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Metabolomic and Proteomic Analysis of the Mesenchymal Stem Cells' Secretome

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Abstract

Mesenchymal stem cells (MSCs) are multipotent stromal cells with a strong potential in human regenerative medicine due to their ability to renew themselves and differentiate into various specialized cell types under certain physiological or experimental conditions. MSCs secrete a broad spectrum of autocrine and paracrine factors (MSCs' secretome) that could exert significant effects on cells in their vicinity. MSCs have been clinically tested and have displayed a great potential in the treatment of bone/cartilage fractures and disorders, diabetes, cardiovascular diseases and immune, neurodegenerative and inflammatory diseases. The therapeutic efficacy of MSCs was initially attributed to their multipotent character and ability to engraft and differentiate at the site of injury. However, in recent years, it has been revealed that either undifferentiated or differentiated MSCs' secretome plays an important role in the therapeutic potential of MSCs. The deciphering of the composition of MSCs' secretome through proteomic and metabolic analyses and implementation of certain advanced analytical (nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), chromatography, etc.) and immunological methods could contribute to the understanding of the mechanisms underlying the therapeutic effects of MSCs.

Keywords: secretome, mesenchymal stem cells, proteomics, metabolomics, umbilical cord blood plasma, growth factors

1. Introduction

Mesenchymal stem cells (MSCs) are one of the most promising types of stem cells for cell-based therapies. The continuously increasing interest in the therapeutic application of MSCs

to various diseases can be linked to their capacity to self-renew and differentiate into several cell types, to secrete soluble factors with paracrine actions and their immunosuppressive and immunomodulatory properties [1–5]. In addition, MSCs can be easily isolated from a wide range of ethically acceptable adult tissues and demonstrate a significant capacity for *ex vivo* expansion [6, 7].

Mesenchymal stem cells (MSCs) were first isolated and described by Friedenstein et al. in the early 1970s as a population of multipotent cells found in the bone marrow that can differentiate into osteocytes, chondrocytes, adipocytes and myoblasts [8, 9]. Currently, MSCs can be found and isolated from various foetal and adult tissues including bone marrow, adipose tissue, skeletal muscle, umbilical cord blood (UCB), umbilical cord tissue or matrix (UCT, Wharton's jelly), peripheral blood, dental pulp and amniotic fluid [10–12]. Despite their presence in different tissue sources, the isolated MSCs exhibit a similar characteristic phenotype [2] with some additional features that reflect their tissue of origin [13].

MSCs are able to renew themselves and differentiate into various specialized cell types such as bone, cartilage, muscle and fat cells under certain physiological or experimental conditions and may serve as a renewable source of cell and tissue replacements [1, 2]. According to the definition agreed by the International Society for Cellular Therapy (ISCT) in 2006, mesenchymal stem cells are characterized by (a) their capacity to adhere to plastic; (b) their expression of specific surface markers, namely, CD73, CD90 and CD105, and no expression of CD14, CD19, CD34, CD45 and HLA-DR. Additionally, according to the ISCT, MSCs are able to undergo tri-lineage differentiation into adipocytes, chondrocytes and osteoblasts [2].

Besides their multipotency, MSCs possess unique characteristics, such as the ability to migrate to sites of injury, inflammation or cancer, the capacity to secrete a broad spectrum of autocrine/paracrine factors that could exert significant effects on cells in their vicinity and the ability to modulate inflammatory and regenerative processes and the immune system. MSCs have been clinically tested for treatment of a variety of pathologies and diseases such as brain paralysis, cardiovascular diseases and myocardial infarction, type I diabetes, multiple sclerosis, Crohn's disease, bone fractures, graft versus host disease (GVHD) in bone marrow transplantation, osteoarthritis and rheumatoid arthritis [3, 14, 15].

The therapeutic effects of MSCs were initially attributed to the ability to migrate to sites of injury and inflammation, engraft into the damaged tissues and differentiate into specialised cell types. However, many studies provide strong evidences that the therapeutic efficacy of transplanted MSCs is not dependent on the physical proximity of the transplanted cells to the damaged tissue and propose that MSCs exert their therapeutic activity through MSCs' secretome [16, 17]. Although these studies, and many others, indicate the potency of MSC-secreted factors in mediating tissue repair and regeneration, the mechanisms of MSCs' action are not completely clear. The scientific community has tried to understand MSCs' biological mechanisms of action considering their capacity to secrete soluble factors with paracrine actions and unveil their potential in cell therapy and regenerative medicine.

Proteomics and metabolomics are omics techniques that employ state of the art analytical instrumentation in conjunction with pattern recognition techniques for comprehensive and

simultaneous systematic analysis of biological systems and monitoring of cellular and systemic proteomic and metabolic fluctuations in response to xenobiotic exposure, environmental factors, physiological stimuli and genetic modifications [18–23].

The studies of the composition of secretion products of MSCs through proteomic and metabolic analyses could contribute to the understanding of the mechanisms underlying the therapeutic effects of MSCs. Currently, certain advanced analytical (nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), chromatography, immunological assays, etc.) and chemometric techniques exist for proteomic and metabolic analyses of bio-fluids, which can be applied in MSC secretomics [20, 22–24].

2. MSCs' conditioned medium and secreted products

The paracrine effect of MSCs and their ability to synthesise and secrete a broad spectrum of growth factors, chemokines and cytokines that could exert significant effects on cells in their vicinity were first described by Haynesworth et al. [25]. This study was followed by other publications in the scientific literature describing therapeutic effects of these secreted factors [26, 27].

The MSCs' secreted factors are principal molecules for intercellular communication involved in most physiological processes, such as cell signalling, differentiation, invasion, metastasis, cell adhesion and binding, angiogenesis and apoptosis [18]. Growth factors, extracellular matrix proteins and extracellular matrix remodelling enzymes, pro-inflammatory, anti-inflammatory and pleiotropic cytokines, chemokines and angiogenic factors have been recently identified in stem cell secretomes including MSCs' one [19].

The compounds synthesised in the process of the proliferation and differentiation of MSCs could be secreted from the cells either constitutively or in the regulated manner and determine the composition of the extracellular environment [28]. Cell treatments *in vitro* could initiate alterations in the amount of particular factors secreted in the culture media [29, 30]. The secreted molecules could act as a possible replacement of stem cells for therapeutic applications that allow precise dosing, low biological variability and overcome of several stem cell related issues including cell origin and immunocompatibility [20]. The therapeutic application of MSCs' secretome could reveal new safe and effective strategies with predictable outcomes as an alternative to the cell therapy.

2.1. Preparation of MSCs' conditioned medium samples

MSCs' secretome studies involve a number of defined stages: (i) cell isolation and characterisation; (ii) cell culture in an appropriate culture medium; (iii) cell expansion and differentiation and (iv) collection of conditioned media.

The preparation of MSCs' conditioned medium has been performed by our research group and published elsewhere and here it is resumed and described [31]. Different batches of human MSCs from Wharton's jelly umbilical cord cryopreserved cells are *in*

vitro cultured and maintained in a humidified atmosphere with 5% CO₂ at 37°C. This process can be applied to previously isolated, expanded and cryopreserved MSCs from a diversity of tissues, with the advantage of a higher number of MSCs obtained in a shorter culture time, not dependent on donor's availability and ethic committee's authorization. During expansion, the cells become long spindle-shaped, and their phenotype is confirmed by flow cytometry analysis for a comprehensive panel of markers, such as platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31), homing cell adhesion molecule (HCAM, CD44), CD45 and Endoglin (CD105). Characterization is also performed with the following antibodies and their respective isotypes: PE anti-human CD105; APC anti-human CD73; PE anti-human CD90; PerCP/Cy5.5 anti-human CD45; FITC anti-human CD34; PerCP/Cy5.5 anti-human CD14; Pacific Blue anti-human CD19 and pacific-blue anti-human HLA-DR. The karyotype of the MSCs should also be determined to ensure that no structural alterations are found, which demonstrate the absence of neoplastic characteristics in these cells, as well as chromosomal stability of the somatic and sexual chromosomes due to the cell culture procedures. Two mesenchymal stem cell media have been tested for the conditioning, namely, PromoCell® medium (LabClinics, Promocell, C-28010, so-called commercial medium further on) and Dulbecco's Modified Eagle Medium (DMEM, Gibco®) supplemented with 10% of foetal bovine serum (FBS, Gibco®), 2 mM glutamine (sigma), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Sigma). Conditioned medium (CM) is normally collected from passage 4 (P4) MSCs. To obtain the desired CM, 4000 cell/cm² are plated and allowed to grow until reaching a minimum of 80% confluence. At this stage, the commercial medium is removed from the T-flasks, and after five washing cycles with Dulbecco's Phosphate Buffered Saline 1× (DPBS) without calcium (Ca²⁺) and magnesium (Mg²⁺) (Gibco®), Dulbecco's Modified Eagle Medium/Nutrient Mixture (DMEM, Gibco®) supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin (Sigma) is added. The cell culture T-flasks are maintained in a humidified atmosphere with 5% CO₂ at 37°C, allowing the adherent cells to be in contact with a serum-free basal medium. The culture medium, added after the confluence is reached, is not renewed and it is collected at different time points (24 and 48 h). For conditioning with Dulbecco's Modified Eagle Medium/Nutrient Mixture (DMEM, Gibco®) supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin (Sigma), samples of the medium are also collected at 24 and 48 h. Upon collection, the CM is frozen at -20°C, being later on thawed on the day of the experiments. ¹H-NMR spectra and Multiplexing LASER Bead analysis were acquired from both 24 and 48 h samples.

3. Human umbilical cord blood plasma as a potential supplement for MSCs' culture and conditioning

In search of a suitable alternative (and animal-component-free) culturing medium for the referred MSCs, we employed similar analytical techniques in order to better characterize hUCBP and assess its viability as a substitute for standard usage in FBS. The utilization of FBS in cellular culture has a series of ethical, technical and biological issues that made challenging

its most extended usage in clinical setting. Since exogenous protein and other factors' supplementation is still a facilitating point in MSCs' expansion (as chemically defined media are mostly expensive and occasionally underperforming), research has focused on finding suitable replacements for the FBS [31–34].

3.1. Preparation of hUCBP samples

These samples were collected from different consenting donors that clinically evaluated according to the Portuguese law 12/2009 (Diário da República, lei 12/2009 de 26 de Março de 2009) and analysed by flow cytometry and for microbiological contamination for aerobic and anaerobic microorganisms and fungi.

Umbilical cord blood (UCB) was collected from the umbilical vein by gravity into a 150 ml volume simple bag (reference 1385.13, Suru, Portugal), containing 21 ml of citrate-phosphate-dextrose (CPD), and stored at $4 \pm 2^\circ\text{C}$ until processing for cryopreservation. UCB samples were transported to the laboratory at refrigerated temperatures ranging between 4 and 22°C , within 48 h after collection. The collected UCB is subjected to a volume reduction process using AXP system[®] (thermogenesis). During the two-step centrifugation, whole blood is separated into three layers that are delivered into a red blood cell (RBC) bag and a freezing bag. Plasma remains in the processing bag which is also known as plasma bag. Samples of the UCB are taken by sampling pillows integrated within the kit for flow cytometry analysis. The total nucleated cell (TNC) count, CD34⁺ cell counts, CD34⁺ cell viability and leucocyte (CD45⁺) viability are determined by samples obtained from the UCB before volume reduction. Microbiological controls are usually performed after volume reduction and before cryopreservation and tested for microbiological contamination using an automated blood culture system (BacT/ALERT[®], bioMérieux) at 35°C for 14 days. TNC and the number of white blood cells (WBC) are counted with a haematology autoanalyser (Ac T diff2[™], Beckman Coulter, Inc.). The CD34⁺ cell number and the CD34⁺ viability are quantified by flow cytometry (BD FACSCalibur[™] 3 CA Becton Dickinson, BD Biosciences); the software for acquisition and analysis was BD CellQuest[™] and BDCellQuest[™] Pro Templates, respectively. The clusters of differentiation (CD) were used to enumerate the total number of CD34⁺ cells and the total number of leucocytes (CD45⁺), and the 7-amino-actinomycin D (7AAD) nucleic acid dye was used for viability measure (BD Stem Cell Enumeration kit, Becton Dickinson, BD Biosciences), according to the manufacture's protocol. The BD stem cell enumeration simultaneously enumerates the total viable dual-positive (CD45⁺/CD34⁺) haematopoietic stem cells in absolute counts of CD34⁺ cells per μl and the percentage (%) of viable leucocytes (CD45⁺) that are CD34 positive (CD34⁺).

The sample collection, sample storage and sample preparation are very important steps in proteomics and metabolomics. If the samples are not collected properly or the samples are not stored or processed uniformly, the metabolomics data generated from these samples could be invalid. Hence, the sample collection, storage and processing procedures are critical for conducting successful metabolomics studies.

4. Proteomic techniques

Proteomics is the omics technique that focuses on proteins and their functions in biological systems. Proteins are involved in all processes of living organisms and possess a complex structure, interactions, dynamics in concentration, degradation and/or modification which are the key factors defining the behaviour of biological systems. Therefore, the main goal of proteomics is the identification, quantification and characterisation of the protein content of biological samples, such as organs, tissues, cells and biological fluids [22].

Well-established methodologies exist for proteomic analysis of bio-fluids that include separation and protein identification techniques, and chemometric methods for data interpretation and visualization [18, 20, 24]. The extensive development of advanced analytical techniques and instrumentation in the last decade has enabled the proteomic analysis of cell secretome and several profiles from different cell types, body fluids and physiological conditions that have been studied and established [20, 35]. The continuously increasing interest in secretome proteomics has been raised due to the pivotal role of these secreted proteins in all physiological processes in living systems, including physiological, pathophysiological and genetic transformations, degenerative processes, disease conditions and progression [23, 24, 28].

4.1. Proteomic analysis of MSCs' secretome

The proteomic profiling of biological systems, including cell secretomes is mainly centred on mass spectrometry (MS), chromatography (liquid chromatography, LC), immunological assays and chemometric techniques [20, 24]. *In vivo* and *ex vivo* studies of MSCs' secretome encounter significant technical difficulties due to the low abundance of secreted proteins relative to total proteins in tissue/bio-fluid, the presence of different cell types including the cells of interest, endothelial cells and fibroblast cells, possibility of contamination by blood and the difficulty of accessing the extracellular medium in a tissue section [20, 23]. Therefore, *in vitro* strategies have been developed to probe the secretome under physiological conditions, where culture medium is conditioned by cells for a certain period of time followed by collection, preparation and processing of the medium for proteomic analysis. The proteomic analysis of MSCs' secreted products involves MSCs' isolation and characterisation; culture medium preparation; MSCs' conditioning; isolation of the conditioned medium; implementation of appropriate proteomic analytical techniques and protocols and data analysis. Proteomic studies of MSCs' secretome are currently performed under *in vitro* conditions as shown in **Figure 1** [20, 23].

Proteomic analysis based on LC, MS, LC-MS, immunological assays and bioinformatics has already been applied in the studies of protein and peptide separation, identification and quantification, screening of posttranslational modifications and characterisation of secreted products [20, 22, 23, 36, 37]. Proteomic studies performed on MSCs' secretome have been recently revised considering the cell origin, conditioning protocols and the analytical techniques used [20]. The studies of the secreted products of MSCs isolated from various foetal and adult tissues including bone marrow, adipose tissue, skeletal muscle, umbilical cord blood and umbilical cord tissue have been performed. Proteomic profiles of secreted products obtained

from culturing of a wide range of human and animal MSCs have been established through implementation of proteomic analytical techniques such as MS, LC, LC/MS, immunological assays and bioinformatics [20, 38–42].

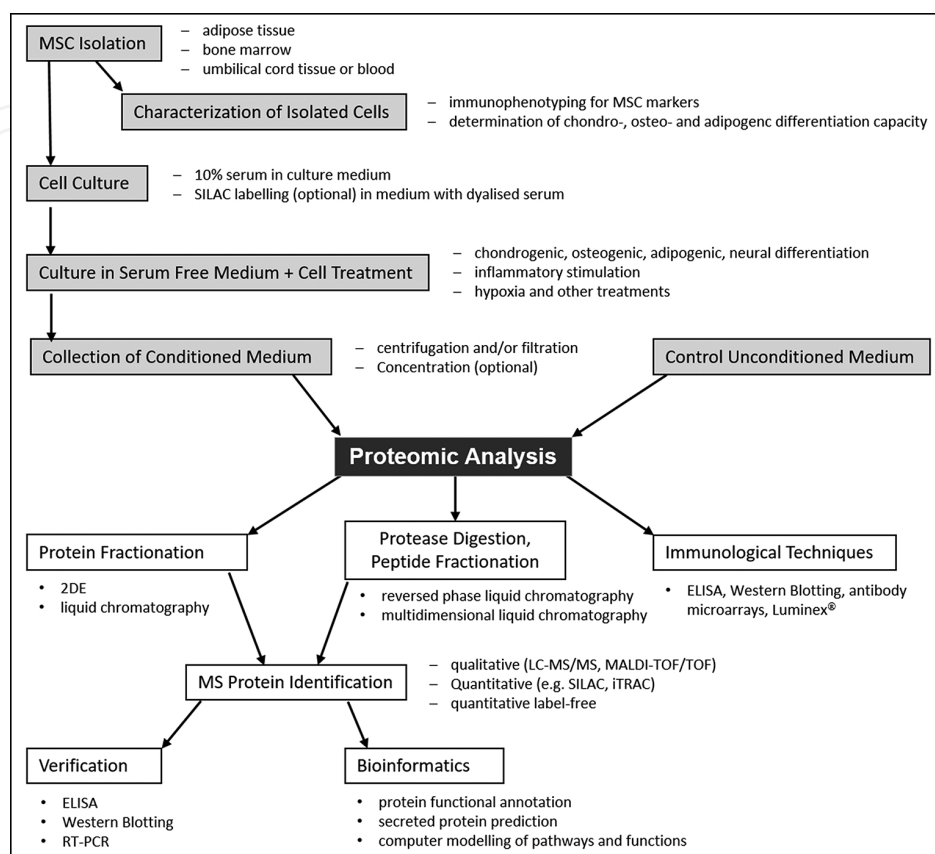


Figure 1. Schematic representation of the steps followed in a proteomic analysis of conditioned medium obtained from MSCs' *in vitro* expansion.

We have recently disclosed proteomic study of several MSCs' conditioned media based on Multiplexing LASER Bead Technology which enable the simultaneous testing of numerous analytic categories such as cytokines, chemokines and growth factors in a single assay [31]. Human Primary Cytokine Array/Chemokine Array 41-Plex Panel (Eve Technologies, Calgary, Alberta, Canada) has been performed to analyse the conditioned media, including the following cytokines, chemokines and growth factors: epidermal growth factor (EGF), eotaxin-1, fibroblast growth factor 2 (FGF-2), fms-related tyrosine kinase 3 ligand (Flt-3L), fractalkine, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), GRO(pan), interferon- alpha 2 (IFN α 2), interferon-gamma (IFN γ), several interleukins (IL-1 α , IL-1 β , IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A), interferon gamma-induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), monocyte chemotactic protein-3 (MCP-3), macrophage-derived chemokine (MDC), macrophage inflammatory protein-1 alpha (MIP-1 α), macrophage inflammatory protein-1 beta (MIP-1 β), platelet-derived growth factor-AA (PDGF-AA),

platelet-derived growth factor-AB/BB (PDGF-AB/BB), chemokine (C-C motif) ligand 5 (RANTES or CCL5), soluble CD40 ligand (sCD40L), transforming growth factor alpha (TGF α), tumour necrosis factor alpha (TNF α), tumour necrosis factor beta TNF β and vascular endothelial growth factor A (VEGF-A). TGF- β 3-Plex Array Multi-Species (Eve Technologies, Calgary, Alberta, Canada) was also performed to analyse the tumour growth factor beta 1, 2 and 3 (TGF- β 1, 2 and 3).

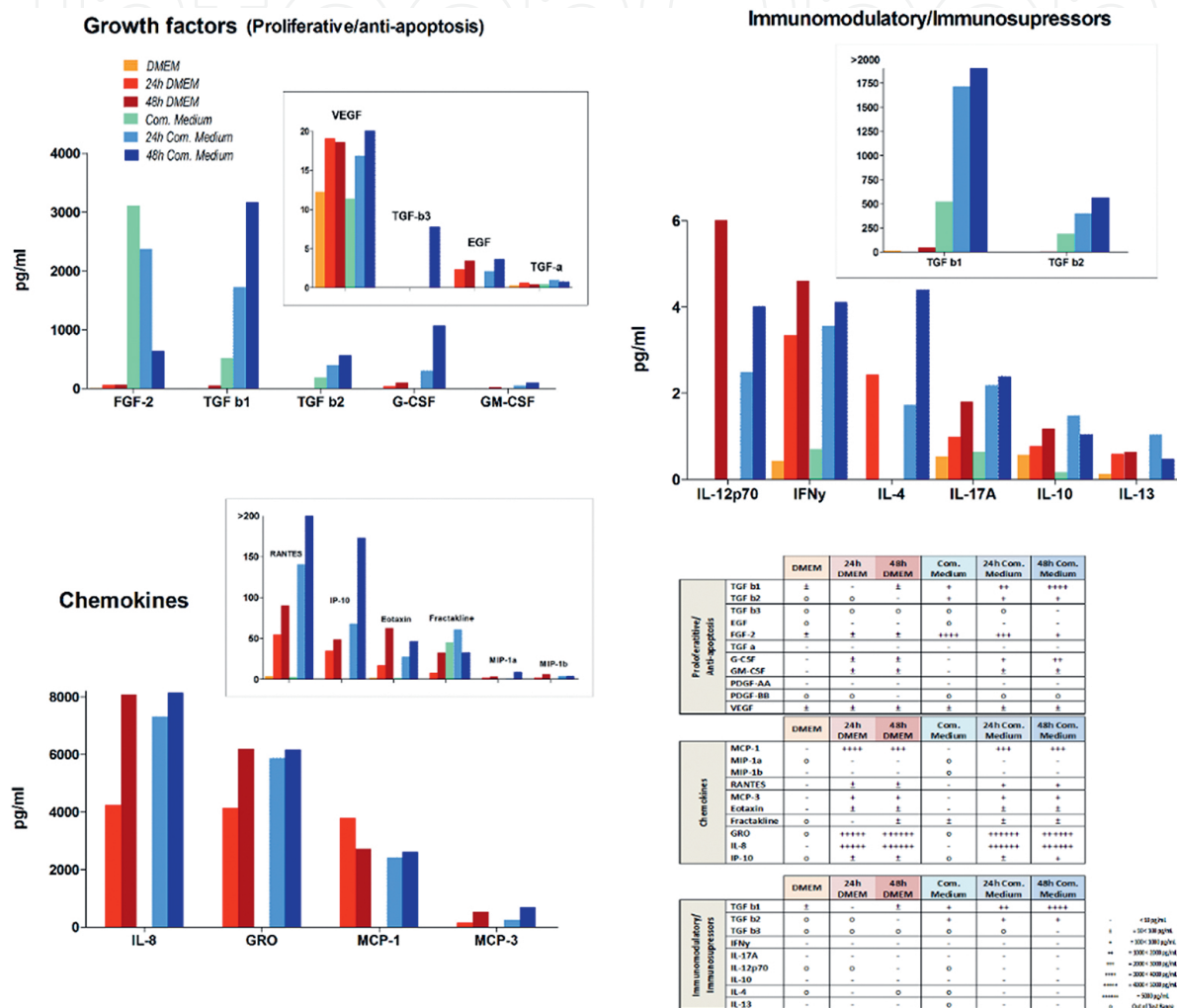


Figure 2. Proliferative and antiapoptotic growth factors, immunomodulatory, immunosuppressive cytokines and chemokines concentrations in unconditioned (Com. Medium* and DMEM) and conditioned media (24/48 h Com. Medium and DMEM). (Multiplexing LASER Bead Analysis (Eve Technologies, Calgary, Alberta, Canada). *Commercial medium from PromoCell (LabClinics, Promocell, reference C-28010), adapted from [31].

The results from the Human Primary Cytokine Array/Chemokine Array 41-Plex Panel together with the TGF- β 3-Plex Array allowed the comprehensive analysis of the cytokines, chemokines and growth factors included in conditioned media in comparison with unconditioned culture media [43]. It was confirmed that the concentration of important proliferative and anti-apoptotic factors increases the process of cell conditioning. This was particularly evident for TGF- β 1, EGF, G-CSF, GM-CSF, PDGF-AA and VEGF. Some of these factors like EGF were even

absent in unconditioned media. It was also confirmed that the content of factors such as TGF- β 1 and G-CSF depends on the duration of the conditioning process (**Figure 2**).

4.2. Proteomic analysis of hUCBP

Following an identical analytical protocol hUCBP depicted impressively high detection levels for TGF- β family members 1–3. Fair concentrations of other factors like VEGF, PDGF-AA, PDGF-BB and EGF were also observed. Other immunosuppressive/immunomodulatory factors can also be found at interesting levels in hUCBP (**Figure 3**).

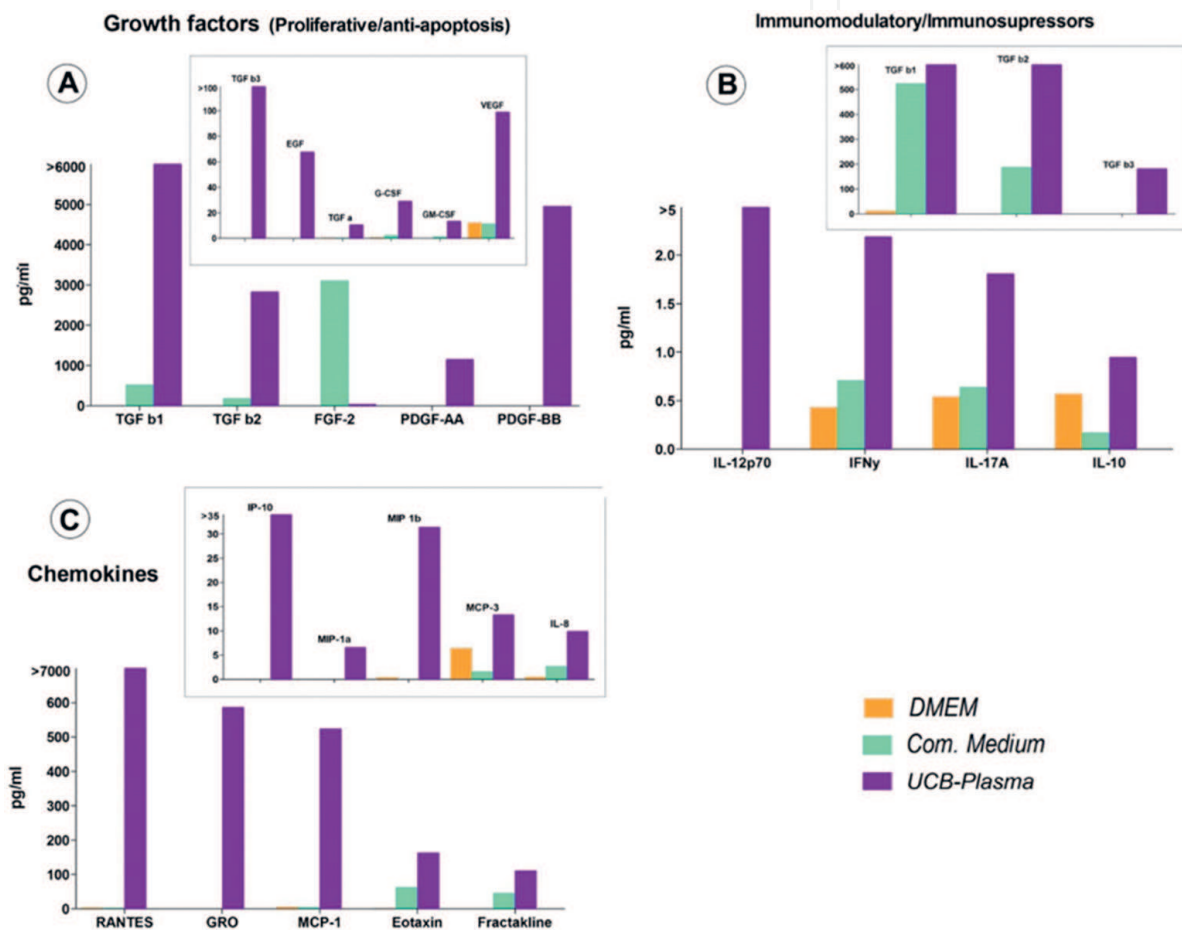


Figure 3. Proliferative and anti-apoptotic growth factors (A), immunomodulatory, immunosuppressive cytokines (B) and chemokines (C) concentrations in UCB Plasma and unconditioned media (Com. Medium* and DMEM). (Multi-plexing LASER Bead Analysis (Eve Technologies, Calgary, Alberta, Canada). *Commercial medium from PromoCell (LabClinics, Promocell, C-28010), as in [31].

5. Metabolomics techniques

Metabolomics is a powerful approach for multicomponent analysis of biological samples that allow the comprehensive and simultaneous systematic profiling of multiple metabolite

concentrations and their cellular and systemic fluctuations in response to xenobiotic exposure, environment, stimuli and genetic modulations. Metabolomics is important and well-established technique that encompasses the multicomponent analysis of metabolites in biological fluids, tissue and cell extracts using advanced analytical techniques [21, 43–46].

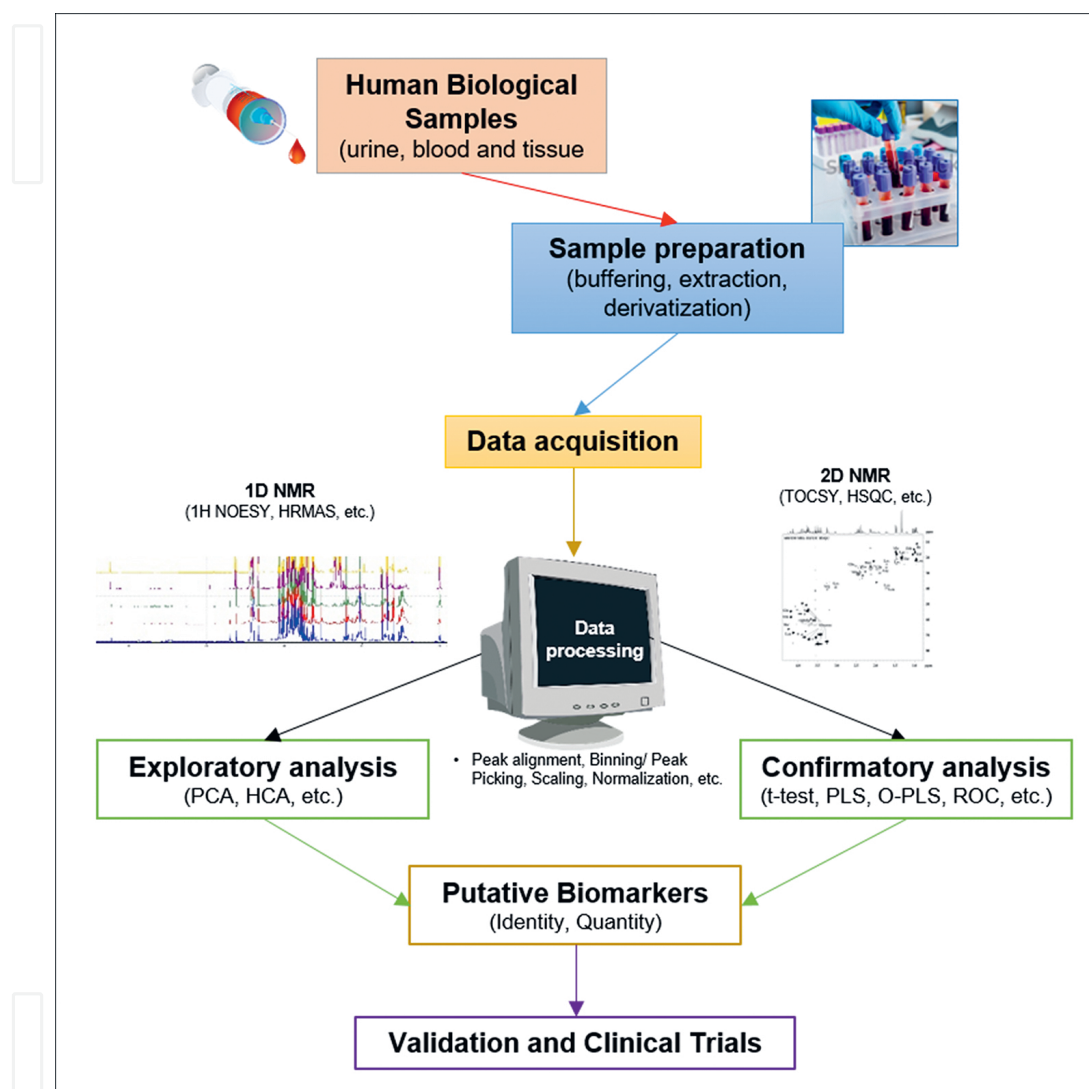


Figure 4. Schematic representation of the steps followed in a metabolomic analysis of conditioned medium obtained from MSCs' *in vitro* expansion.

An analysis of the metabolite spectrum in a biological system provides a detailed information and specific view into cellular metabolic processes under normal and altered conditions. The study of the entire multicomponent metabolic profile of biological systems is a challenging task and relies on the use of reproducible analytical technologies that provide sufficiently high sensitivity, high resolution and wide dynamic range. Metabolomics is mainly based on NMR spectroscopy (NMR) and mass spectrometry (MS), with chromatographic separation usually involved in the latter [43–46]. Both spectroscopic techniques could give valuable information on the structure and quantitative distribution of metabolites in biological systems.

Metabolomics studies usually result in large number of complex multivariate data sets that are typically analysed using chemometric and bioinformatics methods for data interpretation and visualization. These studies have been extensively expanded after implementation of pattern recognition (PR), expert systems and related bio-informatics tools to interpret and classify complex metabolic data sets. Well-established methodologies and protocols for metabolic analysis of biological samples have been extensively developed [21, 43–48].

The metabolomics analysis of biological samples involves several basic steps (**Figures 1 and 4**): sample collection; sample manipulation and storage; sample preparation for metabolic analysis; application of appropriate analytical method(s); data acquisition; data analysis (metabolites identification and quantification) and pattern recognition of spectral data (data interpretation and visualisation).

The development of advanced analytical techniques and instrumentation in the last decades has enabled a comprehensive metabolic analysis of diverse and complex biological samples, such as tissues, cells, tissues and cellular extracts, bio-fluids, etc. Certain advanced analytical techniques such as NMR, MS, LC, LC-MS and bioinformatics exist for metabolic analysis of biological systems, which can be applied in MSC secretomics [21, 43–48].

Studies of the composition of MSCs' secretome could contribute to the understanding of the mechanisms underlying the therapeutic effects of MSCs and assessment of the potential of MSCs' secretome as an alternative to MSCs' cell therapy.

5.1. Metabolic analysis of MSCs' secretome by NMR spectroscopy

NMR spectroscopy being a high reproducible and quantitative analytical technique, with minimal or no sample preparation and the sample can be recovered following the analysis, is one of the main tools for metabolic profiling, identification and quantification of known and unknown metabolites in biological samples. It offers unique opportunities for improving the structural and functional characterization of metabolome, which can be essential for the understanding of many biological processes and physiological changes in biological systems [43–48].

NMR spectroscopy as a quantitative non-destructive, non-invasive analytical technique can be used effectively to screen for metabolite profiles and to monitor structural and physiological changes in biological systems [21, 43, 44]. Nowadays, high-resolution NMR spectroscopy is one of the most powerful analytical techniques available for metabolic profiling of biological systems and could be suitable for metabolic analysis of MSC secretome. One of the advantages of NMR spectroscopy over other methods is that it can generate a large quantity of information concerning the metabolic composition of samples, making possible the simultaneous identification and quantification of structurally diverse metabolites, without the need for individual isolation or no special sample preparation [43, 44].

5.1.1. Samples collection and preparation

As for the proteomic analysis, the sample collection, storage and processing procedures are critical for conducting successful metabolomics studies. As previously studied, the collected

samples of CM or for other biological fluids like umbilical cord blood plasma [31] should be centrifuged to remove cellular detritus and stored at -20 or even -80°C . The cellular systems used in the metabolomics and secretome profile studies should be previously characterised, including the flow cytometry or immunocytochemistry profile and karyotype.

At the end of the cell conditioning period, the conditioned medium is collected and centrifugation and/or filtration are usually applied to remove cell residues. In some cases, larger volumes of conditioned medium can be concentrated to succeed optimal experimental conditions. Upon collection and purification, the conditioned medium can be frozen and stored at -20°C , being later on thawed on the day of the NMR experiments.

The preparation of samples for NMR analysis can involve the addition of buffer to stabilize the pH, deuterated water (D_2O) as a magnetic field lock signal and reference compound to be used as a chemical shift and quantitation standard. The reference compound used in aqueous media is usually sodium trimethylsilyl-[2,2,3,3- d_4]-propionate (TSP), which can be used as an internal or external standard. Absolute concentrations of metabolites can be obtained by the use of internal standard of known concentration added to the sample.

5.1.2. NMR techniques

The metabolomic study of bio-fluids is usually based of ^1H NMR spectroscopy. The NMR data for all samples need to be acquired and processed uniformly. The NMR experimental conditions and procedures are critical for conducting successful metabolomics studies and need to be uniform. The large interfering NMR signal arising from water in all bio-fluids can be eliminated by the use of standard NMR solvent suppression pulse sequences. ^1H NMR spectra using a 1D NOESY (noesygppr1d) pulse sequence (recycle delay- 90° - t_1 - 90° - t_m - acquire) and a Carr-Purcell-Meiboom-Gill (CPMG, cpmgpr1d) pulse sequence (recycle delay- 90° - $[\tau$ - 180° - $\tau]$ n- acquire) can be acquired. The 1D NOESY experiment generates spectra with improved solvent peak (at 4.70 ppm) suppression. Relaxation edited ^1H NMR spectra with T2 (spin-spin relaxation time) filter using CPMG pulse sequence and suppression of water resonance were acquired to facilitate the identification of low molecular weight metabolites, reducing signals from high molecular weight species or systems in intermediate chemical exchange. The generated NMR data should be processed spectra were processed uniformly.

5.1.3. NMR data analysis

NMR spectra of biological systems are usually extremely complex because of the large number of components in the samples, resulting in spectra with complex line shapes and significant overlap of the resonance signals. The analysis of NMR spectra and identification of the metabolites in biological samples may involve a number of spectral techniques, information from databases of known metabolite spectra and NMR assignment software. Though very complex, the NMR spectra of biological samples (in particular ^1H NMR) allow direct assignment of resonance signals of some metabolites based on their chemical shifts, multiplicity and intensity and spectral analysis of appropriate NMR spectroscopic techniques such as two (2D) or multidimensional NMR techniques. 2D NMR spectroscopy can be useful to increase the

signal dispersion in spectra with significant overlap of the resonances and reveal the connectivity between signals ($^1\text{H}/^1\text{H}$ Correlation spectroscopy (COSY); $^1\text{H}/^1\text{H}$ Total Correlation spectroscopy (TOCSY), $^1\text{H}/^{13}\text{C}$ or $^1\text{H}/^{15}\text{N}$ Hetero-correlation spectroscopy (HSQC, HMBC)) and/or to define the multiplicity and coupling pattern of resonances (2D J-resolved spectroscopy), thereby helping to identify metabolites in biological samples [21, 43, 44, 48].

5.1.4. Bioinformatics

In NMR metabolomics, the interpretation and classification of the complex metabolic data sets typically require the use of chemometric and pattern recognition (PR) techniques, and related bio-informatics tools. Pattern recognition techniques such as principal component analysis (PCA) allow for the analysis and classification of the large number of complex NMR spectroscopic data usually acquired for metabolic profiling of biological samples [44, 47, 48].

5.1.5. Applications of NMR-based metabolomics

NMR-metabolomics has been applied to monitor cellular and systemic metabolic fluctuations in response to xenobiotic exposure, environmental factors, physiological stimuli and genetic modifications [21, 43–48]. Many applications of NMR-metabolomics have been published, including the extensive study of physiological variation in experimental animals, age-related changes, dietary modulation, diurnal effects and phenotyping of mutant and transgenic animals [44, 47, 49].

NMR-based metabolomics has found many applications in metabolic analysis of a wide diversity of biological samples and has been recently used for characterization, monitoring and optimisation of stem cell culture medium preparation [50]. NMR spectroscopy was used for metabolomics analysis of human embryonic stem cell (hESCs) conditioning medium characterization. A number of metabolites were identified and quantified in conditioned media. Significantly, higher concentrations for certain metabolites (lactate, alanine, glutamine, glucose and formate) and lower for others (tryptophan, folate and niacinamide) were detected in the conditioned media compared to unconditioned media. Multivariate statistical analysis was applied to classify the data and assess the main metabolic changes in the process of cell conditioning. It was confirmed that NMR could be an accurate and valuable tool for monitoring, controlling and optimising hESC culture medium preparation. In this study, the detailed scrutiny of metabolites involved in a variety of biochemical pathways for potential tissue engineering applications has been facilitated [50].

In a more recent study by the application of proton NMR spectroscopy and implementation of appropriate one-dimensional (1D) and two-dimensional (2D) NMR techniques, it was confirmed that the metabolic composition of Wharton's jellies derived MSCs' conditioned media and changes in the metabolic profile of the culture media in the process of stem cell expansion and differentiation. These changes were found to be affected mainly by the type of the culture media and the duration of the conditioning time [31]. Typical ^1H NMR spectra of two different types of unconditioned and MSCs' conditioned media are presented in **Figure 5**.

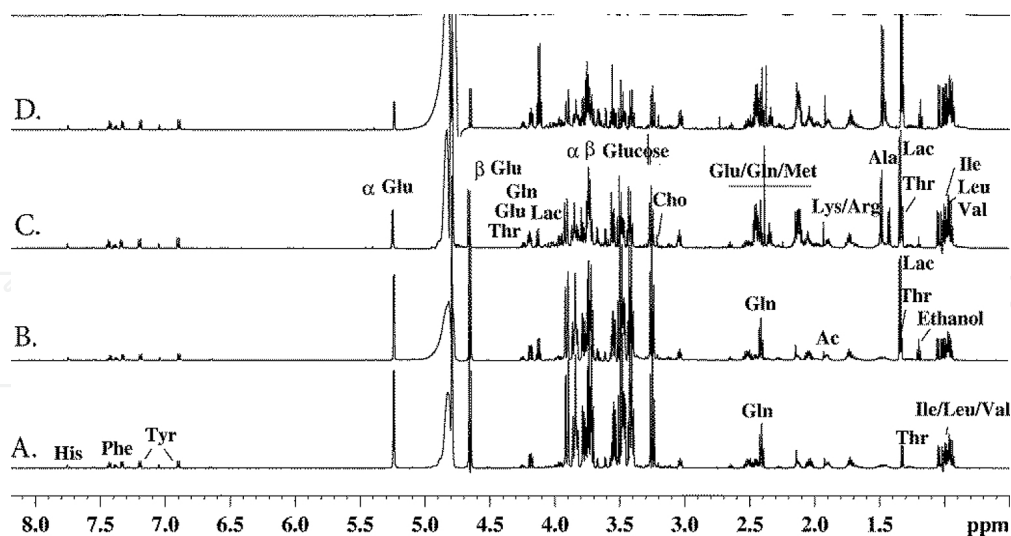


Figure 5. Typical 600 MHz ¹H NMR spectra recorded in H₂O:D₂O (9:1) of (A) Unconditioned medium I, (B) MSCs' conditioned medium I; (C) Unconditioned medium II, (D) MSCs' conditioned medium II. Characteristic signals of metabolites in the samples are marked.

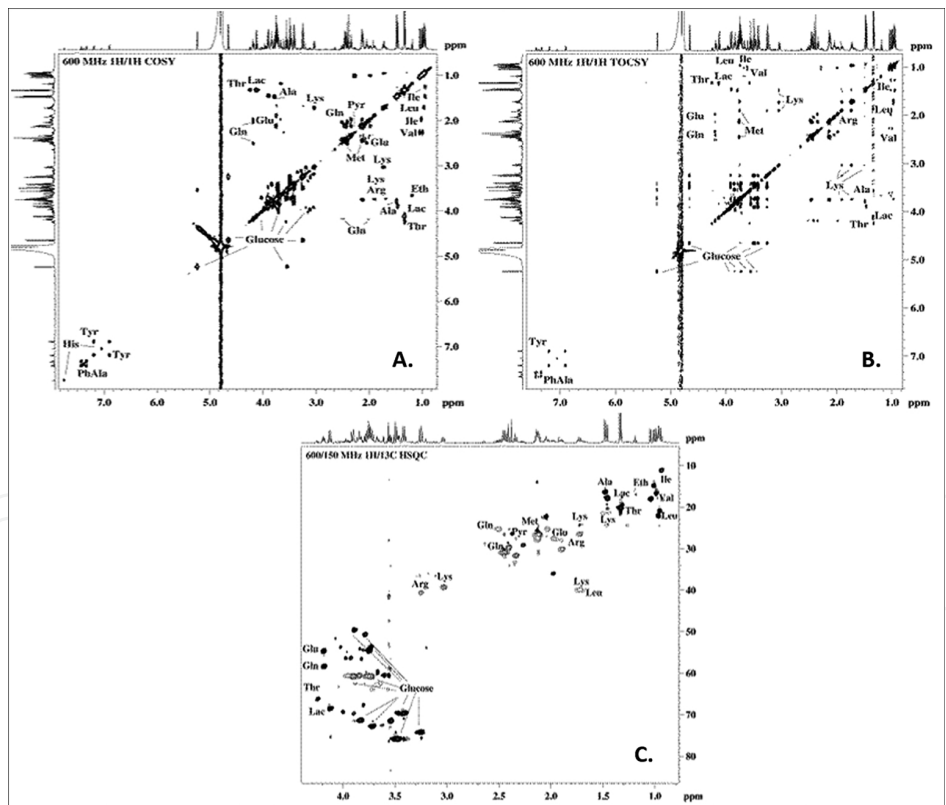


Figure 6. Representative 2D NMR experiments of MSCs' conditioned medium (conditioned medium I): (A) 1H/1H COSY spectrum, (B) 1H/1H TOCSY spectrum, (C) 1H/13C HSQC Spectrum.

The metabolomics study of different types culture media and the corresponding Wharton's jelly derived MSCs' conditioned media isolated at different time points was based on ¹H NMR

spectroscopy. Sodium trimethylsilyl-[2,2,3,3-d₄]-propionate (TSP) was used as an internal reference for the calibration and quantification of NMR spectra. ¹H NMR spectra with suppression of the water resonance signal using a 1D NOESY and CPMG pulse sequences were acquired. The NMR spectra were acquired and processed uniformly using TOPSPIN 3.2 (Bruker Biospin).

Additionally, to confirm the chemical shift assignment of ¹H NMR spectra and facilitate the identification of metabolites presented in both unconditioned and MSCs' conditioned media, the studied 2D ¹H/¹H COSY, ¹H/¹H TOCSY and ¹H/¹³C HSQC spectra were obtained and analysed (**Figure 6**). The results have been compared to the data published in the literature [51].

Metabolites	Abbr.	Chemical shifts (ppm); multiplicity
Isoleucine	Ile	0.95(dd, δCH ₃), 1.27/1.46(m, γCH ₂), 1.98(m, βCH), 1.01(d, βCH ₃), 3.67(d, αCH)
Leucine	Leu	0.95(d, δCH ₃), 0.97(d, δ'CH ₃), 1.69(m, γCH), 1.71(m, βCH ₂), 3.73(d, αCH)
Valine	Val	0.98(d, γCH ₃), 1.05(d, γ'CH ₃), 2.27(m, βCH ₂), 3.61(d, αCH)
Ethanol	Et	1.19 (t, CH ₃), 3.57 (q, CH ₂)
Threonine	Thr	1.33(d, γCH ₃), 4.25(m, βCH ₂), 3.56(d, αCH)
Lactate	Lac	1.34(d, βCH ₃), 4.13(q, αCH)
Alanine	Ala	1.48(d, βCH ₃), 3.78(q, αCH)
Lysine	Lys	3.04(t, εCH ₂), 1.73(m, δCH ₂), 1.47(m, γCH ₂), 1.93(m, βCH ₂), 3.77(t, αCH)
Arginine	Arg	3.23(t, δCH ₂), 1.67(m, γCH ₂), 1.89(m, βCH ₂), 3.77(t, αCH)
Acetate	Ace	1.92(s, CH ₃)
Glutamate	Glu	2.36(t, γCH ₂), 2.08(m, βCH ₂), 3.74 (dd, αCH)
Glutamine	Gln	2.44(t, γCH ₂), 2.51/2.04(m, βCH ₂), 3.76(dd, αCH)
Methionine	Meth	2.2(s, δCH ₃), 2.45(m, γCH ₂), 2.13(m, βCH ₂), 3.75(t, αCH)
Choline	Cho	3.19(s, CH ₃), 3.51(dd, CH ₂), 4.06 (dd, CH ₂),
Pyruvate	CH ₃	2.39 (s)
α-Glucose	α-Glu	5.24(d,H1),3.54(dd,H2),3.72(t,H3),3.41(t,H4), 3.47(dd,H5),3.91/3.72(dd, H6)
β-Glucose	β-Glu	4.65(d,H1),3.24(t,H2),3.49(t,H3), 3.42(t,H4), 3.84(m,H5,6), 3.78(dd,H6)
Tyrosine	Tyr	3.05/3.18 (βCH ₂), 3.93 (αCH), 7.19(d, H2,6), 6.90(d, H3,5)
Phenylalanine	PheAla	3.12/3.26 (βCH ₂), 3.97 (αCH), 7.33(d, H2,6), 7.38(t,H4), 7.43(t, H3,5)
Histidine	His	3.12/3.22 (βCH ₂), 3.97 (αCH), 7.75(s, H2), 7.05(s, H5)
Tryptophan	Try	3.29/3.47 (βCH ₂), 4.05 (αCH), 7.30 (H2), 7.74(d, H4), 7.20(t, H5), 7.28(t, H6), 7.55(d, H7)
Nicotinamide	NA	7.58 (dd, CH), 8.24 (dd, CH), 8.70 (dd, CH), 8.94 (s, CH)
Formate	For	8.46 (CH)

Table 1. ¹H NMR chemical shifts and multiplicity of the main metabolites observed in the spectra of culture medium and MSCs' secretome.

From the analysis of the 1H and 2D (¹H/¹H homonuclear and ¹H, ¹³C heteronuclear correlation spectra) NMR spectra, certain number of metabolites have been identified. The main metabolites identified in the samples are listed in **Table 1** with an assignment of the corresponding resonance signals and their multiplicity in ¹H NMR spectra recorded.

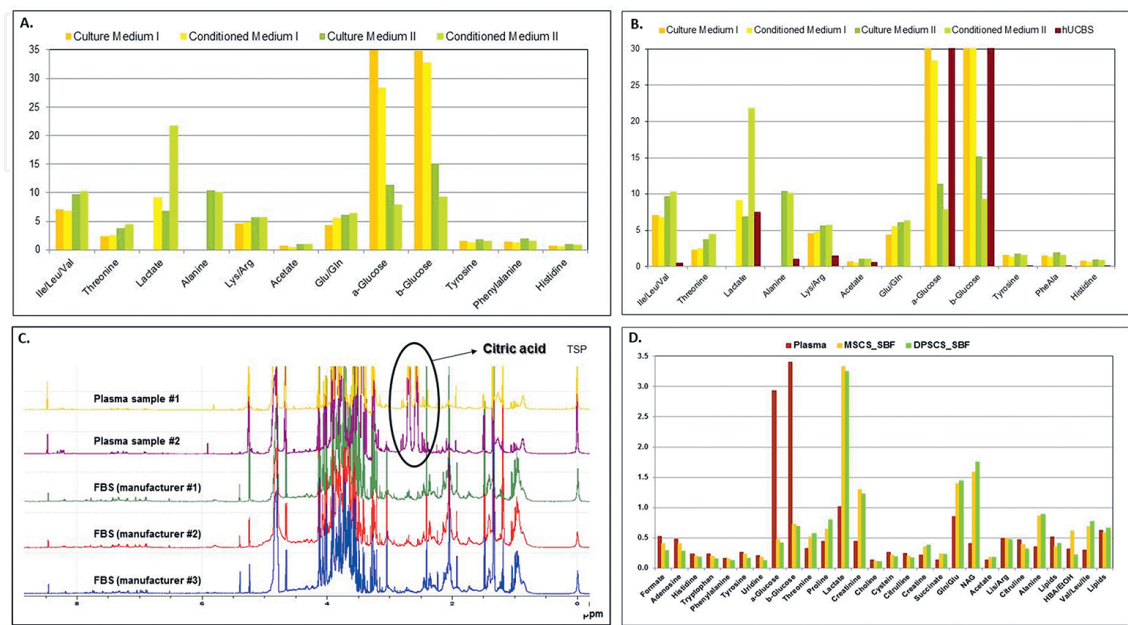


Figure 7. (A) Relative distribution (%) of the main metabolites detected in the culture media I and II and the corresponding MSCs’ conditioned media I and II [31]; (B) Relative distribution (%) of the main metabolites detected in the culture media I and II and the corresponding MSCs’ conditioned media I and II, and umbilical cord blood plasma (hUCBP) [31]; (C) Typical 600 MHz ¹H NMR spectra recorded in H₂O:D₂O (9:1) of two plasma samples obtained from umbilical cord blood (hUCBP) and foetal bovine serum (FBS) from three different manufacturers; (D) The quantitative distribution (ppm) of metabolites determined from the integral intensity of characteristic signals in ¹H NMR spectra considering the number of protons contributing to the intensity of the signal in umbilical cord blood plasma (hUCBP) and foetal bovine serum (SBF) from two different fabricants used of *in vitro* culture of MSCs from the umbilical cord matrix (MSCs_SBF) and dental pulp mesenchymal stem cells (DPSCs_SBF).

The metabolite content in two different types of culture media (culture media I and II) and the corresponding Wharton's jelly derived MSCs’ conditioned media collected at different time points was defined by quantitative NMR analysis, using the relative quantitative method [52]. The quantitative distribution of the NMR-visible metabolites was determined from the relative integral intensity of characteristic signals in ¹H NMR spectra of the samples referenced to the integral intensity of TSP (internal standard), considering the number of the contributing nuclei for that particular resonance. The relative quantitative distribution of the main metabolites detected in the ¹H NMR spectra of the two different types of culture media (culture medium I and culture medium II) and the corresponding Wharton's jelly derived MSCs’ conditioned media collected after 24 h of conditioning is presented graphically in **Figure 7A**.

The results from the NMR metabolomics analysis reveal the presence of important amino acids, glucose and low molecular weight (lactate, acetate, pyruvate and formate) compounds in the basal culture media (types I and II) and the corresponding conditioned media.

Different contents of glucose, lactate and certain amino acids were detected in the two basal culture media (types I and II) and the corresponding conditioned media (**Figures 3 and 7A**). Gathering the results of unconditioned media (types I and II) and comparing them globally with the corresponding conditioned media, it is possible to attest not only to metabolite depletion through cell metabolism (as the glucose example), but also to some metabolites (like lactate, isoleucine, leucine, valine, arginine, lysine, glutamate, formate or pyruvate) presented in higher concentration, reflecting the production and secretion of metabolites. Metabolic differences between samples collected at different time points (24 and 48 h) were also considered [31]. This is because conditioned media could be achieved at different stages after reaching minimal cell confluence.

5.2. Metabolic analysis of hUCBP by NMR spectroscopy

The metabolite profiles of the unconditioned and MSCs' conditioned media (types I and II) were further compared with those of human umbilical cord blood plasma (hUCBP) (**Figure 7B**) which has been considered as a promising culture medium supplement due to the fact that UCB is rich in soluble growth factors that support the growth, proliferation and differentiation of mesenchymal and hematopoietic stem cells [31, 33, 34].

The comparative study of the metabolic profiles of unconditioned media, MSCs' conditioned media and hUCBP reveals the presence of essential proteinogenic amino acids (alanine, arginine, isoleucine, histidine, leucine, lysine, phenylalanine, proline, tyrosine, valine), organic acids and derivatives (lactate, formate and creatine), glucose and nucleotides (adenosine, uridine and guanosine species) in the last. The results strongly suggest that hUCBP might be used as a culture medium supplement in the processes of cell isolation, expansion and cryopreservation and even as an alternative in the cellular therapy [31]. Moreover, hUCBP could be considered as an animal sera substituent that could solve important economic, ethical and scientific issues that have severe complications in the utilization of foetal bovine serum (FBS) for cell culture. In addition, hUCBP are attractive alternatives to FBS due to the worldwide increase in the number of cryopreserved UCB units in Public and Private Cord Blood Banks. However, the substitute medium supplement must possess the animal sera characteristics crucial for the cell expansion and preservation.

Recently, a comparative study of the metabolic composition of hUCBP and FBS, based on ^1H NMR spectroscopy and multivariate statistical analysis has been performed to evaluate the capability of hUCBP as a culture medium supplement and FBS substituent for *in vitro* proliferation and cryopreservation of MSCs. **Figure 7C** shows typical ^1H NMR spectra of hUCBP and FBS.

However, 1D and 2D NMR techniques have been used to identify and quantify the detectible metabolites in the samples. The quantitative distribution of metabolites presented in **Figure 7D** was determined from the integral intensity of characteristic signals in ^1H NMR spectra considering the number of protons contributing to the intensity of the signal.

The results show that the differences between the metabolic composition of hUCBP and FBS are due mainly to the different content of glucose, lactate, acetate, glutamate and alanine. The

much higher content of glucose determined for hUCBP has been attributed to the UCB function to provide energetic support for the foetus during intrauterine development. From the analysis of the NMR spectra it was confirmed the presence of essential proteinogenic amino acids such as alanine, arginine, creatine, glutamine, glutamate, isoleucine, histidine, leucine, lysine, phenylalanine, proline, threonine, tyrosine and valine in hUBCP as well as in FBS. These proteinogenic amino acids are precursors to proteins and could play a crucial role in the growth, proliferation and differentiation of cells. Significantly, higher levels of alanine, glutamate, isoleucine, leucine and valine in the commercial FBS as compared to hUBCP are determined. This seems to be the main difference between the amino acids profiles of human UBC plasma and foetal bovine sera. The increased levels of alanine and glutamine could due to the use of supplements such as Glutamax[®] in the commercial FBS sera [50]. Similar or slightly higher contents of arginine, citrulline, creatine, histidine, lysine, phenylalanine and tyrosine, nucleotides and lipids were found in hUBCP as compared to the FBS samples studied.

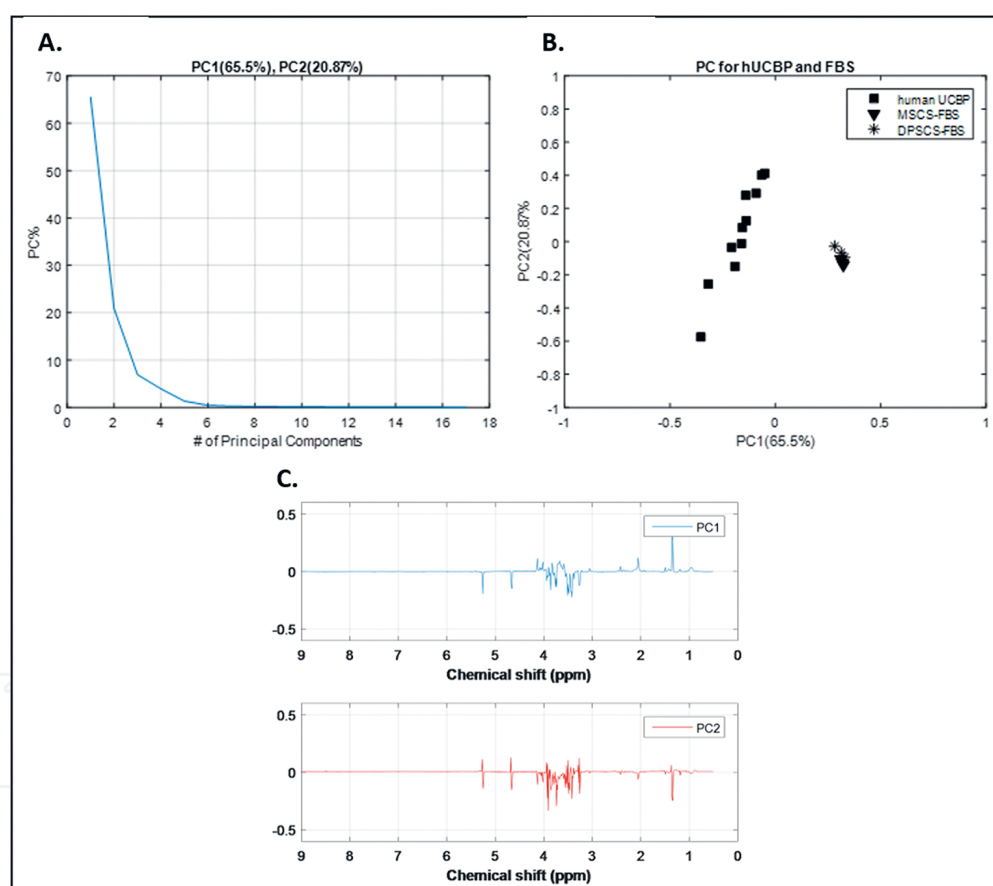


Figure 8. A and B—PCA statistics. PCs distribution of NMR data matrix (A) and Score plots (PC1 versus PC2) (B) and —PC1 and PC2 loading plots (C).

Principal component analysis has been used to interpret and classify the NMR spectral data obtained for a certain number of hUCBP (samples of 11 donors) and FBS samples (MSCs_FBS (3 samples of MSCs FBS and three samples of DPSCS FBS) to evaluate the main factors contributing to the discrimination between the two groups of samples.

The contributions of the principal components (PCs) of the NMR spectral data are depicted in **Figure 8A** and **B**. The first two PCs accumulate more than 85 % of data variance. Therefore statistical analysis based on PC1 and PC2 is legitimate.

The 2D (PC1 versus PC2) PCA score plot is depicted in **Figure 8B**. The results illustrate the close similarity of MSCs_FBS and DPSCs_FBS data sets (a well-defined cluster with overlapping samples) and reveal their separation from the human plasma data (a scattered cluster).

The corresponding loading plots in **Figure 8C** provide evidence that the glucose, lactate and acetates are the metabolites contributing to the divergence between the human plasma and FBS data sets. The results also reveal the close content of essential proteinogenic amino acids in the human plasma and foetal bovine sera. Loading plots around zero line suggest close metabolic profiles in the spectral region from 9.0 to 5.3 ppm of the three groups.

6. Conclusions

In brief conclusion, proteomic and metabolomics studies have demonstrated the richness of MSC' conditioned medium in terms of growth factors with *in situ* action in disease and regeneration processes, as well as in other metabolites that interact with the environment surrounding the mesenchymal cellular populations. Hence, as knowledge is gained in its composition and potential, thought these omics techniques and other assays, their potential on the application for the treatment of number of diseases increases alike.

As for the hUCBP, given its similarity and even superiority (in supplementary proteins and other contents) to promote and sustain MSCs' expansion, it might become a standardized human derived supplement for cellular populations aiming at the production of secretome or direct application in effective clinical treatments.

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