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Optimization of Synovial Fluid Collection and Processing for NMR Metabolomics and LC-MS/MS Proteomics

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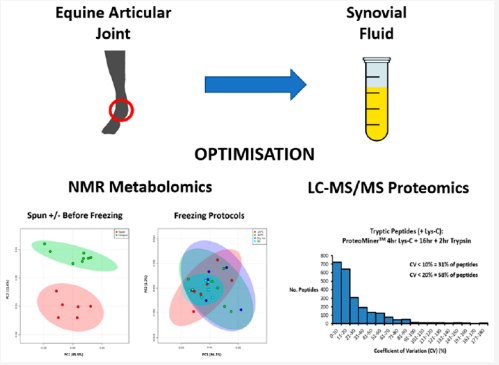
ABSTRACT: Synovial fluid (SF) is of great interest for the investigation of orthopedic pathologies, as it is in close proximity to various tissues that are primarily altered during these disease processes and can be collected using minimally invasive protocols. Multi-"omic" approaches are commonplace, although little consideration is often given for multiple analysis techniques at sample collection. Nuclear magnetic resonance (NMR) metabolomics and liquid chromatography tandem mass spectrometry (LC-MS/MS) proteomics are two complementary techniques particularly suited to the study of SF. However, currently there are no agreed upon standard protocols that are published for SF collection and processing for use with NMR metabolomic analysis. Furthermore, the large protein concentration dynamic range present within SF can mask the detection of lower abundance proteins in proteomics. While combinational ligand libraries (ProteoMiner columns) have been developed to reduce this dynamic range, their reproducibility when used in conjunction with SF, or on-bead protein digestion protocols, has yet to be investigated. Here we employ optimized protocols for the collection, processing, and storage of SF for NMR metabolite analysis and LC-MS/MS proteome analysis, including a Lys-C endopeptidase digestion step prior to tryptic digestion, which increased the number of protein identifications and improved reproducibility for on-bead ProteoMiner digestion.



KEYWORDS: *synovial fluid, metabolomics, nuclear magnetic resonance, proteomics, mass spectrometry, Lys-C endopeptidase*

INTRODUCTION

Synovial fluid (SF) primarily acts as a biological lubricant, reducing friction between synovial joint articular cartilage surfaces, but also functions as a pool of nutrients for surrounding tissues and allows movement of regulatory cytokines.[1,2] SF is of great interest for the investigation of orthopedic pathologies, including osteoarthritis (OA), osteochondrosis, rheumatoid arthritis, and synovial sepsis, as it is in close proximity to various tissues that are primarily altered during these disease processes, with minimally invasive collection protocols.[3,4] Therefore, SF has the potential for improved understanding of underlying disease pathogenesis and biomarker discovery.[5]

Multi-"omic" approaches are commonplace, although little consideration is often given for multiple analysis techniques at sample collection.[6] Nuclear magnetic resonance (NMR) metabolomics and liquid chromatography tandem mass spectrometry (LC-MS/MS) proteomics are two complementary techniques particularly suited to the study of SF. NMR spectroscopy involves very little sample preprocessing and is particularly useful for high-viscosity samples that would require metabolome altering extraction techniques prior to analysis by high performance liquid chromatography (HPLC).[7,8] LC-MS/MS proteomics provides complementary phenotypic information and is suited to SF analysis due to the wide coverage of proteins in typical LC-MS/MS databases.[6,9] There is a growing need to establish sample collection and preprocessing techniques compatible with multi-"omic" analyses, and as such we endeavor to employ both techniques to study the metabolome and proteome of clinical SF samples.

NMR metabolomics is a rapidly expanding field, providing comprehensive metabolite profiling of complex biological samples with high levels of technical reproducibility.[10,11] Several studies have utilized NMR to investigate the SF metabolome of orthopedic diseases in various species, including humans, dogs, pigs, and horses, albeit only few with statistical rigor associated with an "omics" analysis.[3,12,21,13−20] Although the effect of freeze thaw cycles and long-term low temperature storage have been investigated, no studies to date have investigated the impact of different freezing methods on NMR metabolite analysis.[22] Currently,

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there are no agreed standard protocols that are published for SF collection and processing for use with NMR metabolomic analysis.

Various studies have used mass spectrometry (MS) based proteomics approaches to analyze SF, with the development of LC-MS/MS providing a fast and sensitive methodology to identify and quantify proteins within complex biological samples.[23,24] However, due to the multivariate nature of sample analysis, a large number of biological replicates are required in order to achieve an adequately powered study, which for LC-MS/MS may be cost prohibitive. Additionally, the large protein concentration dynamic range present within SF leads to various challenges associated with proteome analysis.[25] A small number of highly abundant proteins, including albumin, can mask the detection of low abundant proteins, thus compromising potential biomarker discovery.[26] Combinational ligand libraries have been developed to reduce this dynamic range, achieving peptide-based depletion while allowing preservation of the whole proteome.[27,28] This methodology has recently been used in the development of ProteoMiner protein enrichment columns (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), depleting highly abundant proteins and enriching those less abundant.[29] This technique was found to generate the largest increase in protein identifications compared to other protein depletion methods when applied to serum.[30] However, the elution solution present within the kit is not compatible with LC-MS/MS analysis, due to the presence of 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS).[31] Although "cleanup" and alternative elution protocols are available, these introduce a further process in sample preparation and subsequently a source of variation. Peffers et al. have previously developed an on-bead trypsin digestion protocol for analysis of ProteoMiner processed SF.[32−35] However, although the reproducibility of ProteoMiner beads when used in conjunction with serum has been examined, reproducibility with SF or on-bead digestions have yet to be investigated.[36]

Lys-C serine endopeptidase is the second most common enzyme used within bottom-up proteomics studies following trypsin, efficiently hydrolyzing the peptide bond of lysine residues on the carboxyl side.[37,38] While trypsin digests peptides at Arg-C or Lys-C residues, unless followed directly by proline, cleavage at the Lys-C site is comparatively poor when compared to Lys-C endopeptidase activity.[38,39] Thus, a combined digestion protocol can produce an overall improved digestion efficiency. When used within an in-solution protein digestion protocol, a Lys-C/trypsin protocol was found to produce significantly less missed cleavages and a more efficient digestion than a tryptic digestion alone.[40] However, on-bead ProteoMiner digestion protocols to date have used trypsin alone, with the potential improved digestion of the addition of Lys-C endopeptidase into this protocol yet to be investigated.

Cartilage breakdown products are generated during orthopedic pathology, i.e., OA, due to elevations in enzymatic activity within synovial joints.[41] These products may be recognized via MS as semitryptic peptides. Identification and quantification of these semitryptic peptides within pathological groups has potential as a method for early OA biomarker discovery enabling disease stratification. However, the reproducibility of semitryptic peptide quantification is yet to be investigated following ProteoMiner processing.

We hypothesize that refining SF collection and processing protocols for NMR metabolomic and LC-MS/MS proteomic analysis will maximize the number of molecule identifications as well as optimize technical reproducibility.

METHODS

Study Overview

A summary of the main NMR and LC-MS/MS protocols investigated during this study for metabolite, protein, and peptide identification and quantification can be found in Table S1.

Ethics

Equine SF samples were collected as a byproduct of the agricultural industry. The Animals (Scientific Procedures) Act 1986, Schedule 2, does not define collection from these sources as scientific procedures and ethical approval was therefore not required. Human SF collection was authorized by the ethics committee at the University of Birmingham via a material transfer agreement and also underwent NHS research ethics service approval (REC 16/SS/0172).

Equine Synovial Fluid Collection

All equine SF was collected post mortem from an abattoir within 8 h of euthanasia in order to ensure a consistent metabolome for all post mortem samples.[42] Metacarpophalangeal (MCP) joints were opened aseptically and SF collected on ice using a 10 mL syringe. For NMR metabolomics analysis, SF was pooled from four MCP joints from four separate donors and vortexed for 1 min. SF was also collected from an additional three equine MCP joints from three donors and processed separately. For proteomic analysis SF was pooled from five MCP joints from five separate donors, pooled and vortexed for 1 min. All joints used for this study were considered to be macroscopically normal and were assigned a score of 0 according to the equine OARSI histopathology initiative scoring system.[43]

Human Synovial Fluid Collection

Following ethical approval (REC 16/SS/0172), SF was collected peri-operatively from the acetabulofemoral joint of nine patients diagnosed with end-stage hip OA undergoing elective total joint replacement surgery at The Royal Orthopaedic Hospital (Birmingham). SF was treated with hyaluronidase and stored at −80 °C. Consent was obtained from all patients. No comparisons were made between living and post mortem samples to ensure all variance was due to sample processing and not post mortem degradation.[42]

NMR Metabolomics

Sample Preparation—Spun vs Unspun. 150 μL of pooled equine SF was aliquoted into nine eppendorfs that did not undergo centrifugation prior to freezing (unspun) and nine eppendorfs that were centrifuged (spun). In the spun group, SF was centrifuged at 2540*g* and 4 °C for 5 min and the supernatant transferred to a new eppendorf. All samples were subsequently snap frozen in liquid nitrogen and stored at −80 °C.

Sample Preparation—Different Freezing Protocols. 150 μL of pooled equine SF was aliquoted into 32 eppendorfs, which were subsequently centrifuged at 2540*g* and 4 °C for 5 min and the supernatant removed. The samples were then divided into four separate groups (eight in each) and frozen either at −20 or −80 °C, placed onto dry ice, or snap frozen in liquid nitrogen. Following freezing, all samples were stored at −80 °C.

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Sample Preparation—Reproducibility of Separate Synovial Fluid Donors. Following collection, equine SF was separated into three separate 150 μL aliquots for each of the three separate donors, nine aliquots in total. SF was then centrifuged at 2540*g* and 4 °C for 5 min, supernatant removed, snap frozen in liquid nitrogen and stored at −80 °C.

Sample Preparation for NMR Spectrometry. 150 μL of each thawed SF sample was diluted to a final volume containing 50% (v/v) SF, 40% (v/v) dd ^1H_2O (18.2 MΩ), 100 mM PO_4^{3-} pH 7.4 buffer (Na_2HPO_4, VWR International Ltd., Radnor, Pennsylvania, USA and NaH_2PO_4, Sigma-Aldrich, Gillingham, UK) in deuterium oxide (^2H_2O, Sigma-Aldrich) and 0.0025% (v/v) sodium azide (NaN_3, Sigma-Aldrich). Samples were vortexed for 1 min, centrifuged at 13 000*g* and 4 °C for 2 min and 200 μL transferred (taking care not to disturb any pelleted material) into 3 mm outer diameter NMR tubes using a glass pipet.

NMR Spectral Acquisition. All SF samples were individually analyzed. 1D ^1H NMR spectra were acquired using a 700 MHz NMR Bruker Avance III HD spectrometer with associated TCI cryoprobe and chilled Sample-Jet autosampler. A Carr−Purcell−Meiboom−Gill (CPMG) pulse sequence was used to attenuate macromolecule signals using a standard cpmgpr1d vendor pulse sequence. All CPMG spectra were acquired at 37 °C with a 15 ppm spectral width, a 4 s interscan delay and 32 transients. Spectral acquisition and processing was carried out using Topsin 3.1 (Bruker Corporation, Billerica, Massachusetts, USA) and IconNMR 4.6.7 (Bruker).

Spectral Quality Control and Bucketing. Following acquisition, all spectra were analyzed to ensure they conformed to community recommended minimum reporting standards.[44] These included flat baseline correction, water suppression, and consistent line widths. Spectra that did not meet these standards were removed from all subsequent analyses. Admissible spectra were aligned to a single formate peak at 8.46 ppm. It is important to note that trimethylsilylpropanoic acid (TSP) and other silica-based reference materials are unsuitable for NMR metabolomics studies of proteinous biofluids due to the propensity to bind to proteins such as albumins and therefore change in chemical shift or attenuate completely.[10] All peaks within each spectrum were then placed into "buckets", excluding the peak generated by water, with each bucket intensity divided by the width in order to negate intensity variance. Spectra for different freezing protocols and spun vs unspun experiments were divided into 144 buckets, and spectra for reproducibility of separate synovial fluid donors were divided into 139 buckets. Buckets were subsequently normalized to the median.

Metabolite Annotation and Identification. Buckets were assigned metabolite identifications using Chenomx NMR Suite 8.2 (330-mammalian metabolite library). Where possible, metabolite identities were confirmed using in-house 1D ^1H NMR and 2D ^1H ^{13}C Heteronuclear Single Quantum Coherence NMR standards. Metabolite assignments, including both Human Metabolome Database identifications and annotation levels, are available within the MetaboLights repository at www.ebi.ac.uk/metabolights/MTBLS1450.

LC-MS/MS Proteomics

Hyaluronidase Treatment Protocol Optimization. 750 μL aliquots of thawed equine SF were supplemented with hyaluronidase (from bovine testes, Sigma-Aldrich) at a final concentration of 0, 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, 1.50, or 2.00 μg/mL and vortexed for 30 s. All samples (bar two 0 μg/mL treated samples) were then incubated at 37 °C for 1 h and rotated. All samples (bar two 0 μg/mL and one 1.00 μg/mL treated samples) were then passed through polypropylene microcentrifuge tube filters with 0.22 μm pore cellulose acetate membranes (Costar Spin-X, Corning, Tokyo, Japan) for 15 min at 5000*g*. One μL of each sample was analyzed by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D SDS PAGE) and stained with Coomassie Blue (Bio-Rad).

Standard Trypsin Protein Digestion Protocol. During this study, previously developed standard trypsin digestion protocols were used for native SF protein digestion and on-bead ProteoMiner protein digestion, or a variation of this protocol as stated[32] (Figure S1). 160 μL of 25 mM ammonium bicarbonate (Fluka Chemicals Ltd., Gillingham, UK) containing 0.05% (w/v) RapiGest (Waters, Elstree, Hertfordshire, UK) was added to ProteoMiner columns. For native SF and ProteoMiner column flow-through, the appropriate volume containing 100 μg of protein was diluted to a final volume of 160 μL 25 mM ammonium bicarbonate (Fluka) containing 0.05% (w/v) RapiGest. Samples were heated for 10 min at 80 °C, 3 mM final concentration DL-Dithiothreitol (Sigma-Aldrich) was added and heated at 60 °C for 10 min, and 9 mM final concentration iodoacetamide (Sigma-Aldrich) was added and incubated at room temperature for 30 min in the dark. Protein digestion was carried out through the addition of 2 μg of proteomics grade trypsin (Sigma-Aldrich), rotation at 37 °C for 16 h with repeated trypsin supplementation for 2 h, again rotating at 37 °C. Columns were centrifuged at 1000*g* for 1 min, and 0.5% (v/v) final concentration trifluoroacetic acid (TFA, Sigma-Aldrich) was added, rotated at 37 °C for 30 min, centrifuged at 13 000*g* and 4 °C for 15 min, and the supernatant was removed.

ProteoMiner Bead Protein Fractions. Pooled equine SF was treated using the standard protocol of adding 1 μg/mL hyaluronidase, heating at 37 °C for 1 h, centrifuging at 1000*g* for 5 min, removing the supernatant and passing through a 0.22 μm cellulose acetate filter at 5000*g* for 15 min. A ProteoMiner Small Capacity bead column (Bio-Rad) was loaded with 3.5 mg of protein and processed according to manufacturer instructions. The sample was rotated at room temperature for 2 h, centrifuged at 1000*g* for 1 min (flow-through also collected), the beads washed in 200 μL phosphate buffered saline (PBS), rotated for 5 min and centrifuged for 1 min at 1000*g* with the wash flow-through collected. The wash step was completed three further times with the final being completed using deionized water. Twenty μL of elution buffer (8 M urea, 5% acetic acid and 2% CHAPS) was added to the column, vortexed several times over 15 min, centrifuged at 1000*g* for 15 min, and the elution collected. The elution step was repeated two further times. All fractions collected during the protocol were analyzed to assess the protein profiles/abundant protein depletion and protein content via 1D SDS PAGE and stained with Coomassie Blue and a Pierce 660 nm protein assay (Thermo Scientific, Waltham, Massachusetts, USA) respectively.

ProteoMiner Bead Protein Loading. Following the standard hyaluronidase treatment protocol, 1.0 mg, 2.5 mg and 5.0 mg of a pooled SF sample and 5.0 mg and 10.0 mg of a separate pooled SF sample were loaded onto separate ProteoMiner columns. Sample incubation and wash steps

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were completed according to manufacturer instructions. However, instead of protein elution, a standard on-bead digestion protocol was undertaken as previously stated. 100 μg of native SF protein and 100 μg of protein column flow-through were also digested using the same reduction, alkylation and digestion steps. Protein profiles were assessed using 1D SDS PAGE and stained with Coomassie Blue. Digests were individually analyzed via LC-MS/MS with 120 min LC gradients.

Gradient Length and Blank Acquisition. SF protein digests used to assess ProteoMiner bead protein loading (100 μg native SF and 2.5 mg column loading) were both analyzed using LC-MS/MS with 60, 90, and 120 min LC gradients and the number of proteins and peptides identified for each protocol recorded. To assess peptide carry-over between successive samples, after each sample a "blank" sample (containing only sample buffer (97% (v/v) HPLC grade H_2O (VWR International), 2.9% acetonitrile (Thermo Scientific) and 0.1% TFA was run on a 30 min LC gradient and the spectra acquired. Following one SF sample a series of five successive blank samples were also run and again the spectra were acquired. The abundance of each peptide identified within the blank sample was calculated as a percentage of the abundance within the previous SF sample, and the median of peptide percentage carry-overs was recorded.

Synovial Fluid Protein Digestion Profiles: Coomassie Brilliant Blue vs Silver Stain. 100 μg of protein of native human SF and 3 mg loaded ProteoMiner columns for the same nine separate human donors underwent a standard 16 h + 2 h trypsin digestion protocol. Digestion profiles were then analyzed via 1D SDS PAGE and stained using both Coomassie Brilliant Blue and silver staining.

Protein Digestion Optimization. Variations of the previously stated standard digestion protocols were used for 100 μg and 2.5 mg loaded ProteoMiner columns using the 16 h + 2 h trypsin digestion method (Table S2). Native and ProteoMiner processed SF was digested using 4, 16, and 16 h + 2 h trypsin protocols. Additionally, for ProteoMiner processed SF, a 16 h + 16 h trypsin on-bead digestion protocol was also investigated as well as 16 h trypsin digests centrifuged at 1000*g* for 1 min and the second stage protein digestion (2 or 16 h trypsin digestion) carried out on the resulting supernatant. Each of these ProteoMiner protocols was also analyzed using a predigest step of Lys-C endopeptidase (FUJIFILM Wako Pure Chemical, Osaka, Japan). Prior to trypsin digestion, 2 μg of Lys-C (10 μg/mL final digest concentration) was added to the column and incubated at 37 °C for 4 h. A longer 16 h Lys-C predigest was also investigated for the standard 16 h + 2 h trypsin digestion protocol. Different ProteoMiner column loading methods were also investigated. After an initial 2 h on-bead sample incubation and centrifugation, a second SF load of equal protein was added to the column and a second 2 h incubation undertaken. Additionally to this, following a 2 h on-bead sample incubation and centrifugation, the resultant flow-through was reloaded onto the column and a second 2 h incubation carried out. As well as on-bead digestion protocols, one column did not undergo protein digestion with the intact proteins eluted using the manufacturer's instructions and elution buffer, as previously described, to compare the protein bound protein profile to that of the digested protein profiles. Following processing, samples and ProteoMiner beads were analyzed by 1D SDS PAGE and silver staining. LC-MS/MS was undertaken with a 1 h LC gradient.

Tryptic Peptide Reproducibility. Using the same pooled equine SF, following hyaluronidase treatment and CoStar processing, 100 μg of protein and 2.5 mg loaded ProteoMiner columns (including ProteoMiner flow-throughs) underwent a standard 16 h + 2 h trypsin digestion protocol with three technical replicates of each. All samples were analyzed using LC-MS/MS with a 2 h LC gradient. Additionally, for each sample type, the same vial was also analyzed three times to investigate the reproducibility of LC-MS/MS alone. ProteoMiner columns loaded with 2.5 mg of pooled SF that underwent a 4 h Lys-C + 16 h + 2 h trypsin protocol were also analyzed, although flow-through and repeated vial analysis was not undertaken.

Semitryptic Peptide Reproducibility. 100 μg protein of native SF underwent 4 h trypsin and 16 h + 2 h trypsin protocols in technical triplicates. 2.5 mg loaded ProteoMiner columns underwent 4 h trypsin, 16 h + 2 h trypsin, 4 h Lys-C + 4 h trypsin, and 4 h Lys-C + 16 h + 2 h trypsin digestion protocols, also in technical triplicate. All digests were analyzed by LC-MS/MS with using 1 and 2 h LC gradients.

1D SDS PAGE. One μL of native SF, 5 μL of digested SF or 8 μL of ProteoMiner beads were used for 1D SDS PAGE for optimal optimization of protein bands. Samples were added to Laemmli loading buffer Novex (Thermo Scientific) with a final concentration of 15% glycerine, 2.5% SDS, 2.5% Tris (hydroxymethyl) aminomethane, 2.5% HCL and 4% β-mercaptoethanol at pH 6.8 and heated for 5 min at 95 °C. Samples were loaded onto a 4−12% Bis-Tris polyacrylamide electrophoresis gel (NuPAGE Novex, Thermo Scientific) with protein separation undertaken at 200 V for 30 min at room temperature.

Coomassie Brilliant Blue Staining (Sensitivity = 100 ng of Protein). Following 1D SDS PAGE, gels were washed three times in ddH_2O for 5 min, stained with Coomassie Brilliant Blue stain (R-250, Bio-Rad) for 1 h, Coomassie stain removed and destained with Coomassie Brilliant Blue destaining solution (R-250, Bio-Rad) for 16 h.

Silver Staining (Sensitivity = 1 ng of Protein). Following 1D SDS PAGE, gels were silver stained according to manufacturer instructions (Thermo Scientific). Gels were washed twice in ddH_2O for 5 min, fixed in 30% (v/v) ethanol (Sigma-Aldrich): 10% (v/v) acetic acid (Sigma-Aldrich): 60% (v/v) ddH_2O for 15 min and the fixing step repeated. Gels were washed twice in 10% (v/v) ethanol: 90% (v/v) ddH_2O for 5 min, twice in 100% ddH_2O for 5 min, incubated in a sensitizer working solution for 1 min and washed twice in 100% ddH_2O for 1 min. The gel was then incubated in a stain working solution for 30 min, washed twice in ddH_2O for 20 s, incubated in developer working solution for 2−3 min until bands appeared, and finally 5% (v/v) acetic acid:95% (v/v) ddH_2O was added and incubated for 10 min.

LC-MS/MS Spectral Acquisition. All digests were individually analyzed via LC-MS/MS on an UltiMate 3000 Nano LC System (Dionex/Thermo Scientific) coupled to a Q Exactive Quadrupole-Orbitrap instrument (Thermo Scientific). Full LC-MS/MS instrument methods are described in the Supporting Information. Tryptic peptides (equivalent to 200 ng of protein) were loaded onto the column and run over a 30, 60, 90, or 120 min LC gradient as stated.

PEAKS Search Parameters. For peptide/protein database searches using PEAKS Studio 8.5 (Bioinformatics Solutions

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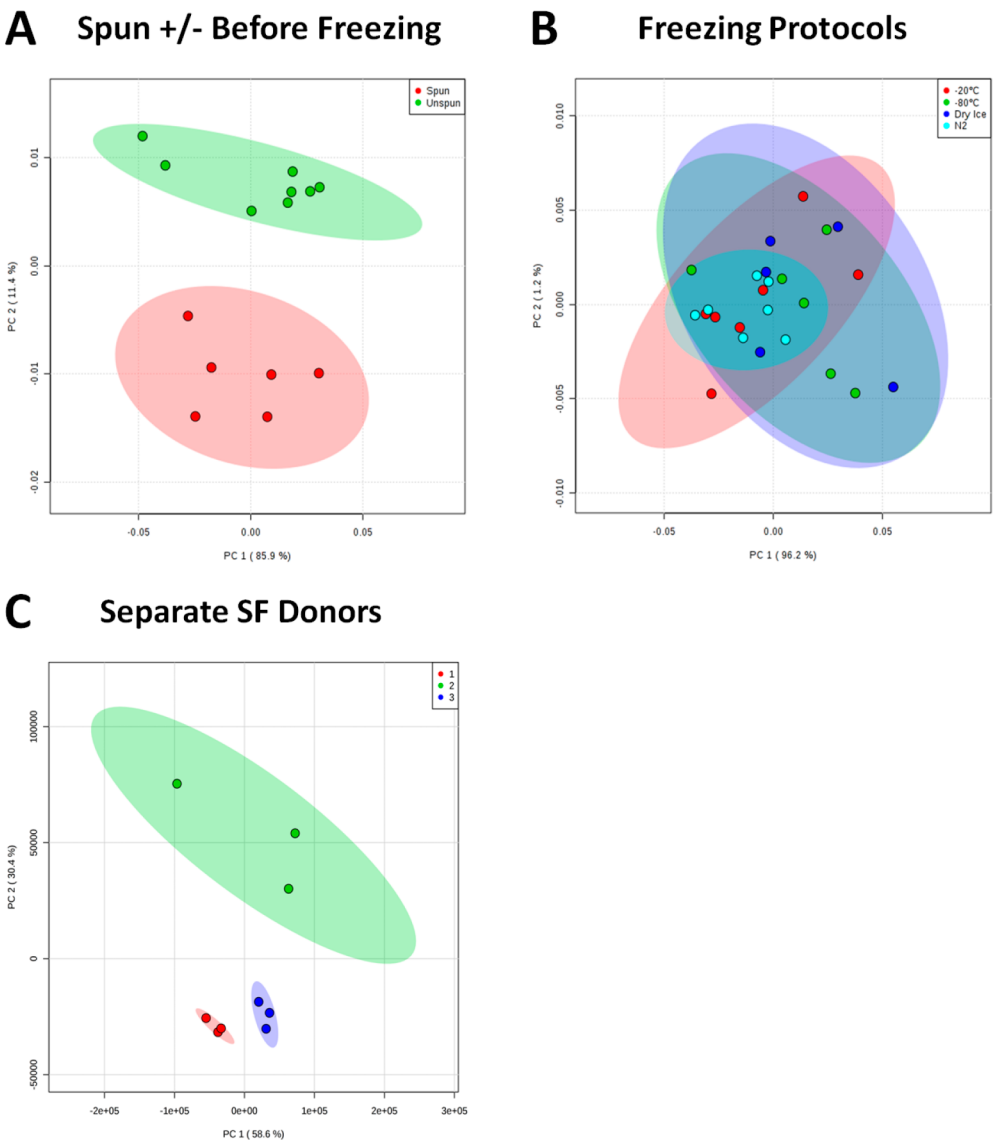

Figure 1. Optimization of equine synovial fluid processing for 1D ^{1}H NMR metabolome analysis. The reproducibility of the metabolome for different processing protocols for equine synovial fluid (SF) was assessed using principal component analysis (PCA). These protocols included (A) with (n = 6) and without (n = 8) a centrifugation step (2540g and 4 °C for 5 min) prior to freezing and (B) the use of different freezing methods (−20 °C (n = 7), −80 °C (n = 6), dry ice (n = 5), and liquid nitrogen (n = 7)). (C) PCA showing reproducibility of the finalized SF processing method (including centrifugation and liquid nitrogen snap freezing) using three separate equine donors with three technical replicates for each donor. PCA shaded regions depict 95% confidence regions.

Inc., Waterloo, Canada) the *Equus caballus* database was used with search parameters including the following: precursor mass error tolerance, 10.0 ppm; fragment mass error tolerance, 0.01 Da; precursor mass search type, monoisotopic; enzyme, trypsin; maximum missed cleavages, 1; nonspecific cleavage, none; fixed modifications, carbamidomethylation; variable modifications, oxidation or hydroxylation and oxidation (methionine). A 1% false discovery rate (FDR) was set and a minimum of 2 unique peptides required for protein identification. No normalization was undertaken. PEAKS searches were used for all peptide and protein identifications except for protein digestion optimization and semitryptic peptide analysis.

Mascot Search Parameters. For peptide/protein database searches using an in-house Mascot server Version 2.6.2[45] the *Equus caballus* database was used with search parameters including the following: peptide mass tolerance, 10.0 ppm; fragment mass tolerance, 0.01 Da; enzyme, trypsin; missed cleavages allowed, one; fixed modifications, carbamidomethylation (cysteine) and variable modifications; oxidation (methionine), oxidation (proline) and oxidation (lysine). Mascot database searches were used for protein digestion optimization and semitryptic peptide analysis.

Semitryptic Peptide Identification and Quantification. Raw spectral files underwent spectral alignment, peak picking and peptide quantification in Progenesis QI 2.0 (Nonlinear Dynamics, Waters). No normalization was undertaken. Peptide identifications were carried out for the top ten spectra of each feature with Mascot, using the same *Equus caballus* search parameters as for tryptic peptides, except a "semitryptic" search was conducted opposed to "tryptic". Peptides, with a 1% FDR correction, were exported from Progenesis and technical replicates of semitryptic peptide abundances compared using the online neopeptide tool.[46]

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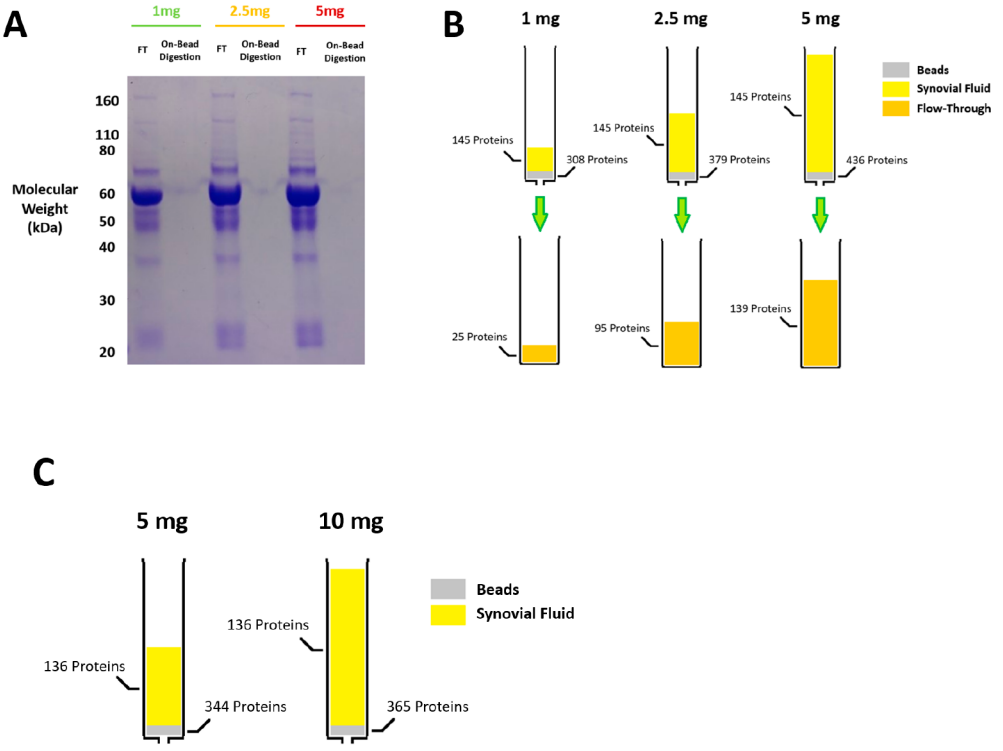

Figure 2. ProteoMiner column loading of synovial fluid. (A) Protein profiles of on-bead digests and flow-through (FT) following 1, 2.5, and 5 mg protein loading. (B) Number of proteins identified via LC-MS/MS following bead enrichment of depleted proteins and column flow-through for 1 mg, 2.5 mg and 5 mg protein loadings, and (C) 5 mg and 10 mg protein loadings for another set of pooled synovial fluid. A full protein gel image can be found in Figure S5.

Statistical Analysis

Prior to multivariate analysis, data sets were Pareto scaled and principal component analysis (PCA) plots conducted using MetaboAnalyst 4.0.[47] *t* tests were conducted in the software package R (https://cran.r-project.org/), box plots were constructed using SPSS 24, and histograms were drawn using Excel 2013. Peptide reproducibility was analyzed using the coefficient of variation (CV) statistic on raw, non-normalized abundance values.

Raw Spectra

All raw metabolomic NMR spectra are available at www.ebi.ac.uk/metabolights/MTBLS1450.[48] The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD017069 and 10.6019/PXD017069.[49]

RESULTS

NMR Metabolomics

Spun vs Unspun. Four spectra did not meet recommended minimum reporting standards and were removed from subsequent analyses. Including a centrifugation step prior to freezing identified clear separation on a PCA plot (Figure 1a). Two distinct metabolomes can be discerned with separation determined by PC2. The PCA loadings for PC2 identified metabolite peaks associated with branched chain amino acid biosynthesis (leucine, valine, and 2-hydroxybutyrate) as higher in concentration in unspun SF vs spun SF (Figure S2). This is likely due to contamination from intracellular material present within the unspun samples.

Different Freezing Protocols. Seven spectra did not meet recommended minimum reporting standards and were removed from subsequent analyses. Unlike the centrifugation protocols, PCA of different SF freezing protocols did not generate separate metabolomic profiles with no distinct groupings identified according to the freezing method used (Figure 1b). However, the SF samples frozen by snap freezing in liquid nitrogen displayed the least variance between technical replicates and this protocol was therefore the most reproducible of those studied.

Reproducibility of Separate Synovial Fluid Donors. The collection and processing protocol was identified as being reproducible with three technical replicates of SF from three macroscopically normal MCP joints from three horses clustering separately on a PCA plot (Figure 1c).

LC-MS/MS Proteomics

Hyaluronidase Treatment Protocol Optimization. SF treated with hyaluronidase at final concentrations of 0−0.50 μg/mL did not fully pass through cellulose acetate membrane filters due to incomplete hyaluronidase degradation of hyaluronic acid, producing <200 μL of flow-through (Figure S3a). Concentrations of 0.75−2.00 μg/mL however all yielded the same greater volume of flow-through (>550 μL). No differences were identified between the global proteome profiles between the different hyaluronidase treatment protocols (Figure S3b).

ProteoMiner Bead Protein Fractions. ProteoMiner columns were found to be effective in equaling the protein concentration dynamic range (Figure S4a). Most of the protein was removed within the initial flow-through with 0.9% attaching to the beads for further analysis of low abundant proteins (Figure S4b).

ProteoMiner Bead Protein Loading. ProteoMiner columns were found to increase the number of identified

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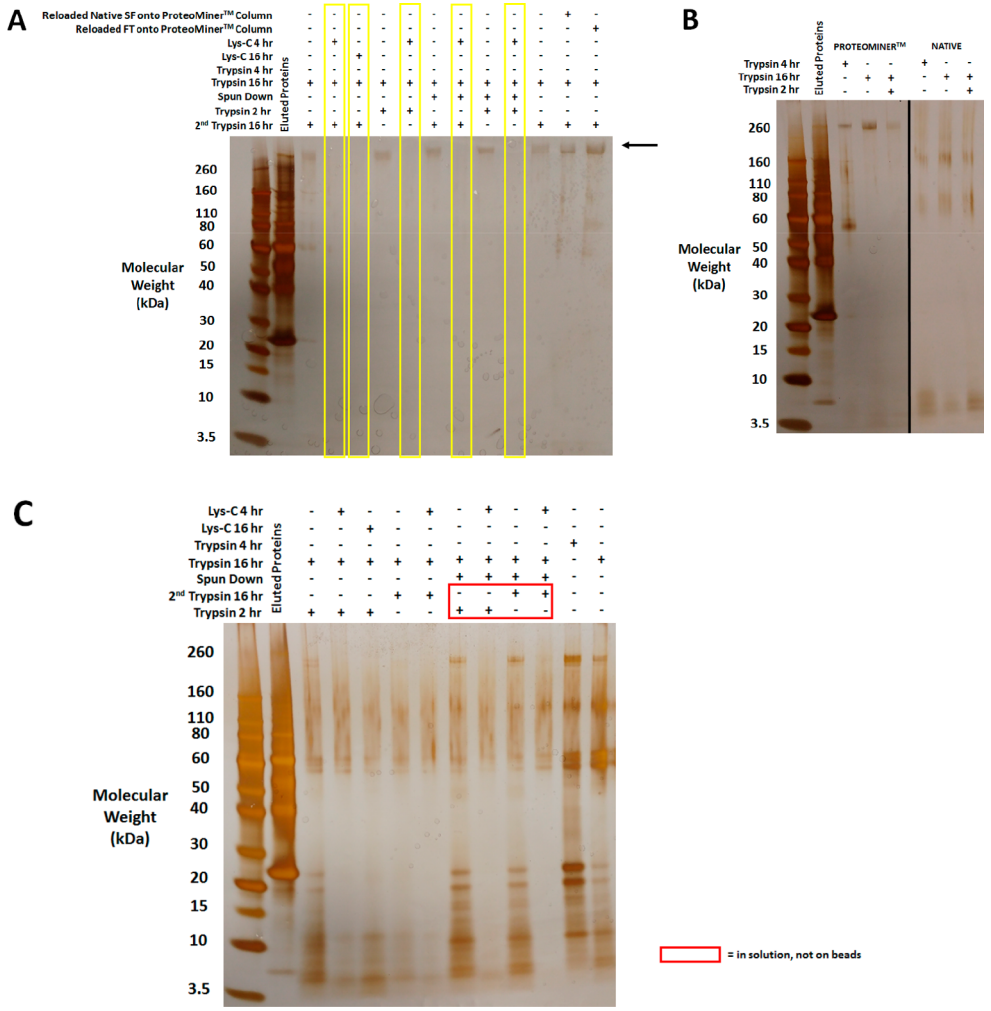

Figure 3. Protein profiles of native and ProteoMiner processed equine synovial fluid following protein digestion. (A) Different ProteoMiner loading and digestion protocols ± Lys-C endopeptidase predigestion (yellow boxes indicate profiles including Lys-C predigestion, arrow indicates a protein band not present following Lys-C predigestion protocols). (B) Trypsin digestion protocols for native and ProteoMiner processed synovial fluid. (C) ProteoMiner bead protein profiles following digestion protocols (red box indicates last stages of digestion were carried out in-solution, not on the beads). Full protein gel images can be found in Figure S8.

proteins within equine SF compared to native SF analysis, with 1, 2.5, and 5 mg column loadings increasing protein identifications by 112%, 161%, and 201%, respectively (Figure 2b). Proteomic analysis of flow-through following 5 mg protein loading identified a similar number of proteins compared to native SF. For a separate pooled equine SF sample set, increasing the protein loading from 5 mg to 10 mg only resulted in a small increase in protein identifications (21 proteins, 6%) (Figure 2c). At the level of Coomassie staining, tryptic digestion was sufficient for LC-MS/MS analysis for all protein loadings analyzed (1−5 mg) (Figure 2a). Intensity of the highly abundant protein bands, 40−80 kDa, increased with increased protein load. SF was loaded at equal concentration, thus indicating a higher proportion of these proteins within the flow-through with increasing protein load.

Gradient Length and Blank Acquisition. For both native and ProteoMiner processed SF, longer LC gradient lengths resulted in increased numbers of identified proteins, with a higher number of proteins identified following ProteoMiner processing (Figure S6). All of the acquired blank samples, bar one, had a low carry-over of peptides from the previous test sample, all with a median percentage carry-over of less than 1 for peptides identified within the blanks. Running a series of consecutive blanks did reduce the number of peptides identified and their peptide abundance carry-over percentage, although this effect was regarded as minimal.

Synovial Fluid Protein Digestion Profiles: Coomassie Brilliant Blue vs Silver Stain. At the level of protein sensitivity of Coomassie Brilliant Blue staining, the 16 h + 2 h trypsin digestion protocol indicated complete digestion of both native and ProteoMiner processed SF (Figure S7). However, the increased sensitivity of silver staining revealed that while native SF digestion was confirmed as complete, incomplete digestion was present on the ProteoMiner column. Additionally, the level of incomplete digestion was not uniform across different donors.

Protein Digestion Optimization. Neither increased length of trypsin digestion nor an additional trypsin supplementation appeared to alter the protein profile of Native SF following digestion (Figure 3b). However, for on-bead ProteoMiner digestion, increased length of trypsin exposure and supplementation improved digestion efficiency, with clear protein bands present at 50−60 kDa and 260 kDa after 4 h trypsin digestion and near complete digestion following a 16 h

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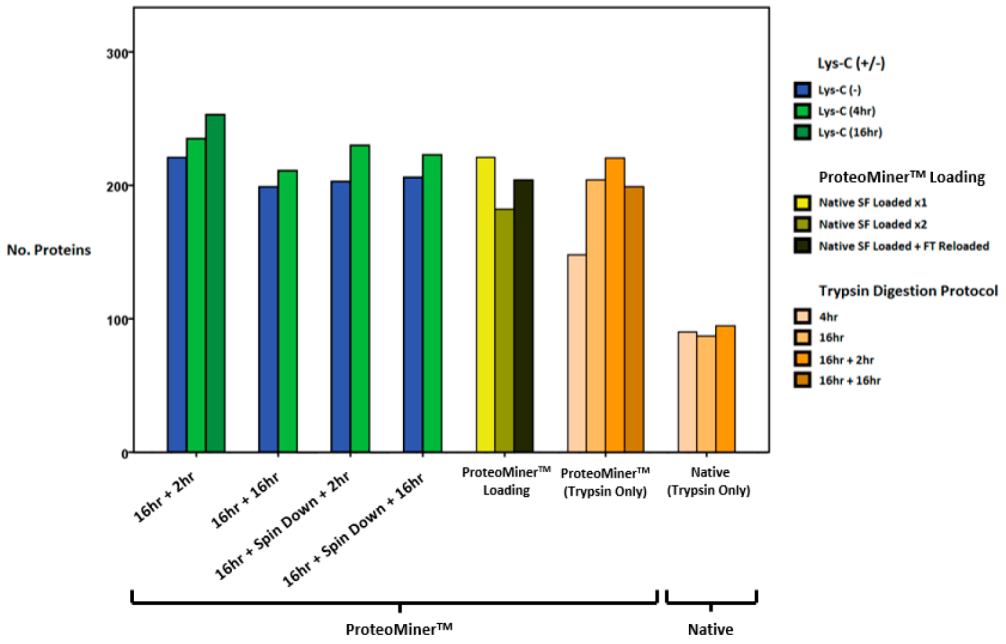

Figure 4. Number of Mascot protein identifications for native and ProteoMiner processed equine synovial fluid following different loading and protein digestion protocols involving trypsin ± Lys-C endopeptidase predigestion. Pilot study, n = 1/digestion protocol.

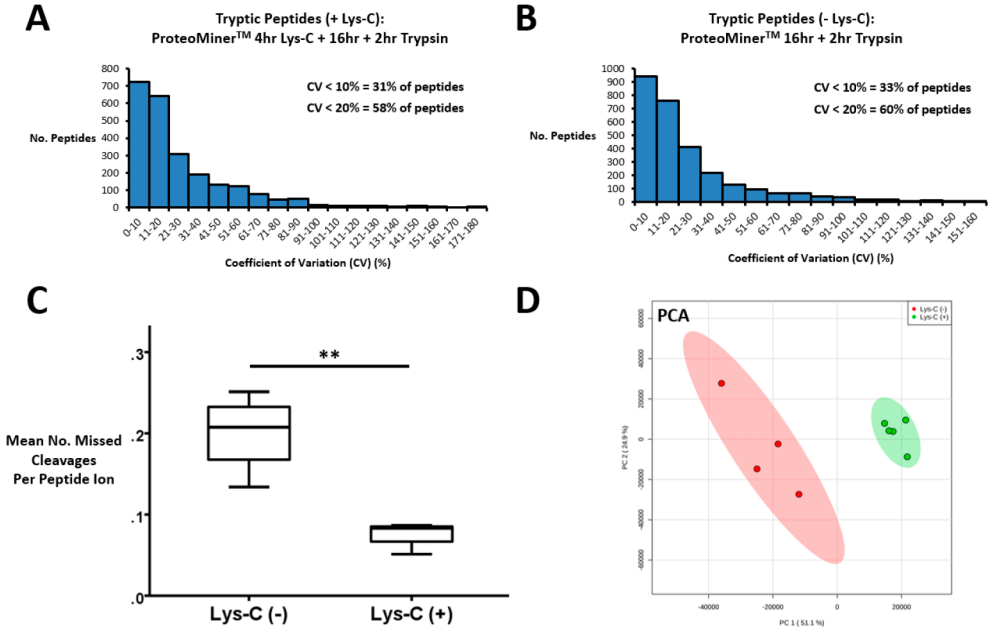

Figure 5. Reproducibility of ProteoMiner processed synovial fluid protein digests with and without Lys-C endopeptidase predigestion. Tryptic peptide reproducibility of three technical replicates (A) with and (B) without a 4 h Lys-C predigestion prior to 16 h + 2 h on-bead trypsin digestion. (C) Average number of missed cleavages per peptide, and (D) principal component analysis (PCA) of tryptic peptide profiles with (green) and without (red) Lys-C predigestion for a series of trypsin digestion protocols. Peptide abundances were analyzed via LC-MS/MS with a 1 h LC gradient. CV = coefficient of variation. $^{**}p < 0.01$.

+ 2 h trypsin digestion protocol. Of the different on-bead tryptic digestion protocols investigated, protein profiles of the digested solutions of equine SF did not reveal as many undigested protein bands as identified previously for human SF (Figure 3a). However, a separate ProteoMiner 16 h + 2 h tryptic digestion of equine SF has also previously led to a series of undigested protein bands, detected at the sensitivity of Coomassie Blue (Figure S9). However, an undigested protein band was detected at >260 kDa for equine SF digests, which was not present for all protocols including the 4 h Lys-C predigest.

For protein profile analysis of the ProteoMiner beads, 4 and 16 h trypsin digestions revealed significant levels of proteins were retained on the beads >50 kDa, indicating significant incomplete digestion (Figure 3c). A 16 h + 2 h tryptic digestion protocol revealed significant retention of undigested proteins bound to the beads, with molecular weights in the range of 3.5−20 kDa. However, the intensity of these bands was significantly reduced with the introduction of the 4 h Lys-

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C predigestion, and to a lesser extent a 16 h Lys-C predigestion step. The intensity of these same bands were less for a 16 h + 16 h trypsin digestion compared to the standard protocol, and were again reduced by the 4 h Lys-C predigestion. Protocols in which the second trypsin digestion was completed in-solution, following an on-bead 16 h trypsin digestion, revealed significant levels of undigested proteins remaining on the beads. However, the 4 h Lys-C predigestion step resulted in complete digestion of these proteins.

Increased trypsin exposure time did not significantly increase the number of proteins identified for native SF (Figure 4). All ProteoMiner processed SF protocols resulted in an increased number of protein identifications compared to unprocessed native SF. Increased time of trypsin exposure increased the number of protein identifications for 4 h to 16 h + 2 h trypsin protocols; however, a reduced number of proteins were identified following a 16 h + 16 h trypsin protocol. Neither repeated loading of native SF onto the column or reloading of flow-through increased the number of protein identifications, with both methods in fact leading to a reduction in the number of identifications. All trypsin digestion protocols in which Lys-C predigestion was included resulted in an increased number of protein identifications compared to the same protocol without a Lys-C predigest. Of all protocols examined, the 16 h Lys-C + 16 h + 2 h trypsin protocol resulted in the highest number of protein identifications.

Tryptic Peptide Reproducibility. When the same trypsin digested sample vial was analyzed via LC-MS/MS three times, reproducibility was high for all sample types, with 67−87% of identified peptides having a CV value of <10% (Figure S10). Triplicate repeats of native SF digests provided a good level of reproducibility, with 78% of identified peptides having a CV value of <20%. Following ProteoMiner processing and an on-bead digestion protocol, reproducibility was reduced to 57% of identified peptides having a CV value of <20%. Analysis of flow-through tryptic peptides provided a reproducibility level between that of native SF and ProteoMiner processing and an on-bead digestion, with 61% of identified peptides having a CV value of <20%.

A 4 h Lys-C predigestion prior to the standard on-bead 16 h + 2 h trypsin digestion protocol did not increase reproducibility in terms of CV values (Figure 5). However, Lys-C predigestion significantly reduced the average number of missed cleavages/peptide during the digestion protocol, with less variation in the number of missed cleavages per sample. Additionally, analysis using a nonsupervised PCA approach, when applying a Lys-C predigestion step, reduced the variability between tryptic digestion protocols, providing a more consistent digestion.

Semitryptic Peptide Reproducibility. For semitryptic peptide quantification, when using variations of a 4 h trypsin digestion protocol with a 1 h LC gradient, a 4 h on-bead trypsin digestion was by far the most reproducible, with 71% of peptides having a CV value of <20% (Figure S11). A 4 h Lys-C predigestion substantially increased the number of identified peptides (38 to 265) although this was accompanied by a significant reduction in reproducibility, with only 29% of peptides having a CV value of <20%. For 16 h + 2 h trypsin digestion protocols with a 2 h gradient, digestion of native SF was the most reproducible, with 74% of peptides having a CV value of <20%. Although ProteoMiner column processing increased the number of identified semitryptic peptides, both with and without a 4 h Lys-C predigestion step, reproducibility dropped significantly, with 33% and 36% of peptides having a CV value of <20% respectively.

DISCUSSION

In this study, protocols were optimized for collection and processing of SF for NMR metabolomic and LC-MS/MS proteomic analysis. Optimal NMR metabolome analysis required SF centrifugation followed by snap freezing in liquid nitrogen. Further investigation into time from death for SF collection is advisable as donor variation in technical triplicates is likely due to continued fluctuation of the post mortem metabolome. Optimization of LC-MS/MS proteomic analysis entailed treatment of SF with 1 μg/mL hyaluronidase and rotational incubation at 37 °C for 1 h with Lys-C endopeptidase predigestion greatly improving on-bead tryptic protein digestion when used in conjunction with small-capacity ProteoMiner column kits. For semitryptic peptide identification, a 16 h + 2 h tryptic digestion of native SF and a 4 h on-bead tryptic digestion were identified as the most reproducible protocols.

SF is an important biofluid to further understand the pathogenesis of articular diseases, and identify specific biomarkers, as it is situated in close proximity to various tissues that are primarily altered by these pathologies.[3,4] The use of global metabolite and protein profiling using systematic approaches, including NMR and LC-MS/MS, are becoming increasingly popular. However, to date, there are no agreed standardized published protocols available for collection and processing of SF for these platforms with reproducibility of on-bead digestions of ProteoMiner columns, used for peptide-based depletion, yet to be investigated.

Centrifugation of SF prior to freezing, removing cells and cellular debris, resulted in a distinct metabolome compared to SF, which did not go through this processing step. It would therefore be recommended to undertake NMR metabolomic analysis on cellular-free SF, avoiding the variation and distinct changes that cell lysis and analysis of cellular contents that may incur on the SF metabolome. This current protocol is however unlikely to remove microvesicles, with a longer and faster centrifugation stage required to achieve this. Further work is required to investigate how the inclusion/exclusion of microvesicles would subsequently affect the SF metabolome. Different freezing method protocols did not result in distinct metabolic profiles; however, snap freezing with liquid nitrogen was found to be the most consistent. Snap freezing with liquid nitrogen would therefore be the recommended gold standard freezing method for future studies. However, if SF has been frozen using a different method, it may be acceptable to be included within the same study, provided that cellular material was removed prior to freezing. It should though be noted that freezing methods not involving liquid nitrogen will result in greater variation, which may affect study results. It has also been found that storage of SF at low temperature for prolonged periods can alter the biochemical profile.[22] Therefore, to optimize study design when freezing is required, analyzed SF should be stored for similar periods of time prior to analysis, with this time period kept to a minimum. Using the centrifugation and liquid nitrogen freezing protocol described in this study, we have also demonstrated this method to be reproducible in identifying consistent separate SF metabolite profiles for individual equine donors.

Hyaluronidase breaks down hyaluronic acid and chondroitin sulfate through the cleavage of β-*N*-acetylhexosamine-(1,4)-

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glycosidic bonds, causing extracellular matrix breakdown and reduced SF viscosity, resulting in an increased number of protein identifications during LC-MS/MS analysis.[50,51] During this study, treatment with 0.75 μg/mL hyaluronidase resulted in sufficient digestion of hyaluronic acid, enabling efficient centrifugation through a 0.22 μm pore cellulose acetate membrane. In order to ensure complete digestion, however, a treatment protocol of 1 μg/mL hyaluronidase would be recommended. However, during this study the effect on the number of proteins identified for each hyaluronidase concentration was not investigated. This therefore may be a relevant area for future study. Within this study hyaluronidase treatment was conducted following thawing of frozen SF. Therefore, downstream NMR metabolomics and LC-MS/MS proteomics analysis can be conducted on the same frozen SF samples, given the collection and processing protocols are identical until freezing.

Small-capacity ProteoMiner column kits recommend a minimum protein loading of 10 mg. However, with a maximum loading capacity of 1 mL, as SF protein concentrations are often less than 10 mg/mL (particularly for post mortem samples) this threshold for protein loading can often not be met.[52] Neither initial loading of 5 mg of protein followed by a repeated 5 mg protein load nor reloading of the resultant column flow-through led to an elevation in the number of identified proteins. However, reduced protein loads of 1 mg, 2.5 mg and 5 mg were all found to significantly increase the number of proteins identified, with a 2.5 mg load also shown to be of acceptable reproducibility when undergoing an on-bead protein digestion protocol. Thus, the small-capacity ProteoMiner column kit is still compatible with SF to achieve protein concentration dynamic range reductions, despite there being suboptimal protein loading.

As expected, for both native and ProteoMiner processed SF, longer LC gradients resulted in an increased number of protein identifications.[53] For native SF a 120 min LC gradient resulted in only a small increase in the number of proteins identified compared to a 90 min LC gradient, 166 compared to 153 proteins. As this small increase in protein identifications is likely to include less abundant proteins, which will also be identified within the ProteoMiner processed samples, if native and ProteoMiner processed SF are to be analyzed within the same study, a 90 min LC gradient is sufficient for native SF analysis. For ProteoMiner processed SF, however, a 120 min gradient identified substantially more proteins than a 90 min gradient, and thus this would be a recommended gradient length for this sample type.

Quantitative proteomic study approaches have become an important methodology for biomarker discovery within complex biological samples.[54] However, due to the multivariate nature of sample analysis, a large number of biological replicates are required in order to achieve an adequately powered study. Thus, when undertaking LC-MS/MS, analyzing sufficient samples to achieve adequate study power can be cost prohibitive. Within this study, peptide carry-over onto the following sample run was found to be minimal and resultant sample contamination can therefore be considered insignificant. Inclusion of a "blank" sample prior to the following run did result in a reduced number of peptides carried over and a reduced carry-over percentage of those identified, although these decreases were minimal. Thus, a gold standard approach would be to include a "blank" sample in between acquired sample spectra. However, excluding intersample blanks will have a minimal impact on experimental analysis and may allow for an increased n number within experimental groups and subsequently a higher powered study and more robust statistical analysis.

For all parameters investigated during this study, predigestion with Lys-C prior to tryptic digestion resulted in improved on-bead digestion, irrespective of the tryptic digestion protocol involved. Lys-C digestion resulted in a reduction of undigested proteins bound to ProteoMiner beads, a reduction in the number of peptide missed cleavages, improved reproducibility of tryptic peptide quantification and an increased number of protein identifications. Any of the protocols investigated during this study that included a Lys-C predigestion step would be acceptable for SF proteome analysis. Although a 16 h Lys-C + 16 h + 2 h trypsin protocol produced the highest number of protein identifications, despite a longer Lys-C incubation time, undigested bound proteins remained bound to the beads, which may introduce variability. Although the reason for increased binding of undigested proteins following a 16 h Lys-C digestion compared to 4 h is unknown. 4 h Lys-C + 16 h trypsin on-bead digestion protocols followed by 2 h trypsin digestion (on-bead and in solution) or 16 h trypsin in solution digestion all resulted in minimal levels of undigested proteins remaining bound to the beads. A second 16 h in-solution digestion did not result in an overall increase in the number of identified proteins. Therefore, our recommended on-bead digestion protocol would be a 4 h Lys-C predigestion + 16 h tryptic digestion followed by a 2 h trypsin supplementation, either on-bead or in-solution.

Reproducibility of quantifying tryptic peptides following protein concentration dynamic range compression of SF via ProteoMiner on-bead digestion has not previously been investigated. Although reproducibility decreased compared to native SF, as expected given the additional selective processing stage, reproducibility was still sufficient to retain confidence in this processing step and is certainly advantageous for biomarker discovery given the increased number of peptides identified. As LC-MS/MS analysis of SF is primarily used for discovery investigations, validation of native SF using orthologous methodologies, including Western blotting and enzyme-linked immunosorbent assays, would also provide greater confidence in the results.

Semitryptic peptides are of interest within SF as increased enzymatic activity and cartilage breakdown during arthropathies, such as OA, lead to peptide degradation products that have potential as a diagnostic aid and disease stratification tool.[55] Of the protocols investigated, a 16 h + 2 h tryptic digestion of native SF and a 4 h on-bead tryptic digestion were found to be the most reproducible for semitryptic peptide quantification. Although these protocols resulted in the fewest semitryptic peptide identifications, increased confidence in the semitryptic peptides identified is more advantageous, particularly given the time, cost, and technical difficulty involved in the development of monoclonal antibodies that might lead on from potential neopeptide discovery.[56] Of these two protocols, a shorter, 4 h trypsin protocol would be recommended as longer trypsin incubations can lead to a greater number of nonspecific cleavages, which may potentially generate false positive biological semitryptic peptide identifications.[57] Further validation of semitryptic peptides of interest would always be recommended, using a multiple reaction monitoring targeted MS/MS approach or carrying out digestion protocols

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in $H_2{}^{18}O$ water to separate biological semitryptic peptides from those generated via miscleavages during tryptic digestion.[58−60]

CONCLUSION

During this study we have optimized collection and processing protocols for NMR metabolomic and LC-MS/MS proteomic analysis of SF. For optimal metabolomic NMR analysis reproducibility, SF should first be centrifuged then frozen via snap freezing in liquid nitrogen. For proteomic analysis, treatment of SF with 1 μg/mL hyaluronidase and rotational incubation at 37 °C for 1 h provided sufficient enzymatic activity to enable efficient centrifugation through a 0.22 μm pore cellulose acetate membrane. Lys-C endopeptidase predigestion was identified to greatly improve on-bead tryptic protein digestion when used in conjunction with small-capacity ProteoMiner column kits, resulting in a reduction of undigested proteins bound to ProteoMiner beads, a reduction in the number of peptide missed cleavages, improved reproducibility of tryptic peptide quantification, and an increased number of protein identifications. To maximize protein identifications using ProteoMiner columns, a 4 h Lys-C predigestion + 16 h tryptic digestion followed by a 2 h trypsin supplementation would be recommended. For semitryptic peptide identification, a 16 h + 2 h tryptic digestion of native SF and a 4 h on-bead tryptic digestion were found to be the most reproducible.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.0c00035.

Liquid Chromatography Tandem Mass Spectrometry—Detailed Methods; Figure S1: Standard trypsin digestion protocol for on-bead ProteoMiner protein digestion, native synovial fluid (SF), and ProteoMiner column flow-through protein digestion; Figure S2: PC2 RMS (Principal component 2 root-mean-square) values for the 25 metabolite peak components with the highest magnitude, differentiating between spun and unspun equine synovial fluid following 1D ^{1}H NMR metabolome analysis; Figure S3: Synovial fluid hyaluronidase treatment optimization; Figure S4: Synovial fluid protein fractions during ProteoMiner column processing; Figure S5: ProteoMiner column loading of synovial fluid (full protein gel image); Figure S6: Number of peptides identified within native and ProteoMiner processed equine synovial fluid using LC-MS/MS for 60, 90, and 120 min LC gradients; Figure S7: Protein profiles of native and ProteoMiner processed human synovial fluid following trypsin digestion and Coomassie Brilliant Blue or silver staining; Figure S8: Full protein gel images for protein profiles of native and ProteoMiner processed equine synovial fluid following protein digestion; Figure S9: Undigested proteins within 12 equine synovial fluid samples following a 16 h + 2 h on-bead tryptic digestion protocol using ProteoMiner beads; Figure S10: Technical reproducibility of tryptic peptide abundances following a 16 h + 2 h trypsin digestion protocol of native equine SF with the same digested sample analyzed three times and digestion triplicates, 2.5 mg protein loaded ProteoMiner columns with the same digested sample analyzed three times and digestion triplicates and their subsequent flow-through, with the same digested sample analyzed three times and digestion triplicates; Figure S11: Reproducibility of semitryptic peptide abundances within equine synovial fluid analyzed by technical triplicates; Table S1: Nuclear magnetic resonance and liquid chromatography-tandem mass spectrometry protocols investigated during this study for metabolite, protein, and peptide identification and quantification; Table S2: Different digestion protocols of native and ProteoMiner processed equine synovial fluid. (PDF)

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Wrote the manuscript (J.A.), revised the manuscript (J.A., M.M.P., P.C., M.J.P., L.R.M., S.J.), sample collection (J.A., S.J.), experimental procedures (J.A., M.M.F), analyzed the data (J.A., M.M.P., M.J.P., M.M.F), experimental design (J.A., M.M.P., P.C., M.J.P., L.R.M.). All authors read and approved the final manuscript.

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Notes

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■ ABBREVIATIONS

1D SDS PAGE, one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis; CHAPS, 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; CPMG, Carr−Purcell−Meiboom−Gill; CV, coefficient of variation; FDR, false discovery rate; FT, flow-through; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; MCP, metacarpophalangeal; MS, mass spectrometry; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PCA, principal component analysis; SF, synovial fluid; TFA, trifluoroacetic acid; TSP, trimethylsilylpropanoic acid.

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