

# Synovial fluid metabolites differentiate between septic and non-septic joint pathologies

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## Abstract

Osteoarthritis (OA), osteochondrosis (OC) and synovial sepsis in horses cause loss of function and pain. Reliable biomarkers are required to achieve accurate and rapid diagnosis, with synovial fluid (SF) holding a unique source of biochemical information. Nuclear magnetic resonance (NMR) spectroscopy allows global metabolite analysis of a small volume of SF, with minimal sample pre-processing using a non-invasive and non-destructive method. Equine SF metabolic profiles from both non-septic joints (OA and OC) and septic joints were analysed using 1D  $^1\text{H}$  NMR spectroscopy. Univariate and multivariate statistical analyses were used to identify differential metabolite abundance between groups. Metabolites were annotated via  $^1\text{H}$  NMR using 1D NMR identification software Chenomx, with identities confirmed using 1D  $^1\text{H}$  and 2D  $^1\text{H}$   $^{13}\text{C}$  NMR. Multivariate analysis identified separation between septic and non-septic groups. Acetate, alanine, citrate, creatine phosphate, creatinine, glucose, glutamate, glutamine, glycine, phenylalanine, pyruvate and valine were higher in the non-septic group whilst glycylproline was higher in sepsis. Multivariate separation was primarily driven by glucose; however PLS-DA plots with glucose excluded demonstrated the remaining metabolites were still able to discriminate the groups. This study demonstrates a panel of synovial metabolites can distinguish between septic and non-septic equine SF, with glucose the principal discriminator.

**Keywords:** metabolomics, equine, synovial fluid, osteoarthritis, osteochondrosis, sepsis, nuclear magnetic resonance

## Introduction

Conditions affecting the articular joints are common in horses resulting in loss of function, chronic pain and/or subsequent inability to work, all of which represent economic and welfare concerns. These pathologies include osteoarthritis (OA), osteochondrosis (OC) and synovial sepsis, which can be life-threatening.<sup>1</sup> Despite these conditions having a high prevalence and clinical relevance, diagnosis, staging, monitoring and determination of an accurate prognosis remain a challenge for practising veterinarians. Therefore, in order to differentiate equine articular joint pathologies, there is a need to identify reliable biomarkers of disease. Synovial fluid (SF) is located within the articular joint cavity, providing a pool of nutrients for surrounding tissues but primarily serving as a biological lubricant, containing molecules with low-friction and low-wear properties to articular surfaces.<sup>2</sup> As SF is in close proximity to articular tissues primarily altered during joint pathology, this bio-fluid is an important source of biomarker discovery.<sup>3,4</sup>

Reduced levels of glucose in human and equine SF, due to an increase in synovial and neutrophil cell glycolytic activity in severe inflammation or infection, have previously been identified, with a serum-synovial glucose difference of  $>2.2$  mmol/L considered supportive of a diagnosis of synovial sepsis.<sup>5,6</sup> However, this parameter is non-specific and can be influenced by multiple variables, including synovial necrosis, diet, pain and white blood cell count. Elevations in lactate during the acute infection phase have previously been identified in SF of septic human and equine joints, due to an increase in the consumption of glucose and subsequent production of lactate within an anaerobic environment.<sup>7,8</sup> However, other studies have not been able to differentiate septic and non-septic arthropathies based on lactate levels alone.<sup>9,10</sup> Synovial D-lactate (produced via bacterial fermentation and a stereoisomer of mammalian L-lactate) was recently found to be unable to aid diagnosis of equine synovial sepsis.<sup>11</sup>

At present no specific biomarkers have been identified for equine OA and therefore none are used as a diagnostic aid in clinical practice. Potential OA markers of interest include cartilage oligomeric matrix proteinase (COMP) and matrix metalloproteinases (MMPs) which have both been identified as elevated in human OA SF.<sup>12</sup> Although detection of active MMPs in horses has demonstrated important potential in diagnosis, conversely decreased levels of COMP have been identified in equine OA and found unable to stage the disease.<sup>13,14</sup> In equine OC, elevated levels of a C-propeptide of cartilage type II procollagen and osteocalcin have shown potential as a diagnostic aid although these markers have not translated to clinical practice.<sup>15-17</sup> However, these protein markers identify pathology following significant cartilage degradation and bone remodelling opposed to an early disease state where potential intervention would prove most beneficial.

Metabolomics encompasses the comprehensive profiling of metabolic changes, including the study of metabolic pathways and quantification of unique biochemical molecules, within living systems.<sup>18</sup> These small molecule metabolites include metabolic intermediates, secondary metabolites, hormones and other signalling molecules.<sup>19</sup> A major advantage of nuclear magnetic resonance (NMR) spectroscopy over other techniques, i.e. mass spectrometry, is the analysis of native samples with a minimal level of sample preparation using a non-invasive and non-destructive method, subsequently producing results which are more reproducible and robust.<sup>20</sup> Thus this methodology holds huge potential in the analysis of bio-fluids to characterise the global metabolic profiles of various pathologies.<sup>21</sup> Huggle *et al.* (2012) previously carried out NMR spectroscopy using human SF, identifying that septic arthritis SF could be distinguished from non-septic arthritis SF via principal component analysis (PCA), although they were unable to identify the specific metabolites

responsible for this discrimination.<sup>22</sup> To date, only one peer reviewed publication has used NMR to investigate the whole metabolic profile of equine SF, comparing normal and osteoarthritic SF.<sup>23</sup> Lacitignola *et al.* identified ten differentially abundant metabolites which included elevated levels of glucose and lactate in OA. No studies have however analysed equine SF using NMR to investigate synovial sepsis or OC, concurrently analysed the metabolic profiles of multiple equine articular pathologies or used multivariate analysis to investigate the metabolic profile as a whole.

In this study we have used global metabolite identification using <sup>1</sup>H NMR to identify potential metabolite biomarkers that allow differentiation between equine articular joint pathologies, potentially aiding accurate diagnosis.

## **Methods**

### *Patient groups and SF collection*

Following ethical approval and owner consent, excess aspirated SF (collected during clinical diagnostic investigations) was analysed from joints of horses presenting to The Philip Leverhulme Equine Hospital, University of Liverpool between 2014 and 2016. SF was aspirated for diagnostic purposes from the affected joints at the start of surgical arthroscopy under general anaesthesia with 500 µl of excess SF submitted for NMR metabolomic analysis. Pathological joints included the femorotibial, glenohumeral, metacarpophalangeal, metatarsophalangeal and tarsocrural joints. Horses were divided into two main groups of joint pathology, septic and non-septic. The septic group was subdivided into six horses with synovial sepsis following a local penetrating wound and one foal diagnosed with synovial sepsis secondary to haematogenous spread. The non-

septic group consisted of four horses diagnosed with OA, two with meniscal tears and concurrent OA (MT) and six with OC. Diagnoses were determined via a combination of radiography, ultrasonography, arthroscopy and SF total protein, cytology and/or bacterial culture as previously described.<sup>11</sup> SF was immediately placed into uncoated 1.5 ml collection tubes and processed within an hour of collection. Particulate and cells were removed from the SF by centrifugation (4°C, 2540 g for 5 mins) then the cell-free supernatant transferred to a clean uncoated 1.5 ml collection tube, snap-frozen using liquid nitrogen and stored at -80°C. Samples were spun to ensure removal of cellular debris which was critical to eliminate variance due to bacterial contamination in septic joints. To ascertain the most reproducible and robust collection protocol, biologically identical SF underwent +/- centrifugation and supernatant removal followed by freezing using various methods at different temperatures. Multivariate statistical analysis of these samples indicated that the above method was most robust at reducing variance due to sample handling/processing (data not shown).

### *Sample preparation*

NMR samples were prepared within 48 hours of acquisition. 300 µl of thawed SF was diluted to a final volume containing 50% (v/v) SF, 40% (v/v) dd <sup>1</sup>H<sub>2</sub>O (18.2MΩ), 10% (v/v) 1M PO<sub>4</sub><sup>3-</sup> pH 7.4 buffer (Na<sub>2</sub>HPO<sub>4</sub>, VWR International Ltd., Radnor, Pennsylvania, USA and NaH<sub>2</sub>PO<sub>4</sub>, Sigma-Aldrich, Gillingham, UK) in deuterium oxide (<sup>2</sup>H<sub>2</sub>O, Sigma-Aldrich) and 0.0025% (v/v) sodium azide (NaN<sub>3</sub>, Sigma-Aldrich). Samples were vortexed for 1 min, centrifuged at 13,000 g and 4°C for 2 mins and 590 µl transferred (taking care not to disturb any pelleted material) into 5 mm outer diameter NMR tubes using a glass pipette.

### *NMR Acquisition*

One dimensional  $^1\text{H}$  NMR spectra with Carr-Purcell-Meiboom-Gill (CPMG) filter to attenuate signals from macromolecules such as albumins were acquired using a standard vendor pulse sequence (cpmgpr1d) on a 600 MHz NMR Bruker Avance III spectrometer with a TCI cryoprobe and chilled Sample-Jet autosampler. Spectra were acquired at 25°C, with a 4 s interscan delay, 32 transients and a 15 ppm spectral width. Software for acquisition and processing was carried out using Topspin 3.1 and IconNMR 4.6.7 with automated phasing and baseline correction and a standard vendor processing routine (exponential window function with 0.3 Hz line broadening).

### *Metabolite Annotation and Identification*

All spectra were scrutinised to ensure spectra met community recommended quality control criteria prior to inclusion in statistical analysis.<sup>24</sup> Quality control criteria included a flat baseline, water signal less than 0.4 ppm wide and line-width half heights of representative beta anomeric glucose doublet all within one standard deviation. Spectra were then divided into spectral regions or 'buckets' according to metabolite annotation from Chenomx NMR Suite 8.2 (330-mammalian metabolite library) with buckets attributed to multiple metabolites where peaks were found to overlap. Each spectrum was divided into 306 buckets with the intensity of each bucket divided by the bucket width to negate the intensity variance. Buckets were normalised to the median and Pareto scaled prior to statistical treatment. Metabolites annotated in Chenomx were also confirmed by a mixture of  $^1\text{H}$  1D NMR and, where possible, to in-house 2D  $^1\text{H}$   $^{13}\text{C}$  Heteronuclear Single Quantum Coherence NMR standards. Spectra and metabolite assignments including HMDB IDs and annotation level is all available in the Metabolights repository ([www.ebi.ac.uk/metabolights/MTBLS543](http://www.ebi.ac.uk/metabolights/MTBLS543)) and shown in table 2.<sup>25</sup> Among the metabolites identified was ethanol, likely through contamination during SF aspiration following surgical

sterilisation. Therefore, for multivariate analysis the buckets attributed to ethanol were omitted.

### *Statistical Analysis*

SF spectra were divided into two groups: non-septic and septic. Further separation of the groups was not possible due to limited sample number in this study. T-tests and partial-least squares discriminant analysis (PLS-DA) were carried out using MetaboAnalyst 3.5 (<http://www.metaboanalyst.ca>) which uses the R package of statistical computing software.<sup>26</sup> For t-tests,  $p < 0.05$  was considered statistically significant following correction for multiple testing using the Benjamini-Hochberg false discovery rate method.<sup>27</sup> Box plots were carried out using the software package SPSS 22. Quantile and PCA plots were produced using standard analytical routines in the software package R ([r-cran.org](http://r-cran.org)).

## **Results**

### *Group limitations*

The patient groups were restricted to two main groups; septic or non-septic (Table 1) due to a lack of sufficient cases of differing sub-diagnoses. The joints from which each sample was collected were noted, however, without a larger study size no separate grouping for differing joints was attempted.



**Table 1.** Table detailing further clinical characteristics of the group patients.

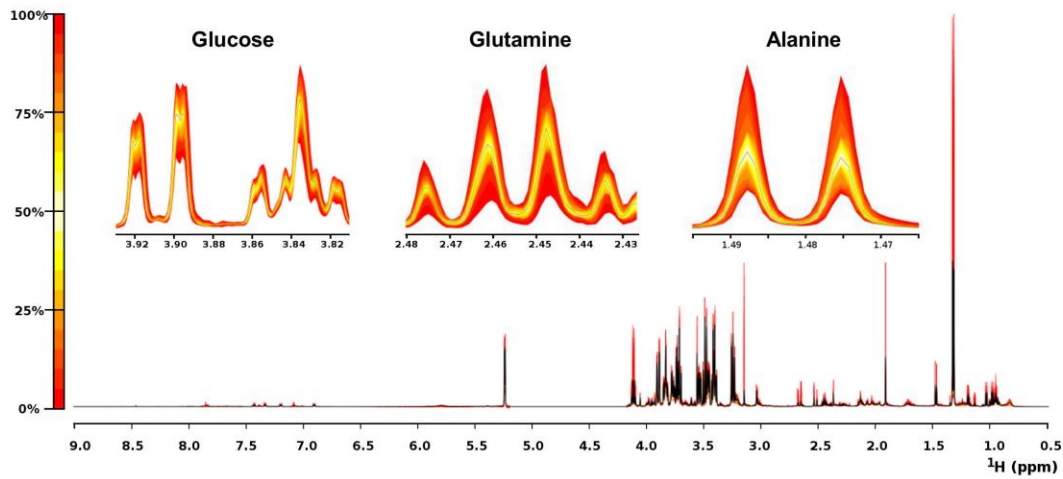
	Main Groups		Sub-Groups				
	Septic	Non-Septic	Septic		Non-Septic		
			Wound Sepsis	Haematogenous Sepsis	OA	MT with OA	OC
<b>Number</b>	7	12	6	1	4	2	6
<b>Mean Age</b>	6 years 10 months	7 years 7 months	7 years 11 months	0 years 2 months	10 years 6 months	12 years 0 months	4 years 6 months
<b>Sex</b>	7M	6F, 6M	6M	1M	1F, 3M	1F, 1M	4F, 2M
<b>Joint</b>	1 x GH 1 x MCP 1 x MTP 4 x TC	2 x GH 3 x MCP 4 x FT 1 x MTP 2 x TC	1 x MCP 1 x MTP 4 x TC	1 x GH	2 x GH 1 x FT 1 x MTP	2 x FT	3 x MCP 1 x FT 2 x TC

*Abbreviations: Groups, OA = Osteoarthritis, MT = Meniscal tear and concurrent OA, OC = Osteochondrosis; Sex, M = Male, F = Female; Joints, GH = Glenohumeral, MCP = Metacarpophalangeal. FT = Femorotibial, MTP = Metatarsophalangeal and TC = Tarsocrural.*

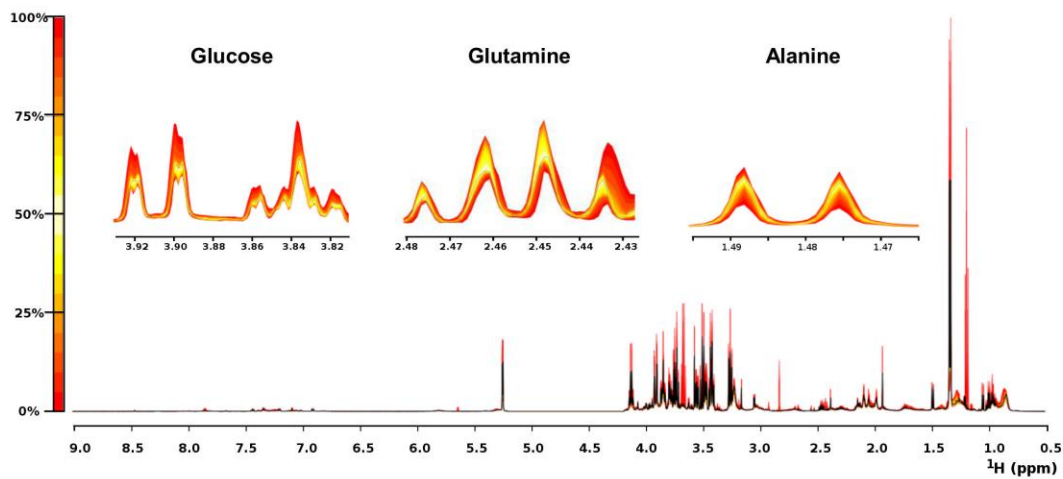
#### *Metabolite annotation and identification*

NMR spectra for all groups showed a consistent set of metabolite signals present with multiple metabolites identified from 1D multiplet pattern overlap (Figure 1). The metabolite abundances within the septic group were less variable than the non-septic group, which is perhaps expected given the non-septic group included samples from two separate diagnoses (OA and OC). Of the 306 spectral bins in each extract, 203 (66%) were annotated to 71 metabolites, with identification confirmed for 55 of these metabolites (Table 2).

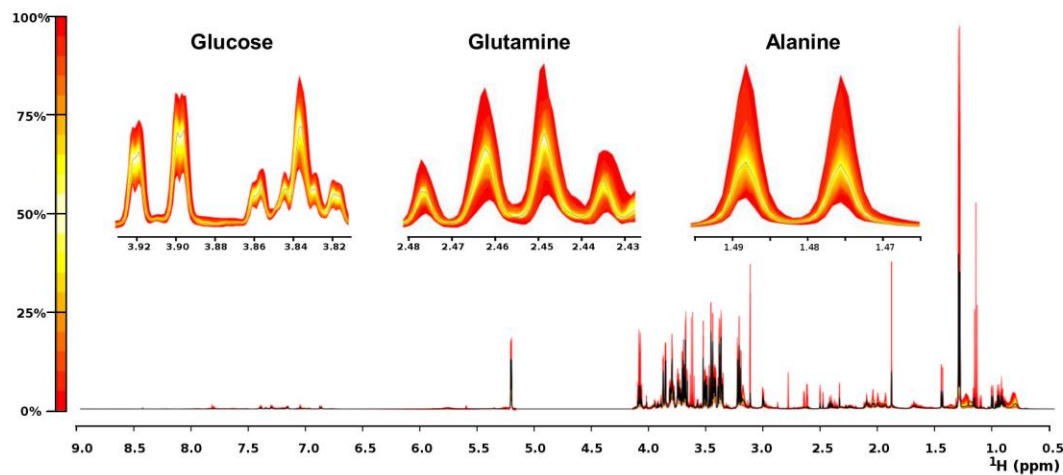
### A - OA & OC (Non-Septic)



### B - Sepsis



### C - All Samples



**Figure 1.** Quantile plots of OA/OC (Non-Septic), sepsis and all spectra depicting the median spectral plot (black line) and variation from the median within each group (yellow to red scale) for the full spectral range (9.0 - 0.5 ppm) and more detailed regions depicting selected peaks from three differentially abundant metabolites, glucose, glutamine and alanine. Variation within the full spectra can most clearly be seen at 0.8 ppm.

**Table 2.** Metabolites annotated in synovial fluid by Chenomx, metabolites subsequently identified using an in-house library are indicated by the Metabolomics Standards Initiative (MSI) level 1.<sup>25</sup>

<b>Database Identifier</b>	<b>Metabolite Identification</b>	<b>Reliability</b>
HMDB00001	1-Methylhistidine	MS Level 2
HMDB59655	2-Hydroxyglutarate	MS Level 2
HMDB11743	2-Phenylpropionate	MS Level 2
HMDB00357	3-Hydroxybutyrate	MS Level 2
HMDB00355	3-Hydroxymethylglutarate	MS Level 2
HMDB01149	5-Aminolevulinate	MS Level 2
HMDB00042	Acetate	MS Level 1
HMDB00060	Acetoacetate	MS Level 2
HMDB01890	Acetylcysteine	MS Level 2
HMDB01432	Agmatine	MS Level 2
HMDB00729	Alpha-Hydroxyisobutyrate	MS Level 2
HMDB00186	Alpha-Lactose	MS Level 2
HMDB00043	Betaine	MS Level 1
HMDB00030	Biotin	MS Level 1
HMDB00094	Citrate	MS Level 1
HMDB00064	Creatine	MS Level 1
HMDB00562	Creatinine	MS Level 1
HMDB00143	D-Galactose	MS Level 2
HMDB00122	D-Glucose	MS Level 1
HMDB00108	Ethanol	MS Level 1
HMDB00142	Formate	MS Level 2
HMDB00663	Glucarate	MS Level 2
HMDB01401	Glucose 6-phosphate	MS Level 2
HMDB00131	Glycerol	MS Level 1
HMDB00123	Glycine	MS Level 1
HMDB00721	Glycylproline	MS Level 2
HMDB00128	Guanidoacetate	MS Level 2
HMDB00870	Histamine	MS Level 2
HMDB00764	Hydrocinnamate	MS Level 2
HMDB00678	Isovalerylglycine	MS Level 2
HMDB00190	Lactate	MS Level 1
HMDB00161	L-Alanine	MS Level 1
HMDB00646	L-Arabinose	MS Level 2
HMDB00062	L-Carnitine	MS Level 2
HMDB00174	L-Fucose	MS Level 2
HMDB00148	L-Glutamate	MS Level 1
HMDB00641	L-Glutamine	MS Level 1
HMDB00177	L-Histidine	MS Level 1
HMDB00687	L-Leucine	MS Level 1

HMDB00159	L-Phenylalanine	MS Level 1
HMDB00167	L-Threonine	MS Level 2
HMDB00158	L-Tyrosine	MS Level 1
HMDB00883	L-Valine	MS Level 1
HMDB01389	Melatonin	MS Level 2
HMDB01238	N-Acetylserotonin	MS Level 2
HMDB02055	o-Cresol	MS Level 2
HMDB00210	Pantothenic acid	MS Level 2
HMDB00821	Phenylacetylglycine	MS Level 2
HMDB01511	Phosphocreatine	MS Level 2
HMDB00239	Pyridoxine	MS Level 2
HMDB00243	Pyruvate	MS Level 1
HMDB00635	Succinylacetone	MS Level 2
HMDB00262	Thymine	MS Level 1
HMDB01878	Thymol	MS Level 2
HMDB00294	Urea	MS Level 1

*Statistical analysis and differentially abundant metabolites.*

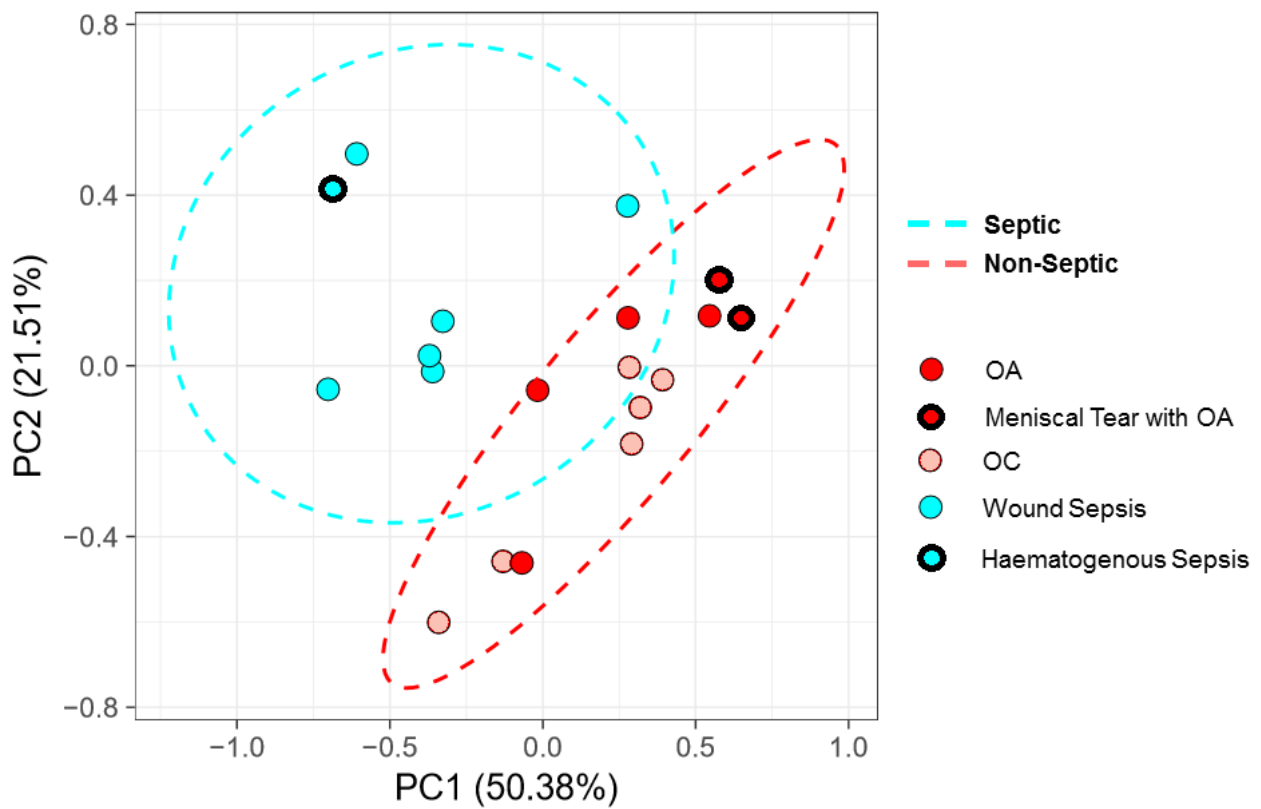
Of the 306 identified peaks, 180 were found to be differentially abundant between septic and non-septic groups when analysed using a univariate t-test,  $p < 0.05$ , with 98 peaks assigned to 26 different metabolites (table S1).

Unsupervised multivariate analysis (PCA) identified clear variance between spectra from septic and non-septic joint pathologies, forming two distinct clusters (Figure 2). 95% of the variance was explained by seven principal components (PCs) with PC1 and PC2 explaining a combined 71.89% of variance within the data. Although the analysis between groups was limited to a binary diagnosis (septic vs non-septic) to explore the variance in metabolite profile for SF aspirated from horses with differing underlying conditions, the PCA score plots were coloured in terms of either main diagnosis (Binary) or sub-diagnosis (five groups). Within the non-septic group, samples obtained from joints with OA and associated meniscal tears were found to cluster together. In addition, despite a separate aetiology, SF obtained from a foal with haematogenous sepsis clustered with SF collected from joints diagnosed with sepsis subsequent to penetrating wounds.

Supervised multivariate discriminant analysis using PLS-DA plots of the known metabolites indicated that the variance was heavily influenced by glucose (Figure 3a and b), with 23 of the 25 most influential buckets attributed to glucose peaks. The optimal model comprised two components with reasonable predictive power ( $R^2 = 0.85$ ,  $Q^2 = 0.72$ ). Omitting glucose signals from the analysis gave rise to a somewhat lower predictive model ( $R^2 = 0.70$ ,  $Q^2 = 0.45$  fit with 1 component) (Figure 3c). From the glucose free model, metabolites acetate, glycyproline, glycine, citrate, creatinine and alanine were among the most influential (Figure 3d).

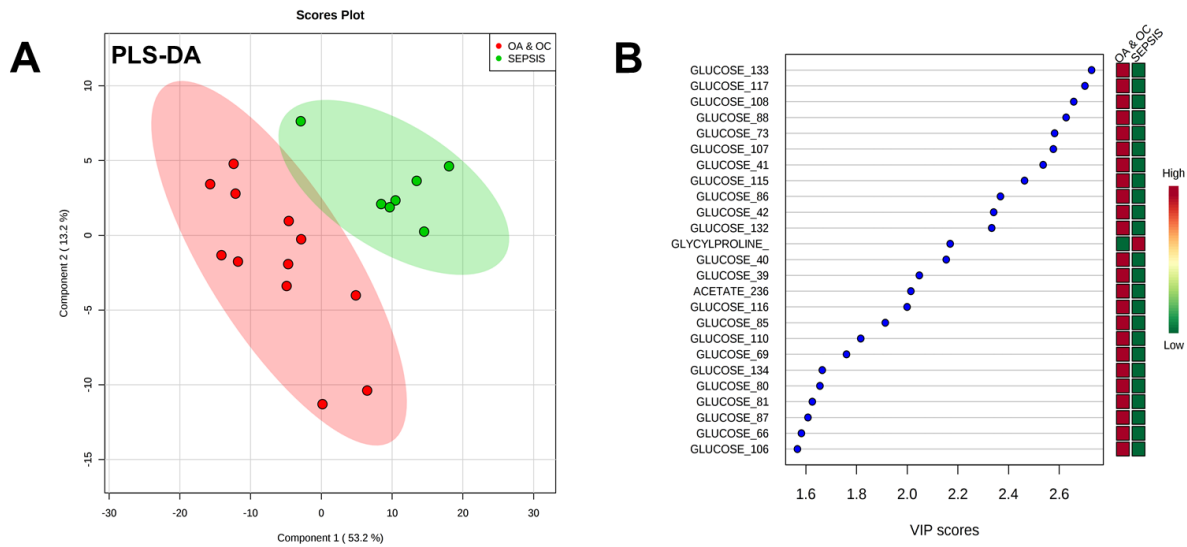
Both univariate and multivariate analysis were compared to identify differentially abundant metabolites. Metabolite peaks identified as either significantly different ( $p < 0.05$ ) via t-test or in the top 25 variables of influence (VIP) via glucose excluded PLS-DA were then attributed to a genuine differentially abundant metabolite change if the individual metabolite peak changes correlated. In total a panel of 13 metabolites were identified as being differentially abundant between septic and non-septic groups. Acetate, alanine, citrate, creatine phosphate, creatinine, glucose, glutamate, glutamine, glycine, phenylalanine, pyruvate and valine were higher in the non-septic group whilst glycyproline was higher in synovial sepsis.

PCA analysis of OA and OC sub-groups suggested that, bar one sample, they may cluster separately and may therefore demonstrate distinctive metabolomes. However, with this modest group size no significant differences in metabolites ( $p < 0.05$ ) were identified by univariate analysis (data not shown).

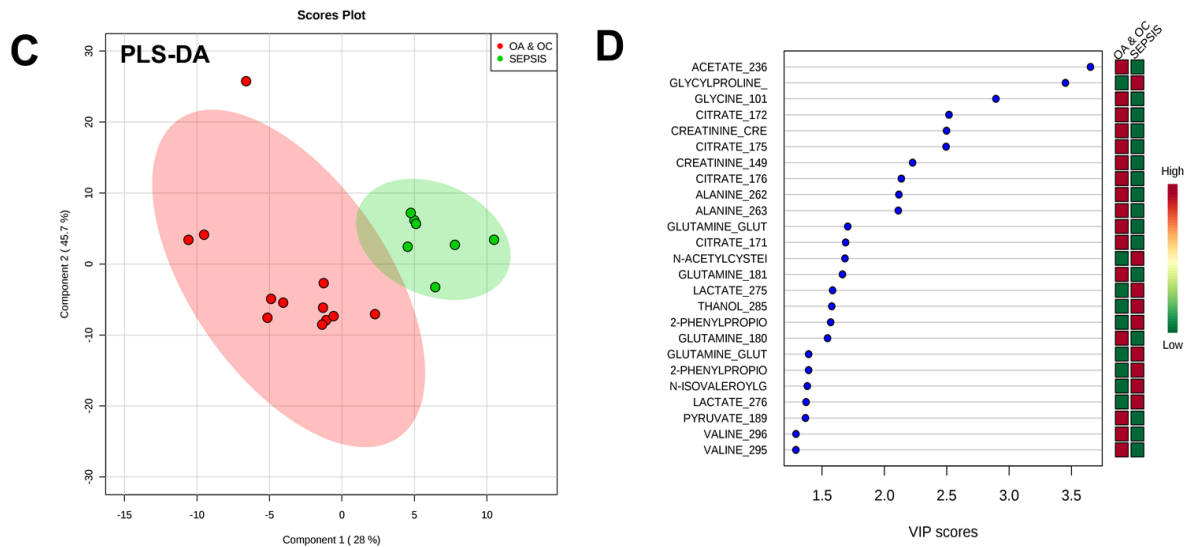


**Figure 2.** Principal component analysis (PCA) scores plot of septic (blue) and non-septic (red) SF samples. Samples with meniscal tear or haematogenous sepsis sub-diagnoses for OA and sepsis respectively are highlighted in each group with a thick black outline, OC are distinguished from OA by highlight shade.

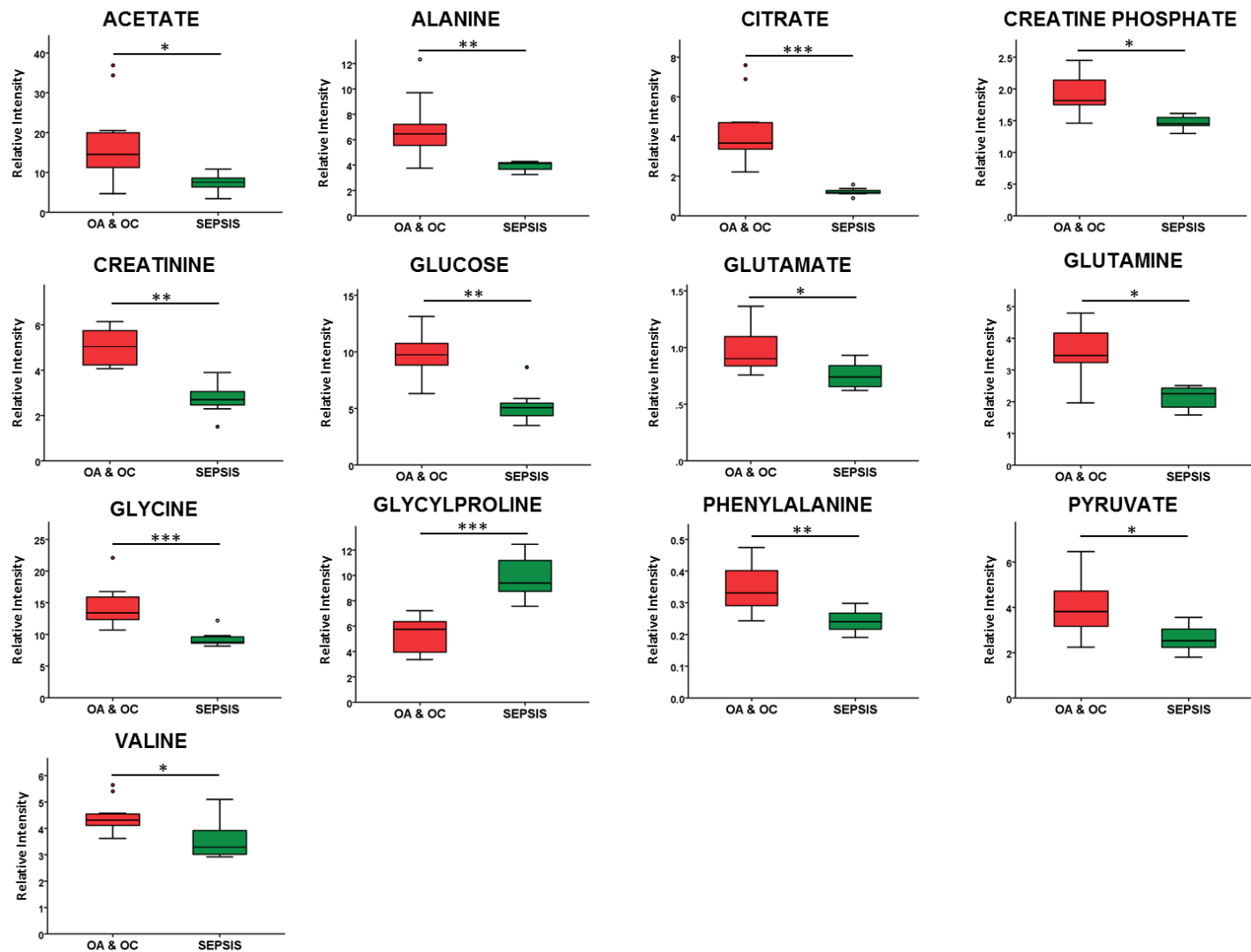
## Glucose Included



## Glucose Excluded



**Figure 3.** (a) PLS-DA plots of OA/OC vs sepsis using only metabolite annotated buckets - first two components shown out of a total of two components used to fit the model ( $R^2 = 0.85$ ,  $Q^2 = 0.72$ ). (b) VIP scores for the 25 most influential buckets of PLS-DA. (c) PLS-DA plots of OA/OCD vs sepsis using only metabolite annotated/identified buckets with all glucose buckets excluded - first two components shown. It should be noted that only one component was used to fit the model ( $R^2 = 0.70$ ,  $Q^2 = 0.45$ ). (d) VIP scores for the 25 most influential buckets of glucose-excluded PLS-DA.



**Figure 4.** Boxplots of key metabolites; shown as relative intensities corresponding to the most representative peak for each metabolite. T-test: \* =  $p < 0.05$ , \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$ .



## Discussion

Despite OA, OC and synovial sepsis having a high prevalence and clinical relevance, diagnosis, staging, monitoring and determination of an accurate prognosis remain a challenge for practising veterinarians. Therefore, to differentiate equine articular joint pathologies, there is a need to identify reliable biomarkers of disease. To aid equine synovial sepsis diagnosis, currently recognised markers of glucose and lactate are considered to be non-specific and unreliable respectively.<sup>8,10</sup> In addition, clinically assessing response to treatment of septic joints can be challenging, with differentiating horses with continued lameness from a non-resolving sepsis to those with a resolving sepsis but ongoing significant inflammation, difficult. Therefore, reliable metabolic markers of sepsis may aid as part of a longitudinal clinical assessment.

In this study it has been shown that the metabolite profile of equine SF is able to discriminate between septic and non-septic joint pathologies. A panel of thirteen metabolites was identified which were differentially abundant between these two groups. These metabolites may in turn prove beneficial in an equine clinical setting as a diagnostic aid as well as monitoring response to treatment and assessing prognosis. Ideally this could be carried out through field tests, similar to those produced and used for equine biofluids for metabolites glucose and lactate, urinalysis and the protein inflammatory marker serum amyloid A.<sup>28-31</sup> No metabolite abundances were identified to be statistically different between OA and OC sub-groups. However, the PCA analysis suggested that, bar one sample, OC and OA metabolite profiles were different and thus may demonstrate distinctive metabolomes sufficient to apply robust statistical models such as PLS-DA. This can only be tested with a larger sample size which was not possible on this modest subset of patients.

The PLS-DA VIP scores revealed spectral peaks assigned to glucose were highly represented as a discriminant of joint sepsis (23/25 of the most influential peaks). Reduced levels of glucose in human and equine SF, due to an increase in synovial and neutrophil cell glycolytic activity in severe inflammation or infection, have previously been identified, with a serum-synovial glucose difference of >2.2 mmol/L considered supportive of a diagnosis of synovial sepsis.<sup>5,6</sup> Glucose is, however, a non-specific parameter and can be influenced by multiple other variables.<sup>8</sup> However, PLS-DA analysis with all glucose influenced spectral peaks omitted revealed there to still be a modest separation between the groups, thus indicating the differentiated metabolomes are driven by a panel of metabolites, and potential septic markers, opposed to glucose alone.

Previous studies have described mixed results as to whether elevated synovial lactate can be used to help distinguish synovial sepsis<sup>7-10</sup>. In this study we did not identify lactate abundance to be different between the septic and non-septic groups, therefore providing further evidence that lactate is an unreliable marker for synovial sepsis. <sup>1</sup>H NMR spectroscopy is not able to distinguish D-lactate from L-lactate and consequently we are unable to draw any conclusions on the specific levels of bacterially derived D-lactate within the groups.

In this study the majority of differentially abundant metabolites were found to be reduced in sepsis compared to the non-septic group. However, glycyproline was elevated in synovial sepsis. Glycyproline is a dipeptide end product of the catabolism of collagen.<sup>32</sup> Following synovial infection and an insufficient immunological response, collagen catabolism is upregulated through a high cytokine concentration increasing the release of matrix metalloproteinases and other collagen-degrading enzymes from within the host.<sup>33</sup> Elevated

levels in markers of collagen destruction in synovial sepsis over non-septic joint pathologies have been identified previously, with one study demonstrating increased synovial levels of the collagen degradation marker crosslinked C-telopeptide fragments of type II collagen (CTX-II) in longitudinal samples of a patient with septic arthritis compared to osteoarthritic patients' SF.<sup>34</sup> Thus glycyproline, in combination with other metabolite/protein derived markers of collagen catabolism, may aid clinical diagnosis of synovial sepsis. Following on from this study, further investigation using mass spectrometry analysis would complement these results as the identified degradation of extracellular matrix may provide lipid and carbohydrate profiles of interest.

All raw NMR spectral data and associated annotations which contributed to this study have been deposited with the open access online repository Metabolights, curated by the European Bioinformatics Institute (EBI), including acquisition and processing parameters with all metabolites identified clearly reported. The authors feel this transparent, gold standard approach to data submission to independent bioinformatics specialists, such as the EBI, should be further encouraged within the NMR metabolomics field, in line with other 'omics' journal submissions.

### *Study limitations*

Modest sample size prohibited further analysis of metabolome differences between OA and OC or between wound sepsis and haematogenous sepsis. Similarly, SF with OA and associated meniscal tears were found to cluster together although we are unable to conclude that SF collected from femorotibial joints with meniscal tears form a distinct metabolome as only two samples were available for inclusion within this study. This is of particular interest as a diagnostic aid for meniscal tears would have a practical clinical

implication within veterinary medicine which could also translate to human medicine. It is our hope that additional studies will provide further information and enable more stringent analyses to be undertaken. Furthermore, due to limited sample size SF was aspirated from a range of joints which may lead to variation in data due to biomechanical differences between joints. Again, a larger sample size would enable a greater understanding as to what influence joint location has on the metabolome and a stricter control of site of sample collection (if necessary) and thus enable further information to be extracted from the metabolic profiles. In addition, during the joint pathologies studied there are likely to be changes to exercise and diet which may alter the metabolome of SF, although this influence is likely to be minimal compared to the significant metabolic influence of the localised joint pathology.

During this study we were unable to compare the SF of the joint pathologies of interest to a normal group. In equine clinical practice only pathological joints are aspirated during diagnostic investigations. Thus, under current UK ethical guidelines, no normal SF from living horses was available for analysis. Post-mortem SF cannot be defined as having a normal metabolome due to the anaerobic changes that take place following death, including artefactual changes in the abundance of glucose and lactate, two key metabolites of interest during sepsis.

### *Conclusion*

This paper is the first to use NMR-led metabolomics to analyse equine SF with multiple pathologies and demonstrates that NMR-led metabolomics is an effective technique for analysis of equine SF collected within a clinical veterinary environment. Furthermore, this study demonstrates a panel of synovial metabolites which can distinguish between septic

and non-septic equine SF, with glucose the principal discriminator. To translate this into a diagnostic aid a wider study with participants with less common diagnoses are required to improve the diagnostic power.

## **Supporting Information**

Table S1. Statistically significant differentially abundant peaks between septic and non-septic equine synovial fluid.

## **Ethics**

University of Liverpool Ethics approval ref: VREC175

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## **Author contributions**

Wrote the manuscript (JA, MMP), revised the manuscript (JA, MMP, PC, MJP, LRM), analysed the data (JA, MMP), experimental design (JA, MMP, PC, MJP, LRM), collected clinical samples (LRM). All authors read and approved the final manuscript.

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## **Notes**

The authors declare no competing financial interest.

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## **Abbreviations**

COMP, cartilage oligomeric matrix proteinase; CPMG, Carr-Purcell-Meiboom-Gill; CTX-II, C-telopeptide fragments of type II collagen; EBI, European Bioinformatics Institute; FT, femorotibial; GH, glenohumeral; MCP, metacarpophalangeal; MMPs, matrix

metalloproteinases; MSI, Metabolomics Standards Initiative; MT, meniscal tear and concurrent OA; MTP, metatarsophalangeal; NMR, nuclear magnetic resonance; OA, osteoarthritis; OC, osteochondrosis; PCA, principal component analysis; PC, principal components; PLS-DA, partial-least squares discriminant analysis; SF, synovial fluid; TC, tarsocrural; VIP, variables of influence.

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## ABS/TOC Graphic

