^{1}H NMR lipidomic analysis of synovial fluid in equine osteochondrosis

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Osteochondrosis (OC) is a significant developmental disorder seen in growing horses and affects the cartilage and bone within the joints, subsequently causing pain, lameness and reduced athletic ability. Synovial fluid is located within the joint cavity and acts to reduce friction and thereby enhance the mobility of the joint. Synovial fluid is predominately affected by the health status of the joint, with the lipid (or fat) content of synovial fluid reflecting this, implying that synovial fluid is a good source for biomarker discovery and therefore the biofluid of choice to investigate OC. Synovial fluid samples were analysed using nuclear magnetic resonance (NMR). NMR enables the analysis of the molecular structure of a sample by measuring the interaction of nuclear spins when inserted into a powerful magnetic field. It allows for efficient analysis of small volumes of synovial fluid, requires minimal sample preparation and is non-invasive and non-destructive, providing robust and reproducible results. Statistical analyses were utilised to identify any differences in the lipid content of synovial fluid obtained from the joints of horses with and without OC. No statistically significant differences were detected. Time limitations and limited access to specialised software prevented the identification of the individual lipids within the synovial fluid samples. Future studies will focus their attention on identifying these lipids. While this study was unable to identify any significant differences between the joints with and without OC, it was the first study to our knowledge to investigate the synovial fluid lipid profiles with NMR.

Abstract

Osteochondrosis is a significant developmental disorder of horses and is caused by the disruption or failure of the endochondral ossification process within epiphyseal articular cartilage or growth plates, resulting in pain, lameness and reduced athletic performance. Synovial fluid is located in close proximity to articular tissues and is predominantly affected by joint pathology. The lipid profile of synovial fluid is said to reflect the health status of the joints, therefore presenting a potential source of biomarker discovery. Nuclear magnetic resonance (NMR) allows for efficient analysis of the molecular structure of small volumes of synovial fluid with minimal sample preparation as well as being non-invasive and non-destructive, providing robust and reproducible results. ^{1}H NMR was utilised to analyse and compare the lipid profiles of synovial fluid from horses with osteochondrosis (n=five) and controls without osteochondrosis (n=four), and was shown to be a sufficient technique to investigate this biofluid. Univariant and multivariant statistical analyses found there were no significant differences between the lipid profiles of the two groups ($p>0.05$). Time limitations and limited access to specialised software prevented identification of the lipids within the synovial fluid samples. Any future studies would need to focus their attention on identifying these lipids. While this study was unable to identify any significant differences between the two groups, it is the first study to use NMR lipidomics to investigate equine synovial fluid's role in osteochondrosis.

Introduction

Osteochondrosis (OC) is a significant developmental orthopaedic disease of growing horses (Mendoza *et al.*, 2015) and is characterised by the focal disturbance of endochondral ossification within epiphyseal articular cartilage (De Grauw *et al.*, 2011, Rejnö & Strömberg, 1978) and growth plates (Ytrehus *et al.*, 2007). This disturbance can result in detached fragments (osteochondrosis dissecans, OCD), subchondral bone cysts or fissures forming on the epiphyseal growth cartilage (Rejnö & Strömberg, 1978, Ytrehus *et al.*, 2007). In addition, the blood supply to epiphyseal cartilage channels can be interrupted resulting in focal regions of chondronecrosis which forms clefts that extend through the articular cartilage into subchondral bone (McCoy *et al.*, 2013). One of the most common outcomes of OC is OCD. OCD is associated with the failure of cellular differentiation in growing cartilage, causing it to become thicker or retained, the development of fissures and subsequent cartilaginous flaps from the joint (Carlson *et al.*, 1995). These detached fragments can subsequently lead to lameness, pain and diminished athletic performance in young horses (McIlwraith, 1993, Verwilghen *et al.*, 2013). Biomechanics, exercise, nutrition, rapid growth as well as genetics (Philipsson, 1996) have been identified as the main risk factors associated with OC (Jeffcott, 1991), although how they influence endochondral ossification remains unclear (Donabédian *et al.*, 2008, McCoy *et al.*, 2013). It commonly affects the tarsocrural joint, with an incidence of up to 30-40% in some breeds (Philipsson, 1996) and has a prevalence of 23% in Thoroughbreds (Russell *et al.*, 2017).

Synovial fluid is located within articular joints, providing a pool of nutrients to neighbouring tissues as well as lubricating articular cartilage, resulting in frictionless movement of the joint (Blewis *et al.*, 2007). The synovial fluid lipid profile is associated with the joint's health status (Kosinska *et al.*, 2016), with alterations to the lipid profile resulting in reduced lubrication and altered inflammatory state of the joint (Kosinska *et al.*, 2013, Kosinska *et al.*, 2014). Elevated synovial fluid concentrations of prostaglandin E2 and leukotriene B4 were identified in horses with clinical signs of OC (Billinghurst *et al.*, 2004, De Grauw *et al.*, 2006, Donabédian *et al.*, 2008),

demonstrating that these substances have the potential for diagnostic purposes. Synovial fluid is an essential biofluid for investigating OC due to neighbouring articular tissues which are primarily affected during joint pathology, thus presenting a potential source of biomarker discovery (Anderson *et al.*, 2018).

Previously, high-resolution ^{1}H nuclear magnetic resonance (^{1}H NMR), has been utilised to analyse metabolites, lipids and small molecules in various body fluids including cerebrospinal fluid (Musteata *et al.*, 2013), plasma (Le Moyec *et al.*, 2014, Nicholson *et al.*, 1984) and synovial fluid (Anderson *et al.*, 2018, Lacitignola *et al.*, 2008). The main advantages of NMR analysis of biological fluids, compared with previously utilised techniques, are that it requires minimal sample preparation and with its non-destructive and non-invasive nature produces results which are robust and reproducible (Keun & Athersuch, 2011). To date, ^{1}H NMR has been used to analyse metabolite biomarkers within synovial fluid (Anderson *et al.*, 2018) however, this technique is yet to be used to investigate the lipid profile of synovial fluid from OC joints.

This study aimed to use ^{1}H NMR to identify lipid biomarkers in synovial fluid to enable differentiation between horses with and without OC, thereby advancing diagnostic and prognostic capabilities and enhancing equine health and welfare.

Methods

Sample cohorts and collection

Adhering to owner consent and ethical approval, synovial fluid was aspirated from the joints of horses presented to The Philip Leverhulme Equine Hospital, University of Liverpool, between 2014 and 2016. It was aspirated from the affected joints at the start of surgical arthroscopy under general anaesthetic. 500 µL of each sample was submitted for NMR lipidomic analysis. Affected joints comprised of femorotibial, glenohumeral, metacarpophalangeal, metatarsophalangeal and tarsocrural. Horses were organised into two groups: OC (n=5) and controls (n=4). The controls consisted of four horses which suffered from non-joint related disorders. OC diagnosis occurred via a combination of arthroscopy, radiography and ultrasonography. Synovial fluid was immediately deposited into uncoated 1.5 mL collection tubes and processed within an hour of collection. Centrifugation (4°C, 2450 g for 5 min) removed particulate matter from the synovial fluid and the cell-free supernatant was placed into a clean, uncoated 1.5 mL collection tube, snap-frozen with liquid nitrogen and stored at -80°C.

Sample preparation

Following thawing on ice, the synovial fluid samples underwent centrifugation (4°C, 14000 g for 10 min). The supernatant was removed and treated with 1 µg/ml hyaluronidase (bovine origin, Sigma-Aldrich) at 37°C for 1 hour and subsequently centrifuged at 2500 g for 10 min. 200 µL of the sample was combined with 250 µL deuterated chloroform and incubated on ice for 5 min. The mixture was vortexed for 30 s, centrifuged at 1200 g and 4° C for 1 min and incubated on ice for a further 1 min to allow the aqueous and lipid phase to separate. Using a glass pipette 200 µL of the lower (lipid) phase was transferred into 3 mm outer diameter clean NMR tubes.

NMR optimisation, acquisition and processing

One dimensional ^{1}H NMR spectra with Nuclear Overhauser Effect Spectroscopy (NOSEY) filter was utilised to attain signals from lipids on a 700 MHz NMR Bruker Avance III spectrometer. Spectra were obtained at 15°C, with a 4 s interscan delay, 256 transients and a 26 parts per million (ppm) spectral width. Topspin 3.6.2 and IconNMR 4.67 software with automated baseline and phasing correction as well as standard vendor processing routine were used to attain and process data.

Lipid annotation and identification

All spectra were screened to guarantee they met the recommended quality control criteria before statistical analysis. The quality control criteria included a line-width half height which is of representative residual ^{1}H chloroform peak aligned to 7.26 ppm within one standard deviation and a flat baseline. The spectra were separated into 'bins' or 'buckets' according to the lipid annotation from TameNMR (Grauslys, n.d.). Each spectrum was separated into 99 bins and the intensity of each bin divided by the bin width to counteract the intensity variance. Before statistical analysis, the bins were normalised to the median spectrum and Pareto scaled in MetaboAnalyst (MetaboAnaylst, n.d.). Residual ^{1}H chloroform ($C^{1}HCl_3$) was identified in the spectra as it is present in the deuterated chloroform ($C^{2}HCl_3$ (<1%)) and was used to dilute the synovial fluid sample. Therefore, for multivariant analysis, the bins assigned to chloroform were excluded.

Lipidomic statistical analysis

The synovial fluid spectra were divided into two groups: OC and controls. MetaboAnalyst 4.0 was used for normalisation, t-tests, principal component analysis (PCA) scores plots as well as box plots used the R package of statistical computing software (Chong *et al.*, 2019). For t-tests, $p \leq 0.05$ was considered statistically significant.

Results

NMR spectra quality control

Spectra were successfully acquired from the nine samples. Upon an initial overview of the NMR spectra, three distinct, large peaks were identified (Figure 1A). On closer inspection, these were determined to be due to contamination with deuterated chloroform at 7.26 ppm (peak 1), an artefact from water suppression at 5.0 ppm (peak 2) and water at 1.6 ppm (peak 3) (Figure 1B). As these were deemed contaminants they were subsequently omitted from further analysis. The spectra representing the 99 lipid metabolite peaks from all nine synovial fluid samples were subsequently analysed.

Data normalisation

Data were normalised to the median and Pareto scaled. Figure 2 shows the data before and after normalisation.

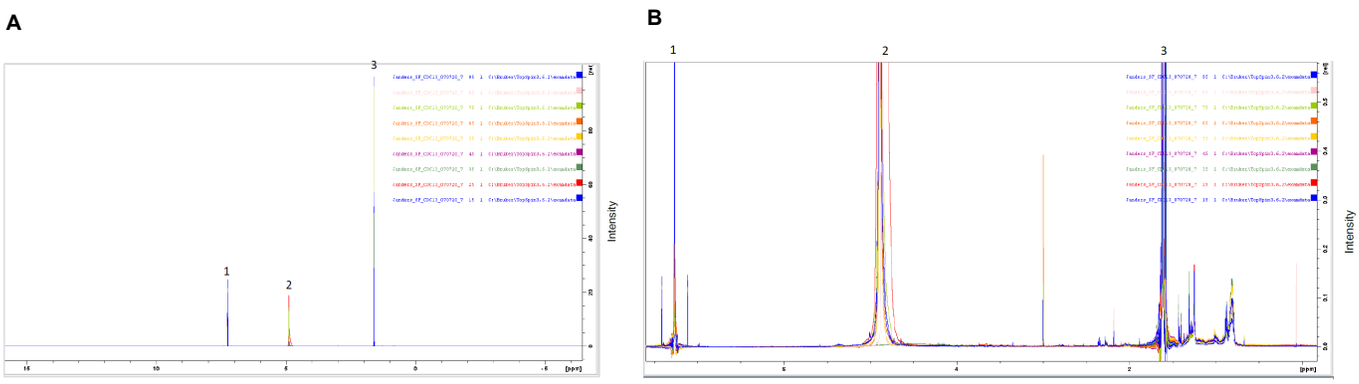

Figure 1. (A) Initial lipidomic NMR spectra of synovial fluid from four control and five OC donors with annotated contaminated peaks. Contaminant annotations include: chloroform (1) and water (2 and 3). These contaminants were omitted from analysis. All nine samples are represented in this NMR spectra with their intensity along the y-axis and parts per million (ppm) along the x-axis. **(B)** Expanded lipidomic NMR spectra of synovial fluid from all samples. This NMR spectra ranges from 1-7.5 ppm and shows 99 peaks. The contaminant chloroform at 7.26 ppm (1), artefact of water suppression at 5.0 ppm (2) and water peak at 1.6 ppm (3) can be seen in this spectra. All nine samples are represented in this NMR spectra with their intensity along the y-axis and ppm along the x-axis.

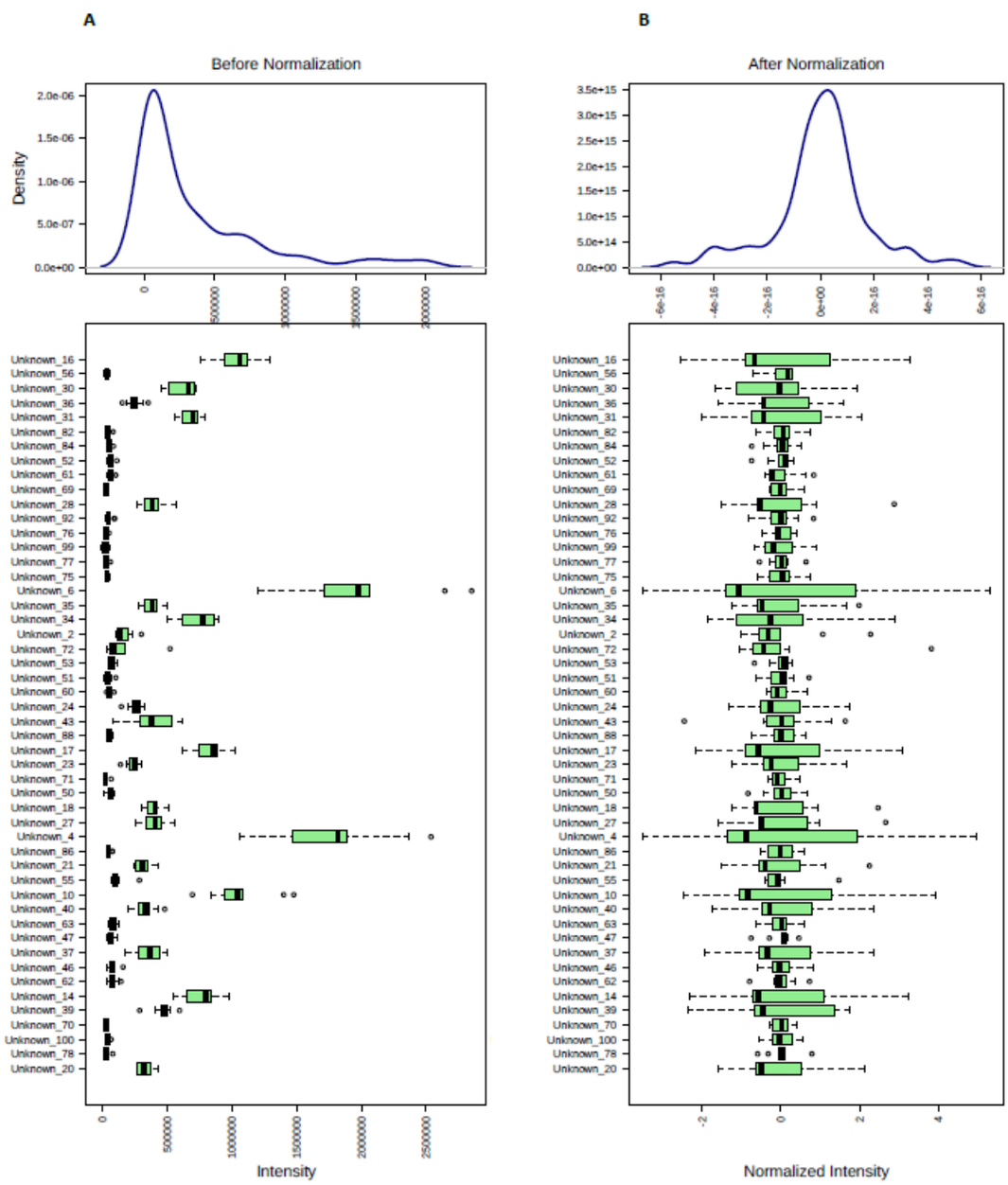

Figure 2. NMR data of the OC and control groups before and after normalisation. **(A)** Prior to normalisation. **(B)** After normalisation. The data were normalised to the median and Pareto scaled prior to statistical analysis to ensure that the data were directly comparable. The density is displayed in the graphs with the intensity of each lipid peak displayed directly below as green box pots.

Prior to normalisation, the density is skewed to the left and the lipid boxplots vary in intensity with Unknown_4 and Unknown_6 displaying the greatest intensity. Following normalisation, the density curve is near-symmetrical and the boxplot intensities are all central to 0.

Lipidomic statistical analysis

Unsupervised multivariate principal component analysis (PCA) scores were utilised to identify significant differences in the lipid profile between the synovial fluid aspirated from OC and control joints. No clear separation was observed between the different groups (Figure 3A). There was variation in the shape and size of the two clusters, with the control group being more clustered together. In the synchronised 3D plot, the coloured markers show that there is some separation between the two groups along the principal component (PC) 3 axis (Figure 3B). However, this axis accounts for 4.5% of the variance, thus does not display any statistical significance.

Data were further analysed using a loadings plot. Figure 4A represents the loading plot associated with the PCA scores described in Figure 3A and enabled comparison of each lipid peak identified in control and OC samples. The loadings plot showed that Unknown_43 (highlighted in the blue box) (p=0.71) had the most significant effect (Figure 4A). Expression of Unknown_43 was higher in the OC group although not statistically significant (Figure 4B).

Univariate t-test analysis of the spectra determined no significant differences (p>0.05) between the lipid profile of the 99 peaks from the synovial fluid obtained from the two groups.

Discussion

OC is a developmental disorder caused by a disturbance to endochondral ossification (De Grauw *et al.*, 2011, Rejnö and Strömberg, 1978) subsequently affecting the cartilage of joints. Due to its close association with articular tissue and being influenced by joint pathology, synovial fluid is an important source of biomarker discovery, thereby an essential biofluid to investigate OC (Anderson *et al.*, 2018).

The lipid profile of synovial fluid was investigated as it is related to the health status of the joint (Kosinska *et al.,* 2016), with modifications affecting the inflammatory state and lubrication of the joint (Kosinska *et al.*, 2013, Kosinska *et al.*, 2014). It is thought this is the first report of the use of NMR lipidomics to interrogate synovial fluid in OC.

No lipids were found to be statistically different between the OC and control groups. Synovial fluid collected from the control group was more clustered, suggesting that these samples shared similar lipid profiles. It also implies that there is more variation in the lipid biomarkers associated with equine OC. Similarly, Zhang *et al.,* (2020) raised the possibility of lipid biomarkers being used for disease diagnosis with Alzheimer's disease (AD) mice displaying significant changes in many lipid species and clear separation between AD and control mice.

Loadings plots are used to analyse which variables have the greatest effect on each component, in this case being OC and control horses. From the loadings plot, Unknown_43 was identified as a significant driver and appears to be associated with the OC group. Further investigations would be needed to identify this lipid and thus its role in OC.

It is imperative to identify contaminants in spectra prior to further analysis. On the NMR spectra, chloroform and water were identified as contaminants and thus omitted from further investigation. To confirm that chloroform was the peak at 7.26 ppm, the same process, as stated in the methods, should be utilised to analyse chloroform alone and determine whether this peak matches. Due to the separation of the aqueous and lipid portions of synovial fluid prior to NMR analysis, water is a probable contaminant. It would be difficult to obtain a pure sample without water peaks.

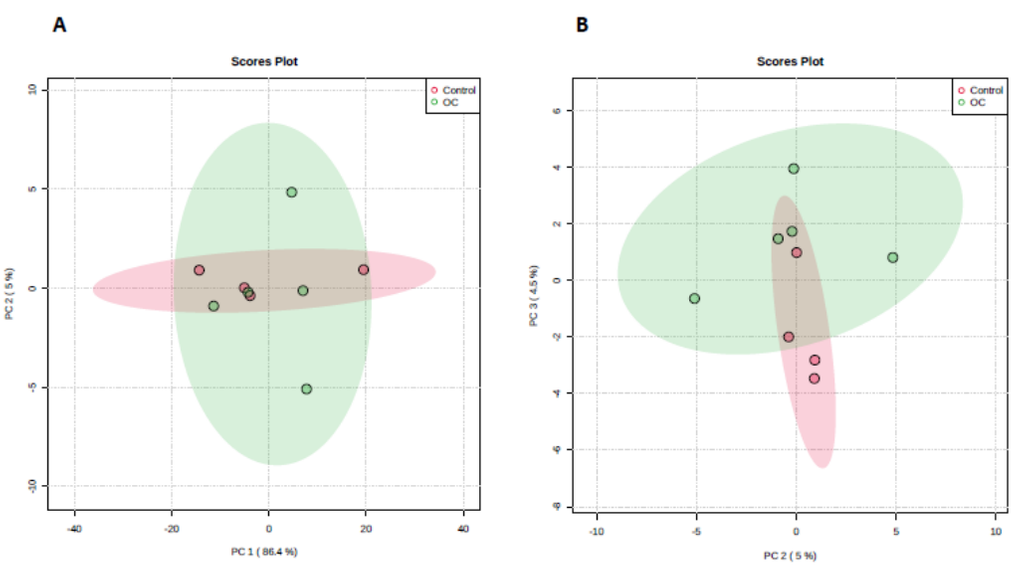

Figure 3. Principle component analysis (PCA) of lipid peaks identified within synovial fluid aspirated from OC affected horses (n=5) and controls (n=4) using ^{1}H NMR. **(A)** Equine synovial fluid lipidomic 2D PCA plot. Groups are represented as green (OC) and red (control) coloured areas. PC1 and PC2 account for 89.4% variance. Shading represents 95% of the confidence region. **(B)** Equine synovial fluid synchronised 3D plot shown in 2D orientation. Groups are represented as green (OC) ore red (control). PC2 and PC3 account for 9.5% variance.

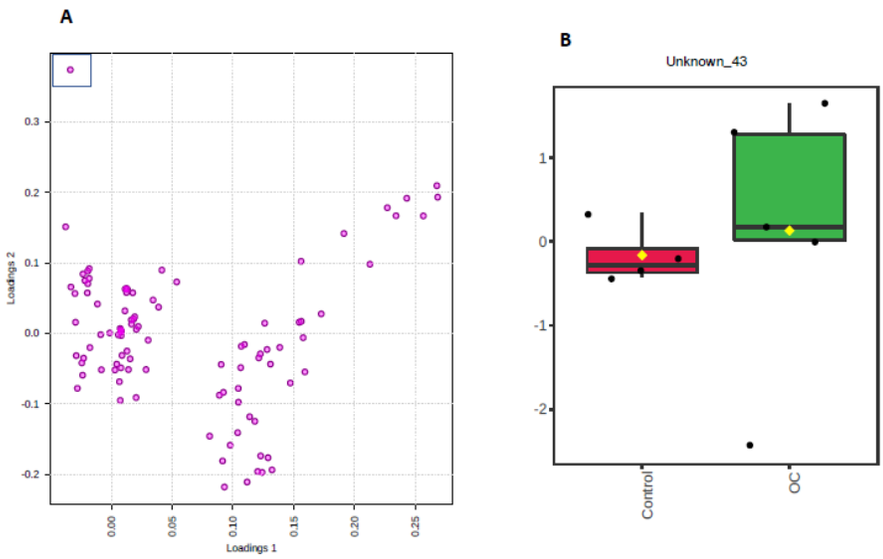

Figure 4. Loadings plots of lipid peaks identified within synovial fluid aspirated from OC affected horses (n=5) and controls (n=4) using ^{1}H NMR. **(A)** Equine synovial fluid loadings plot. The peak most different, Unknown_43, is highlighted. **(B)** Unknown_43 normalised box plot comparing the effect of Unknown_43 between control and OC groups.

Before further analysis, it is vital to normalise data so that all the samples are directly comparable (Craig *et al.*, 2006, Dieterled *et al.*, 2006). Normalising the data using the median and Pareto scaling proved to be sufficient, with the normalised data producing a near symmetrical curve. Additionally, methyl (CH_3) normalisation has also been recommended for lipidomic statistical analysis compared with other methods, such as probabilistic quotient normalisation, as they do not skew normalised data (Morgan, 2019).

This pilot study used a small sample size, nine samples, and this is a major limitation. The preliminary data could be used in a power calculation for future work to determine the number of subjects and/or samples required to detect a significant effect. A larger sample size would be beneficial to identify any statistical significance in future studies.

Due to time limitations and limited access to specialised software, lipid metabolites could not be identified. Therefore, a follow up to this study would be the identification of lipids within the synovial fluid aspirated from the two groups by comparing it with spectra of known lipids or mass spectrometry to determine the lipid landscape. Additional studies should compare and contrast the lipid profiles of synovial fluid aspirated from OC and osteoarthritic equine joints as they commonly cause pain and lameness in horses.

Conclusion

This is the first study to utilise ^{1}H NMR to study lipidomics of synovial fluid from horses with osteochondrosis. Although we were unable to discriminate between OC affected and non-affected joints, this study is a pilot for this area of research, with the potential for significant biomarker discovery within the near future.

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