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Research Article

Lipoaspirate fluid proteome: A preliminary investigation by LC-MS top-down/bottom-up integrated platform of a high potential biofluid in regenerative medicine

The lipoaspirate fluid (LAF) is emerging as a potentially valuable source in regenerative medicine. In particular, our group recently demonstrated that it is able to exert osteoinductive properties *in vitro*. This original observation stimulated the investigation of the proteomic component of LAF, by means of LC-ESI-LTQ-Orbitrap-MS top-down/bottom-up integrated approach, which represents the object of the present study. Top-down analyses required the optimization of sample pretreatment procedures to enable the correct investigation of the intact proteome. Bottom-up analyses have been directly applied to untreated samples after monodimensional SDS-PAGE separation. The analysis of the acid-soluble fraction of LAF by top-down approach allowed demonstrating the presence of albumin and hemoglobin fragments (i.e. VV- and LVV-hemorphin-7), thymosins β4 and β10 peptides, ubiquitin and acyl-CoA binding protein; adipogenesis regulatory factor, perilipin-1 fragments, and S100A6, along with their PTMs. Part of the bottom-up proteomic profile was reproducibly found in both tested samples. The bottom-up approach allowed demonstrating the presence of proteins, listed among the components of adipose tissue and/or comprised within the ASCs intracellular content and secreted proteome. Our data provide a first glance on the LAF molecular profile, which is consistent with its tissue environment. LAF appeared to contain bioactive proteins, peptides and paracrine factors, suggesting its potential translational exploitation.

Keywords:
Lipoaspirate fluid / Liquid chromatography / Mass spectrometry / Proteomics / Regenerative medicine
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1 Introduction

Adipose tissue (AT) is a specialized connective tissue, featured in different varieties across somatic and visceral body compartments. Rather than being exclusively a fat storage and energy reservoir, AT is currently considered an endocrine organ, able to secrete paracrine factors that influence and regulate several biological functions, in both healthy and disease conditions [1, 2].

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Abbreviations: **AMBIC**, ammonium bicarbonate; **ASCs**, adipose-derived stem cells; **AT**, adipose tissue; **BpB**, blue bromophenol; **DDS**, data-dependent scan; **FA**, formic acid; **FDR**, false discovery rate; **GO**, gene ontology; **IA**, iodoacetamide; **LAF**, lipoaspirate fluid; **LTQ**, linear trap quadrupole; **MeOH**, methanol; **SVF**, stromal vascular fraction; **RT**, retention time; **Tβ4**, thymosin beta 4; **Tβ10**, thymosin beta 10; **iTRAQ**, isobaric tags for relative and absolute quantitation

AT structure comprises fat lobules, made up of differentiated lipid storage cells (adipocytes) supported by a connective stroma (stromal vascular fraction, SVF). The SVF houses collagen fibers and blood vessels, plus wide and heterogeneous cell populations. In particular, adult stem cells with mesenchymal-like phenotype, namely adipose-derived stem cells (ASCs), are known to reside here in perivascular location, and make AT a valuable resource in regenerative medicine [3].

ASCs are multipotent stromal stem cells that share significant molecular and functional features with bone-marrow stromal stem cells [4]. In particular, they have been proved to be able to differentiate along the osteogenic lineage *in vitro* and to induce successful bone healing *in vivo* [5, 6].

AT is commonly harvested from subcutaneous depots through lipoaspiration and is used for autologous transplantation in fat grafting techniques. Lipoaspiration procedures

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cause the mechanical disaggregation of fat lobules, causing the liposucted AT to be a semiliquid compound. This can be separated into three layers, by centrifugation: an "oily" upper layer containing disrupted adipocytes, a tissue fraction (grossly corresponding to the SVF) in the intermediate layer, and a fluid/blood fraction. ASCs are commonly isolated from the tissue fraction through enzymatic digestion, which requires intensive and time-consuming processing, and potentially increases the risk of contamination. In addition, the costs for clinical-grade collagenase, along with the debated residual toxicity, hamper a broader exploitation of ASCs in the clinical practice.

Interestingly, multipotent somatic stem cells, with ASC-like features, have been found also in the fluid portion of lipoaspirates (lipoaspirate fluid, LAF) [7–9].

LAF can be isolated from lipoaspirate specimens by either centrifugation/washing procedures [10], or using automated systems, recently described elsewhere [11, 12]. This portion contains an ASC-like population (namely, LAF cells) suspended in blood/saline fluid, which reasonably contains the secretome of cells comprised in a lipoaspirate, among other components.

Our group recently described that LAF, separated from lipoaspirate specimens through a clinical-grade closed device, retains valuable osteoinductive properties in an *in vitro* co-culture system [12]. Reasonably, these features can be due to the secretome released by LAF-cells. These observations stimulated the interest in investigating the proteomic profile of LAF, which represents the aim of the present study, given that no previous data are currently available regarding LAF protein/peptide composition.

Here we report the results of a pilot investigation on cell-free LAF proteome and peptidome of two independent specimens, performed by means of a top-down/bottom-up LC-MS integrated platform.

2 Materials and methods

2.1 Chemicals and reagents

Iodoacetamide (IA) ≥ 99 %, DL- dithiothreitol (DTT) for electrophoresis ≥ 99 %, ammonium bicarbonate powder (AMBIC) reagent plus ≥ 99 %, acetone ≥ 99 %, glycerol for electrophoresis ≥ 99 %, sodium dodecyl sulfate (SDS) for electrophoresis ≥ 96 %, trypsin (from Porcine pancreas – type IX –S, 90–100 % protein), acetonitrile (ACN) LC-MS hypergrade 99.9 % and Blue bromophenol (BpB) RPE grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA) HPLC grade > 99.5 %, and primary standard TrisHCl buffer ≥ 99 %, were obtained from Fluka (Sigma-Aldrich, Buchs, Switzerland).

Chloroform (RPE grade), suprapur 98–100 % formic acid (FA), acetic acid RPE, and methanol (MeOH) LC-MS hypergrade 99.9 %, were purchased respectively from Prolabo (Fontenay-sous-Bois, France), J.T Baker (Deventer, Holland), Carlo Erba (Milan, Italy) and Merck (Darmstadt, Germania). Ultrapure water was obtained using the P.Nix Power System apparatus (Human, Seoul, Korea).

2.2 Platforms and apparatuses

Monodimensional SDS-PolyAcrylamide Gel Electrophoresis (PAGE) separation was performed using a Criterion Cell apparatus (Bio-Rad, Hercules, CA, USA). Each sample was run twice onto 12% precast gel Criterion XT Bis-Tris, using a 12 wells comb, 11 cm in length, plus one well for molecular weight standard (13.3 × 8.7×0.1 cm, width x length x thickness; Bio-Rad), with a concentration of 5% (w/v) of bisacrylamide crosslinker.

HPLC-ESI-MS/MS analyses were carried out on LTQ Orpitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with ESI ion source coupled to an Ultimate 3000 Micro HPLC (Dionex, Sunnyvale, CA, USA) equipped with a FLM-3000-Flow manager module. The protein and peptide separation was performed on Zorbax 300 SB-C8 (3.5 μm, 1.0 id x 150 mm) and Zorbax 300 SB-C18 (3.5 μm, 1.0 id x 150 mm) chromatographic columns (Agilent technologies, Santa Clara, CA, USA) for top-down and bottom-up analyses, respectively.

2.3 LAF samples processing

2.3.1 Sample collection

Lipoaspirate fluid (LAF) was obtained from two female donors (A and B donor-specimens) through lipoaspiration from the abdominal region. LAF portion was separated from the lipoaspirate using the MyStem Evo® kit device (see Cicione et al. [12], for details), which allowed obtaining an output sample of 50 mL from each specimen. This was subsequently centrifuged at 27 216 x *g* 5 min (4°C) to remove the cellular components. The supernatant was stored at –80°C, until further analysed.

2.3.2 Sample pretreatment

The LAF sample A, underwent four alternative pretreatment procedures, namely methods M1, M2, M3, and M4, to set up the optimum protein extraction procedure, which was therefore applied also to LAF sample B. M1 was a simple and rapid procedure, already described in our previous papers [13–15]. Briefly, the samples were thawed at room temperature, acidified with 0.1 % (v/v) TFA aqueous solution and 2x volumes of ACN were added to deplete the most abundant and interfering proteins. After centrifugation, the resulting supernatant was liquid/liquid extracted with 2x volumes of chloroform, to remove possible residual lipids in the sample.

M2-4 pretreatments were based on fast protein fractionation by precipitation using acetone. Details of the methods are reported below.

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In the M2 method, protein precipitation was performed adding 4x volume of cold (–20°C) acetone to each sample aliquot, hence vortex-mixed (1 min), incubated for 60 min at –20°C and then centrifuged for 10 min at 21 952 x *g*. The supernatant was discarded without dislodging the protein pellet. The remaining acetone was left to evaporate at room temperature for 30 min. The protein pellet was resuspended in aqueous TFA 0.4 % (v/v). Chloroform (2x volumes) was added to remove the sample lipid component possibly still present in the sample. After vortex-mixing (1 min) the sample was centrifuged (12 045 x g 2 min) at room temperature, and the aqueous phase was collected.

In the M3 method a preliminary extraction of the lipid fraction from untreated LAF was performed, using 2x volumes of chloroform, before accomplishing protein precipitation using acetone as described for the M2.

Method M4 consisted in a single treatment of protein precipitation with acetone, as described above, without chloroform treatment. Sample B was subjected exclusively to the M1 method of choice.

For 1D SDS-PAGE analysis, the sample was diluted 1:1 (v/v) with SDS buffer (Tris-HCl 0.05 M pH 6.8, 2 % (v/v) SDS, 10 % (v/v) glycerol and 100mM DTT). Thereafter, it was sonicated 3×10 s, and incubated first at 100°C in a water bath for 5 min, hence at 37°C for 55 min in a thermomixer (Eppendorf, Hamburg, Deutschland). After centrifugation (700 x *g* 25°C, 15 min), two phases were obtained: an organic phase containing the lipid fraction, and an aqueous phase with hydrophilic proteins. The aqueous phase was used for SDS-PAGE analysis. Protein quantification in the aqueous phase was performed with 2D-QuantKIT (GE Healthcare Bio-Sciences Corporation, Little Chalfont, USA). The SDS-PAGE separation was carried out loading 50 μg of protein in duplicate on a Bis-Tris Criterion XT 12 % precast gels and proteins were visualized with Coomassie Brilliant Blue R-250 staining. Gel images were acquired by Quantity One software (version 4.3.1; Bio-Rad, Hercules, CA, USA).

Cut gel lanes were washed with water, destained with 200 μL of 25 mM NH_4HCO_3/ ACN (1:1), then dehydrated with 200 μL of ACN 100 % (v/v) for 5 min. After organic solvent removal, samples were reduced with the addition of 200 μl of 10 mM DTT solution in 100 mM NH_4HCO_3 at 56°C for 45 min. Samples were then alkylated, using 200 μL of 55 mM IAA in 100 mM NH_4HCO_3, in the dark at room temperature, for 30 min. After complete dehydration, 100 μL of trypsin solution 0.01 μg/μL in 100 mM NH_4HCO_3 was added for in gel protein digestion at 37°C for 16 h. The reaction was stopped by adding 1 % (v/v) aqueous TFA solution in order to obtain a 0.1 % (v/v) final concentration. Digestion peptides were extracted from gel by treating slices at 37°C and slight stirring with aqueous TFA 1 % (v/v) (20 min) followed by water/ACN (40:60, v/v) solution containing 0.1 % (v/v) TFA (15 min) and 100 % (v/v) ACN. The resulting supernatants were collected, pooled and lyophilized and then resuspended with 40 μL of water/ACN solution (97:3, v/v) containing 0.1 % (v/v) FA before LC-MS analysis.

2.4 Top-down/bottom-up HPLC-ESI-LTQ-Orbitrap-MS analyses

2.4.1 Top-down HPLC-MS analysis

Top-down analyses were performed by μHPLC coupled to high resolution LTQ-Orbitrap mass spectrometry with an ESI source. Proteins and peptides were separated using on an RP-C8 column in gradient elution, using aqueous FA 0.1 % (v/v) as eluent A and ACN/H_2O (80:20, v/v) 0.1 % FA (v/v) as eluent B. The following step gradient was applied: from 5 to 55 % B (40 min); from 55 % to 100 % B (8 min); from 100 % to 5 % B (9 min) (% values, v/v) at flow rate of 80 μL/min. The injection volume was 20 μL. The following MS parameters were set: capillary temperature 250°C, source voltage 4 kV, capillary voltage 37 V, tube lens voltage 245 V. The acquisition of high resolution full scan MS and MS/MS spectra were carried out in data-dependent scan mode (DDS) with a resolution of 60 000 and 30 000, respectively, in 300–2000 m/z range of acquisition, selecting the three most intense multiply charged ions acquired every 3 ms scans and fragmenting them by collision-induced dissociation (CID) (35% normalized collision energy).

2.4.2 Bottom-up HPLC-MS analysis

For the bottom-up HPLC-ESI-LTQ-Orbitrap analyses, a RP-C18 chromatography column was used.

The analyses were performed using an aqueous solution of FA (0.1 %, v:v) as eluent A, and ACN/water (80:20, v/v) with 0.1 % (v/v) FA as eluent B. Chromatographic separation was carried out in a three steps gradient elution: from 5 to 55 % of eluent B (40 min), from 55 % to 100 % of eluent B (8 min), from 100 % of eluent B to 5 % (9 min) (% values, v/v) at a flow rate of 80 μL/min. The injection volume was 20 μL.

MS acquisition parameters were the same used for top down analysis above reported.

2.4.3 MS data analysis

The top-down MS and MS/MS spectra collected were analysed manually using the HPLC-MS apparatus management software (Xcalibur 2.2 SP1.48, Thermo Fisher Scientific), along with license-free tools for proteomics analysis (www.expasy.org). The bottom-up data were elaborated using Proteome Discoverer 1.4.0.288 (2012, Thermo Fisher Scientific), based on SEQUEST HT cluster as search engine (University of Washington, USA, licensed to Thermo Electron Corp., San Jose, CA, USA) against Swiss-Prot human proteome database (uniprot-homo+sapiens+reviewed_2014_08, released August 2014). The setting parameters were as follows: retention time window 0–61 minutes; minimum precursor mass 300 Da; maximum precursor mass 10000 Da; total intensity threshold 0.0; minimum peak count 5; Signal

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to Noise (S/N) threshold 3.0; precursor mass tolerance 10.0 ppm; fragment mass tolerance 0.6 Da; use average precursor mass False; use average fragment mass False; maximum retention time difference 0.5 minutes. Trypsin was used as proteolytic enzyme. Bottom-up data were processed setting static carbamidomethylation (+57.021 Da) on cysteine residues and oxidation (+15.995 Da) on methionine residues as dynamic modification. The strict target false discovery rate (FDR) value was set to 0.01, while the relaxed value was set to 0.05.

3 Results

LAF samples from two different donors (A and B) were analysed by LC-MS for protein characterization, using a top-down and bottom-up integrated platform. The use of different approaches was successful in complementing the proteomic data, as it allowed characterizing both small proteins and peptides with their PTMs, through top-down strategy, and large molecules through bottom-up analysis of tryptic digests.

For top-down analysis, different sample pretreatment procedures were tested on the same sample, namely LAF sample A, in order to evaluate the optimum protein extraction procedure. The selected procedure was therefore applied to LAF sample B. The bottom-up analysis was, instead, directly applied to both untreated LAF A and B samples.

3.1 Top-down proteomic analysis

3.1.1 LAF pretreatment procedure optimization

Four different pretreatment methods (M1-4) have been tested on different aliquots of the LAF sample A and compared in order to set the optimal procedure for peptides and proteins extraction for LC-MS analyses by top-down proteomic approach.

The first method (M1) consists in a simple procedure previously applied by our group to other bodily fluids [14, 15]. In this procedure the resulting extract represents the acid-soluble fraction of LAF, purified from abundant proteins and depleted from possible lipid residues. The other three tested methods, namely M2, M3 and M4, were based on protein fractionation by cold acetone precipitation. They differed from one another in the liquid/liquid chloroform extraction step, which was applied either after protein precipitation and acidic resuspension (M2), or directly on LAF sample before the protein precipitation (M3) or not applied at all (M4).

The first comparison among the different pretreatments was based on the evaluation of the total protein concentration by Bradford assay. The highest yield, 2.00 μg/μL, was obtained with M3. The M2 and M4 methods, also based on protein precipitation, showed a comparable result: total protein concentration was 0.79 and 1.10 μg/μL, respectively. Finally, M1 yielded the lowest concentration (0.48 μg/μL). The higher protein content obtained with M3 can be due to the addition of chloroform before protein precipitation. In fact, the addition of the organic solvent to the untreated LAF may facilitate the breaking down of lipoprotein complexes and other aggregates, increasing the total protein content of the aqueous phase. In M2 the chloroform was added to the soluble acidic fraction resulting from dissolution of protein precipitate, still in presence of the insoluble pellet, probably containing lipoprotein complexes. Once pelleted, these complexes result probably less affected by the chloroform breaking up action, explaining the lower total protein content. These results suggest that the total protein content is deeply influenced by chloroform treatment on the LAF specimen; this yielded better output when performed before the protein precipitation step. Indeed the total protein content obtained with M4 was comparable to that yielded with M2.

The M1 provided the most purified sample, representing only the acid-soluble fraction of LAF proteome, depleted of both (most abundant) high molecular weight proteins and lipids. This explains the lower total protein content observed in these samples. In this procedure, the chloroform treatment had a dual role: i) purifying the sample from possible lipids still present and ii) removing the ACN, in order to collect the undiluted purified acidic aqueous phase.

Thereafter, the total ion current (TIC) plots obtained from the alternative methods of LC-MS chromatographic analyses, were also compared and discussed (Fig. 1).

The LC-MS analysis was carried out by injecting for each sample the same total protein content corresponding to 5 μg. Due to the diverse protein yield achieved with the alternative extraction methods (see previous section), different dilutions (with aqueous 0.4 % (v/v) TFA) have been made: 1:1 for M1, 1:2 for M2, 1:8 for M3, and 1:3 for M4.

The extraction methods based on acetone protein precipitation (M2, M3 and M4) showed very similar TIC profiles in the 35–50 minutes elution window, where the most intense signals were recorded. The LC-MS profile, obtained with the first method, showed higher resolved signals in the same retention time region, probably due to the higher purification of the LAF's acid-soluble protein fraction, obtained through the combination of ACN and chloroform pretreatments.

Relevant differences were observed in the 19–35 minutes retention time window, generally characterized by the elution of peptides and more hydrophilic proteins, as it is shown in the grey magnified views in Fig. 1. In this region all four methods revealed a different TIC profile.

The sample obtained with M3 extraction showed a very poor LC-MS profile (Fig. 1). The absence of peaks at retention time that generally characterizes peptides, could be due to chloroform addition to the unacidifed untreated LAF sample. This observation could be possibly explained by the different solubility of peptides based on the pH. The chloroform extraction performed, under physiological pH conditions, on untreated LAF could increase the rate of partitioning of hydrophobic or less polar peptides into the organic phase. Indeed, peptides are generally less polar than proteins, being less structured, and hindering less hydrophobic sites to the aqueous environment. Therefore, although showing the highest protein content, M3 did not result a

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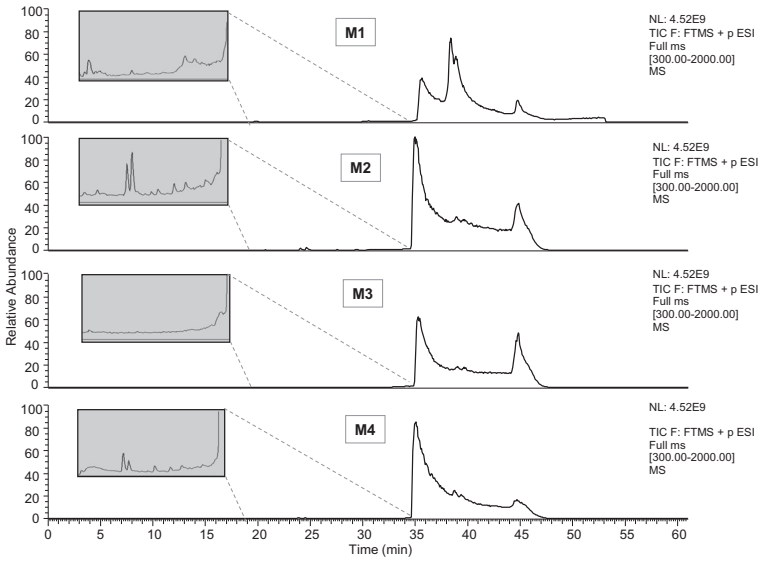

Figure 1. LC-ESI-LTQ-Orbitrap-MS Full scan TIC profiles of LAF sample A obtained by M1-M4 pretreatment procedures (for experimental details see the Materials and Methods section). For each profile an enlarged view of the elution window 19–35 min is also shown.

suitable extraction method for top-down analysis. The other three LC-MS profiles, related to M1, M2 and M4, showed instead many resolved peaks (Fig. 1), within the same elution window (19-35 minutes), belonging to potential peptides and protein presents in the sample. In fact the addition of TFA before the chloroform treatment, producing peptides protonation, probably increased their affinity for the aqueous phase.

Particularly, M2 and M4 provided comparable chromatographic profiles, though characterized by different intensities. Although generally showing lower signals, the M1 allowed the characterization of several small proteins and peptides and showed an improved peak resolution in the 35–45 elution window characterized by the most abundant signals (Fig. 1). This suggested that the M1 method may represent a good compromise, yielding good LC-MS resolution, accomplished by a very rapid and simple pretreatment procedure.

The combination of both ACN and chloroform extraction in acidic environment in M1, produced a purified sample suitable for the identification of small proteins, peptides, and minor components, within a wide chromatographic elution time range. For these reasons, despite yielding the lowest amount of proteins, M1 proved as the method of choice for LAF proteomic analysis by top-down approach.

3.1.2 Top-down protein identification

The M1 method was therefore applied to both A and B LAF samples in order to provide the original identification of their intact proteome. The two samples exhibited different LC-MS TIC profiles compatible with the wide inter-individual variability that characterizes biological specimens.

Table 1 lists the proteins and peptides identified, in the two LAF samples, by top-down proteomic analysis, with corresponding experimental molecular mass (M_r), chromatographic retention time (RT), Uniprot accession, protein name, and characterized PTMs data.

The acid-soluble fraction of LAF, besides albumin fragments, showed the presence of several hemoglobin fragments belonging to both the β- and α-globin chains. Some of these fragments, namely the M_r 1194.62 peptide (VV- hemorphin-7) and M_r 1307.70 peptide (LVV- hemorphin-7), have documented biological activities. They are non-classical opioid peptides specifically found in the central nervous system (CNS), exhibiting several biological actions, including putative roles in blood pressure regulation, learning and memory, intracellular calcium variation and protein phosphorylation [16,17]. A role in cellular homeostasis [18] and tumor cytotoxic and antiproliferative capacity [19] have been also reported, along with a potential role as prognosis biomarker in posterior cranial fossa pediatric brain tumors [20]. The latter was also recognized for the other hemoglobin fragments of M_r of 3274.75, 3325.70, 3472.77 and 3900.96 also identified in LAF.

LAF featured also the thymosin beta 4 (Tβ4) and beta 10 (Tβ10) peptides, along with their C-terminal-truncated forms. Tβ4 is the major G-actin sequestering peptide [21] involved in regulation of G-actin polymerisation/depolymerisation process and cytoskeleton organization [22]. In addition to promote angiogenesis, wound healing and tissues repair [22,23], Tβ4 also exhibits an anti-inflammatory role [24]. Recent papers also evidenced a role of Tβ4 in relation to odontogenic differentiation [25], tooth development [26] and bone formation [27,28]. Conversely, the inhibition of osteogenic differentiation towards promotion of the adipogenic one has also been reported in mesenchymal stem cells [29]. The Tβ4 e Tβ10 C-terminal truncated form have been already characterized by our group in different tissues; however, their biological role is still unclear [30].

Along with the full length protein, also for ubiquitin protein, different C-terminal des-GG and des-RGG proteoforms were detected. Their role is still under investigation: both

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Table 1. Proteins and peptides identified in LAF by top-down LC-MS proteomic analysis

M_r (Da)	RT (min)	Uniprot accession	Protein name	PTMs	Sample A	Sample B
1194.62	25.40	P68871	Hemoglobin chain β **Fragment (34-42)**	–	✓	✓
1307.70	27.59	P68871	Hemoglobin chain β **Fragment (33-42)**	–	✓	✓
2540.28	21.04	H7C013	Albumin **Fragment (27-48)**	–	–	✓
2752.43	24.86	H7C013	Albumin **Fragment (27-50)**	–	✓	✓
2936.56	26.96	H7C013	Albumin **Fragment (27-52)**	–	–	✓
3217.79	37.55	P69905	Hemoglobin chain α **Fragment (107-137)**	–	–	✓
3274.75	29.25	P68871	Hemoglobin chain β **Fragment (2-32)**	–	✓	✓
3325.70	24.10	P69905	Hemoglobin chain α **Fragment (2-33)**	–	✓	✓
3386.83	31.34	P68871	Hemoglobin chain β **Fragment (2-33)**	–	✓	✓
3426.84	34.81	P69905	Hemoglobin chain α **Fragment (111-142)**	–	✓	✓
3472.77	27.57	P69905	Hemoglobin chain α **Fragment (2-34)**	–	✓	✓
3574.86	21.28	O60240	Perilipin-1 **Fragment (458-493)**	–	✓	✓
3900.96	30.05	P68871	Hemoglobin chain β **Fragment (112-147)**	–	✓	✓
4351.35	32.44	P01011	α-1 Antichymotrypsin **Fragment (387-423)**	–	✓	–
4464.43	32.44	P01011	α-1 Antichymotrypsin **Fragment (386-423)**	–	✓	–
4563.44	35.07	P68871	Hemoglobin chain β **Fragment (2-42)**	–	✓	✓
4733.41	20.38	P63313	Thymosin β10 **truncated(-IS C-terminale)**	Acetylation N-terminal	✓	✓
4744.42	19.66	P62328	Thymosin β4 **truncated(-ES C-terminale)**	Acetylation N-terminal	✓	✓
4933.53	20.78	P63313	Thymosin β10	Acetylation N-terminal	✓	✓
4960.49	19.66	P62328	Thymosin β4	Acetylation N-terminal	✓	✓
7074.53	43.94	Q15847	Adipogenesis regulatory factor **Fragment (2-70)**	Acetylation K3	✓	–
7349.70	43.94	Q15847	Adipogenesis regulatory factor **Fragment (2-72)**	Acetylation K3	✓	–
7406.70	44.00	Q15847	Adipogenesis regulatory factor **Fragment (2-73)**	Acetylation K3	✓	✓
7429.84	32.83	P68871	Hemoglobin chain β **Fragment (43-111)**	–	–	✓
7758.03	30.72	P69905	Hemoglobin chain α **Fragment (34-104)**	–	–	✓
7827.07	31.75	P69905	Hemoglobin chain α **Fragment (35-106)**	–	–	✓
7974.14	31.75	P69905	Hemoglobin chain α **Fragment (34-106)**	–	–	✓
8087.22	32.48	P69905	Hemoglobin chain α **Fragment (34-107)**	–	–	✓
8289.50	30.55	P0CG48	Ubiquitin **truncated(-RGG C-terminale)**	–	✓	✓
8400.44	33.60	P69905	Hemoglobin chain α **Fragment (34-110)**	–	–	✓
8445.60	30.55	P0CG48	Ubiquitin **truncated (-GG C-terminale)**	–	✓	✓
8559.64	30.55	P0CG48	Ubiquitin	–	✓	✓
9949.01	30.65	P07108	Acyl-CoA-binding protein	Acetylation N-terminal	✓	✓
10084.48	42.75	P06703	S100A6	des Met1 Acetylation N-terminal	✓	–
11173.88	40.21	P69905	Hemoglobin chain α **Fragment (34-137)**	–	–	✓
11311.86	37.59	P68871	Hemoglobin chain β **Fragment (43-117)**	–		✓
11653.18	39.21	P69905	Hemoglobin chain α **Fragment (34-141)**	–	✓	✓
11809.28	39.25	P69905	Hemoglobin chain α **Fragment (34-142)**	–	–	✓
14961.75	41.67	P69905	Hemoglobin chain α	des-Arg C-terminal	✓	
15116.92	38.62	P69905	Hemoglobin chain α	–	✓	✓
15857.27	38.62	P68871	Hemoglobin chain β	–	✓	✓

forms have been identified by our group in paediatric brain tumour tissues [31]. In a previous study the des-GG was reported to mark a specific breast cancer histotypes [32].

Figure 2 shows the distribution of β-thymosins and ubiquitin proteoforms within the two analysed LAF specimens. Generally the entire forms resulted prevalent over the relative truncated proteoforms with the exception of sample A where the C-terminal des-RGG truncated ubiquitin was largely present.

S100A6 was already identified in ASCs secretome, as being increased upon *in vitro* culture passages [33]. Acyl-CoA binding protein resulted among the proteins mainly upregulated in SVF secretome during adipogenesis [34]. The des-Met N-terminal proteoform of S100A6, N-terminal acetylated on Ala residue, is not yet reported in Uniprot database. The protein was characterized by sequencing a portion of its C-terminal, and by comparing theoretical/experimental MS^2 spectra. This confirmed the hypothesis of N-terminal acetylation, possibly explaining the delta mass observed with respect to the theoretical M_r value.

S100A6 (calcyclin) belongs to the S100 Ca^{2+} binding protein family, having different actions in both intracellular and extracellular compartments [35]. In particular, it was reported to regulate osteoblastic function and bone formation, due to

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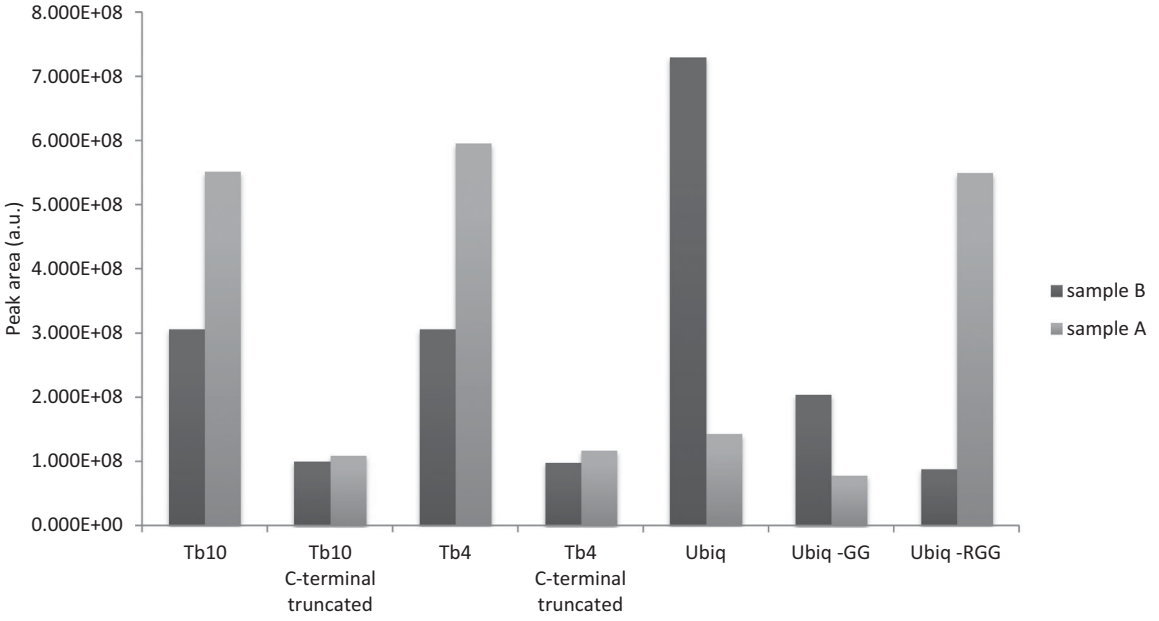

Figure 2. Distribution of the thymosin beta 4 (Tb4), thymosin beta 10 (Tb10) and ubiquitin (Ubiq) proteoforms in LAF samples A and B. In Y-axis the peak area values of the relative extracted ion current (XIC) plots are reported.

the capability of stimulating cells to sense extracellular cations [36]. More recently, S100A6 was identified as a protein expressed in osteoblasts and inhibited upon adipocyte-induced suppression of osteoblastogenesis, in a co-culture model [37]. Also, it was identified as a putative target of glucocorticoid-induced apoptosis in osteoblast progenitors [38].

Top-down analysis of LAF also identified two different C-terminal fragments (387-423 and 386–423) of alpha-1-antichymotrypsin, or SERPINA3, the perilipin-1 fragment 458–493 and three fragments of adipogenesis regulatory factor (2-70, 2–72 and 2–73) all presenting the loss of initial methionine and carrying N-terminal Ala acetylation, PTMs not reported in Uniprot database.

3.2 Bottom-up proteomic analysis

Bottom-up proteomics of LAF samples was based on monodimensional SDS-PAGE separation in coupling with LC-ESI-LTQ-Orbitrap MS of digested bands. Figure 3 shows the gel electrophoresis separation of the two LAF samples. The two samples exhibited similar separation patterns, however different band intensities were observed.

The LC-MS analysis of the separate digested bands of each sample followed by data elaboration by Proteome Discoverer 1.4, filtering for two peptides per proteins and high confidence identification, allowed the identification of several protein species, in part shared by both samples. Figure 4 shows the Venn diagrams (Venny 2.0.2"Computational Genomics Service) and the name and Uniprot accession number of common (i.e. found in both samples) and exclusive (found individually in either A or B sample) proteins. Out of the 89 proteins identified, 46 were common to both samples, while 17 and 26 resulted exclusive of sample A and B, respectively.

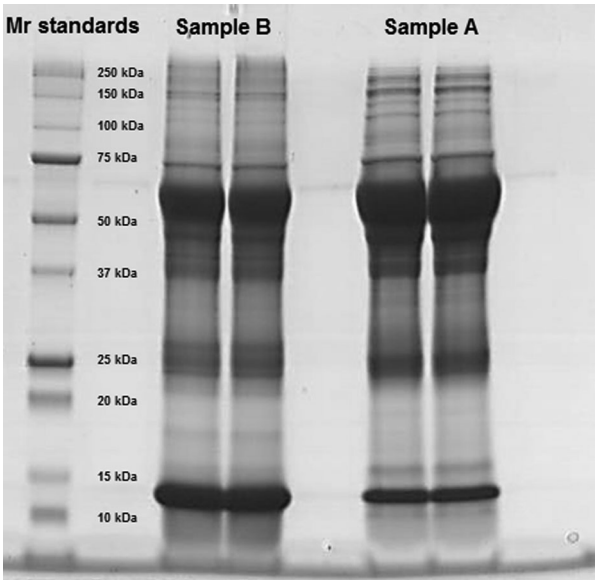

Figure 3. Monodimensional SDS-PAGE separation of LAF sample A and B (for detailed experimental conditions see the Materials and Methods section).

In addition, Fig. 5 shows the gene ontology (GO) classification of the molecular function and biological process annotations obtained by PANTHER (Protein ANalysis THrought Evolutionary Relationships version 9.0) for the common (panels A and B) and exclusive (panel A or panel B) identified proteins.

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Uniprot accession	Protein (sample A and B)
P01834	Ig kappa chain C region
P01859	Ig gamma-2 chain C region
P01857	Ig gamma-1 chain C region
P01876	Ig alpha-1 chain C region
P15090	Fatty acid-binding protein, adipocyte
P69905	Hemoglobin subunit alpha
P68871	Hemoglobin subunit beta
P02042	Hemoglobin subunit delta
P02766	Transthyretin
P62937	Peptidyl-prolyl cis-trans isomerase A
P02792	Ferritin light chain
P32119	Peroxiredoxin-2
P02763	Alpha-1-acid glycoprotein 1
P00915	Carbonic anhydrase 1
P62258	14-3-3 protein epsilon
P02647	Apolipoprotein A-I
P06727	Apolipoprotein A-IV
P04114	Apolipoprotein B-100
P04406	Glyceraldehyde-3-phosphate dehydrogenase
P07195	L-lactate dehydrogenase B chain
P00338	L-lactate dehydrogenase A chain
P21695	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic
P51884	Lumican
P07355	Annexin A2
P04083	Annexin A1
P00325	Alcohol dehydrogenase 1B
P60709	Actin, cytoplasmic 1
P00738	Haptoglobin
P01009	Alpha-1-antitrypsin (Serpina1)
P02790	Hemopexin
P10909	Clusterin
P02774	Vitamin D-binding protein
P08670	Vimentin
P04217	Alpha-1B-glycoprotein
P05155	Plasma protease C1 inhibitor
P35527	Keratin, type I cytoskeletal 9
P04264	Keratin, type II cytoskeletal 1
P02768	Serum albumin
P02787	Serotransferrin
P00751	Complement factor B
P06396	Gelsolin
P19827	Inter-alpha-trypsin inhibitor heavy chain H1
P00450	Ceruloplasmin
P01023	Alpha-2-macroglobulin
P01024	Complement C3
P0C0L4	Complement C4-A

Uniprot accession	Protein (sample B)
P02652	Apolipoprotein A-II
P0CF74	Ig lambda-6 chain C region
P01620	Ig kappa chain V-III region SIE
P01766	Ig heavy chain V-III region BRO
P69891	Hemoglobin subunit gamma-1
P23528	Cofilin-1
P02511	Alpha-crystallin B chain
Q06830	Peroxiredoxin-1
P30043	Flavin reductase (NADPH)
P30041	Peroxiredoxin-6
P00918	Carbonic anhydrase 2
P08758	Annexin A5
P63267	Actin, gamma-enteric smooth muscle
P04220	Ig mu heavy chain disease protein
P06733	Alpha-enolase
P01011	Alpha-1-antichymotrypsin (serpina 3)
P07437	Tubulin beta chain
P68363	Tubulin alpha-1B chain
P02679	Fibrinogen gamma chain
P02675	Fibrinogen beta chain
P02671	Fibrinogen alpha chain
P02730	Band 3 anion transport protein
P08603	Complement factor H
P04040	Catalase
P13645	Keratin, type I cytoskeletal 10
P35908	Keratin, type II cytoskeletal 2 epidermal

Uniprot accession	Protein (sample A)
P0CG05	Ig lambda-2 chain C regions
P0CG04	Ig lambda-1 chain C regions
P01600	Ig kappa chain V-I region Hau
P01598	Ig kappa chain V-I region EU
P01617	Ig kappa chain V-II region TEW
P01861	Ig gamma-4 chain C region
P02753	Retinol-binding protein 4
P63104	14-3-3 protein zeta/delta
P25311	Zinc-alpha-2-glycoprotein
P40925	Malate dehydrogenase, cytoplasmic
P02765	Alpha-2-HS-glycoprotein
Q6NZI2	Polymerase I and transcript release factor
P19823	Inter-alpha-trypsin inhibitor heavy chain H2
P49327	Fatty acid synthase
Q15323	Keratin, type I cuticular Ha1
O43790	Keratin, type II cuticular Hb6
P78385	Keratin, type II cuticular Hb3

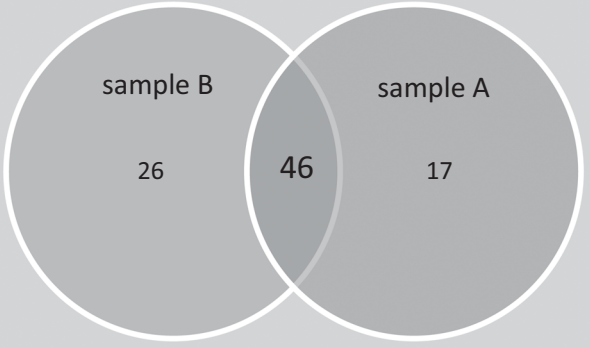

Figure 4. Lists (name and Uniprot accession number) and Venn diagram (Venny 2.0.2″Computational Genomics Service) of the common (i.e. found in both samples A and B) and exclusive (found individually in A or B sample) proteins identified in LAF samples.

The prevalent molecular function, in both common and sample-exclusive proteins, was 'catalytic activity'. Biological processes annotations were more diversified, but showed a large predominance of metabolic and cellular processes. By comparing the GO data of the exclusive proteins, a wider variety of molecular functions and biological processes seemed to characterize the sample B (Fig. 5 panels B) compared with sample A (Fig. 5 panels A).

Among the large number of common proteins identified, several have been reported to be directly or indirectly involved in osteogenic processes or bone related disorders, such as ferritin light chain [39, 40], peroxiredoxin-2 [41, 42], glyceraldeide-3-phosphate dehydrogenase [41], lumican [43, 44], haptoglobin [45, 46], vitamin D-binding protein [46, 47], 14-3-3 protein epsilon and gelsolin [48], serotransferrin [42], complement C3 [41, 42, 46, 49–51], annexin A1 and A2 [48, 51–55], and vimentin [41, 56].

Noteworthy, different isoforms of vimentin, which is involved in the formation of lipid droplets, have been characterized in ASCs [57], ASCs secretome [58] and adipose tissue, suggesting a role for this protein in metabolic alterations occurring in different nosological conditions [59].

Although annexins are generally considered intracellular proteins, the A1, A2 and A5 types were also found in the extracellular compartment and in blood [60]. This is consistent with their identification in the LAF.

Indeed,several other proteins, within our list, have been already described in adipose tissue, being either expressed by cellular components or part of their secretome.

Different cytokeratins, belonging to the keratin type I and II cytoskeletal family, have been identified in both tested LAF samples. In a previous study, the same proteins have been found overexpressed in visceral adipose tissue, compared with subcutaneous depots, being produced by mesothelial cells of the peritoneum surrounding fat lobules [59].

The adipokine retinol binding protein 4, identified in sample A, and the related alcohol dehydrogenase 1B, identified in both samples, have been found expressed in visceral adipose tissue [59]. Moreover, retinol binding protein 4, fatty acid binding protein, peroxiredoxin-1 an peptidyl-prolyl-cis-trans-isomerase A, were reported in SVF-derived secretome and upregulated during adipogenesis [57]. Retinol binding protein, transthyretin, albumin and serpins have been identified in ASCs' secretome [61] together with lumican

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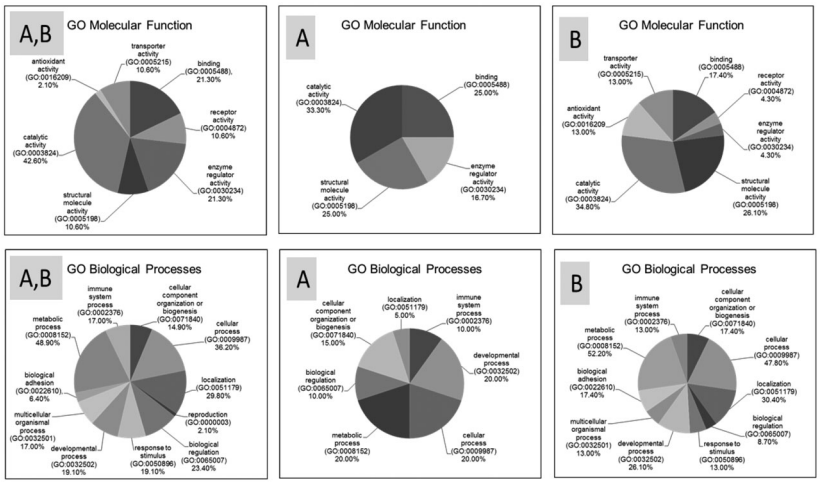

Figure 5. Gene Ontology (GO) molecular function and biological processes classification of the common and exclusive proteins identified in the analysed LAF samples. Panels A, B: protein identified in both LAF samples A and B. Panels A: proteins exclusive of LAF sample A. Panels B: proteins exclusive of LAF sample B.

and beta actin [38]. The annexin A1 and A5, keratin type II cytoskeletal I and type I cytoskeletal 10, alpha crystallin B chain, beta actin, hemoglobin alpha and beta globin chains, resulted abundant and differentially expressed in mature adipocytes of aged-versus-young obese individuals [62].

Lumican, clusterin, annexin A2 and retinol binding protein 4 have been numbered among the 68 most conserved proteins in ASC secretome [62]. Finally, gelsolin and haptoglobin were also identified in ASCs' secretome [58].

4 Discussion

The characterization of adipose tissue proteome and secretome has recently gained an increasing attention. The first study on human adipose tissue secretome appeared in 2007 [62]. Since then, several others have been published focusing on proteomic characterization of either whole adipose tissue, or mature adipocytes, or SVF, or its individual cellular components (including progenitors, preadipocytes, endothelial cells, adipose derived stem cells (ASCs) and blood cells), recently reviewed [2, 58, 59, 63]. However, to the best of our knowledge, no data to date have been reported regarding LAF characterization.

The biological properties of LAF, along with its rapid and easy isolation, make this fluid suitable and attractive for regenerative medicine applications, as a "minimally manipulated tissue" in grafting procedures [12].

In all studies performed so far, proteomic analyses followed the bottom-up approach, by mono- or bidimensional gel electrophoresis and MALDI or LC-MS/MS characterization, also performing quantitative analysis and correlations to diseases.

A different protein expression was found in visceral and subcutaneous adipose tissue depots [64] and in mature adipocytes of obese individuals in relation to age [62]. Kheterpal et al. [66] compared the SVF and mature adipocytes proteome by 2DE in coupling to nanoLC-QTOF analysis evidencing the prevalence of common proteins over the sample-exclusive ones.

A shotgun proteomic study of SVF and subcutaneous depot adipocytes, demonstrated the role of secretory factors, mostly involved in Wnt and TGF-β signalling pathways, in regulating the adipogenic process [34]. Several proteins characterized in SVF secretome resulted upregulated during adipogenesis. A differential expression of several secreted proteins was also found during differentiation of preadipocyte into mature adipocytes by iTRAQ-based quantitative proteomics [67].

Particularly, K. Lee and co-workers [38] studied the ASCs protein expression and secretome in relation to the osteoinductive effect observed after their transplantation in ovariectomized mice: several proteins and cytokines related to osteogenesis and bone regeneration processes were identified. The human ASCs' secretome was characterized *in vitro* by M.J. Lee et al. [68] by shotgun proteomic analysis, aimed at studying the associated-inflammation profile after recombinant human tumor necrosis factor-α exposure. Among the 187 secreted proteins identified, 118 were highly expressed under treatment including inflammatory cytokines, chemokines, proteases and proteases inhibitors involved in monocytes chemotactic migration.

The LAF component originally analysed in this study, may be rationally considered as the acellular fraction of liposuctioned adipose tissue, hence containing a heterogeneous cocktail of biologically active molecules. The proteomic and peptidomic analysis of LAF, performed by an LC-MS top-down/bottom-up integrated platform, evidenced the presence of several protein and peptide components, involved in a variety of biological processes, which may reasonably explain the osteoinductive properties of LAF previously described by our group [12].

Some of the proteins identified in LAF, have been already described as components of either the whole adipose tissue, or the SVF, or part of the ASCs' intracellular and secreted proteome. This evidence may originally demonstrate that

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LAF features a molecular profile that is consistent with its tissue environment. In particular, we have demonstrated that it contains bioactive proteins and peptides produced by adipose tissue cytotypes - including somatic stem cells of the stroma (ASCs) - and relevant paracrine factors of different origins, which may account for putative exploitation in regenerative medicine applications.

The two proteomic platforms employed in this study provided complementary information for the characterization of the LAF proteome. These methods allow investigating the entire proteome also focusing on protein PTMs relevantly modulated during health/pathological transition states, explaining the missing correlations between the genes and their expression product. The top-down strategy analyses protein and peptides in their intact naturally occurring state. This allowed identifying several peptides belonging to hemoglobin fragments, some exhibiting specific biological properties, together with β-thymosin peptides, important in wound healing processes [24], S100A6, and other proteins together with their PTMs. The bottom-up approach, analysing trypsin digested fragments, supported and complemented the top-down findings, allowing the characterization of higher molecular weight proteins, some of them reported in literature to be correlated to osteogenesis or bone diseases. The application of LysC/trypsin digestion protocol for bottom-up analysis in future experiments could enhance the number of identified proteins also allowing, due to the high reproducibility of fragmentation, an accurate comparative quantitation between specimens, important to correlate the protein profiling with the different osteogenic effects recognized for individual samples.

Some of the identified proteins in LAF have been already characterized in the secretome of ASCs, extensively studied for their regenerative properties on bone [5]. The osteogenic properties exhibited by LAF would therefore confirm and specify the role of the osteoinductive compounds featured in the adipose tissue cells' secretome.

These data, besides providing a preliminary insight into the LAF proteome, represent the starting point for further studies. Based on our results, upcoming experiments could be devoted to the isolation and characterization of LAF protein fractions, to be tested *in vitro* to obtain a functional validation of their biological properties. In particular, the identification of protein components involved in osteogenesis or related processes, could pave the way to future possible exploitation of LAF as a bioactive fluid in the design and development of novel cell-free bone regenerative medicine applications.

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The authors declare no conflict of interests.

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