Determining predictive metabolomic biomarkers of meniscal injury in dogs with cranial cruciate ligament rupture.

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Abstract

Objectives

This study used hydrogen nuclear magnetic resonance spectroscopy (^{1}H NMR) for the first time to examine differences in the metabolomic profile of stifle joint synovial fluid from dogs with cranial cruciate ligament rupture with and without meniscal injuries, in order to identify biomarkers of meniscal injury. Identifying a biomarker of meniscal injury could then ultimately be used to design a minimally invasive diagnostic test for meniscal injuries in dogs.

Methods

Stifle joint synovial fluid was collected from dogs undergoing stifle joint surgery or arthrocentesis for lameness investigations. We used multivariate statistical analysis using principal component analysis and univariate statistical analysis using one-way analysis of variance and analysis of co-variance to identify differences in the metabolomic profile between dogs with cranial cruciate ligament rupture and meniscal injury, cranial cruciate ligament rupture without meniscal injury, and neither cranial cruciate ligament rupture nor meniscal injury, taking into consideration clinical variables.

Results

154 samples of canine synovial fluid were included in the study. 64 metabolites were annotated to the ^{1}H NMR spectra. Six spectral regions were found to be significantly altered (false discovery rate adjusted p-value <0.05) between groups with cranial cruciate ligament rupture with and without meniscal injury, including three attributed to NMR mobile lipids (mobile lipid -CH_3 [p=0.016], mobile lipid -n$(CH_3)_3$ [p=0.017], mobile unsaturated lipid [p=0.031]).

Clinical Significance

We identified an increase in NMR mobile lipids in the synovial fluid of dogs with meniscal injury which are of interest as potential biomarkers of meniscal injury.

Introduction

Cranial cruciate ligament rupture (CCLR), either partial or complete, is one of the most common causes of pelvic limb lameness in dogs (Witsberger *et al.*, 2008). It presents a significant cause of morbidity amongst the canine population, and it has been estimated that dogs with CCL disease account for 0.56% of all cases presented to primary care veterinary practices in the UK (Taylor-Brown *et al.*, 2015). One sequelae of joint instability caused by a loss of CCL function is tears to the menisci, occurring in approximately 50% of cases at time of CCLR surgery (Bennett and May, 1991). The menisci are a pair of C-shaped fibrocartilaginous structures located between the tibial plateau and femoral condyles (Kambic and McDevitt, 2005). They have several important functions including load bearing, load distribution and shock absorption, as well as contributing to joint stability, proprioception and joint lubrication (Arnoczky et al., 1980, Pozzi et al., 2010).

Meniscal injuries can also occur post-operatively after CCLR surgery due to residual joint instability (Metelman *et al.*, 1995). Failure to diagnose meniscal injuries at the time of surgery for CCLR can also lead to poor post-operative outcomes (Metelman *et al.*, 1995). The prevalence of late meniscal injuries varies from 2.8% to 13.8% (Metelman et al., 1995, Fitzpatrick and Solano, 2010). Late meniscal injuries can be a cause of recurring stifle joint pain and lameness, and are challenging for the veterinary practitioner to diagnose (Dillon *et al.*, 2014). Affected dogs often present with a recurring lameness on the operated limb weeks or months after CCLR surgery, with clinical examination potentially revealing pain on stifle flexion, and/or a “click” on stifle flexion (Dillon *et al.*, 2014, Case *et al.*, 2008). The presence of a meniscal click has been found to be an unreliable diagnostic sign (McCready and

Ness, 2016). Radiographs, useful in ruling out other causes of recurring lameness post-operatively, cannot show meniscal injuries directly. Further diagnostic imaging techniques for late meniscal injuries include low field or high field magnetic resonance imaging (MRI), computed tomography (CT) with arthrography, or ultrasound examination (McCready and Ness, 2016). Depending on the study, the sensitivity of these techniques in diagnosing meniscal injuries in dogs is 64-100% for low field MRI (Böttcher *et al.*, 2010, Gonzalo-Orden *et al.*, 2001), 75-100% (Olive *et al.*, 2014, Blond *et al.*, 2008) for high field MRI, 71% for CT arthrography (Samii *et al.*, 2009) and 90% for ultrasonography (Mahn *et al.*, 2005). All of these imaging techniques require either expensive specialised equipment, and/or advanced technical expertise, limiting the availability of these diagnostics in veterinary practice, and amount to a considerable cost. Surgical methods of diagnosis include either stifle joint arthroscopy or arthrotomy (Pozzi et al., 2008). Diagnosis of meniscal injuries by surgical intervention holds inherent risks including the risk of post-operative morbidity (Hoelzler et al., 2004). Furthermore, using surgery as a means of diagnosis has the risk of the animal undergoing an unnecessary surgical procedure if no meniscal injury is found (Blond *et al.*, 2008). The development of a simple, inexpensive, minimally invasive diagnostic test for meniscal injuries in dogs would be useful when determining whether invasive surgical intervention is necessary. For example, knowledge of whether a meniscal injury is present or not would help with the decision to perform an arthrotomy, either when planning extra-articular or per-articular surgical techniques to treat CCLR (Comerford et al., 2013), or when there is a suspicion of late meniscal injury post-operatively.

Currently, there are no biomarkers of meniscal injury that can be used as a diagnostic aid. One potential source of biomarkers of stifle joint pathologies is synovial fluid (SF) (Boffa et al., 2020). SF is a viscous fluid, that is a dialysate of plasma, and functions as a joint lubricant (Ghosh, 1994). It contains a unique source of biomarkers of joint disease, due to its close proximity to structures within joints (Anderson et al., 2018b).

Metabolomics allows the identification and quantification of small molecule metabolites and analysis of metabolic pathways within a variety of biofluids, cells and tissues (Bujak *et al.*, 2015). Nuclear magnetic resonance (NMR) is a tool for metabolomics studies, having the benefits of being rapid, non-destructive and relatively inexpensive compared to other metabolomics tools such as mass spectrometry (Clarke *et al.*, 2020). ^{1}H NMR has been used successfully to investigate changes in the SF metabolomic profile in humans and horses with joint pathologies such as rheumatoid arthritis, osteoarthritis (OA), and septic arthritis (Anderson *et al.*, 2018a, Anderson *et al.*, 2018b, Clarke *et al.*, 2020). In addition to detecting breakdown products of proteins, ^{1}H NMR spectroscopy can also detect resonances arising from lipid species (Soininen et al., 2009). NMR mobile lipids are resonances on an NMR spectrum that arise from methyl or methylene groups of lipid acyl chains (Delikatny et al., 2011). These arise primarily from triglycerides, fatty acids and cholesteryl esters in lipid droplets, and also from phospholipidic acyl chains if not embedded in lipid membrane bilayers (Mannechez *et al.*, 2005). A previous NMR lipidomic study in SF from canine and human OA affected joints found an increase in numerous lipid species in OA compared to healthy controls in both species (Kosinska et al., 2016). Alterations in lipid profiles of SF from joints with meniscal injury have not yet been investigated. Therefore, there is promise for using NMR spectroscopy to investigate biomarkers of joint pathology within canine SF, including CCLR and meniscal injuries.

We hypothesise that the metabolomic profile of canine stifle joint SF will alter depending on the presence of CCLR and depending on the presence of concurrent meniscal injuries. Metabolomic changes within SF linked to the presence of CCLR and meniscal injuries could be due to alterations in pathways linked to degeneration in the CCL, inflammatory responses and/or traumatic tears to the meniscal tissue. Metabolomic biomarkers of CCLR and meniscal injuries could then potentially allow for the development of a simple, minimally invasive diagnostic test (for example via arthrocentesis) more reliable at detecting meniscal injuries, and late meniscal injuries, than pre-existing non-surgical diagnostic techniques. This diagnostic test could then reduce the need for invasive surgical methods of meniscal injury diagnosis.

Materials and methods

Ethical approval

Ethical approval for the collection of canine SF for use in this study was granted (VREC634) as surplus clinical waste under the generic approval RETH00000553.

Synovial fluid collection

Canine SF was collected from dogs undergoing surgery for CCLR, either with or without concurrent meniscal injuries, from dogs undergoing surgery for patella luxation, or as excess clinical waste from dogs undergoing arthrocentesis as part of lameness investigations from March 2018 to June 2021. Cases were divided into three groups, namely, group 1) CCLR with meniscal injury; Group 2) CCLR without meniscal injury; and group 3) neither CCLR nor meniscal injury (the control group). Cases were recruited with informed consent from three veterinary practices in the north-west of England. SF was collected by stifle joint arthrocentesis as per the BSAVA guide to procedures in small animal practice (Bexfield and Lee, 2014). A 21-gauge to 23-gauge needle attached to a 2 to 5 mL sterile syringe (depending on the size of the dog) was inserted into the stifle joint space either medially or laterally to the patella ligament after sterile preparation of the skin, prior to first surgical incision. After aspiration of the SF, samples were placed in sterile 1.5 mL Eppendorf tubes (Eppendorf UK Ltd, Stevenage, UK), and immediately refrigerated at 4^{o}C.

Synovial fluid processing

SF samples were transported on ice to the laboratory within 48 hours of collection. Samples stored for longer than 48 hours before processing were excluded from the study based on previous data examining metabolomic changes in the SF with elongated refrigerated storage time (Pye, 2021). Any SF samples with a large amount of blood contamination, or that had a haemorrhagic discolouration were excluded from the study. A small number of SF samples with minor iatrogenic blood contamination from arthrocentesis (seen as blood "streaks" that are not completely blended with the SF sample (Clements, 2006)) were included. Samples were centrifuged at 2540*g* at 4°C for 5 minutes. The supernatant was pipetted into 200 µl aliquots, and snap frozen in liquid nitrogen before storing at -80°C (Anderson *et al.*, 2020).

Clinical information on the canine participants

Inclusion criteria for this study were dogs undergoing surgery for either partial or complete CCLR (with or without concurrent meniscal injury), dogs undergoing surgery for patella luxation, or dogs that had stifle joint arthrocentesis as part of clinical investigations into hindlimb lameness. There were no exclusion criteria based on other clinical attributes of the dogs.

Clinical information from the dogs used in this study was collected. This information included breed, age, sex and neuter status, body weight, body condition score (Laflamme, 1997), presence and degree of CCLR (whether partial or complete CCLR), presence of meniscal injury, location and type of meniscal injury (Bennett and May, 1991), presence of patella luxation, length of time of lameness, co-morbidities, medication being received by the dog and radiographic level of OA using two separate scoring systems (Innes *et al.*, 2004, Wessely *et al.*, 2017).

Orthogonal radiographs (medio-lateral view and caudo-cranial views) of the stifle joint of each dog included in the study were analysed. Radiographs were performed either as pre-operative radiographs

146 or as part of lameness investigations, less than 21 days before arthrocentesis of the SF sample. These
147 were analysed either by a veterinary surgeon with a postgraduate certificate in small animal surgery,
148 or by a veterinary student who had received training in radiographic OA scoring of the stifle joint and
149 was overseen by the aforementioned veterinary surgeon. Two separate scoring systems were initially
150 used to assess difference between the three groups in terms of their radiographic OA score (Innes *et*
151 *al.*, 2004, Wessely *et al.*, 2017). These scoring systems use either a 10-point scale (Innes *et al.*, 2004),
152 or a 45-point scale (Wessely *et al.,* 2017). A global assessment score from zero (no OA) to three (severe
153 OA) as described by Innes *et al.* (2004) was then used when assessing metabolomic differences in the
154 stifle joint SF based on level OA in order to group the level of OA for ANOVA testing (see Statistical
155 Analysis section below).

156 NMR Metabolomics

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158 *Sample preparation for NMR metabolomics*

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160 SF samples were thawed on ice immediately prior to sample preparation for NMR spectroscopy. 100
161 µL of each thawed SF sample was diluted to a final volume containing 50% (v/v) SF, 40% (v/v) dd $^{1}H_2O$
162 (18.2 MΩ), 100 mM phosphate buffer, pH 7.4 (Na_2HPO_4, VWR International Ltd., Radnor, Pennsylvania,
163 USA and NaH_2PO_4, Sigma-Aldrich, Gillingham, UK) in deuterium oxide ($^{2}H_2O$, Sigma-Aldrich) and
164 0.0025% (v/v) sodium azide (NaN_3, Sigma-Aldrich). Samples were vortexed for 1 minute, centrifuged
165 at 13,000*g* and 4 °C for 5 minutes and 180 µL transferred (taking care not to disturb any pelleted
166 material) into 3 mm outer diameter NMR tubes using a glass Pasteur pipette.

167

168 *NMR metabolomics spectral acquisition*

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Spectra were acquired using a 700MHz Bruker Avance III spectrometer (Bruker Corporation, Billerica, Massachusetts, USA) with associated triple resonance inverse (TCI) cryoprobe and chilled Sample Jet auto-sampler. Software used for spectral acquisition and processing were Topspin 3.1 (Bruker Corporation, Billerica, Massachusetts, USA) and IconNMR 4.6.7 (Bruker Corporation).

1D ^{1}H NMR spectra were acquired using a Carr-Purcell-Meiboom-Gill (CPMG) filter to suppress background signals from proteins and other endogenous macromolecular constituents, and allow acquisition specifically of small molecule metabolite signals (Carr and Purcell, 1954, Meiboom and Gill, 1958). A vendor-supplied standard pulse sequence was used to achieve this (cpmgpr1d) with water suppression carried out by pre-saturation (Hoult, 1976). The CPMG spectra were acquired at 37 °C with a 15 ppm spectral width, a four second interscan delay and 32 transients (Anderson *et al.*, 2020).

The spectra acquired in this study are available in the MetaboLights (Haug et al., 2020) repository (https://www.ebi.ac.uk/metabolights/MTBLS6050).

NMR metabolomics spectral quality control

1D ^{1}H NMR spectra were individually assessed to ensure minimum reporting standards were met (Sumner *et al.*, 2007). The steps for quality control included: 1) Assessing the spectral baseline to ensure minimal curvatures or deviations; 2) Assessing the quality of water suppression, to ensure the water peak at 4.7 ppm was no more than 0.4 ppm wide; 3) Aligning the spectra to the glucose beta anomeric doublet at 5.24 ppm; and 4) Measurement of the line-width half height of the glucose peak at 5.24 ppm, with any spectrum where the width of this peak at half the height of the peak exceeded more than one standard deviation from the mean being regarded as a failure of quality control. Any samples that were deemed to have failed quality control were re-ran on the spectrometer up to a

maximum of three spectral acquisitions. Any samples that failed after the third spectral acquisition were excluded from the study.

Metabolite annotation and identification

The NMR spectra were divided into spectral regions (termed "bins") using Topspin 3.1 (Bruker Corporation, Massachusetts, USA), with each bin representing either single metabolite peaks or multiple metabolite peaks where peaks overlapped on the spectra. These bins were also examined using TameNMR (hosted by Github: https://github.com/PGB-LIV/tameNMR), an "in-house" toolkit built within the galaxy framework (Afgan et al., 2018). Bins were altered accordingly upon visualising the fit to the overlaid spectra to ensure the area under the peak was represented by the bin.

Metabolites were annotated to the spectra using Chenomx NMR Suite Profiler version 7.1 (Chenomx, Edmonton, Canada), a reference library of 302 mammalian metabolite NMR spectra. When metabolite peaks overlapped, multiple metabolites were annotated to the bin. When peaks were unable to be annotated to a metabolite, they were classed as being an "unknown" metabolite. Previous literature specifying metabolite chemical shifts and spectral appearance were examined to aid annotation of unknown areas. Downstream unique peak metabolite identification and in-house NMR metabolite standards were examined to confirm metabolite identities where possible. Metabolites were assigned a level of identification according to the Metabolite Standards Initiative (MSI) levels. Level 1 identified metabolites require two or more orthogonal properties of a standard component to be analysed using the same spectrometer and experimental conditions as the experimental spectra. Level 2a metabolite identifications are made after matching one property of a standard component analysed using the same spectrometer and experimental conditions. Level 2b are putatively identified metabolites using reference libraries of standard compounds obtained from external laboratories. Level 3 are putatively

annotated compound classes, used when the molecule can only be annotated to a class rather than a specific metabolite (Sumner *et al.* 2007).

A pattern file was created of the spectral bins and metabolites annotated to that bin. This is a spreadsheet outlining the bin boundaries in ppm, and the metabolites annotated to that bin. The pattern file and the Bruker spectra files were input into TameNMR, in order to create a spreadsheet of the integrals from binned spectra, with the relative intensities of each bin for each sample, which could then be used for statistical analysis of the spectra.

Statistical analysis

Differences in clinical variables of the canine participants

Analysis of the differences in clinical features between the groups in terms of age, sex and neuter status, BCS, the length of time of lameness on the affected hindlimb, and radiographic OA scores using both scoring systems and the global assessment of radiographic OA from zero to three (Innes *et al.*, 2004; Wessely *et al.*, 2017) were undertaken in the following way. Firstly, normality was tested using Shapiro-Wilk test, as well as visualising histograms and quantile-quantile (QQ) plots of the data for each variable. Kruskal-Wallis tests were undertaken on non-parametric data, with Dunn's *post-hoc* test. A Benjamini-Hochberg false discovery rate (FDR) adjustment was carried out for all tests, and significance set at $p<0.05$. These analyses and creation of boxplots to visualise this data was undertaken using R (R Core Team, 2020).

Metabolomics data analysis

Sample size power calculations were completed using data from a previous unpublished small cohort study (n=5 with CCLR and meniscal injury and n=7 with CCLR without meniscal injury), with a specified FDR of 0.05 using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca), a software based on a metabolomics data analysis package written in R (the MetaboAnalystR package) (Pang *et al.*, 2021).

Metabolomics data was normalised using probabilistic quotient normalisation (PQN) (Dieterle *et al.*, 2006), and Pareto scaled using R prior to statistical analysis (R Core Team, 2020). Unsupervised multivariate analysis was carried out using principal component analysis (PCA) on the normalised and scaled data using R. The variance between canine phenotypes was investigated through analysis of principal components 1 through 10 using one-way ANOVAs or linear models depending on the data type. Briefly, CCLR, sex, neuter status, BCS, radiographic OA score and batch were numerically encoded and assessed against each principal component using a one-way ANOVA. Age, Length of time of lameness, weight, length of time of storage pre-processing which were already numeric variables were assessed against each principal component using a linear model. All p values were corrected using FDR (Bejamini Hochberg) correction. Correlation matrices between phenotypes were computed using the spearmans correlation using the *cor* function in R and visualised using a heatmap generated with the *pheatmap* function in R (Kolde, 2012).

Univariate analysis was carried out using one-way ANOVAs and one-way analysis of co-variance (ANCOVAs) using R. To account for multiple testing across all 236 metabolite bins FDR correction was applied to the F-Test p value of each metabolite, significance was accepted at $p < 0.05$. For metabolites with an FDR < 0.05 Tukey's honest significant difference *post-hoc* test was applied to assess between group variances. Metabolite differences were separately analysed with respect to age, weight (divided into groups of 10 kg intervals), BCS, global assessment of radiographic OA score (0-3) (Innes et al., 2004), length of time of clinical lameness (divided into groups less than one month, one to three

months, three to six months and six to twelve months), site of collection, partial *versus* complete *versus* no CCLR and between the three groups (group 1: CCLR with meniscal injury, group 2: CCLR without meniscal injury and group 3: control group with neither CCLR nor meniscal injury). Age adjusted one-way ANCOVAs were applied to each metabolite to assess differences between the three groups (1) CCLR with meniscal injury, 2) CCLR without meniscal injury and 3) control group with neither CCLR nor meniscal injury) , FDR adjustment was applied as a above. Boxplots to visualise the changes in metabolite abundances were created using *ggplot2* package within R.

Results

Sample size calculations revealed a sample size of n=60 per group, namely group 1) CCLR with meniscal injury, 2) CCLR without meniscal injury and 3) control group with neither CCLR nor meniscal injury, would give a predictive power of 0.83 when plotted on a predictive power curve.

For the metabolomic study, 191 samples of canine stifle joint SF were collected and submitted for NMR spectroscopy. Of these, 14 samples had been stored for longer than 48 hours prior to collection for processing, and were subsequently excluded from the study. Four samples were from cases in which the meniscal injury status was unknown, and were also excluded from the study. Nineteen samples were excluded as they failed to meet minimum reporting standards (Sumner *et al.*, 2007) after three spectral acquisitions.

In total, 154 canine stifle joint SF samples were included in the statistical analysis. These were divided into three groups, namely group 1) CCLR with meniscal injury (n=65), group 2) CCLR without meniscal injury (n=72), and group 3) control group with neither CCLR nor meniscal injury present (n=17). The two groups of CCLR cases included dogs with either partial or complete CCLR. The control group consisted of 13 cases of patella luxation, three cases from arthrocentesis of the stifle joints during

lameness investigations which subsequently were found to have no pathology, and one sample from a case with fraying of the caudal cruciate ligament.

Differences in signalment of the canine participants between groups

Information regarding the signalment of the dogs in each group is shown in Table 1. There was a significant difference between the control group and both the CCLR groups with or without meniscal injury in terms of age, weight, and radiographic OA score using both the Innes *et al.* (2004) and Wessely *et al.* (2017) scoring systems. There was no significant differences between the three groups in terms of BCS of the dogs and length of time of clinical lameness (Figure 1). There was no significant difference between groups CCLR with meniscal injury and CCLR without meniscal injury in terms of these clinical variables, although age was closest to reaching significance between the two groups (p=0.13, mean difference=0.86 years [0.01 to 1.73 95% CI]).

Metabolite annotation and identification.

Spectra were divided into 246 bins. Of these, 84 (34%) remained with an unknown metabolite identification, and 162 (66% of bins) were annotated to one or more metabolites. In total, 65 metabolites were annotated to the spectra (Table 2). Any bins containing ethanol peaks were excluded from the statistical analysis, due to ethanol being considered a contaminant in NMR, usually either during the collection of the SF from the sterilisation of skin with alcohol-based solutions (Hutchinson, 2012)or during the processing steps (van der Sar *et al.*, 2015). Propylene glycol, a metabolite found in solvents used in pharmaceuticals (Zar *et al.*, 2007) was found in one spectrum, and so those bins were excluded so as to not bias the statistical analysis.

313

314 Metabolomic statistical analysis results

315

316 *Analysis of canine synovial fluid metabolome with respect to weight, age, radiographic OA score,*
317 *length of time of lameness, BCS, site of collection and degree of CCLR of the canine participants*

318

319 Analysis of metabolite changes with respect to clinical variables found significantly altered metabolites
320 with differing weight (Supplementary information Figure S2), age (Supplementary information Figure
321 S3) and radiographic OA score of the dogs using the global assessment score (0-3) within Innes *et al.*
322 (2004) (Supplementary information Figure S4). This included an increase in glutamine with increasing
323 weight of the dogs (Supplementary information Figure S2). Four mobile lipid regions on the spectra
324 were significantly increased with increasing age of the dogs (Supplementary information Figure S3).
325 There were no significant metabolite differences depending on the length of time the dog had clinical
326 signs of lameness or due to the BCS of the dog. There were also no significant difference between dogs
327 with a partial CCLR *versus* dogs with a complete CCLR.

328

329 *Multivariate analysis of canine synovial fluid metabolome with respect to CCLR and meniscal injury*
330 *status*

331

332 Multivariate PCA was undertaken to compare the differences in the overall metabolome between the
333 groups, namely: group 1) CCLR with meniscal injury, group 2) CCLR without meniscal injury and group
334 3) no CCLR and no meniscal injury (the control group) (Figure 2). Over principal components (PC) one
335 and two, there were overlapping clustering of the groups, indicating little overall difference in the
336 metabolome over these PCs (Figure 2a). Associations between different phenotypes of the canine

participants and PC one to ten found that PC three and four were primarily associated with CCLR and meniscal injury (supplementary material Figure S1). PCA of the groups plotted over PC three and four showed some samples from the control group were separated from the groups CCLR with and without meniscal injury, indicating that the control group appears to have a wider variation with some samples exhibiting a differing metabolome from the other two groups (Figure 2b).

Univariate analysis of canine synovial fluid metabolome with respect to CCLR and meniscal injury status

Univariate analysis of metabolomic differences between the three groups 1) CCLR with meniscal injury, 2) CCLR without meniscal injury and 3) control group with neither CCLR nor meniscal injury was then undertaken.

Between groups 1) CCLR with meniscal injury, and group 2) CCLR without meniscal injury, there were six spectral bins that were below the threshold of significance ($p<0.05$), and two others that neared the threshold ($p<0.06$) after one-way ANOVA testing with FDR adjusted p-values and Tukey's HSD *post-hoc* test (Table 3). These included the four spectral bins related to mobile lipids.

It was noted that mobile lipids were also significantly increased with increasing age of the canine participants (supplementary information Figure S3), and that groups CCLR with meniscal injury and CCLR without meniscal injury had a slight, although insignificant ($p=0.13$, mean difference=0.86 years [0.01 to 1.73 95% CI]) difference in terms of age of the canine participants in each group with the group CCLR with meniscal injury being older (Figure 1). There was no difference in other variables (including weight, radiographic OA score or BCS) between dogs in groups CCLR with meniscal injury and CCLR without meniscal injury (Figure 1). ANCOVAs were therefore undertaken to control for age. The results of these ANCOVAs controlling for age are shown in Table 4. After controlling for age, three out of four spectral regions annotated to mobile lipids were significantly higher in the group CCLR

with meniscal injury compared to the group CCLR without meniscal injury (Figure 3). These regions were attributed to mobile lipid -CH_3 (p=0.016), mobile lipid -n$(CH_3)_3$ (p=0.017) and mobile unsaturated lipid (p=0.031). A complete list of the ANCOVA outputs are included in the supplementary information (Supplementary information Table S2).

After controlling for age, there were 49 spectral bins, relating to 31 metabolites that were found to be significantly altered between group 1 (CCLR with meniscal injury) and group 3 (control group). 48 out of these 49 bins, related to the same 31 metabolites, were also found to be significantly altered between group 2(CCLR without meniscal injury) and group 3 (control group) (Supplementary information Table S2). However, as the control group differed to the other two groups in terms of other variables, such as weight and radiographic OA score, as well as it being of a smaller sample size, it was not possible to accurately assess whether these metabolomic changes were based on the presence of CCLR alone.

Discussion

This is the first study of its kind to use NMR metabolomics to investigate biomarkers of meniscal injury within the stifle joint SF of dogs. It is also the first study to use NMR metabolomics to investigate biomarkers of meniscal injury within the SF of any species, including humans. It was found that NMR mobile lipids were significantly increased in the stifle joint SF of dogs with CCLR and meniscal injury, compared with dogs with CCLR but no meniscal injury, or dogs with neither CCLR nor meniscal injury. Mobile lipids are NMR lipid resonances that arise from isotropically tumbling, relatively non-restricted molecules such as methyl and methylene resonances belonging to lipid acyl chains (Hakumäki and Kauppinen, 2000, Delikatny *et al.*, 2011). These arise primarily from triglycerides, fatty acids and cholesteryl esters in lipid droplets, and also from phospholipidic acyl chains if not embedded in lipid

membrane bilayers (Mannechez *et al.*, 2005). Lipids serve various important functions in biological systems, including as components of cell membranes and other cellular organelles, acting as an energy source, and having a crucial role in signalling and regulation of cellular processes (Onal *et al.*, 2017). Many biological processes have been associated with changes in NMR mobile lipids, including cell necrosis and apoptosis, malignancy, inflammation, proliferation and growth arrest (Hakumäki and Kauppinen, 2000). Lipid analysis of SF in humans have found differential abundance of lipids with different disease states, including OA, rheumatoid arthritis and trauma (Wise *et al.*, 1987). A more recent NMR lipidomic study in SF from canine and human OA affected joints found an increase in numerous lipid species in OA compared to healthy controls in both species (Kosinska et al., 2016).

There are a number of possible hypotheses for the increase in NMR mobile lipid resonances found in the SF of dogs with CCLR and concurrent meniscal injury compared to CCLR without meniscal injury in this study. Injury to the meniscus could lead to damage to cellular phospholipid membranes, resulting in the release of lipids into the SF. Human menisci have also been found to contain lipid debris that could have an impact on SF lipid concentrations in meniscal injury (Ghadially and Lalonde, 1981). Also, lipid droplets could be released from the intracellular environment due to cell necrosis or apoptosis in the damaged meniscal tissue (Uysal *et al.*, 2008), leading to an increased concentration of lipid droplets in the SF. Lipid droplets have been found to play a key role in inflammation, as such it may be that meniscal tears lead to a release of lipid droplets to facilitate in the inflammatory response within the joint (Melo *et al.*, 2011). As lipid droplets contain mediators of inflammation such as pro-inflammatory cytokines, lipids could also potentiate inflammatory changes in meniscal injury affected joints (Ichinose *et al.*, 1998). However, other metabolites linked to inflammation that were identified within the SF in this study, such as metabolites of glycolysis and the tricarboxylic acid (TCA) cycle (including lactic acid, glucose, pyruvate and citrate) (Anderson *et al.,* 2018a) were not significantly altered in dogs with CCLR and meniscal injury compared to those with CCLR but without meniscal injury. Alterations in SF lipid composition and lipid species can also have a role in affecting the lubricating ability of the SF (Antonacci *et al.*, 2012). The concentration of phospholipid species in

412 human SF have been found to be increased in OA affected joints, therefore the observed increase in
413 lipids could also be an attempt to improve lubrication of the SF after meniscal injury in order to have
414 protective effects on the articular cartilage (Kosinska et al., 2015).

415 Amongst the other differentially abundant metabolites between groups with CCLR with and without
416 meniscal injury, was methanol. Although methanol could be considered a contaminant in NMR
417 (Fulmer *et al.*, 2010), it has also been found to be a naturally occurring metabolite in humans, either
418 through dietary consumption in various fruit and vegetables, the artificial sweetener aspartame,
419 alcohol, or through actions of gut microbiota (Dorokhov *et al.*, 2015). Some of these sources cannot
420 be ruled out, and therefore the decision not to remove methanol from analysis was made. However,
421 its association with meniscal injury remains unclear.

422 One of the spectral bins that also showed a significant increase in canine SF in dogs with CCLR and
423 meniscal injury compared to CCLR without meniscal injury was a region that had overlapping NMR
424 peaks annotated to glycylproline, isoleucine, and an unknown metabolite. This region also requires
425 further work to confirm the identity of the specific metabolites attributed to this area although it is
426 likely given its correlation with other mobile lipid regions to derive from the same source. Fatty acyl
427 chains have been previously noted to be attributed to resonances in this spectral region (Delikatny *et*
428 *al.*, 2011). This would correlate with the findings of increases in mobile lipids with meniscal injury.

429 Spectral overlap and limited SF ^{1}H NMR studies resulted in a number of metabolite peaks that are, as
430 yet, unidentified on the canine SF spectra, including one that was found to be significantly altered with
431 meniscal injury. SF has been relatively understudied compared to other biofluids such as serum, and
432 it is possible that SF contains metabolites that have yet to be reported in the literature, although the
433 use of SF for NMR metabolomic studies of joint disease has been increasing in recent years (Clarke *et*
434 *al.,* 2021). It could also be possible that there may be canine-specific metabolites, or breakdown
435 products within canine SF that are different to other species due to the gait and physiology of the dog,
436 that are currently not reported in the literature. Further work is required in identifying these regions,

437 such as undertaking a 2D NMR experiment, or spiking SF with authentic standards (Dona et al., 2016).
438 Alternatively, using complimentary methods of metabolite analysis, such as mass spectrometry, would
439 improve the number of metabolite annotations and also potentially identifications in the SF samples.

440 One of the limitations of our study was the lack of a balanced control group to compare with the CCLR
441 affected joints. There are several reasons for this. Firstly, collection of "normal" SF via arthrocentesis
442 from joints without pre-existing pathology involves a level of risk, including introducing infection into
443 the joint, and the need for sedation or anaesthetic for the protocol (Bexfield and Lee, 2014). Therefore,
444 this would have ethical implications, and was outside the ethical approval for this study. SF from dogs
445 with no stifle joint pathology collected post-mortem would have been subjected to metabolite
446 changes that would have compromised the comparison to the diseased groups (Donaldson and
447 Lamont, 2015). Control samples in this study were collected from dogs undergoing surgery for patella
448 luxation, or excess SF from dogs undergoing arthrocentesis from investigations of lameness. These
449 were cases without CCLR or meniscal injuries, but also are likely not to have been completely without
450 pathological changes, as patella luxation can be cause of OA and synovitis (Roush, 1993). Patella
451 luxation also tends to be more common in smaller breeds of dogs, and as primarily a congenital
452 disease, cases often show clinical signs of lameness at a younger age than CCLR affected dogs (LaFond
453 *et al.*, 2002, Rudd Garces *et al.*, 2021). Both these factors meant the control group were on average
454 younger and smaller than the CCLR groups, with less osteoarthritic changes. This, along with the fewer
455 samples collected in the time constraints of this study affected the ability to infer conclusions from
456 the metabolite changes between the control and other groups in terms of CCLR alone. The inclusion
457 of more donors in the control group of healthy, non-diseased canine stifle joint SF would be of value
458 in future work to allow analysis of metabolomic changes due to CCLR and OA in the canine stifle joint.
459 However, for the investigation of biomarkers of meniscal injury in dogs with CCLR, a "healthy" control
460 group may not be essential, as the comparison of groups CCLR with meniscal injury against CCLR
461 without meniscal injury would be adequate to aid diagnosis.

462 Another potential limitation was the inclusion of some SF samples with minor blood contamination
463 caused by arthrocentesis. As this study aimed to find biomarkers of meniscal injury within SF that
464 could be used as a clinical diagnostic test, it was decided to include these samples as minor iatrogenic
465 blood contamination of SF during sampling can be an occasional occurrence (Clements, 2006). Future
466 studies could involve more detailed analysis of the level of blood contamination, including red blood
467 cell counts, and how this may alter the SF metabolome. All samples were centrifuged to remove any
468 cellular content prior to NMR spectroscopy.

469 There were factors such as diet and level of exercise that have been found to affect the metabolome
470 of human serum that were not been accounted for in this study (Esko *et al.*, 2017, Sakaguchi *et al.*,
471 2019). However, unlike humans, dogs tend to have a less variable diet, and also exercise is likely to be
472 similar between the canine participants, as the standard advice for dogs affected by CCLR is to limit
473 exercise. Medications were found to be too heterogeneous between the dogs in this study from which
474 to make any statistical conclusions but are known to affect the metabolomic profile of biofluids (Um
475 *et al.*, 2009).

476 This study is the first of its kind in using ^{1}H NMR spectroscopy to identify biomarkers of meniscal injury
477 within SF. SF lipid species appear to be of interest in the study of biomarkers of meniscal injury, and
478 future work to identify the lipid species involved by undertaking a lipidomics experiment, such as NMR
479 or Liquid Chromatography coupled Mass Spectrometry (LC-MS) lipidomics using lipid extracts from
480 the SF samples. A simple, minimally invasive, inexpensive diagnostic test for meniscal injury in dogs by
481 means of arthrocentesis could reduce the need for invasive surgical methods of meniscal injury
482 diagnosis. This work could prove useful in exploring the potential for targeted assays to establish a
483 diagnostic marker of meniscal injury in canine SF.

484

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Figure Legends

Figure 1. Clinical characteristics of the canine participants between groups. Box and whisker plots show differences in a) Age, b) Weight, c) body condition score (BCS), d) The length of time of clinical lameness on the affected hindlimb, e) The radiographic osteoarthritis score using the radiographic scoring system as described by Wessely *et al.* (2017), and f) the global assessment of radiographic osteoarthritis from 0 to 3 as described by Innes *et al.* (2004). The box indicates the interquartile range (IQR) around the median. Each whisker extends to the furthest data point that is above or below 1.5 times the IQR. Possible outliers are data points outside of this distance. Boxplot colours indicate different groups: Grey = CCLR with meniscal injury (n=65), Orange = CCLR without meniscal injury (n=72), Light blue = control group with neither CCLR nor meniscal injury (n=17). Significance testing was performed using Kruskal-Wallis testing with Dunn's *post-hoc* test. (CCLR=cranial cruciate ligament rupture, MI=meniscal injury, OA= osteoarthritis, ns=not significant, *=p<0.05, **=p<0.01, ***=p<0.001).

Figure 2. Principal component analysis (PCA) 2D scores plot of metabolite profiles of canine stifle joint synovial fluid by NMR. Samples grouped by CCLR and meniscal injury status. Group 1 (grey): CCLR with meniscal injury (n=65), Group 2 (orange) = CCLR without meniscal injury (n=72), Group 3 (light blue) = control group with neither CCLR nor meniscal injury (n=17). Plotted over A) PC1 and PC2 and B) PC3 and PC4.

Figure 3. Altered mobile lipids on ^{1}H NMR with respect to meniscal injury status in canine stifle joint synovial fluid from dogs. Box and whisker plots show the normalised relative metabolite abundance on the Y axis and group on the X axis. The box indicates the interquartile range (IQR) around the median. Each whisker extends to the furthest data point that is above or below 1.5 times the IQR. Possible outliers are data points outside of this distance. Boxplot colours indicate different groups:

709 Grey with circular points = CCLR with meniscal injury (n=65), Orange with triangle points = CCLR
710 without meniscal injury (n=72), Light blue with square points = control group with neither CCLR nor
711 meniscal injury (n=17). Significance testing was completed using one-way ANCOVAs controlling for
712 age of the canine participants in each group with Tukey's HSD *post-hoc* test for multiple comparisons.
713 Significance values given are the false discovery rate adjusted p-values.

714 Conflict of Interest Statement

715

716 No conflicts of interest have been declared

Determining predictive metabolomic biomarkers of meniscal injury in dogs with cranial cruciate ligament rupture

Abstract

Objectives

This study used hydrogen nuclear magnetic resonance spectroscopy (^{1}H NMR) for the first time to examine differences in the metabolomic profile of stifle joint synovial fluid from dogs with cranial cruciate ligament rupture with and without meniscal injuries, in order to identify biomarkers of meniscal injury. Identifying a biomarker of meniscal injury could then ultimately be used to design a minimally invasive diagnostic test for meniscal injuries in dogs.

Methods

Stifle joint synovial fluid was collected from dogs undergoing stifle joint surgery or arthrocentesis for lameness investigations. We used multivariate statistical analysis using principal component analysis and univariate statistical analysis using one-way analysis of variance and analysis of co-variance to ~~Samples underwent ^{1}H NMR spectroscopy and metabolite identification. We used multivariate and univariate statistical analysis to~~ identify differences in the metabolomic profile between dogs with cranial cruciate ligament rupture and meniscal injury, cranial cruciate ligament rupture without meniscal injury, and neither cranial cruciate ligament rupture nor meniscal injury, taking into consideration clinical variables.

Results

154 samples of canine synovial fluid were included in the study. 64 metabolites were annotated to the ^{1}H NMR spectra. Six spectral regions were found to be significantly altered (false discovery rate adjusted p-value <0.05) between groups with cranial cruciate ligament rupture with and without

meniscal injury, including three attributed to NMR mobile lipids (mobile lipid -CH_3 [p=0.016], mobile lipid -n$(CH_3)_3$ [p=0.017], mobile unsaturated lipid [p=0.031]).

Clinical Significance

We identified an increase in NMR mobile lipids in the synovial fluid of dogs with meniscal injury which are of interest as potential biomarkers of meniscal injury.

Introduction

Cranial cruciate ligament rupture (CCLR), either partial or complete, is one of the most common causes of pelvic limb lameness in dogs (Witsberger *et al.*, 2008). It presents a significant cause of morbidity amongst the canine population, and it has been estimated that dogs with CCL disease ~~CCLR~~ account for 0.56% of all cases presented to primary care veterinary practices in the UK (Taylor-Brown *et al.*, 2015). One sequelae of joint instability caused by a loss of CCL function is tears to the menisci, occurring in approximately 50% of cases at time of CCLR surgery (Bennett and May, 1991). The menisci are a pair of C-shaped fibrocartilaginous structures located between the tibial plateau and femoral condyles (Kambic and McDevitt, 2005). They have several important functions including load bearing, load distribution and shock absorption, as well as contributing to joint stability, proprioception and joint lubrication (Arnoczky et al., 1980, Pozzi et al., 2010).

Meniscal injuries can also~~–~~ occur post-operatively after CCLR surgery ~~,~~ due to ~~either~~ residual joint instability (Metelman *et al.*, 1995). Failure to diagnose meniscal injuries at the time of surgery for CCLR can also lead to poor post-operative outcomes (Metelman *et al.*, 1995).~~, or failure to diagnose at the time of surgery (Metelman *et al.*, 1995).~~ The prevalence of ~~these~~ late meniscal injuries varies from 2.8% to 13.8% (Metelman et al., 1995, Fitzpatrick and Solano, 2010). Late meniscal injuries can be a cause of recurring stifle joint pain and lameness, and are challenging for the veterinary practitioner to

diagnose (Dillon *et al.*, 2014). Affected dogs often present with a recurring lameness on the operated limb weeks or months after CCLR surgery, with clinical examination potentially revealing pain on stifle flexion, and/or a "click" on stifle flexion (Dillon *et al.*, 2014, Case *et al.*, 2008). The presence of a meniscal click has been found to be an unreliable diagnostic sign (McCready and Ness, 2016). Radiographs, useful in ruling out other causes of recurring lameness post-operatively, cannot show meniscal injuries directly. Further diagnostic imaging techniques for late meniscal injuries include low field or high field magnetic resonance imaging (MRI), computed tomography (CT) with arthrography, or ultrasound examination (McCready and Ness, 2016). Depending on the study, the sensitivity of these techniques in diagnosing meniscal injuries in dogs is 64-100% for low field MRI (Böttcher *et al.*, 2010, Gonzalo-Orden *et al.*, 2001), 75-100% (Olive *et al.*, 2014, Blond *et al.*, 2008) for high field MRI, 71% for CT arthrography (Samii *et al.*, 2009) and 90% for ultrasonography (Mahn *et al.*, 2005). All of these imaging techniques require either expensive specialised equipment, and/or advanced technical expertise, limiting the availability of these diagnostics in veterinary practice, and amount to a considerable cost. Surgical methods of diagnosis include either stifle joint arthroscopy or arthrotomy (Pozzi et al., 2008). Diagnosis of meniscal injuries by surgical intervention holds inherent risks including the risk of post-operative morbidity (Hoelzler et al., 2004). Furthermore, using surgery as a means of diagnosis has the risk of the animal undergoing an unnecessary surgical procedure if no meniscal injury is found (Blond *et al.*, 2008). The development of a simple, inexpensive, minimally invasive diagnostic test for meniscal injuries in dogs would be useful when determining whether invasive surgical intervention is necessary. For example, knowledge of whether a meniscal injury is present or not would help with the decision to perform an arthrotomy, either when planning extra-articular or per-articular surgical techniques to treat CCLR (Comerford et al., 2013), or when there is a suspicion of late meniscal injury post-operatively.

Currently, there are no biomarkers of meniscal injury that can be used as a diagnostic aid. ~~Also, no biomarkers of CCLR exist that could lead to earlier intervention or target preventative treatment in "at risk" stifles, such as the contralateral stifles of high-risk breeds such as Labrador retrievers (Guthrie~~

et al., 2012). One potential source of biomarkers of stifle joint pathologies is synovial fluid (SF) (Boffa et al., 2020). SF is a viscous fluid, that is a dialysate of plasma, and functions as a joint lubricant (Ghosh, 1994). It contains a unique source of biomarkers of joint disease, due to its close proximity to structures within joints (Anderson et al., 2018b).

Metabolomics allows the identification and quantification of small molecule metabolites and analysis of metabolic pathways within a variety of biofluids, cells and tissues (Bujak *et al.*, 2015). Nuclear magnetic resonance (NMR) is a tool for metabolomics studies, having the benefits of being rapid, non-destructive and relatively inexpensive compared to other metabolomics tools such as mass spectrometry (Clarke *et al.*, 2020). ^{1}H NMR has been used successfully to investigate changes in the SF metabolomic profile in humans and horses with joint pathologies such as rheumatoid arthritis, osteoarthritis (OA), and septic arthritis (Anderson *et al.*, 2018a, Anderson *et al.*, 2018b, Clarke *et al.*, 2020). In addition to detecting breakdown products of proteins, ^{1}H NMR spectroscopy can also detect resonances arising from lipid species (Soininen et al., 2009). NMR mobile lipids are resonances on an NMR spectrum that arise from methyl or methylene groups of lipid acyl chains (Delikatny et al., 2011). These arise primarily from triglycerides, fatty acids and cholesteryl esters in lipid droplets, and also from phospholipidic acyl chains if not embedded in lipid membrane bilayers (Mannechez *et al.*, 2005). A previous NMR lipidomic study in SF from canine and human OA affected joints found an increase in numerous lipid species in OA compared to healthy controls in both species (Kosinska et al., 2016). Alterations in lipid profiles of SF from joints with meniscal injury have not yet been investigated. Therefore, there is promise for using NMR spectroscopy to investigate biomarkers of joint pathology within canine SF, including CCLR and meniscal injuries.

We therefore hypothesise that the metabolomic profile of canine stifle joint SF will alter depending on the presence of CCLR and depending on the presence of concurrent meniscal injuries.

Metabolomic changes within SF linked to the presence of CCLR and meniscal injuries could be due to alterations in pathways linked to degeneration in the CCL, inflammatory responses and/or traumatic tears to the meniscal tissue. Metabolomic biomarkers of CCLR and meniscal injuries could then potentially allow for the development of a simple, minimally invasive diagnostic test (for example via arthrocentesis) more reliable at detecting meniscal injuries, and late meniscal injuries, than pre-existing non-surgical diagnostic techniques. This diagnostic test could then reduce the need for invasive surgical methods of meniscal injury diagnosis.

~~We therefore hypothesise that the metabolomic profile of canine stifle joint SF will alter depending on the presence of CCLR and depending on the presence of concurrent meniscal injuries.~~

Materials and methods

Ethical approval

Ethical approval for the collection of canine SF for use in this study was granted ~~by the University of Liverpool Veterinary Research Ethics Committee~~ (VREC634) as surplus clinical waste under the generic approval RETH00000553.

Synovial fluid collection

~~Canine SF was collected from dogs undergoing surgery for CCLR or patella luxation, or as excess clinical waste from dogs undergoing arthrocentesis as part of lameness investigations from March 2018 to June 2021.~~ Canine SF was collected from dogs undergoing surgery for CCLR, either with or without concurrent meniscal injuries, from dogs undergoing surgery for patella luxation, or as excess clinical waste from dogs undergoing arthrocentesis as part of lameness investigations from March 2018 to June 2021. Cases were divided into three groups, namely, group 1) CCLR with meniscal injury; Group 2) CCLR without meniscal injury; and group 3) neither CCLR nor meniscal injury (the control group). Cases were recruited with informed consent from three veterinary practices in the north-west of England. SF was collected by stifle joint arthrocentesis as per the BSAVA guide to procedures in small animal practice (Bexfield and Lee, 2014). A 21-gauge to 23-gauge needle attached to a 2 to 5 mL ~~two to five millilitre~~ sterile syringe (depending on the size of the dog) was inserted into the stifle joint space either medially or laterally to the patella ligament after sterile preparation of the skin, prior to first surgical incision. After aspiration of the SF, samples were placed in sterile 1.5 mL~~ml~~ Eppendorf tubes (Eppendorf UK Ltd, Stevenage, UK), and immediately refrigerated at 4°C.

Synovial fluid processing

SF samples were transported on ice to the laboratory within 48 hours of collection. Samples stored for longer than 48 hours before processing were excluded from the study based on previous ~~unpublished~~ data examining metabolomic changes in the SF~~synovial fluid~~ with elongated refrigerated storage time (Pye, 2021). Any SF samples with a large amount of blood contamination, or that had a haemorrhagic discolouration were excluded from the study. A small number of SF samples with minor iatrogenic blood contamination from arthrocentesis (seen as blood "streaks" that are not completely blended with the SF sample (Clements, 2006)) were included~~(Clements, 2006).~~ Samples were centrifuged at 2540*g* at 4°C for 5 minutes. The supernatant was pipetted into 200 µl aliquots, and snap frozen in liquid nitrogen before storing at -80°C (Anderson *et al.*, 2020).

142

143 Clinical information on the canine participants

144

145 Inclusion criteria for this study were dogs undergoing surgery for either partial or complete CCLR (with
146 or without concurrent meniscal injury), dogs undergoing surgery for patella luxation, or dogs that had
147 stifle joint arthrocentesis as part of clinical investigations into hindlimb lameness. There were no
148 exclusion criteria based on other clinical attributes of the dogs.

149 Clinical information from the dogs used in this study was collected. This information included breed,
150 age, sex and neuter status, body weight, body condition score (Laflamme, 1997), presence and degree
151 of CCLR (whether partial or complete CCLR), presence of meniscal injury, location and type of meniscal
152 injury (Bennett and May, 1991), presence of patella luxation, length of time of lameness, co-
153 morbidities ~~s~~, medication being received by the dog and radiographic level of OA using two separate
154 scoring systems (Innes *et al.*, 2004, Wessely *et al.*, 2017).

155 Orthogonal radiographs (medio-lateral view and caudo-cranial views) of the stifle joint of each dog
156 included in the study were analysed. Radiographs were performed either as pre-operative radiographs
157 or as part of lameness investigations, less than 21 days before arthrocentesis of the SF sample. These
158 were analysed either by a veterinary surgeon with a postgraduate certificate in small animal surgery,
159 or by a veterinary student who had received training in radiographic OA scoring of the stifle joint and
160 was overseen by the aforementioned veterinary surgeon. Two separate scoring systems were initially
161 used to assess difference between the three groups in terms of their radiographic OA score (Innes *et*
162 *al.*, 2004, Wessely *et al.*, 2017). These scoring systems use either a 10-point scale (Innes *et al.*, 2004),
163 or a 45-point scale (Wessely *et al.*, 2017). A global assessment score from zero (no OA) to three (severe
164 OA) as described by Innes *et al.* (2004) was then used when assessing metabolomic differences in the
165 stifle joint SF based on level OA in order to group the level of OA for ANOVA testing (see Statistical
166 Analysis section below).

NMR Metabolomics

Sample preparation for NMR metabolomics

SF samples were thawed on ice immediately prior to sample preparation for NMR spectroscopy. 100 µL of each thawed SF sample was diluted to a final volume containing 50% (v/v) SF, 40% (v/v) dd $^{1}H_2O$ (18.2 MΩ), 100 mM phosphate buffer, ~~PO43−~~ pH 7.4 ~~buffer~~ (Na_2HPO_4, VWR International Ltd., Radnor, Pennsylvania, USA and NaH_2PO_4, Sigma-Aldrich, Gillingham, UK) in deuterium oxide ($^{2}H_2O$, Sigma-Aldrich) and 0.0025% (v/v) sodium azide (NaN_3, Sigma-Aldrich). Samples were vortexed for 1 minute, centrifuged at 13,~~-~~000*g* and 4 °C for 5 minutes and 180 µL transferred (taking care not to disturb any pelleted material) into 3 mm outer diameter NMR tubes using a glass Pasteur pipette.

NMR metabolomics spectral acquisition

Spectra were acquired using a 700MHz Bruker Avance III spectrometer (Bruker Corporation, Billerica, Massachusetts, USA) with associated triple resonance inverse (TCI) cryoprobe and chilled Sample Jet auto-sampler. Software used for spectral acquisition and processing were Topspin 3.1 (Bruker Corporation, Billerica, Massachusetts, USA) and IconNMR 4.6.7 (Bruker Corporation).

1D ^{1}H NMR spectra were acquired using a Carr-Purcell-Meiboom-Gill (CPMG) filter to suppress background signals from proteins and other endogenous macromolecular constituents, and allow acquisition specifically of small molecule metabolite signals (Carr and Purcell, 1954, Meiboom and Gill, 1958). A vendor-supplied standard pulse sequence was used to achieve this (cpmgpr1d) with water suppression carried out by pre-saturation (Hoult, 1976). The CPMG spectra were acquired at 37 °C with a 15 ppm spectral width, a four second interscan delay and 32 transients (Anderson *et al.*, 2020).

The spectra acquired in this study are available in the MetaboLights (Haug et al., 2020) repository (https://www.ebi.ac.uk/metabolights/MTBLS6050).

NMR metabolomics spectral quality control

1D ^{1}H NMR spectra were individually assessed to ensure minimum reporting standards were met (Sumner *et al.*, 2007). The steps for quality control included: 1) Assessing the spectral baseline to ensure minimal curvatures or deviations; 2) Assessing the quality of water suppression, to ensure the water peak at 4.7 ppm was no more than 0.4 ppm wide; 3) Aligning the spectra to the glucose beta anomeric doublet at 5.24 ppm; and 4) Measurement of the line-width half height of the glucose peak at 5.24 ppm, with any spectrum where the width of this peak at half the height of the peak exceeded more than one standard deviation from the mean being regarded as a failure of quality control. Any samples that were deemed to have failed quality control were re-ran on the spectrometer up to a maximum of three spectral acquisitions. Any samples that failed after the third spectral acquisition were excluded from the study.

Metabolite annotation and identification

The NMR spectra were divided into spectral regions (termed "bins") using Topspin 3.1 (Bruker Corporation, Massachusetts, USA), with each bin representing either single metabolite peaks or multiple metabolite peaks where peaks overlapped on the spectra. These bins were also examined using TameNMR (hosted by Github: https://github.com/PGB-LIV/tameNMR), an "in-house" toolkit built within the galaxy framework (Afgan et al., 2018). Bins were altered accordingly upon visualising the fit to the overlaid spectra to ensure the area under the peak was represented by the bin.

Metabolites were annotated to the spectra using Chenomx NMR Suite Profiler version 7.1 (Chenomx, Edmonton, Canada), a reference library of 302 mammalian metabolite NMR spectra. When metabolite peaks overlapped, multiple metabolites were annotated to the bin. When peaks were unable to be annotated to a metabolite, they were classed as being an "unknown" metabolite. Previous literature specifying metabolite chemical shifts and spectral appearance were examined to aid annotation of unknown areas. Downstream unique peak metabolite identification and in-house NMR metabolite standards were examined to confirm metabolite identities where possible. Metabolites were assigned a level of identification according to the Metabolite Standards Initiative (MSI) levels. Level 1 identified metabolites require two or more orthogonal properties of a standard component to be analysed using the same spectrometer and experimental conditions as the experimental spectra. Level 2a metabolite identifications are made after matching one property of a standard component analysed using the same spectrometer and experimental conditions. Level 2b are putatively identified metabolites using reference libraries of standard compounds obtained from external laboratories. Level 3 are putatively annotated compound classes, used when the molecule can only be annotated to a class rather than a specific metabolite (Sumner *et al.* 2007).

A pattern file was created of the spectral bins and metabolites annotated to that bin. This is a spreadsheet outlining the bin boundaries in ppm, and the metabolites annotated to that bin. The pattern file and the Bruker spectra files were input into TameNMR, in order to create a spreadsheet of the integrals from binned spectra, with the relative intensities of each bin for each sample, which could then be used for statistical analysis of the spectra.

Statistical analysis

Differences in clinical variables of the canine participants

Analysis of the differences in clinical features between the groups in terms of age, sex and neuter status, BCS, the length of time of lameness on the affected hindlimb, and radiographic OA scores using both scoring systems and the global assessment of radiographic OA from zero to three (Innes *et al.*, 2004; Wessely *et al.*, 2017) were undertaken in the following way. Firstly, normality was tested using Shapiro-Wilk test, as well as visualising histograms and quantile-quantile (QQ) plots of the data for each variable. Kruskal-Wallis tests were undertaken on non-parametric data, with Dunn's *post-hoc* test. A Benjamini-Hochberg false discovery rate (FDR) adjustment was carried out for all tests, and significance set at $p<0.05$. These analyses and creation of boxplots to visualise this data was undertaken using R (R Core Team, 2020).

~~Analysis of the differences in clinical features between the groups in terms of age, sex and neuter status, body condition score, and radiographic OA score was undertaken using one-way analysis of variance (ANOVA) with a Benjamini-Hochberg false discovery rate (FDR) adjustment, and significance set at $p<0.05$. When any variable did not fit to ANOVA assumptions of having a homogeneous variance, Brown-Forsythe and Welch ANOVA tests with Benjamini-Hochberg FDR adjustment was carried out. These analyses and creation of graphs to visualise this data was undertaken using . (R Core Team, 2020)GraphPad Prism 9.1.0 (GraphPad Software, San Diego, CA, USA).~~

Metabolomics data analysis

~~Sample size power calculations were completed using data from a previously unpublished small cohort~~ Sample size power calculations were completed using data from a previous unpublished small cohort study (n=5 with CCLR and meniscal injury and n=7 with CCLR without meniscal injury), with a specified

FDR of 0.05 ~~study,~~ using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca), a software based on a metabolomics data analysis package written in R (the MetaboAnalystR package) (Pang *et al.*, 2021).

Metabolomics data was normalised using probabilistic quotient normalisation (PQN) (Dieterle *et al.*, 2006), and Pareto scaled using R prior to statistical analysis (R Core Team, 2020). Unsupervised multivariate analysis was carried out using principal component analysis (PCA) on the normalised and scaled data using R. The variance between canine phenotypes was investigated through analysis of principal components 1 through 10 using o~~O~~ne-w~~W~~ay ANOVAs or linear models depending on the data type. Briefly, CCLR, sex, neuter status, BCS, radiographic OA score and batch were numerically encoded and assessed against each principal component using a ~~O~~one-~~W~~way ANOVA. Age, Length of time of lameness, weight, length of time of storage pre-processing which were already numeric variables were assessed against each principal component using a linear model. All p values were corrected using FDR (Bejamini Hochberg) correction. Correlation matrices between phenotypes were computed using the spearmans correlation using the *cor* function in R and visualised using a heatmap generated with the *pheatmap* function in R (Kolde, 2012).

Univariate analysis was carried out using o~~O~~ne-w~~W~~ay ANOVAs and o~~O~~ne-w~~W~~ay analysis of co-variance (ANCOVAs) using R. To account for multiple testing across all 236 metabolite bins FDR correction was applied to the F-Test p value of each metabolite, significance was accepted at $p < 0.05$. For metabolites with an FDR < 0.05 Tukey's honest significant difference *post-hoc* test was applied to assess between group variances. Metabolite differences were separately analysed with respect to age, weight (divided into groups of 10 kg intervals), BCS, global assessment of radiographic OA score (0-3) (Innes et al., 2004), length of time of clinical lameness (divided into groups less than one month, one to three months, three to six months and six to twelve months), site of collection, partial *versus* complete *versus* no CCLR and between the three groups (group 1: CCLR with meniscal injury, group 2: CCLR without meniscal injury and group 3: control group with neither CCLR nor meniscal injury). Age adjusted o~~O~~ne-~~W~~way ANCOVAs were applied to each metabolite to assess differences between the

three groups (1) CCLR with meniscal injury, 2) CCLR without meniscal injury and 3) control group with neither CCLR nor meniscal injury) ~~meniscal and no meniscal injury groups~~, FDR adjustment was applied as a above. Boxplots to visualise the changes in metabolite abundances were created using *ggplot2* package within R. ~~in MetaboAnalyst 5.0 (https://www.metaboanalyst.ca).~~

Results

Sample size calculations revealed a sample size of n=60 per group, namely ~~–~~group 1) CCLR with meniscal injury, 2) CCLR without meniscal injury and 3) control group with neither CCLR nor meniscal injury, would give a predictive power of 0.83 when plotted on a predictive power curve.

For the metabolomic study, 191 samples of canine stifle joint SF were collected and submitted for NMR spectroscopy. Of these, 14 samples had been stored for longer than 48 hours prior to collection for processing, and were subsequently excluded from the study. Four samples were from cases in which the meniscal injury status was unknown, and were also excluded from the study. Nineteen samples were excluded as they failed to meet minimum reporting standards (Sumner *et al.*, 2007) after three spectral acquisitions.

In total, 154 canine stifle joint SF samples were included in the statistical analysis. These were divided into three groups, namely group 1) CCLR with meniscal injury (n=65), group 2) CCLR without meniscal injury (n=72), , and group 3) ~~CCLR without meniscal injury (n=72), CCLR with meniscal injury (n=65), and~~ control group with neither CCLR nor meniscal injury present (n=17). The two groups of CCLR cases included dogs with either partial or complete CCLR. The control group consisted of 13 cases of patella luxation, three cases from arthrocentesis of the stifle joints during lameness investigations which subsequently were found to have no pathology, and one sample from a case with fraying of the caudal cruciate ligament.

313

314 Differences in signalment of the canine participants between groups

315

316 Information regarding the signalment of the dogs in each group is shown in Table 1. There was a
317 significant difference between the control group and ~~the~~ both the CCLR groups with or without
318 meniscal injury in terms of age, weight, and radiographic OA score using both the Innes *et al.* (2004)
319 and Wessely *et al.* (2017) scoring systems~~,~~. There was no significant differences between the three
320 groups in terms of ~~but not with~~ BCS of the dogs and length of time of clinical lameness (Figure 1).
321 There was no significant difference between groups CCLR with meniscal injury and CCLR without
322 meniscal injury in terms of these clinical variables, although age was closest to reaching significance
323 between the two groups (p=0. ~~0~~137~~7~~, m~~M~~ean ~~D~~difference=0.86 years [0.01 to 1.73 95% CI]).

324

325 Metabolite annotation and identification.

326

327 Spectra were divided into 246 bins. Of these, 84 (34%) remained with an unknown metabolite
328 identification, and 162 (66% of bins) were annotated to one or more metabolites. In total, 65
329 metabolites were annotated to the spectra (Table 2). Any bins containing ethanol peaks were excluded
330 from the statistical analysis, due to ethanol being considered a contaminant in NMR, usually either
331 during the collection of the SF from the sterilisation of skin with alcohol-based solutions (Hutchinson,
332 2012)~~surgical spirit,~~ or during the processing steps (van der Sar *et al.*, 2015). Propylene glycol, a
333 metabolite found in solvents used in pharmaceuticals (Zar *et al.*, 2007) was found in one spectrum,
334 and so those bins were excluded so as to not bias the statistical analysis.~~.~~

335

Metabolomic statistical analysis results

Analysis of canine synovial fluid metabolome with respect to weight, age, radiographic OA score, length of time of lameness, BCS, site of collection and degree of CCLR of the canine participants

Analysis of metabolite changes with respect to clinical variables found significantly altered metabolites with differing weight (Supplementary information Figure S2), age (Supplementary information Figure S3) and radiographic OA score of the dogs using the global assessment score (0-3) within Innes *et al.* (2004) (Supplementary information Figure S4). This included an increase in glutamine with increasing weight of the dogs (Supplementary information Figure S2). Four mobile lipid regions on the spectra were significantly increased with increasing age of the dogs (Supplementary information Figure S3). There were no significant metabolite differences depending on the length of time the dog had clinical signs of lameness or due to the BCS of the dog. There were also no significant difference between dogs with a partial CCLR *versus* dogs with a complete CCLR.

Multivariate analysis of canine synovial fluid metabolome with respect to CCLR and meniscal injury status

Multivariate PCA was undertaken to compare the differences in the overall metabolome between the groups, namely: group 1) CCLR with meniscal injury, group 2) CCLR without meniscal injury and group 3) no CCLR and no meniscal injury (the control group). ~~Multivariate PCA was undertaken to compare the differences in the overall metabolome between the groups (no CCLR and no meniscal injury [control group], CCLR with meniscal injury, CCLR without meniscal injury)~~ (Figure 2). Over principal components (PC) one and two, there were overlapping clustering of the groups, indicating little overall difference in the metabolome over these PCs (Figure 2a). Associations between different phenotypes

of the canine participants and PC one to ten found that PC three and four were primarily associated with CCLR and meniscal injury (supplementary material Figure S1). PCA of the groups plotted over PC three and four showed some samples from the control group were separated from the groups CCLR with and without meniscal injury, indicating that the control group appears to have a wider variation with some samples exhibiting a differing metabolome from the other two groups (Figure 2b).

~~*Univariate analysis of canine synovial fluid metabolome with respect to weight, age and radiographic OA score of the canine participants*~~

~~Analysis of metabolite changes with respect to clinical variables found significantly altered metabolites with differing weight (Supplementary information Figure S2), age (Supplementary information Figure S3) and radiographic OA score of the dogs (Supplementary information Figure S4). This included an increase in glutamine with increasing weight of the dogs (Supplementary information Figure S2). Four mobile lipid regions on the spectra were significantly increased with increasing age of the dogs (Supplementary information Figure S3).~~

Univariate analysis of canine synovial fluid metabolome with respect to CCLR and meniscal injury status

Univariate analysis of metabolomic differences between the three groups 1) CCLR with meniscal injury, 2) CCLR without meniscal injury and 3) control group with neither CCLR nor meniscal injury ~~groups (no CCLR and no meniscal injury [control group], CCLR with meniscal injury and CCLR without meniscal injury)~~ was then undertaken.

Between ~~the~~ groups 1) CCLR with meniscal injury, and group 2) CCLR without meniscal injury, there were six spectral bins that were below the threshold of significance ($p<0.05$), and two others that neared the threshold ($p<0.06$) after one-way ANOVA testing with FDR adjusted p-values and Tukey's HSD *post-hoc* test (Table 3). These included the four spectral bins related to mobile lipids.

It was noted that mobile lipids were also significantly increased with increasing age of the canine participants (supplementary information Figure S3), and that groups CCLR with meniscal injury and CCLR without meniscal injury had a slight, although insignificant ($p=0.1307$, mMean dDifference=0.86 years [0.01 to 1.73 95% CI]) difference in terms of age of the canine participants in each group with the group CCLR with meniscal injury being older (Figure 1). ~~.~~There was no difference in other variables (weight, radiographic OA score or BCS) between dogs in groups CCLR with meniscal injury and CCLR without meniscal injury (Figure 1). ANCOVAs were therefore undertaken to control for age. The results of these ANCOVAs controlling for age are shown in Table 4. After controlling for age, three out of four spectral regions annotated to mobile lipids were significantly higher in the group CCLR with meniscal injury compared to the group CCLR without meniscal injury (Figure 3). These regions were attributed to mobile lipid -CH_3 ({~~[~~$p=0.016$)~~]~~}, mobile lipid -$n(CH_3)_3$ ({~~[~~$p=0.017$~~]~~}) and mobile unsaturated lipid ({~~[~~$p=0.031$)~~]~~}. A complete list of the ANCOVA outputs are included in the supplementary information (Supplementary information Table S2).

After controlling for age, ~~T~~there were 49 spectral bins, relating to 31 metabolites that were found to be significantly altered between ~~the~~ group 1 (CCLR with meniscal injury) and group 3 (control group). 48 out of these 49 bins, related to the same 31 metabolites, were also found to be significantly altered between group 2(CCLR without meniscal injury) and group 3 (control group)~~two groups with CCLR (with or without meniscal injury) compared to the control group (no CCLR no meniscal injury) after controlling for age~~ (Supplementary information Table S2). However, as the control group differed to the other two groups in terms of other variables, such as weight and radiographic OA score, as well as

it being of a smaller sample size, it was not possible to accurately assess whether these metabolomic changes were based on the presence of CCLR alone.

Discussion

This is the first study of its kind to use NMR metabolomics to investigate biomarkers of meniscal injury within the stifle joint SF of dogs. It is also the first study to use NMR metabolomics to investigate biomarkers of meniscal injury within the SF of any species, including humans. It was found that NMR mobile lipids were significantly increased in the stifle joint SF of dogs with CCLR and meniscal injury, compared with dogs with CCLR but no meniscal injury, or dogs with neither CCLR nor meniscal injury. Mobile lipids are NMR lipid resonances that arise from isotropically tumbling, relatively non-restricted molecules such as methyl and methylene resonances belonging to lipid acyl chains (Hakumäki and Kauppinen, 2000, Delikatny *et al.*, 2011). These arise primarily from triglycerides, fatty acids and cholesteryl esters in lipid droplets, and also from phospholipidic acyl chains if not embedded in lipid membrane bilayers (Mannechez *et al.*, 2005). Lipids serve various important functions in biological systems, including as components of cell membranes and other cellular organelles, acting as an energy source, and having a crucial role in signalling and regulation of cellular processes (Onal *et al.*, 2017). Many biological processes have been associated with changes in NMR mobile lipids, including cell necrosis and apoptosis, malignancy, inflammation, proliferation and growth arrest (Hakumäki and Kauppinen, 2000). Lipid analysis of SF in humans have found differential abundance of lipids with different disease states, including OA, rheumatoid arthritis and trauma (Wise *et al.*, 1987). A more recent NMR lipidomic study in SF from canine and human OA affected joints found an increase in numerous lipid species in OA compared to healthy controls in both species (Kosinska et al., 2016).

There are a number of possible hypotheses for the increase in NMR mobile lipid resonances found in the SF of dogs with CCLR and concurrent meniscal injury compared to CCLR without meniscal injury in this study. Injury to the meniscus could lead to damage to cellular phospholipid membranes, resulting in the release of lipids into the SF. Human menisci have also been found to contain lipid debris that could have an impact on SF lipid concentrations in meniscal injury (Ghadially and Lalonde, 1981). Also, lipid droplets could be released from the intracellular environment due to cell necrosis or apoptosis in the damaged meniscal tissue (Uysal *et al.*, 2008), leading to an increased concentration of lipid droplets in the SF. Lipid droplets have been found to play a key role in inflammation, as such it may be that meniscal tears lead to a release of lipid droplets to facilitate in the inflammatory response within the joint (Melo *et al.*, 2011). As lipid droplets contain mediators of inflammation such as pro-inflammatory cytokines, lipids could also potentiate inflammatory changes in meniscal injury affected joints (Ichinose *et al.*, 1998). However, other metabolites linked to inflammation that were identified within the SF in this study, such as metabolites of glycolysis and the tricarboxylic acid (TCA) cycle (including lactic acid, glucose, pyruvate and citrate) (Anderson *et al.,* 2018a) were not significantly altered in dogs with CCLR and meniscal injury compared to those with CCLR but without meniscal injury. Alterations in SF lipid composition and lipid species can also have a role in affecting the lubricating ability of the SF (Antonacci *et al.*, 2012). The concentration of phospholipid species in human SF have been found to be increased in OA affected joints, therefore the observed increase in lipids could also be an attempt to improve lubrication of the SF after meniscal injury in order to have protective effects on the articular cartilage (Kosinska et al., 2015).

Amongst the other differentially abundant metabolites between groups with CCLR with and without meniscal injury, was methanol. Although methanol could be considered a contaminant in NMR (Fulmer *et al.*, 2010), it has also been found to be a naturally occurring metabolite in humans, either through dietary consumption in various fruit and vegetables, the artificial sweetener aspartame, alcohol, or through actions of gut microbiota (Dorokhov *et al.*, 2015). Some of these sources cannot

be ruled out, and therefore the decision not to remove methanol from analysis was made. However, its association with meniscal injury remains unclear.

One of the spectral bins that also showed a significant increase in canine SF in dogs with CCLR and meniscal injury compared to CCLR without meniscal injury was a region that had overlapping NMR peaks annotated to glycylproline, isoleucine, and an unknown metabolite. This region also requires further work to confirm the identity of the specific metabolites attributed to this area although it is likely given its correlation with other mobile lipid regions to derive from the same source. Fatty acyl chains have been previously noted to be attributed to resonances in this spectral region (Delikatny *et al.*, 2011). This would correlate with the findings of increases in mobile lipids with meniscal injury.

Spectral overlap and limited SF ^{1}H NMR studies resulted in a number of metabolite peaks that are, as yet, unidentified on the canine SF spectra, including one that was found to be significantly altered with meniscal injury. SF has been relatively understudied compared to other biofluids such as serum, and it is possible that SF contains metabolites that have yet to be reported in the literature, although the use of SF for NMR metabolomic studies of joint disease has been increasing in recent years (Clarke *et al.*, 2021). It could also be possible that there may be canine-specific metabolites, or breakdown products within canine SF that are different to other species due to the gait and physiology of the dog, that are currently not reported in the literature. Further work is required in identifying these regions, such as undertaking a 2D NMR experiment, or spiking SF with authentic standards (Dona et al., 2016). Alternatively, using complimentary methods of metabolite analysis, such as mass spectrometry, would improve the number of metabolite annotations and also potentially identifications in the SF samples.

One of the limitations of our study was the lack of a balanced control group to compare with the CCLR affected joints. There are several reasons for this. Firstly, collection of “normal” SF via arthrocentesis from joints without pre-existing pathology involves a level of risk, including introducing infection into the joint, and the need for sedation or anaesthetic for the protocol (Bexfield and Lee, 2014). Therefore, this would have ethical implications, and was outside the ethical approval for this study. SF from dogs

with no stifle joint pathology collected post-mortem would have been subjected to metabolite changes that would have compromised the comparison to the diseased groups (Donaldson and Lamont, 2015). Control samples in this study were collected from dogs undergoing surgery for patella luxation, or excess SF from dogs undergoing arthrocentesis from investigations of lameness. These were cases without CCLR or meniscal injuries, but also are likely not to have been completely without pathological changes, as patella luxation can be cause of OA and synovitis (Roush, 1993). Patella luxation also tends to be more common in smaller breeds of dogs, and as primarily a congenital disease, cases often show clinical signs of lameness at a younger age than CCLR affected dogs (LaFond *et al.*, 2002, Rudd Garces *et al.*, 2021). Both these factors meant the control group were on average younger and smaller than the CCLR groups, with less osteoarthritic changes. This, along with the fewer samples collected in the time constraints of this study affected the ability to infer conclusions from the metabolite changes between the control and other groups in terms of CCLR alone. The inclusion of more donors in the control group of healthy, non-diseased canine stifle joint SF would be of value in future work to allow analysis of metabolomic changes due to CCLR and OA in the canine stifle joint. However, for the investigation of biomarkers of meniscal injury in dogs with CCLR, a "healthy" control group may not be essential, as the comparison of groups CCLR with meniscal injury against CCLR without meniscal injury would be adequate to aid diagnosis.

Another potential limitation was the inclusion of some SF samples with minor blood contamination caused by arthrocentesis. As this study aimed to find biomarkers of meniscal injury within SF that could be used as a clinical diagnostic test, it was decided to include these samples as minor iatrogenic blood contamination of SF during sampling can be an occasional occurrence (Clements, 2006). Future studies could involve more detailed analysis of the level of blood contamination, including red blood cell counts, and how this may alter the SF metabolome. All samples were centrifuged to remove any cellular content prior to NMR spectroscopy.

There were factors such as diet and level of exercise that have been found to affect the metabolome of human serum that were not been accounted for in this study (Esko *et al.*, 2017, Sakaguchi *et al.*, 2019). However, unlike humans, dogs tend to have a less variable diet, and also exercise is likely to be similar between the canine participants, as the standard advice for dogs affected by CCLR is to limit exercise. Medications were found to be too heterogeneous between the dogs in this study from which to make any statistical conclusions but are known to affect the metabolomic profile of biofluids (Um *et al.*, 2009).

This study is the first of its kind in using ^{1}H NMR spectroscopy to identify biomarkers of meniscal injury within SF. SF lipid species appear to be of interest in the study of biomarkers of meniscal injury, and future work to identify the lipid species involved by undertaking a lipidomics experiment, such as NMR or Liquid Chromatography coupled Mass Spectrometry (LC-MS) lipidomics using lipid extracts from the SF samples. A simple, minimally invasive, inexpensive diagnostic test for meniscal injury in dogs by means of arthrocentesis could reduce the need for invasive surgical methods of meniscal injury diagnosis. This work could prove useful in exploring the potential for targeted assays to establish a diagnostic marker of meniscal injury in canine SF. ~~.~~

~~The work presented here is also of translational value into metabolomics studies in human and other mammalian species. No SF biomarker has been found to date in human SF with meniscal injury, therefore this research could also lead to the investigation of biomarkers of meniscal injury in human SF.~~

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Figure Legends

Figure 1. Clinical characteristics of the canine participants between groups. Box and whisker plots show differences in a) Age, b) Weight, c) body condition score (BCS), d) The length of time of clinical

lameness on the affected hindlimb, e) The radiographic osteoarthritis score using the radiographic scoring system as described by Wessely *et al.* (2017, and f) the global assessment of radiographic osteoarthritis from 0-3 as described by Innes *et al.* (2004). The box indicates the interquartile range (IQR) around the median. Each whisker extends to the furthest data point that is above or below 1.5 times the IQR. Possible outliers are data points outside of this distance. Boxplot colours indicate different groups: Grey = CCLR with meniscal injury (n=65), Orange = CCLR without meniscal injury (n=72), Light blue = control group with neither CCLR nor meniscal injury (n=17). ~~Column Bar graphs represent mean and standard deviation. Groups= Control (n=17), CCLR with meniscal injury (n=65), CCLR without meniscal injury (n=72). Significance testing performed with either one-way ANOVA, or Brown-Forsythe and Welch ANOVA (depending on whether data had common variance) with Benjamini-Hochberg false discovery rate adjustment~~ Significance testing was performed using Kruskal-Wallis testing with Dunn's *post-hoc* test. (CCLR=cranial cruciate ligament rupture~~disease~~, MI=meniscal injury, OA= osteoarthritis, ns=not significant, *=p<0.05, **=p<0.01, ***=p<0.001).

Figure 2. Principal component analysis (PCA) 2D scores plot of metabolite profiles of canine stifle joint synovial fluid by NMR. Samples grouped by CCLR and meniscal injury status. Group 1 (grey): CCLR with meniscal injury (n=65), Group 2 (orange) = CCLR without meniscal injury (n=72), Group 3 (light blue) = control group with neither CCLR nor meniscal injury (n=17). Plotted over A) PC1 and PC2 and B) PC3 and PC4.~~Meniscal injury status plotted over A) PC1 and PC2 and B) PC3 and PC4. Blue (control)=no CCLR, no meniscal injury; red (no)=CCLR without meniscal injury; green (yes)=CCLR with meniscal injury.~~

Figure 3. Altered mobile lipids on ^{1}H NMR with respect to meniscal injury status in canine stifle joint synovial fluid from dogs. Box and whisker plots show the normalised relative metabolite abundance

on the Y axis and group on the X axis. The box indicates the interquartile range (IQR) around the median. Each whisker extends to the furthest data point that is above or below 1.5 times the IQR. Possible outliers are data points outside of this distance. Boxplot colours indicate different groups: Grey with circular points = CCLR with meniscal injury (n=65), Orange with triangle points = CCLR without meniscal injury (n=72), Light blue with square points = control group with neither CCLR nor meniscal injury (n=17). ~~Control (red)= no CCLR or meniscal injury. No (green)= CCLR without meniscal injury. Yes (blue)= CCLR with meniscal injury. Red stars above boxplots denote significance against control group. Blue stars above boxplots denote significance against 'No' group.~~ Significance testing was completed using one-way ANCOVAs controlling for age of the canine participants in each group with Tukey's HSD *post-hoc* test for multiple comparisons. Values given are the false discovery rate adjusted p-values ~~(*=p<0.05, **=p<0.01, ***=p<0.0001)~~.

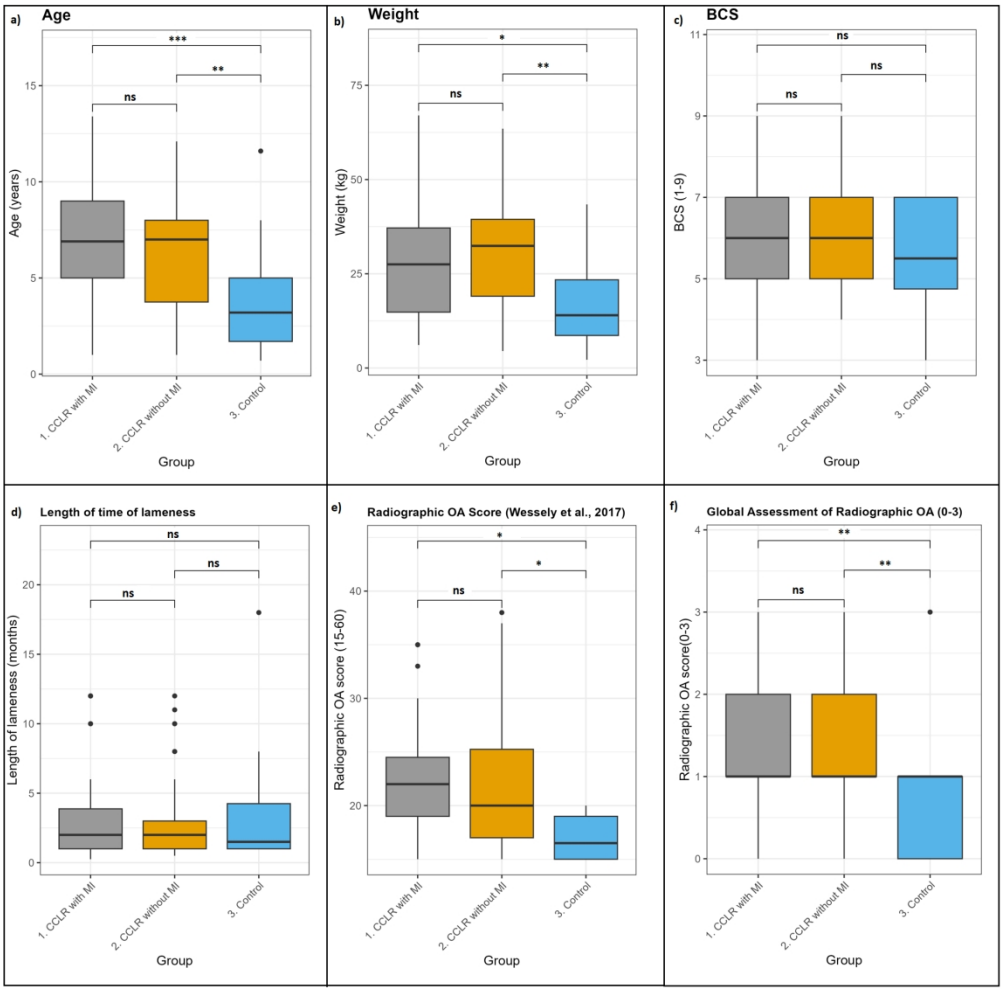
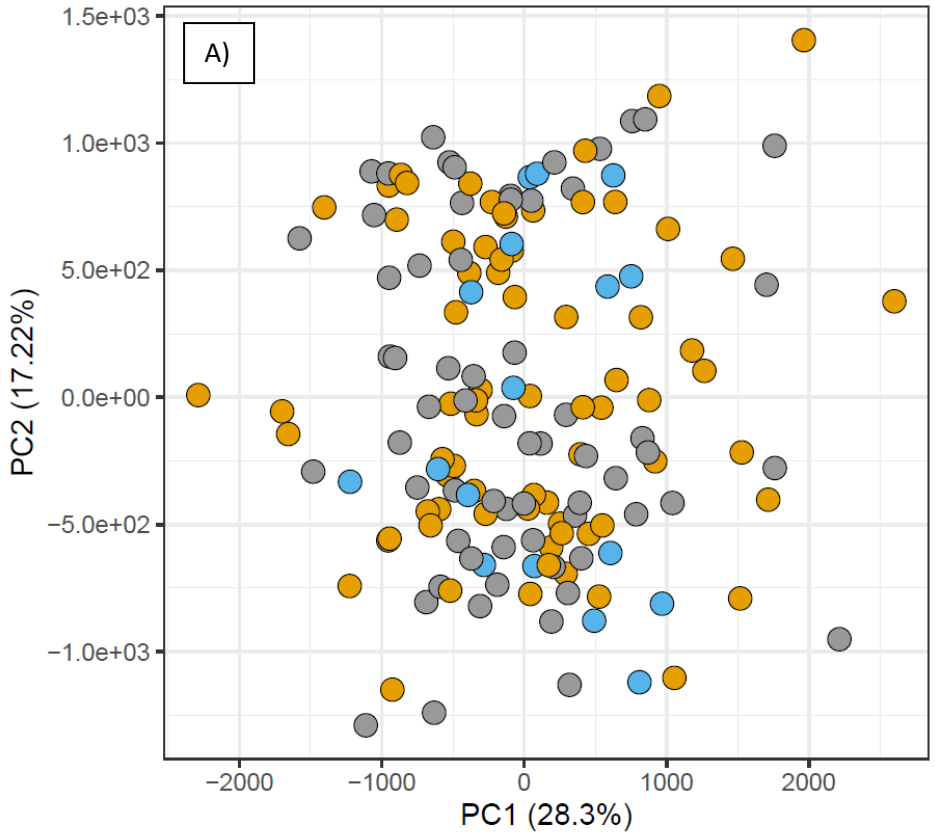
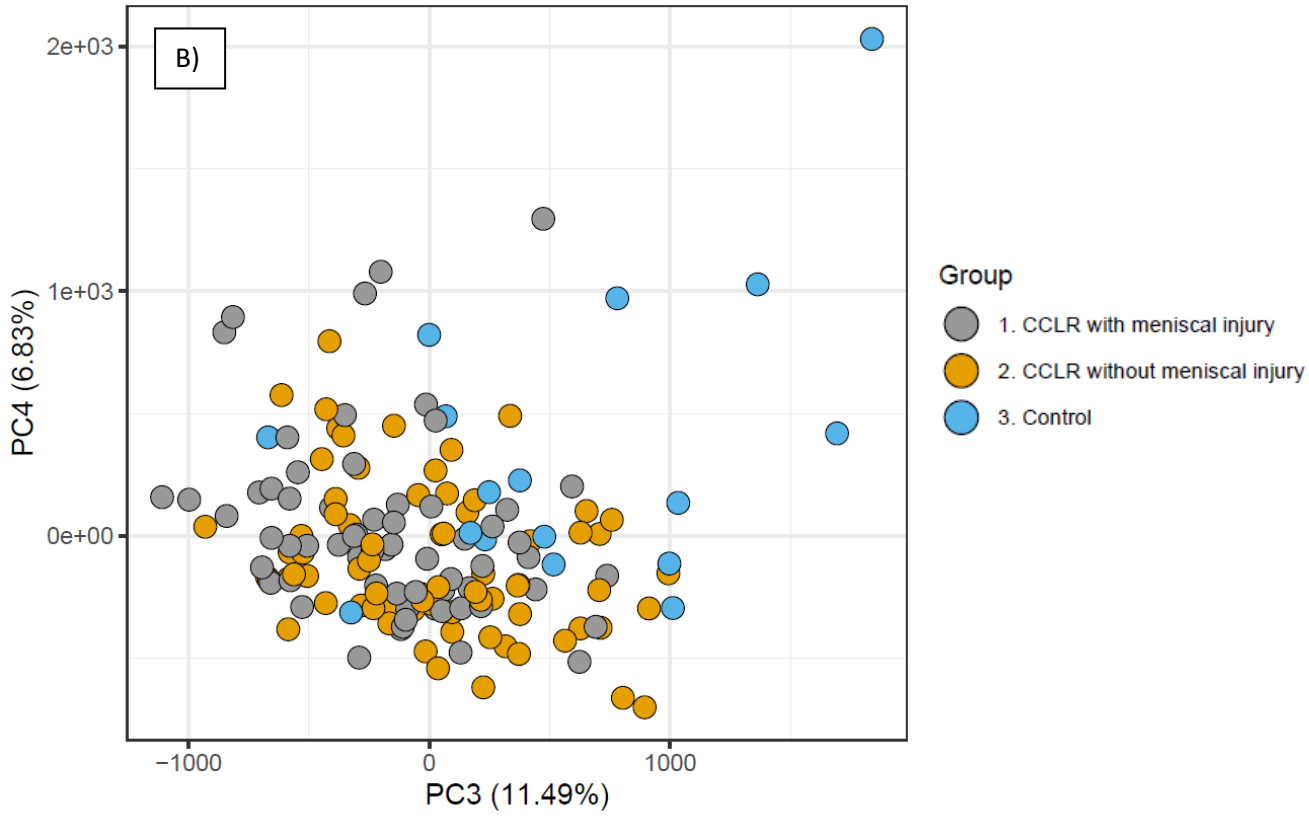


Figure 1. Clinical characteristics of the canine participants between groups. Box and whisker plots show differences in a) Age, b) Weight, c) body condition score (BCS), d) The length of time of clinical lameness on the affected hindlimb, e) The radiographic osteoarthritis score using the radiographic scoring system as described by Wessely et al. (2017), and f) the global assessment of radiographic osteoarthritis from 0 to 3 as described by Innes et al. (2004). The box indicates the interquartile range (IQR) around the median. Each whisker extends to the furthest data point that is above or below 1.5 times the IQR. Possible outliers are data points outside of this distance. Boxplot colours indicate different groups: Grey = CCLR with meniscal injury (n=65), Orange = CCLR without meniscal injury (n=72), Light blue = control group with neither CCLR nor meniscal injury (n=17). Significance testing was performed using Kruskal-Wallis testing with Dunn's post-hoc test. (CCLR=cranial cruciate ligament rupture, MI=meniscal injury, OA= osteoarthritis, ns=not significant, *=p<0.05, **=p<0.01, ***=p<0.001).

1133x1116mm (47 x 47 DPI)


A)
1.5e+03
1.0e+03
5.0e+02
0.0e+00
−5.0e+02
−1.0e+03
PC2 (17.22%)
−2000
−1000
0
1000
2000
PC1 (28.3%)
Group
1. CCLR with meniscal injury
2. CCLR without meniscal injury
3. Control


B)
2e+03
1e+03
0e+00
PC4 (6.83%)
−1000
0
1000
PC3 (11.49%)
Group
1. CCLR with meniscal injury
2. CCLR without meniscal injury
3. Control

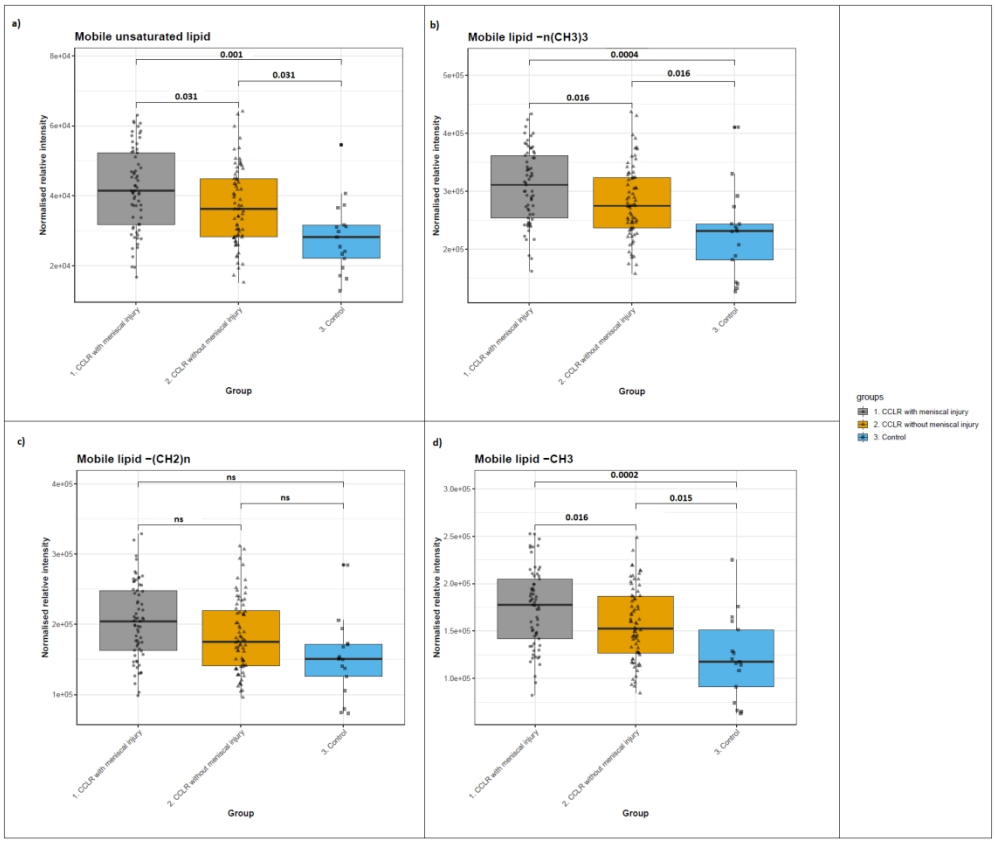


Figure 3. Altered mobile lipids on 1H NMR with respect to meniscal injury status in canine stifle joint synovial fluid from dogs. Box and whisker plots show the normalised relative metabolite abundance on the Y axis and group on the X axis. The box indicates the interquartile range (IQR) around the median. Each whisker extends to the furthest data point that is above or below 1.5 times the IQR. Possible outliers are data points outside of this distance. Boxplot colours indicate different groups: Grey with circular points = CCLR with meniscal injury (n=65), Orange with triangle points = CCLR without meniscal injury (n=72), Light blue with square points = control group with neither CCLR nor meniscal injury (n=17). Significance testing was completed using one-way ANCOVAs controlling for age of the canine participants in each group with Tukey's HSD post-hoc test for multiple comparisons. Significance values given are the false discovery rate adjusted p-values.

979x832mm (47 x 47 DPI)

Table 1. Clinical characteristics of the canine participants included in the nuclear magnetic resonance (NMR) metabolomic study of biomarkers of meniscal injury in canine stifle joint synovial fluid. Canine participants were divided in three groups depending on the presence of CCLR with meniscal injury (n=65), CCLR without meniscal injury (n=72) or neither CCLR nor meniscal injury (n=17).

	Group		
	CCLR with meniscal injury	**CCLR without meniscal injury**	**Control (no CCLR, no meniscal injury)**
Sample size (*n*)	65	72	17 (n=13 cases of patella luxation, n=3 cases of lameness of unknown cause, n=1 case of fraying of the caudal cruciate ligament)
Age, years (median, IQR)	6.9 (4.00)	7.0 (4.25)	3.2 (3.30)
Weight, kg (median, IQR)	27.5 (22.32)	32.4 (20.40)	14.0 (14.76)
Sex, *n* (%)	FE= 7 (11%) FN=26 (40%) ME=12 (19%) MN=18 (28%)	FE= 8 (11%) FN=28 (29%) ME=5 (7%) MN=30 (42%)	FE=2 (12%) FN=3 (18%) ME=7 (41%) MN=5 (29%)
BCS, 1-9 (median, IQR)	6.0 (2.00)	6.0 (2.00)	5.5 (2.25)
Radiographic OA score (15-60) (Wessely *et al.*, 2017) (median, IQR)	22.0 (5.50)	20.0 (8.25)	16.5 (4.00)
Radiographic OA score (0-10) (Innes *et al.*, 2004) (median, IQR)	4 (2)	4 (2.25)	3 (1.25)
Length of time of lameness, months (median, IQR)	2.0 (2.88)	2.0 (2.00)	1.5 (3.25)
Partial vs complete CCLR, *n*	Partial =9 Complete=55 Unknown=1	Partial =29 Complete=42 Unknown=1	N/A

Abbreviations= Cranial cruciate ligament rupture (CCLR), female entire (FE), female neutered (FN), male entire (ME), male neutered (MN), body condition score (BCS), kilograms (kg) standard deviation (SD), osteoarthritis (OA), not applicable (N/A), IQR=interquartile range.

Table 2. Metabolites annotated or identified to canine stifle joint synovial fluid nuclear magnetic resonance spectra, including HMDB identification number where possible, and level of identification according to the Metabolomics Standard Initiative (Sumner et al., 2007).

AMINO ACIDS			FATTY AND ORGANIC ACIDS		
Metabolite name	**HMDB number**	**MSI ID Level**	**Metabolite name**	**HMDB number**	**MSI ID Level**
ACETYLCYSTEINE	HMDB0001890	Level 2b	2-HYDROXYVALERIC ACID	HMDB0001863	Level 2b
AMINOADIPIC ACID	HMDB0000510	Level 2b	2-METHYLGLUTARATE	HMDB0000422	Level 2b
ANSERINE	HMDB0000194	Level 2b	2-PHENYLPROPIONATE	HMDB0011743	Level 2b
BETAINE	HMDB0000043	Level 2b	3 HYDROXYISOVALERATE	HMDB0000754	Level 2b
CREATINE	HMDB0000064	Level 2a	4-PYRIDOXATE	HMDB0000017	Level 2b
CREATINE PHOSPHATE	HMDB0001511	Level 2b	ACETIC ACID	HMDB0000042	Level 2b
CREATININE	HMDB0000562	Level 2a	ACETOACETIC ACID	HMDB0000060	Level 2b
CREATININE PHOSPHATE	HMDB0041624	Level 2b	AZELATE	HMDB0000784	Level 2b
GLYCINE	HMDB0000123	Level 2b	CITRIC ACID	HMDB0000094	Level 2a
L-ALANINE	HMDB0000161	Level 2a	FORMIC ACID	HMDB0000142	Level 2b
L-ALLOISOLEUCINE	HMDB0000557	Level 2b	GLYCEROL	HMDB0000131	Level 2b
L-GLUTAMINE	HMDB0000641	Level 2a	GLYCOCHOLIC ACID	HMDB0000138	Level 2b
L-HISTIDINE	HMDB0000177	Level 2a	GLYCOLATE	HMDB0000115	Level 2b
L-ISOLEUCINE	HMDB0000172	Level 2a	GLYCYLPROLINE	HMDB0000721	Level 2b
L-LEUCINE	HMDB0000687	Level 2a	ISOBUTYRIC ACID	HMDB0001873	Level 2b
L-LYSINE	HMDB0000182	Level 2a	L-CARNITINE	HMDB0000062	Level 2b
L-METHIONINE	HMDB0000696	Level 2a	L-GLUTAMIC ACID	HMDB0000148	Level 2b
L-PHENYLALANINE	HMDB0000159	Level 2a	L-LACTIC ACID	HMDB0000190	Level 2a
L-THREONINE	HMDB0000167	Level 2a	METHYLSUCCINIC ACID	HMDB0001844	Level 2b
L-TYROSINE	HMDB0000158	Level 2a	MOBILE LIPIDS	N/A	Level 3
L-VALINE	HMDB0000883	Level 2a	PYRUVIC ACID	HMDB0000243	Level 2a
SUGARS			**OTHERS**		
Metabolite name	**HMDB number**	**MSI ID Level**	**Metabolite name**	**HMDB number**	**MSI ID Level**
D-GALACTOSE	HMDB0000143	Level 2b	1-METHYLHISTIDINE	HMDB0000001	Level 2b
D-GLUCOSE	HMDB0000122	Level 2a	3-HYDROXY-3-METHYLGLUTARATE	HMDB0041199	Level 2b
D-MANNOSE	HMDB0000169	Level 2a	3-METHYLHISTIDINE	HMDB0000479	Level 2b
FRUCTOSE	HMDB0000660	Level 2b	ACETAMINOPHEN	HMDB0001859	Level 2b
GLUCITOL	HMDB0000247	Level 2b	ACETONE	HMDB0001659	Level 2b
MANNITOL	HMDB0000765	Level 2b	ACETYLCHOLINE	HMDB0000895	Level 2b
			CHOLINE	HMDB0000097	Level 2b
			DIMETHYL SULFONE	HMDB0004983	Level 2b
			DTTP	HMDB0001342	Level 2b
			ETHANOL	HMDB0000108	Level 2a
			HISTAMINE	HMDB0000870	Level 2b
			GLYCEROPHOSPHOCHOLINE	HMDB0000086	Level 2b
			O-CRESOL	HMDB0002055	Level 2b
			P-CRESOL	HMDB0001858	Level 2b
			PROPYLENE GLYCOL	HMDB0001881	Level 2b
			TRIGONELLINE	HMDB0000875	Level 2b
			XANTHINE	HMDB0000292	Level 2b

Abbreviations: HMDB=Human metabolome database; MSI= Metabolomics standards initiative

Table 3. Metabolites found to be significantly altered in canine stifle joint synovial fluid between those dogs with CCLR and with meniscal injury (n=65) and those with CCLR but without meniscal injury (n=72) using ANOVA testing with Tukey's honestly significant difference *post-hoc* test.

Bin number	Chemical shift (ppm)	Metabolite(s) annotated to bin	Mean difference (RI)	95% CI	FDR adjusted p-value
145	3.268-3.272	Unknown	-46.57	-80.45 to -12.69	0.004
230	1.071-1.080	Methylsuccinate and/or 2-Methylglutarate	21.97	5.91 to 38.04	0.004
129	3.362-3.371	Methanol	-40.04	-74.27 to -5.80	0.017
210	1.936-2.020	Glycylproline, Isoleucine and unknown	37.96	2.79 to 73.12	0.031
152	3.203-3.238	Mobile lipid -n$(CH_3)^3$	104.42	4.85 to 203.98	0.037
246	0.789-0.891	Mobile lipid-CH_3	82.25	3.37 to 161.13	0.039
37	5.212-5.353	Mobile unsaturated lipid	42.04	-0.06 to 84.14	0.050
224	1.199-1.312	Mobile lipid–(CH_2)n	88.78	-2.63 to 180.19	0.059

Abbreviations: ppm= parts per million; RI=relative intensity, CI= confidence interval; FDR= false discovery rate

Table 4. Metabolites found to be significantly altered (p<0.05) in canine stifle joint synovial fluid between those dogs with CCLR and with meniscal injury (n=65) and those with CCLR but without meniscal injury (n=72) using ANCOVA testing controlling for age of the dogs with Tukey's honestly significant difference *post-hoc* test.

Bin number	Chemical shift (ppm)	Metabolite(s) annotated to bin	Mean difference (RI)	95% CI	FDR adj p-value
145	3.268-3.272	Unknown	46.94	18.6 to 75.3	0.004
129	3.362-3.371	Methanol	40.01	11.3 to 68.7	0.009
246	0.789-0.891	Mobile lipid -CH_3	-78.88	-142.84 to -14.91	0.016
152	3.203-3.238	Mobile lipid-n$(CH_3)^3$	-99.38	-179.03 to -19.73	0.017
210	1.936-2.020	Glycylproline, Isoleucine and unknown	-36.35	-64.7 to -7.97	0.019
37	5.212-5.353	Mobile unsaturated lipid	-40.06	-73.96 to -6.16	0.031

Abbreviations: ppm= parts per million; RI = Relative intensity; CI= confidence interval; FDR= false discovery rate; adj= adjusted.

Supporting information

Determining predictive metabolomic biomarkers of meniscal injury in dogs with cranial cruciate ligament rupture

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Supplementary figures

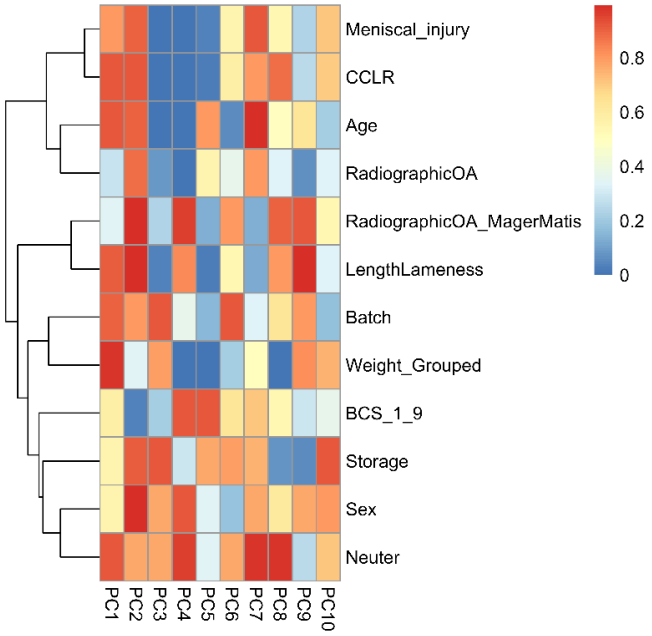

Figure S1. Heat map showing association of clinical variables of dogs whose synovial fluid was submitted for metabolomic analysis in the study with the first ten principal components in a principal component analysis. The key on the right denotes FDR corrected p-values from 0 (blue) to 1 (red). Meniscal injury and cranial cruciate ligament rupture (CCLR) appear to be associated primarily with principal components (PC) three to five. (OA= osteoarthritis, BCS= body condition score, Radiographic OA= global assessment of radiographic OA score (0-3), RadiographicOA_MagerMatis= Mager and Matiss radiographic osteoarthritis score (15-60) as described by Wessely *et al* (2017), LengthLameness= length of time of lameness, Storage= time stored at four degrees Celsius prior to processing and freezing).

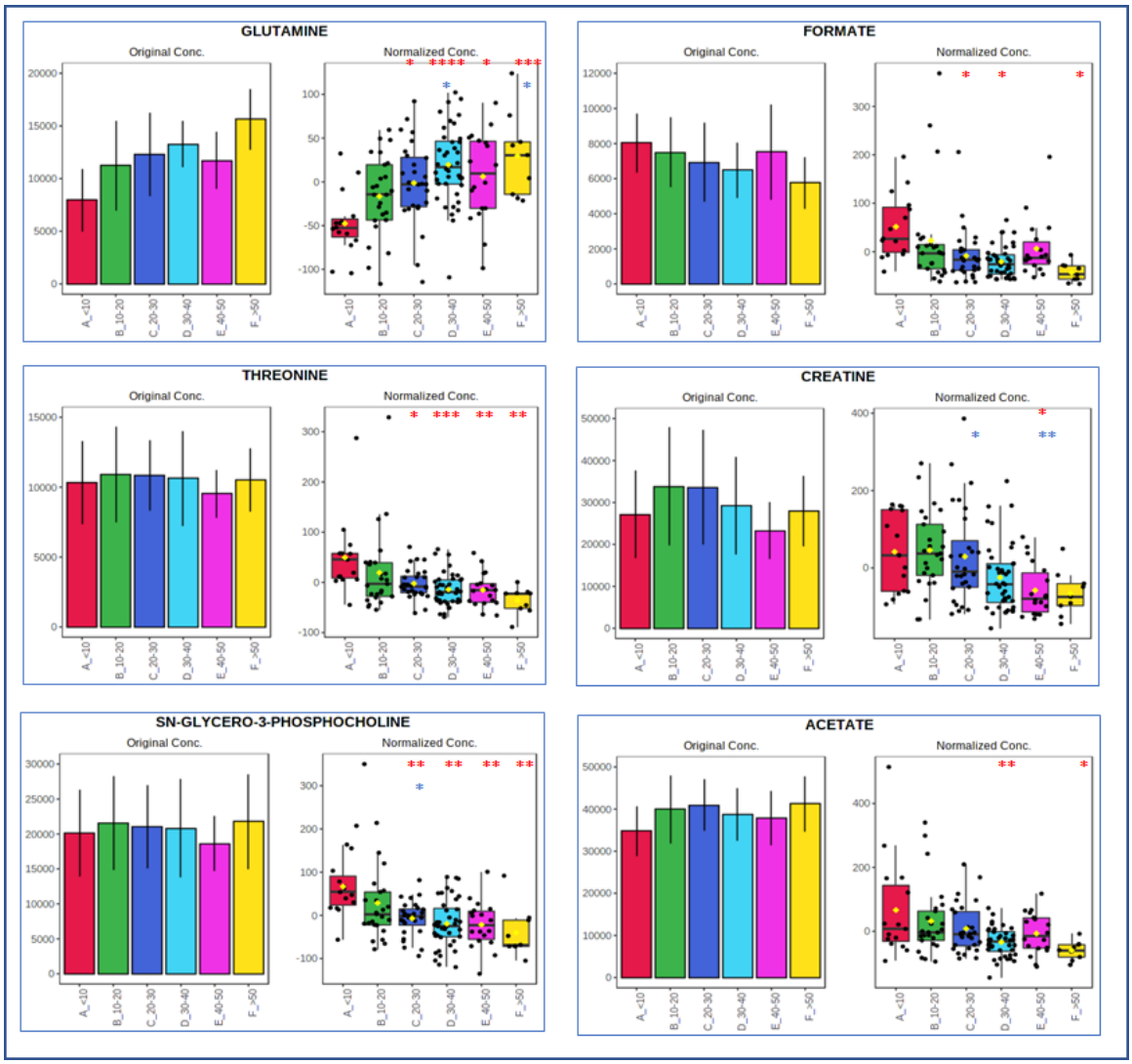

Figure S2. Bar plots and box and whisker plots of representative metabolites identified from univariate analysis as significantly different in canine stifle joint synovial fluid from dogs with cranial cruciate ligament rupture with increasing body weight of the canine participants. Y-axis represents peak intensity. The x axis shows the weight groups in Kg. Bar plots on the left show the original peak intensity (mean +/- SD), and box and whisker plots on the right show the normalised peak intensity after probabilistic quotient normalisation and pareto scaling. Key to colours of bar charts: Red= <10kg, Green = 10-20kg, Navy blue= 20-30kg, Light blue= 30-40kg, Pink=40-50kg, Yellow= >50kg. Red stars above the boxplots denote significance in comparison with the <10kg group, blue stars above the box plots denote significance in comparison with the 10-20kg group; *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

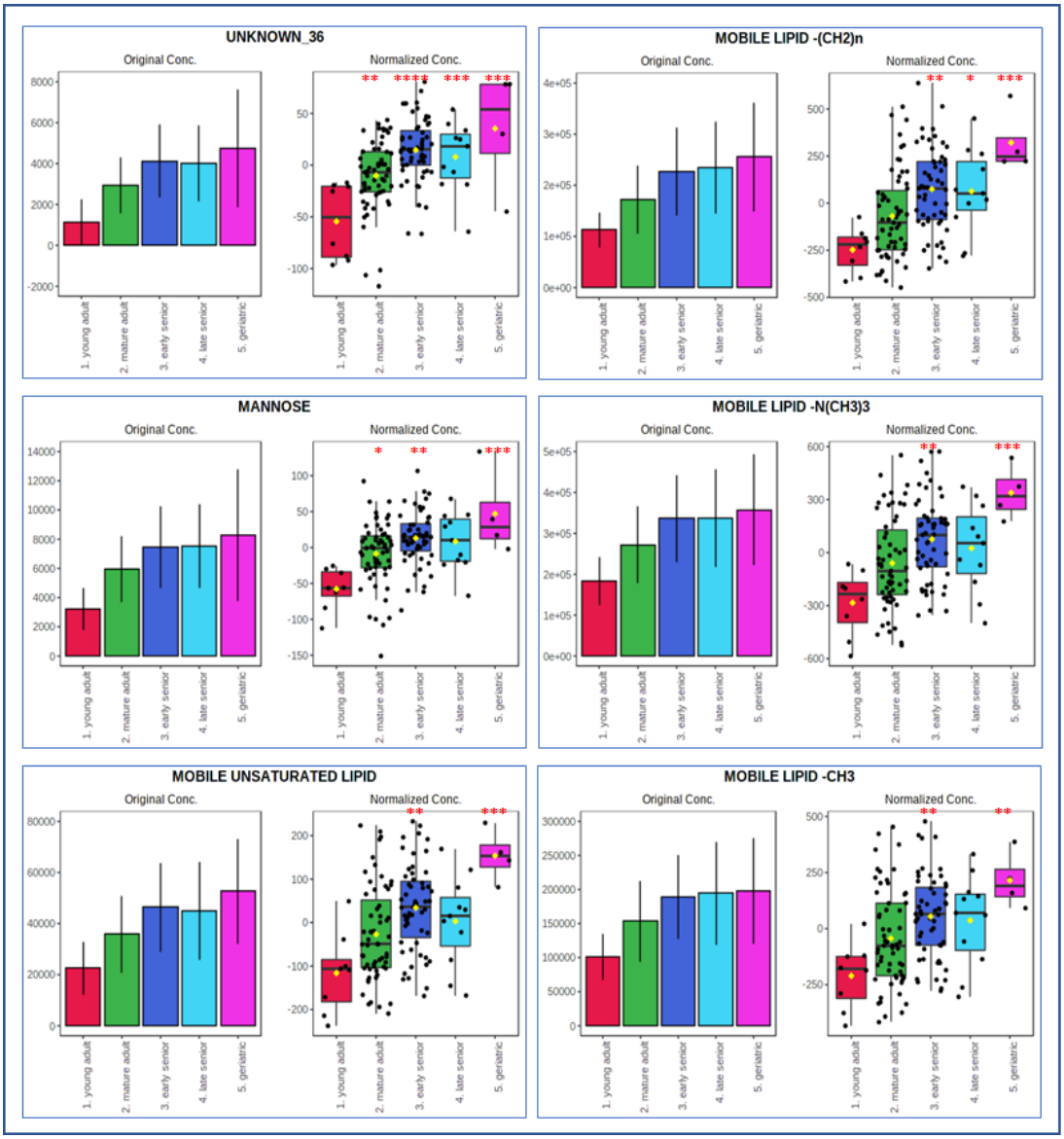

Figure S3. Bar plots and box and whisker plots of representative metabolites identified from univariate analysis as significantly different in canine stifle joint synovial fluid from dogs with cranial cruciate ligament rupture with increasing age of the canine participants. Y-axis represents peak intensity. The x axis shows the age groups from young adult to geriatric. Bar plots on the left show the original peak intensity (mean +/- SD), and box and whisker plots on the right show the normalised peak intensity after probabilistic quotient normalisation and pareto scaling. Key to colours of bar charts: Red=young adult, Green =mature adult, Navy blue=early senior, Light blue=late senior, Pink=geriatric. Red stars above boxplots denote significance in comparison with group 1 (young adult); *=p<0.05,

**=p<0.01, ***=p<0.001, ****=p<0.0001. Unknown_36= bin number 36 on the pattern file, representing an unidentified metabolite(s).

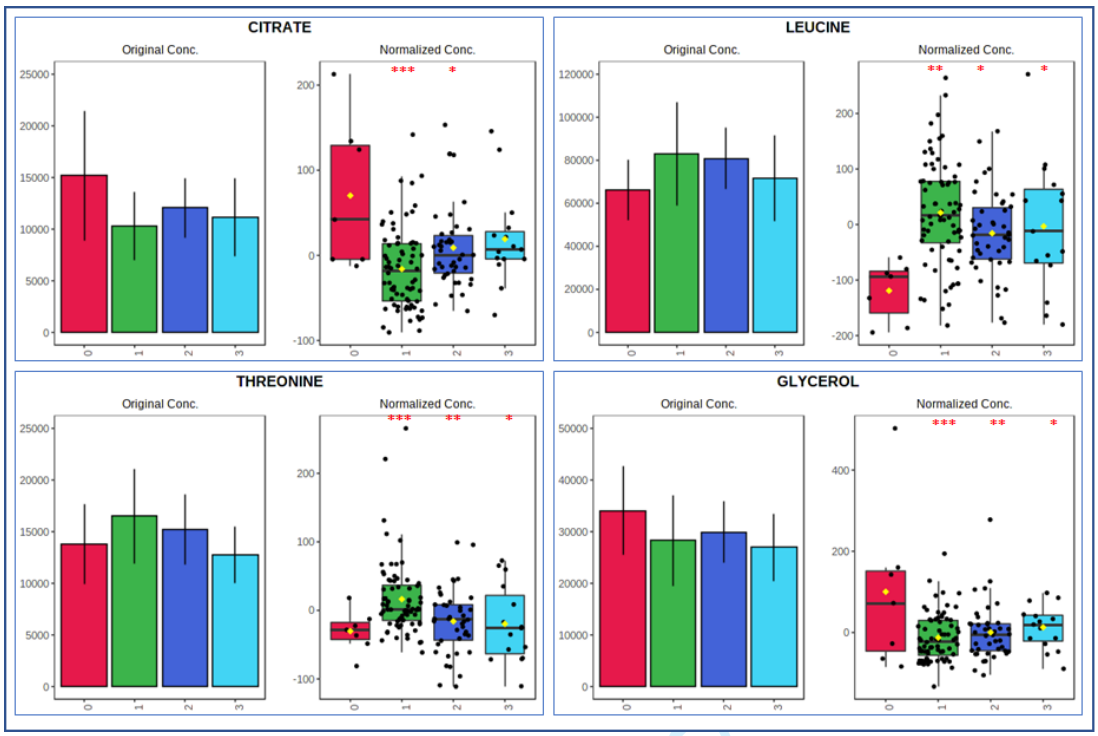

Figure S4. Bar plots and box and whisker plots of representative metabolites identified from univariate analysis as significantly different in canine stifle joint synovial fluid from dogs with cranial cruciate ligament rupture with increasing radiographic osteoarthritis score (based on a global assessment from 0-3) in the affected stifle joint of the canine participants. Y-axis represents peak intensity. The x axis shows OA scores from 0-3 (Red=0, Green=1, Navy blue=2, Light blue=3). Bar plots on the left show the original peak intensity (mean +/- SD), and box and whisker plots on the right show the normalised peak intensity after probabilistic quotient normalisation and pareto scaling. Stars above boxplots denote significance against OA score 0 (*=p<0.05, **=p<0.01, ***=p<0.001).

Supplementary tables

Table S1. All outcomes of Tukey HSD post-hoc test from metabolites found to be significant between groups (group 1=CCLR with meniscal injury [n=65], group 2=CCLR without meniscal injury [n=72], group 3=control group without CCLR or meniscal injury [n=17]) after ANCOVA to control for age of the canine participants.

Group comparison	Bin number	Metabolite(s) annotated to bin	Difference in means	SE	95% CI (low)	95% CI (high)	FDR adjusted p-value	Significance level
2. CCLR without meniscal injury vs 3. control	180	GLUTAMINE	-99.89	19.62	-138.66	-61.11	3.17E-06	****
	181	GLUTAMINE	-148.54	29.61	-207.04	-90.04	3.60E-06	****
	179	GLUTAMINE	-57.16	11.41	-79.69	-34.62	4.52E-06	****
	182	GLUTAMINE	-150.17	30.62	-210.67	-89.67	4.68E-06	****
	120	GLYCEROL	131.66	27.94	76.47	186.86	8.28E-06	****
	183	GLUTAMINE	-97.10	20.56	-137.72	-56.48	9.63E-06	****
	31	UNKNOWN	24.45	5.23	14.12	34.77	1.92E-05	****
	184	GLUTAMINE	-57.83	12.85	-83.23	-32.43	2.19E-05	****
	227	UNKNOWN	171.21	37.07	97.97	244.45	2.47E-05	****
	147	BETAINE	366.02	82.59	202.83	529.21	2.69E-05	****
	200	GLUTAMINE_ACETAMINOPHEN	-67.43	14.78	-96.63	-38.23	3.12E-05	****
	189	GLUTAMATE_3-HYDROXYISOVALERATE	118.69	27.17	65.01	172.37	3.48E-05	****
	190	GLUTAMATE	113.55	27.26	59.69	167.42	7.84E-05	****
	223	LACTATE_THREONINE	324.03	75.67	174.51	473.55	9.87E-05	****
	199	GLUTAMINE_2-METHYLGLUTARATE_AZELATE_SEBACATE	-35.15	8.47	-51.89	-18.42	1.66E-04	***
	204	GLUTAMINE_GLUTAMATE	-74.23	18.14	-110.07	-38.39	2.08E-04	***
	55	LACTATE	137.94	34.04	70.67	205.21	2.44E-04	***
	3	4-PYRIDOXATE_ANSERINE	17.57	4.55	8.57	26.56	2.52E-04	***
	209	UNKNOWN_GLUTAMATE	93.52	23.59	46.90	140.14	3.41E-04	***
	185	GLUTAMINE	-37.40	9.99	-57.14	-17.65	3.89E-04	***
	211	ACETATE	97.85	26.74	45.01	150.69	5.25E-04	***

	134	UNKNOWN	-28.55	7.91	-44.18	-12.93	6.23E-04	***
	135	PI-METHYLHISTIDINE	-30.06	8.03	-45.92	-14.20	7.69E-04	***
	27	TYROSINE_ACETAMINOPHEN_O-CRESOL_GLUTAMINE	41.98	11.85	18.56	65.39	7.92E-04	***
	8	UNKNOWN	14.83	4.22	6.49	23.16	8.79E-04	***
	206	GLUTAMATE	71.22	20.52	30.68	111.76	0.001	**
	13	PHENYLALANINE_2-PHENYLPROPRIONATE	21.29	6.15	9.13	33.44	0.001	**
	207	GLUTAMATE	69.80	20.37	29.55	110.04	0.001	**
	1	FORMATE	71.63	19.77	32.57	110.68	0.001	**
	21	TYROSINE	27.23	8.05	11.33	43.14	0.001	**
	203	METHIONINE_GLUTAMINE_GLUTAMATE	-82.38	24.80	-131.38	-33.39	0.003	**
	118	UNKNOWN_GLYCEROL	75.11	24.88	25.94	124.27	0.004	**
	202	GLUTAMINE_GLUTAMATE	-55.23	17.12	-89.05	-21.41	0.005	**
	188	PYRUVATE	101.20	32.08	37.82	164.58	0.005	**
	16	PHENYLALANINE	26.07	9.05	8.20	43.95	0.007	**
	218	LYSINE	-38.81	13.48	-65.45	-12.17	0.007	**
	35	UNKNOWN	34.18	11.28	11.89	56.47	0.009	**
	242	ALLOISOLEUCINE_ISOLEUCINE	-53.71	19.91	-93.06	-14.37	0.012	*
	148	UNKNOWN_BETAINE_GLUCOSE	88.83	32.99	23.64	154.03	0.012	*
	246	MOBILE LIPID -CH3	-135.95	52.03	-238.76	-33.14	0.015	*
	152	MOBILE LIPID -N(CH3)3	-157.14	64.79	-285.16	-29.12	0.016	*
	34	UNKNOWN	22.33	9.14	4.26	40.40	0.024	*
	30	UNKNOWN	16.58	6.80	3.15	30.01	0.024	*
	174	CITRATE	41.36	17.34	7.09	75.62	0.028	*
	37	MOBILE UNSATURATED LIPID	-60.15	27.57	-114.63	-5.67	0.031	*
	210	GLYCYLPROLINE_ISOLEUCINE_UNKNOWN	-49.38	23.08	-94.98	-3.77	0.034	*
	129	METHANOL	49.14	23.34	3.03	95.25	0.037	*
	243	ISOLEUCINE	-46.89	21.50	-89.37	-4.41	0.046	*
	145	UNKNOWN	20.74	23.04	-24.79	66.27	0.370	ns

1. CCLR with meniscal injury vs 3.control	181	GLUTAMINE	-147.28	30.02	-206.60	-87.96	3.60E-06	****
	180	GLUTAMINE	-97.34	19.90	-136.66	-58.02	3.82E-06	****
	182	GLUTAMINE	-150.45	31.05	-211.80	-89.10	4.68E-06	****
	120	GLYCEROL	141.71	28.33	85.74	197.69	4.69E-06	****
	147	BETAINE	408.25	83.75	242.77	573.73	8.24E-06	****
	189	GLUTAMATE_3-HYDROXYISOVALERATE	134.21	27.55	79.78	188.65	8.36E-06	****
	190	GLUTAMATE	133.93	27.64	79.31	188.56	9.38E-06	****
	183	GLUTAMINE	-97.51	20.85	-138.70	-56.32	9.63E-06	****
	179	GLUTAMINE	-52.37	11.57	-75.22	-29.52	1.81E-05	****
	184	GLUTAMINE	-58.42	13.04	-84.18	-32.67	2.19E-05	****
	31	UNKNOWN	22.41	5.30	11.94	32.88	6.10E-05	****
	3	4-PYRIDOXATE_ANSERINE	19.62	4.62	10.50	28.74	1.12E-04	***
	211	ACETATE	113.21	27.12	59.63	166.79	1.51E-04	***
	227	UNKNOWN	146.56	37.59	72.29	220.83	2.18E-04	***
	246	MOBILE LIPID -CH3	-214.82	52.76	-319.07	-110.58	2.26E-04	***
	204	GLUTAMINE_GLUTAMATE	-69.39	18.39	-105.73	-33.05	3.48E-04	***
	185	GLUTAMINE	-38.40	10.13	-58.42	-18.37	3.89E-04	***
	13	PHENYLALANINE_2-PHENYLPROPRIONATE	24.43	6.24	12.10	36.75	4.08E-04	***
	152	MOBILE LIPID -N(CH3)3	-256.53	65.70	-386.34	-126.71	4.27E-04	***
	223	LACTATE_THREONINE	284.65	76.73	133.03	436.27	4.38E-04	***
	199	GLUTAMINE_2-METHYLGLUTARATE_AZELATE_SEBACATE	-31.74	8.59	-48.71	-14.77	4.61E-04	***
	200	GLUTAMINE_ACETAMINOPHEN	-55.23	14.98	-84.84	-25.63	4.77E-04	***
	55	LACTATE	126.46	34.52	58.25	194.68	5.17E-04	***
	134	UNKNOWN	-30.01	8.02	-45.86	-14.17	6.23E-04	***
	209	UNKNOWN_GLUTAMATE	85.68	23.92	38.41	132.96	6.91E-04	***
	129	METHANOL	89.15	23.66	42.39	135.90	7.09E-04	***
	8	UNKNOWN	16.11	4.28	7.65	24.56	7.19E-04	***
	27	TYROSINE_ACETAMINOPHEN_O-CRESOL_GLUTAMINE	44.92	12.02	21.18	68.66	7.89E-04	***

	206	GLUTAMATE	72.26	20.80	31.16	113.37	0.001	**
	210	GLYCYLPROLINE_ISOLEUCINE_UNKNOWN	-85.72	23.41	-131.97	-39.48	0.001	**
	218	LYSINE	-49.86	13.67	-76.88	-22.84	0.001	**
	207	GLUTAMATE	73.38	20.65	32.56	114.19	0.001	**
	37	MOBILE UNSATURATED LIPID	-100.21	27.96	-155.46	-44.97	0.001	**
	21	TYROSINE	27.42	8.16	11.29	43.55	0.001	**
	135	PI-METHYLHISTIDINE	-26.81	8.14	-42.89	-10.73	0.002	**
	1	FORMATE	65.78	20.04	26.17	105.38	0.002	**
	148	UNKNOWN_BETAINE_GLUCOSE	112.59	33.46	46.48	178.70	0.003	**
	242	ALLOISOLEUCINE_ISOLEUCINE	-67.80	20.19	-107.70	-27.91	0.003	**
	118	UNKNOWN_GLYCEROL	83.95	25.23	34.10	133.81	0.003	**
	188	PYRUVATE	97.60	32.53	33.33	161.86	0.005	**
	16	PHENYLALANINE	29.03	9.17	10.90	47.15	0.006	**
	145	UNKNOWN	67.68	23.37	21.51	113.85	0.007	**
	202	GLUTAMINE_GLUTAMATE	-50.11	17.36	-84.41	-15.82	0.007	**
	35	UNKNOWN	32.07	11.44	9.46	54.67	0.009	**
	243	ISOLEUCINE	-65.90	21.80	-108.98	-22.83	0.009	**
	203	METHIONINE_GLUTAMINE_GLUTAMATE	-65.86	25.14	-115.54	-16.18	0.015	*
	34	UNKNOWN	24.77	9.27	6.45	43.09	0.024	*
	30	UNKNOWN	18.44	6.89	4.83	32.06	0.024	*
	174	CITRATE	45.03	17.59	10.28	79.78	0.028	*
1.CCLR with meniscal injury vs 2. CCLR without meniscal injury	145	UNKNOWN	46.94	14.34	18.61	75.27	0.004	**
	129	METHANOL	40.01	14.52	11.32	68.70	0.010	**
	246	MOBILE LIPID -CH3	-78.88	32.37	-142.84	-14.91	0.016	*
	152	MOBILE LIPID -N(CH3)3	-99.38	40.31	-179.04	-19.73	0.016	*
	210	GLYCYLPROLINE_ISOLEUCINE_UNKNOWN	-36.35	14.36	-64.72	-7.97	0.019	*

37	MOBILE UNSATURATED LIPID	-40.06	17.16	-73.96	-6.16	0.031	*
243	ISOLEUCINE	-19.02	13.38	-45.45	7.41	0.157	ns
200	GLUTAMINE_ACETAMINOPHEN	12.20	9.19	-5.97	30.36	0.187	ns
218	LYSINE	-11.05	8.39	-27.63	5.52	0.190	ns
190	GLUTAMATE	20.38	16.96	-13.14	53.90	0.231	ns
148	UNKNOWN_BETAINE_GLUCOSE	23.76	20.53	-16.81	64.32	0.249	ns
242	ALLOISOLEUCINE_ISOLEUCINE	-14.09	12.39	-38.57	10.39	0.257	ns
203	METHIONINE_GLUTAMINE_GLUTAMATE	16.52	15.43	-13.96	47.00	0.286	ns
227	UNKNOWN	-24.65	23.06	-70.22	20.92	0.287	ns
211	ACETATE	15.35	16.64	-17.52	48.23	0.358	ns
189	GLUTAMATE_3-HYDROXYISOVALERATE	15.52	16.90	-17.88	48.92	0.360	ns
223	LACTATE_THREONINE	-39.38	47.08	-132.41	53.65	0.404	ns
147	BETAINE	42.23	51.39	-59.30	143.77	0.412	ns
13	PHENYLALANINE_2-PHENYLPROPRIONATE	3.14	3.83	-4.42	10.70	0.413	ns
3	4-PYRIDOXATE_ANSERINE	2.05	2.83	-3.55	7.65	0.470	ns
179	GLUTAMINE	4.78	7.10	-9.24	18.81	0.501	ns
135	PI-METHYLHISTIDINE	3.25	4.99	-6.62	13.11	0.517	ns
199	GLUTAMINE_2-METHYLGLUTARATE_AZELATE_SEBACATE	3.42	5.27	-7.00	13.83	0.518	ns
31	UNKNOWN	-2.04	3.25	-8.46	4.39	0.532	ns
120	GLYCEROL	10.05	17.38	-24.29	44.39	0.564	ns
118	UNKNOWN_GLYCEROL	8.84	15.48	-21.75	39.43	0.569	ns
55	LACTATE	-11.48	21.18	-53.33	30.38	0.589	ns
209	UNKNOWN_GLUTAMATE	-7.83	14.68	-36.84	21.17	0.594	ns
16	PHENYLALANINE	2.95	5.63	-8.17	14.08	0.600	ns
8	UNKNOWN	1.28	2.63	-3.91	6.47	0.626	ns
202	GLUTAMINE_GLUTAMATE	5.12	10.65	-15.92	26.16	0.631	ns
1	FORMATE	-5.85	12.30	-30.15	18.45	0.635	ns
30	UNKNOWN	1.87	4.23	-6.49	10.22	0.660	ns
204	GLUTAMINE_GLUTAMATE	4.84	11.28	-17.45	27.14	0.668	ns

	34	UNKNOWN	2.44	5.69	-8.80	13.68	0.669	ns
	27	TYROSINE_ACETAMINOPHEN_O-CRESOL_GLUTAMINE	2.94	7.37	-11.63	17.51	0.691	ns
	174	CITRATE	3.67	10.79	-17.65	24.99	0.734	ns
	35	UNKNOWN	-2.12	7.02	-15.99	11.75	0.763	ns
	134	UNKNOWN	-1.46	4.92	-11.18	8.26	0.767	ns
	207	GLUTAMATE	3.58	12.67	-21.46	28.62	0.778	ns
	180	GLUTAMINE	2.55	12.21	-21.58	26.67	0.835	ns
	188	PYRUVATE	-3.60	19.96	-43.03	35.83	0.857	ns
	185	GLUTAMINE	-1.00	6.22	-13.28	11.29	0.873	ns
	206	GLUTAMATE	1.04	12.77	-24.18	26.27	0.935	ns
	184	GLUTAMINE	-0.59	8.00	-16.40	15.21	0.941	ns
	181	GLUTAMINE	1.26	18.42	-35.14	37.66	0.946	ns
	21	TYROSINE	0.18	5.01	-9.72	10.08	0.971	ns
	183	GLUTAMINE	-0.41	12.79	-25.68	24.86	0.974	ns
	182	GLUTAMINE	-0.28	19.05	-37.93	37.36	0.988	ns