LETTER TO THE EDITOR

Beyond pre-analytical and analytical concerns in the study of synovial fluid proteome: description of an optimized Gel-based protocol

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To the Editor,

Synovial fluid (SF) is a highly viscous and mucinous substance, whose viscosity mainly depends on its high concentration of hyaluronic acid. SF is produced by the synovium, a specialized connective tissue that lines diarthrodial joints. SF mainly aims to lubricate joints, but it also allows the circulation of nutrients and catabolites between the avascular articular cartilage and the vascularized synovial membrane (1). SF is an ultrafiltrate of plasma, thus synovial proteome includes both plasmatic proteins and proteins secreted from the surrounding tissues (e.g. the articular cartilage and synovium) (1-3).

As SF is in the closest proximity with the joint tissues, which are primarily altered during articular diseases, it could provide relevant information in orthopaedic and rheumatologic diseases (4,5). However, the lack of normal SF samples is a big concern in the study of this biologic fluid.

Proteome analysis or "proteomics" is the large-scale protein-based systematic analysis of the protein content, from a cell, tissue or entire organism (5), Proteomic plays a key role in the search for disease-specific biomarkers, which could be also selectively modulated with specific drugs (5,6).

Several studies have focused on the study of SF fluid proteome in joint diseases, however, the lack of consensus arises on the preanalytical issues, including i) the SF collection (e.g. several studies have analyzed *post-mortem* SF samples or animal samples), ii) its storage, iii) the treatment to overcome its viscosity (e.g. SF is treated with different concentrations of hyaluronidase, that breaks down hyaluronic acid and chondroitin sulfate) and analytical, issues mainly related to the strategies used to obtain highly abundant SF proteins depletion (6). Hence, SF has a large protein concentration dynamic range (3), that

Keywords: synovial fluid; proteomics; two-dimensional electrophoresis (2DE); hyaluronidase

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0393-974X (2021)

could mask the detection of low abundant proteins thus compromising SF proteome analysis (7).

This study aims to define an optimized sample processing protocol for human synovial fluid proteome analysis, employing a gel-based proteomics approach.

MATERIAL AND METHODS

Patient selection

In the present study, 16 SF samples were collected from patients undergoing knee arthroscopy for acute meniscal (mean age: 24.65 years old) at the Orthopaedic and Trauma Unit, University of Bari "Aldo Moro", between January 2019 and January 2020. Exclusion criteria: diabetes mellitus; coagulopathies; rheumatoid arthritis or other autoimmune arthritis; chronic inflammatory diseases; previous surgery of the affected knee; previous intra-articular injections in the last 12 months; osteoarthritis; obesity; the history of joint infection.

All the patients underwent a preoperative clinical, radiographic and laboratory evaluation, following the current clinical practice standards. During enrolment, anthropometric data, drugs assumption and comorbidities were recorded.

Ethical clearance was obtained from our centre's clinical research ethics as per the 1964 Declaration of Helsinki (code: SINOVIALE-20), all participants signed a consent form before enrollment in the study.

Synovial fluid sample collection

The patient underwent local anaesthesia and then was positioned in supine decubitus with a pneumatic tourniquet applied to the thigh and set at 100 mmHg over the systolic pressure, to avoid blood contamination. The SF samples were obtained through knee joint arthrocentesis, before starting the arthroscopic surgical procedure. A heparinized syringe with an 18 gauche needle was inserted into the suprapatellar pouch of the knee and 2-4 mL of synovial fluid were aspirated and aliquoted into 500µl amounts. Each 500 µl aliquoted sample was centrifuged at 400´g at 4°C for 5 min to remove cells and debris. The aliquoted samples were then stored at -80°C in liquid nitrogen until analysis. A single frozen sample was thawed and used only once.

Experimental Protocol 1: the search for the optimal hyaluronidase concentration

Cell-free SF samples from non-arthritic patients (i.e. patients undergoing knee arthroscopy for acute meniscal injury) were treated with 1 µg/mL hyaluronidase (Sigma-Aldrich, Gillingham, UK) at 37°C for 1 h, or with 1 mg/mL hyaluronidase (Sigma-Aldrich, Gillingham, UK) at 37°C for 1 h, to assess the optimal hyaluronidase concentration. All samples were then centrifuged at 10.000´g, at 5°C for 10 min. The total protein concentration of SF was measured by a colourimetric assay based on the Bradford dye-binding method (BioRad Protein assay) yielding a mean of 13µg/µL (±1.4).

Experimental Protocol 2: the search for the optimal high-abundance protein depletion technique

The SF samples treated with 1 µg/mL hyaluronidase underwent two different high-abundance protein depletion techniques. All the samples were divided into two aliquots, one of which was treated with affinity columns (ProteoPrep® Immunoaffinity Albumin and IgG Depletion Kit, Sigma-Aldrich, Gillingham, UK) and processed according to the manufacturer's instructions.

The remaining SF aliquot was concentrated by 3-kDa cut-off Amicon filter devices (Millipore, Billerica, MA) to reach the concentration of about 25 µg/µL and treated with ProteoMiner™ Small Capacity beads (Bio-Rad Laboratories, Hercules, California, USA). 5 mg of SF proteins were appropriately adapted to its spin column and processed according to the manufacturer's instructions.

Following both the high-abundance protein depletion techniques, all SF samples were then centrifuged (10.000´g, at 5°C for 10 min) and the protein concentration was determined by Bradford assay.

Two-dimensional gel electrophoresis (2DE)

SF proteins enriched with the two different methods were then analyzed by 2D-PAGE, as previously described by Taurino et al.(6), with minor modifications. Briefly, isoelectrofocusing (IEF) was carried out using 7 cm pH 3-10 NL gradient Immobiline IPG strips, previously rehydrated with 125µL IEF buffer overnight at 20°C. Forty µg of SF proteins were loaded onto rehydrated IPG strips and focused for a total of 22 kVh produced by overnight run (PROTEAN IEF cell, Bio-Rad) and then stored at -70°C.

After strips equilibration, the second-dimension run was carried out using NuPAGE™ 4-12% Bis-Tris ZOOM™ Protein Gels (Thermo Fisher Scientific Waltham, Massachusetts, USA). Both NuPAGE™

Table I. *Patients' data.*

Patients (n)	16
Age	
Mean ±SD	24.65±4.73
Range	19-34
Gender	
Male, n (%)	11 (68.75%)
Female, n (%)	5 (31.25%)
BMI (Kg/m^2)	
Mean ±SD	22.16 ±1.7
Smoking status	
# of smokers, n (%)	2 (12.5%)

BMI= Body Mass Index; CRP= C-Reactive Protein; SD= Standard Deviation.

MES SDS Running Buffer and NuPAGE™ MOPS SDS Running Buffer were used. Gels were stained with SYPRO® Ruby (Invitrogen™, Carlsbad, CA) according to the manufacturer's protocol and acquired with a PROXPRESS 2D scanner (Perkin ElmerLife Sciences, Cambridge, UK). Image Master Platinum 2D software was used for image analysis of analytical 2D gels as previously described (7). 2DE gels of all SF samples were run at the list in duplicate.

Statistical analysis

The results of the quantitative variables were expressed as mean ± SD. Statistical analysis was performed using SPSS software (version 23; IBM Corp, Armonk, NY). The Mann-Whitney U test was used. Statistical significance was set at $p<0.05$ level of confidence.

RESULTS

The demographic and clinical data of the patients enrolled in the study are summarized in Table I. Sixteen patients were recruited, with a mean age of 24.65 years old; 31.5% of patients were females and 68.75% males.

First, we evaluate the impact of two very different hyaluronidase concentrations on SF samples, and we found that 1 µg/mL was enough to reduce the viscosity of SF, making it suitable for subsequent proteomic analysis.

Table II shows the mean protein spots number in samples treated with different hyaluronidase concentrations. Image analysis provided a significantly lower protein spots number with 1mg/mL compared with 1 µg/mL hyaluronidase-treated SF ($p=0.001$; black arrows in Figure-1A). Subsequently, we evaluated the efficacy of two major methods for abundant plasma proteins depletion in SF. Specifically, we assessed the effect of albumin and IgG depletion kit and ProteoMiner™ on the number of protein spots identified in the 2DE proteomic map of SF.

Table III shows the mean protein spots number in SF samples without depletion, in SF samples treated with affinity columns or ProteoMiner™. A significantly higher protein spots number was obtained in SF samples treated with ProteoMiner™, compared with albumin and IgG-depleted kit ($p= 0.001$). The non-depleted SF samples shared 56.6% protein spots with immune-depleted samples (mean=215) and 59.8% protein spots with ProteoMiner™ treated samples (mean=277, Table III). Moreover, 273 mean protein spots were shared between immune-depleted and ProteoMiner-treated SF samples and 171 mean protein spots were shared among the three differently treated SF aliquots.

Table II. *Mean protein spots number in samples treated with different concentrations of hyaluronidase: comparison between groups (Mann-Whitney U test).*

	Samples treated with 1µg/mL hyaluronidase	Samples treated with 1mg/mL hyaluronidase	***p*-value**
Protein spots number (Mean ±SD)	418±57	288±40	**0.001***

*significant p-value

Table III. *Mean protein spots number in samples without depletion, treated with affinity columns (ProteoPrep® Immunoaffinity Albumin and IgG Depletion Kit) or treated with ProteoMiner™.*

	Samples without depletion	Samples treated with affinity columns only (ProteoPrep®)	Sample treated with ProteoMiner™	*p*-value§	Contrasts *p*-value°
Protein spots number (Mean ±SD)	367±50	351±47	611±70	**0.003***	0.783 undepleted samples vs ProteoPrep **0.001*** undepleted samples vs ProteoMiner **0.001*** ProteoPrep vs ProteoMiner
Number of protein spots shared with untreated samples Mean (%)	-	215 (56.6%)	277 (59.8%)	-	-

*§Kruskal-Wallis test; °Mann-Whitney U test; *significant p-value*

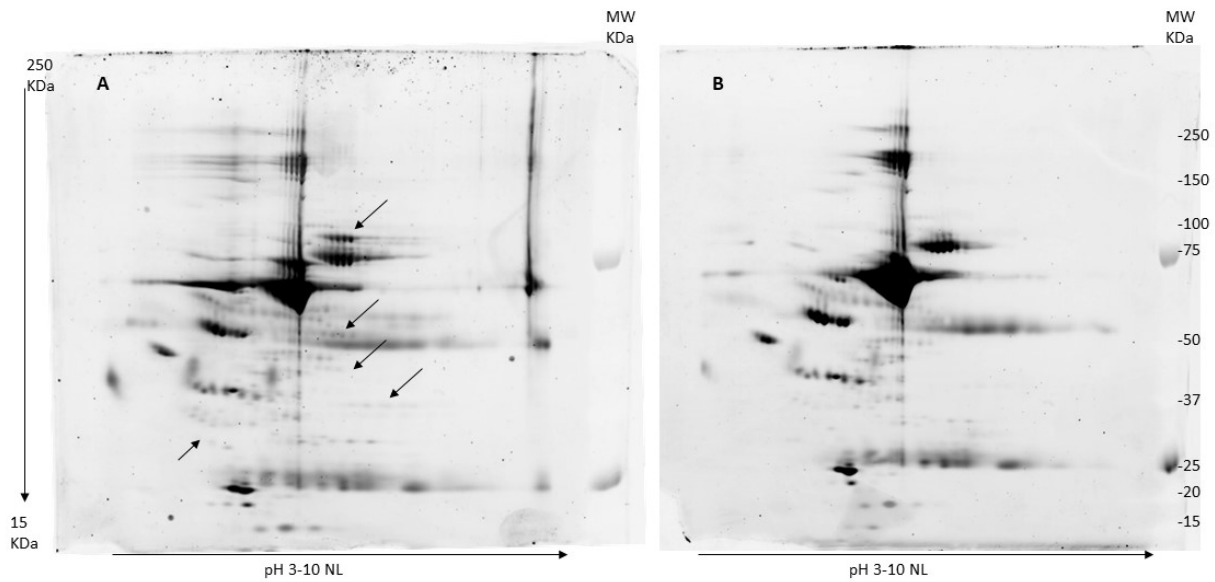

Fig. 1. *Two-dimensional gels of two SF aliquots of the same sample, stained with SYPRO Ruby. Representative 2D gel of SF diluted (1:1, v/v) with solubilization buffer containing **A)** 1 µg/mL of hyaluronidase or **B)** 1 mg/mL of hyaluronidase. MW markers: Precision Plus dual colour (Bio-Rad). The arrows indicate protein spots more expressed in the 2D gel of SF proteome obtained treating the sample with a lesser amount of hyaluronidase.*

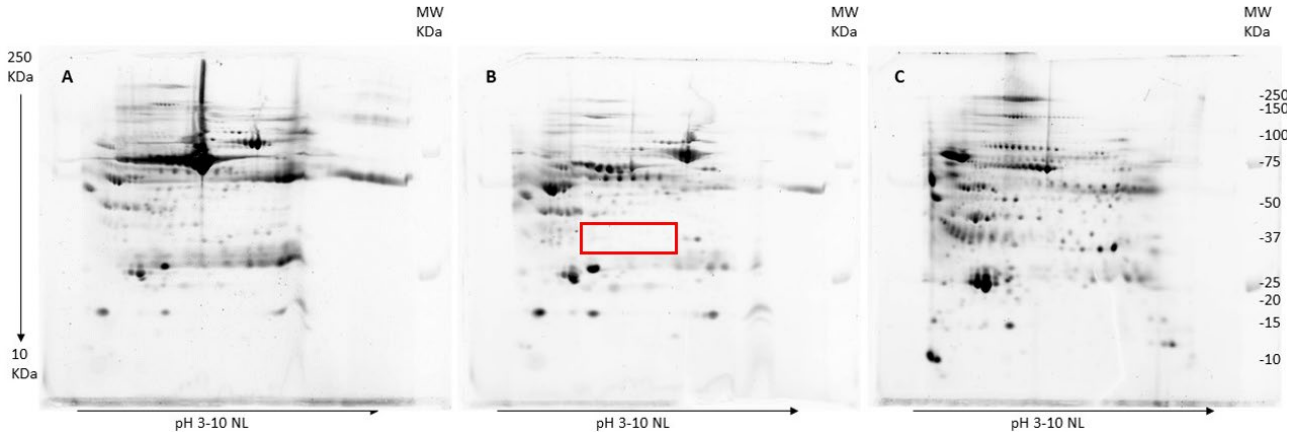

Fig. 2. *Two-dimensional gels of three aliquots of the same SF samples, stained with SYPRO Ruby.* ***A)*** *Representative 2D gel of SF;* ***B)*** *albumin depleted SF by ProteoPrep;* ***C)*** *SF treated by ProteoMiner™. MW markers: Precision Plus dual colour (Bio-Rad, Hercules, California, USA). The red box indicates protein spots less expressed in the 2D gel of SF proteome albumin and IgG depleted by affinity column.*

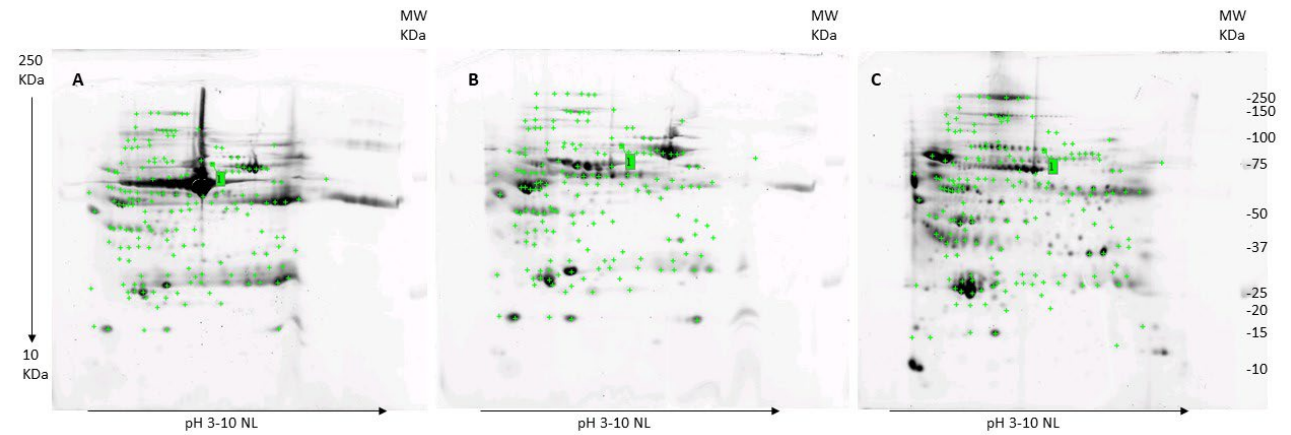

Fig. 3. *Two-dimensional gels of three aliquots of the same SF sample, stained with SYPRO Ruby.* ***A)*** *Representative 2D gel of SF;* ***B)*** *albumin depleted SF by ProteoPrep (Sigma);* ***C)*** *SF treated by ProteoMiner™ (Bio-Rad). MW markers: Precision Plus dual colour (Bio-Rad, Hercules, California, USA). The green crosses indicate the protein spots shared (pairs) among the three maps.*

Figure 2 and Figure 3 show representative 2D gels of three differently treated SF aliquots of the same sample: without depletion (Figure 2A and 3A); treated with the Immunoaffinity Albumin and IgG Depletion Kit (Figure 2B and 3B); treated with the ProteoMiner™ (Figure 2C and 3C).

DISCUSSION

The study of the synovial fluid proteome has been gaining importance in recent years, since it has shown the up-regulation of several components of the classic complement pathway in osteoarthritic knee SF (1-3, 8-10). To the best of our knowledge, this is the first study aiming at describing an optimized protocol for SF analysis, using a proteomic gel-based approach.

SF sample selection plays a key role in SF proteome analysis, spanning from animal-derived SF samples, *post-mortem* SF samples, purchased samples to pathological samples – i.e., samples from OA or RA patients – (1, 8-10). In the current study,

16 SF samples from non-OA knees were analyzed.

Reducing SF viscosity with hyaluronidase is mandatory, but there is no consensus about the optimal hyaluronidase concentration. Therefore, in the present study, we compared the 2DE proteome profiles of SF samples treated with two different hyaluronidase concentrations (11). We found hyaluronidase 1 μg/mL is the best concentration to reduce SF viscosity, while a high hyaluronidase concentration (i.e. 1mg/mL) may have proteolytic activity.

The next steps aimed to solve one of the major concerns in SF proteome analysis, i.e. high-abundant proteins (e.g. albumin and IgG) depletion, since these proteins may mask the detection of low-abundant ones, on a 2DE gel. We tested two different strategies to overcome this problem, namely affinity depletion and ProteoMiner™. We found a significantly higher protein spots number in ProteoMiner™-enriched SF samples providing 334 unique mean protein spots; these findings are consistent with those reported by Pisanu et al. in serum proteomics (12). The optimized standard sample processing protocol for human SF proteome analysis described in the present paper could be helpful also in the gel-free proteome approach of SF analysis (i.e. shotgun analysis).

Our findings are relevant in light of the increasing interest in SF study in orthopaedics and rheumatology, to improve the diagnosis and the management of several joint diseases, including osteoarthritis, rheumatoid arthritis, other autoimmune non-rheumatoid arthritis, osteochondrosis, articular infection and periprosthetic joint infection.

REFERENCES

1. Vicenti G, Bizzoca D, Carrozzo M, Solarino G, Moretti B. Multi-omics analysis of synovial fluid: a promising approach in the study of osteoarthritis. J Biol Regul Homeost Agents 2018; 32(6 Suppl. 1):9–13.
2. Vicenti G, Bizzoca D, Nappi V, et al. Serum biomarkers in the diagnosis of periprosthetic joint infection: consolidated evidence and recent developments. Eur Rev Med Pharmacol Sci 2019; 23(2 Suppl):43–50. doi:10.26355/eurrev_201904_17473
3. Balakrishnan L, Nirujogi RS, Ahmad S, et al. Proteomic analysis of human osteoarthritis synovial fluid. Clin Proteomics 2014; 11(1):6. doi:10.1186/1559-0275-11-6
4. Salari P, Grassi M, Cinti B, Onori N, Gigante A. Synovial fluid calprotectin for the preoperative diagnosis of chronic periprosthetic joint infection. J Arthroplasty 2020; 35:534–7. doi:10.1016/j.arth.2019.08.052
5. Corigliano A, Preianò M, Terracciano R, et al. C3f is a potential tool for the staging of osteoarthritis. J Biol Regul Homeost Agents 2017; 31(4 Suppl. 1):29–35.
6. Taurino F, Stanca E, Siculella L, et al. Mitochondrial proteome analysis reveals depression of the Ndufs3 subunit and activity of complex I in diabetic rat brain. J Proteomics 2012; 75(8):2331–41.
7. Rocchetti MT, Alfarano M, Varraso L, et al. Two dimensional gel phosphoproteome of peripheral blood mononuclear cells: comparison between two enrichment methods. Proteome Sci 2014; 12(1):46. doi:10.1186/s12953-014-0046-1
8. Peffers MJ, McDermott B, Clegg PD, Riggs CM. Comprehensive protein profiling of synovial fluid in osteoarthritis following protein equalization. Osteoarthr Cartil 2015; 23:1204–13. doi:10.1016/j.joca.2015.03.019
9. Fernández-Puente P, Calamia V, González-Rodríguez L, et al. Multiplexed mass spectrometry monitoring of biomarker candidates for osteoarthritis. J Proteomics 2017; 152:216–25. doi:10.1016/j.jprot.2016.11.012
10. Yamagiwa H, Sarkar G, Charlesworth MC, McCormick DJ, Bolander ME. Two-dimensional gel electrophoresis of synovial fluid: Method for detecting candidate protein markers for osteoarthritis. J Orthop Sci 2003; 8(4):482–90. doi:10.1007/s00776-003-0657-3
11. Anderson JR, Phelan MM, Rubio-Martinez LM, et al. Optimization of synovial fluid collection and processing for NMR metabolomics and LC-MS/MS proteomics. J Proteome Res 2020; 19(7):2585-97. doi:10.1021/acs.jproteome.0c00035
12. Pisanu S, Biosa G, Carcangiu L, Uzzau S, Pagnozzi D. Comparative evaluation of seven commercial products for human serum enrichment/depletion by shotgun proteomics. Talanta 2018; 185:213–20. doi:10.1016/j.talanta.2018.03.086