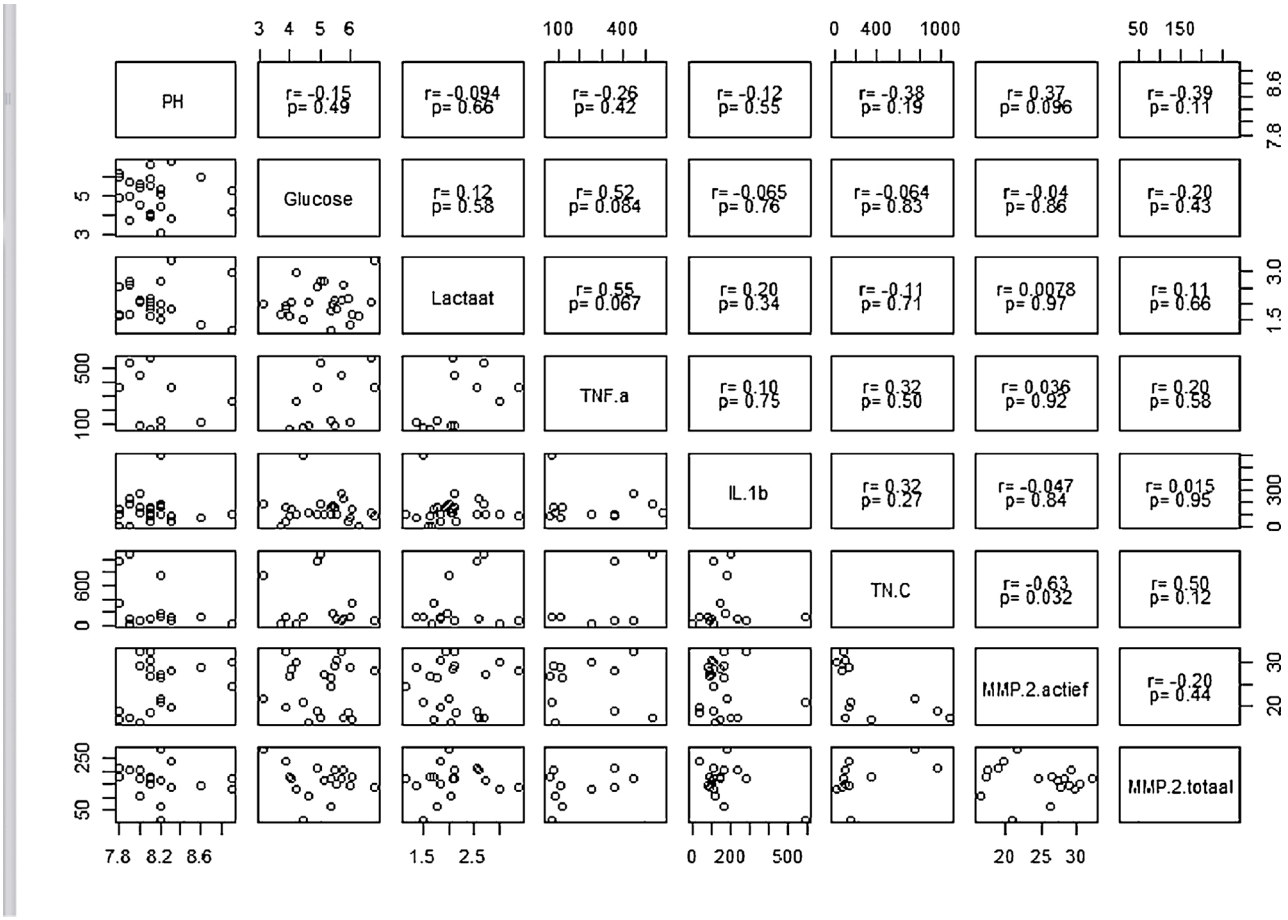

Fig. 4. Graphic presentation of the correlation between metabolic (pH, glucose and lactate), pro-inflammatory (TNF-alpha and IL-1beta) and degenerative (TN-C and MMP-2) biomarkers. Due to multiple testing, Spearman correlation coefficients are significant if $p \leq 0.05/28 = 0.00178$. No significant correlation can be found between any of these biomarkers.

approved the design of our study. Based on the Belgian and European legislation (EU directive 2010/63/EU), this study was not considered to be a "procedure", hence not requiring prior ethical approval. In analogy with this, the chairperson of our institutional ethics committee approved the procedure for verbal consent. It was not considered necessary to provide a written consent, as this is also not necessary for radiographs taken for diagnostic purposes. Per patient, it was noted in the clinical program that a verbal consent was obtained from the owner.

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Declaration of Competing Interest

On behalf of all the authors, I declare that none of us have known competing financial interests or personal relationships that could inappropriately influence or bias the content of the paper.

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