Metabolomic applications in stem cell research: a review

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Abstract

This review describes the use of metabolomics to study stem cell (SC) characteristics and function (excluding SCs in cancer research, suited to a fully dedicated text). The interest in employing metabolomics in SC research has consistently grown and emphasis is, here, given to developments reported in the past five years. This text informs on the existing methodologies and their complementarity regarding the information provided, comprising untargeted/targeted approaches, which couple mass spectrometry or nuclear magnetic resonance spectroscopy with multivariate analysis (and, in some cases, pathway analysis and integration with other omics), and more specific analytical approaches, namely isotope tracing to highlight particular metabolic pathways, or in tandem microscopic strategies to pinpoint characteristics within a single cell. The bulk of this review covers the existing applications in various aspects of mesenchymal SC behavior, followed by pluripotent and neural SCs, with a few reports addressing other SC types. Some of the central ideas investigated comprise the metabolic/biological impacts of different tissue/donor sources and differentiation conditions, including the importance of considering 3D culture environments, mechanical cues and/or media enrichment to guide differentiation into specific lineages. Metabolomic analysis has considered cell endometabolomes and exometabolomes (fingerprinting and footprinting, respectively), having measured both lipid species and polar metabolites involved in a variety of metabolic pathways. This review clearly demonstrates the current enticing promise of metabolomics in significantly contributing towards a deeper knowledge on SC behavior, and the discovery of new biomarkers of SC behavior and function with potential translation to *in vivo* clinical practice.

Abbreviations

AA, arachidonic acid; **ADA**, alginate di-aldehyde; **ALA**, α-linolenic acid; **AST**, astaxanthin; **BMP-2**, bone morphogenetic protein 2; **CE**, capillary electrophoresis; **CNS**, central nervous system; **CoA**, coenzyme A; **DAG**, diacylglycerols; **Dex**, dexamethasone; **DFO**, desferrioxamine; **DHA**, docosahexaenoic acid; **DI**, direct infusion; **DPSCs**, dental pulp; **EBs**, embryoid bodies; **ECM**, extracellular matrix; **EFs**, embryonic fibroblasts; **EpSCs**, epidermal Stem cells; **ESCs**, Embryonic stem cells; **FAs**, fatty acids; **FLS**, fibroblast-like synoviocyte; **GC**, gas chromatography; **GdnF**, Glial cell line-derived neurotrophic factor; **GHK**, glycine-histidine-lysine; **GPC**, glycerophosphocholine; **GPMVs**, of giant plasma membrane vesicles; **GWAS**, Genome-Wide-Association-Studies; **hAMSCs**, Human adipose-derived mesenchymal stem cells; **hBMMSCs**, Human bone marrow mesenchymal stem cells; **hBTSCs**, human biliary tree stem/progenitor cells; **hHBs**, human hepatoblasts; **hHpSCs**, hepatic stem cells; **hPDLSCs**, Human periodontal ligaments stem cells ; **hPMSCs**, Human placenta-derived mesenchymal stem cells; **hPSC-CMs**, Cardiomyocytes derived from human pluripotent stem cell; **HRMAS**, high resolution magic angle spinning; **HSCs**, haematopoietic stem cells; **hSGSCs**, Human salivary gland stem cells; **htt**, Huntington locus; **IB**, inclusion bodies; **IFN-γ**, interferon gamma ; **ILK**, integrin linked kinase; **iMSCs**, mesenchymal stem cells derived from iPSCs; **iPSCs**, induced pluripotent stem cells; **LA**, linoleic acid; **LC**, liquid chromatography; **LIPUS**, low-intensity pulsed ultrasound; **mESCs**, murine Embryonic stem cells; **MRI**, magnetic resonance imaging; **MS**, Mass Spec-

trometry; **MSC**, Mesenchymal stem cell; **MVA**, multivariate analysis; **Mw**, molecular weight; **NAM**, nicotinamide; **NMR**, Nuclear Magnetic Resonance; **nMSCs**, native mesenchymal stem cells; **NSCs**, Neural stem cells; **OECs**, Olfactory Ensheathing Cells; **P13K**, phosphoinositide 3-kinase; **PC**, phospchocholine; **PCA**, Principal component analysis; **PD**, Pompe disease; **PDH**, pyruvate dehydrogenase; **PKB**, protein kinase B; **PLC**, poly(DL-lactide-ε-caprolactone); **PLs**, phospholipids; **PLS-DA**, Partial-least squares discriminant analysis; **PPP**, pentose phosphate pathway; **PSCs**, pluripotent stem cells; **PTCs**, phosphatidylcholines; **PTEs**, phosphatidylethanolamines; **PTGs**, phosphatidylglycerols; **PUFA**, polyunsaturated fatty acid; **ROCK**, Rho kinase inhibitor; **SC**, stem cell; **SCRM**, single-cell Raman microspectroscopy; **SMs**, sphingomyelins; **SR-FTIR**, synchrotron radiation-based Fourier transform infrared; **SSCs**, spermatogonial stem cells; **SVZ**, subventricular zone; **TAG** , triacylglycerols; **TCA**, tricarboxylic acid; **TRPV**, transient receptor potential vallanoid sub-family 1; **UCMSC**, Umbilical Cord-Derived Mesenchymal Stem Cell; **VOCs**, volatile organic compounds; **YAP**, Yes-associated protein.

1. Introduction

Metabolomics has been increasingly employed within systems biology approaches to deepen the knowledge on stem cell (SC) characteristics and function. Compared to other omics, such as genomics or proteomics, metabolomics has more recently begun to be preferable applied in SC research [1] as it reflects not only upstream cell characteristics (genes, RNA, proteins), but also serves as a highly sensitive probe to external stimuli and SCs cross talk within their particular niche. This review provides an updated account of the main advances in metabolomics in SC research, with particular emphasis (although not exclusively) on findings reported in the last 5 years. This text begins with an introduction to the definition of metabolomics, the general analytical and statistical approaches usually involved, and the adaptation of typical metabolomics protocols (often applied to human biofluids/tissues) to the analysis of cells. An schematic brief account of SC metabolomic studies carried out to date will follow, while the main body of the review will focus on the metabolomic studies of the function and behaviour of different SC types, in different conditions. Metabolomic studies of SCs in cancer research were considered out of the scope of this review due to their specificity/extension (relevant information may be found elsewhere [2]), as were the wide range of studies of the impact of drugs, contaminants, immunomodulators and other agents on SC metabolism.

2. Cell metabolomics and brief overview of applications in stem cell research

Metabolomics consists of the comprehensive analysis of large numbers of low molecular weight endogenous molecules, or metabolites, in biological systems, using advanced profiling analytical techniques, typically Nuclear Magnetic Resonance (NMR) spectroscopy or Mass Spectrometry (MS), combined with multivariate statistical analysis (MVA) [3]. Metabolomics aims at determining fluctuations on metabolite levels, in biological matrixes (biofluids, tissues or cells), usually to probe the origin/impact of diseases or other perturbations (*e.g.*, therapy, diet, contaminants) on a living organism. Deviant metabolic features may pave the way to further understand organism response and unveil metabolic biomarkers of biological status. Metabolomics can either follow an untargeted approach, usually employing the more holistic NMR technique, or target a particular family of compounds for which prior knowledge on their relevance exists. Targeted metabolomics often benefits from the higher sensitivity of MS methodologies (<picomolar, compared to sub-millimolar in NMR), usually in tandem with a previous separation step, while untargeted NMR strategies offer higher reproducibility, non-destructiveness, and simple sample preparation (associated to automation and high-throughput characteristics well suited to omic approaches). Both analytical strategies are complementary and provide large amounts of data, which require MVA (either unsupervised, *e.g.* principal component analysis, PCA, or supervised, *e.g.* partial-least squares discriminant analysis, PLS-DA) to extract relevant and robust information on metabolic response. Less often, targeted metabolomics may also involve i) isotopic tracing protocols that highlight particular metabolic pathways and help unambiguous conclusions to be drawn on their regulation, as shown for SCs either using NMR or MS [4, 5] and ii) single-cell localized lipid analysis, through MS or vibrational spectroscopy in tandem with microscopy [1, 6], both subjects also mentioned in this review. Although the main use of metabolomics has involved human biofluids/tissues [3],

adequate protocols for cell harvesting and metabolism quenching have been developed for metabolomics [7], usually for analysis of cell extracts or endometabolome (*fingerprinting*) and/or of cell media or exometabolome, including cell secretome (*footprinting*). NMR offers the additional possibility to directly analyze cell pellets, using high-resolution-magic-angle-spinning (HRMAS) and, thus, minimize extraction-related metabolite alterations or loss, while assessing cell characteristics near to their native status [8]. A typical workflow for cell metabolomics (Fig. 1) usually begins with the challenging need to harvest large enough cell numbers and perform several independent assays, to enable a dataset that allows suitable statistical validation and unveil new metabolic information.

In its application to SCs, metabolomics has addressed many aspects of these cells´ properties and function. SCs are unspecialized (or undifferentiated) cells that can reproduce extensively (self-renewal) and have the ability to differentiate into specialized cells (potency) [9, 10]. These cells are usually classified with basis on: a) source - embryonic SCs (ESC, obtained from blastocysts in their early stages of development), and adult SCs (further classified depending on their location and/or type of lineages they can originate), and b) differentiation potency – totipotent (can give rise to embryonic and extraembryonic cell types), pluripotent (capable of producing any type of adult cell), multipotent (with multilineage potential) and unipotent (able to differentiate into one lineage alone). Within the scientific community, a rising and sustained interest in SC metabolomics has become clear over recent years (Fig. 2A), this account not including metabolic studies which measure only specific metabolites (and which thus do not qualify as metabolomic). Such studies have included untargeted and targeted strategies, with MS approaches predominating, compared to NMR. Most studies have addressed changes in SC metabolic profile due to different origins, or donors, or differentiation conditions, followed by studies of varying culture conditions (*e.g.* hypoxia or varying media composition). Some reports have addressed cellular aging, senescence or autophagy, or helped to support disease models (Fig. 2B). Mesenchymal SCs (MSC, multipotent adult SCs, isolated from different tissues, and which mainly differentiate into mesenchymal lineages, *e.g.* fat, bone and cartilage cells, although differentiation into other lineages is also possible) have been the target of most reports in all contexts, except disease models, whereas a consistent interest has become clear in the metabolism of pluripotent SCs (PSCs, that include embryonic SCS, or ESCs, and induced PSCs, or iPSCs, which are reprogrammed directly from a somatic cell through co-expression of defined pluripotency factors). Neural SCs (NSCs, multipotent adult SCs that give rise to neuronal and glial cells) have been the subject of fewer reports, along with other SCs (Fig. 2B). A few recent review papers (Table 1) have highlighted the prospective value of metabolomics in SC research, having discussed complementarity of NMR/MS metabolomics in MSC analysis and its potential compared to other omics [11], recognizing MS-based lipidomics as important to characterize adipocytes [12] and study lipid-mediated SC regulation [13], and including a critical global account of existing methods [1] while drawing attention to the need to carry out, not only typical cell metabolomic studies, but also metabolic flux analysis (or isotope tracing) and single-cell metabolomics (with in tandem microscopy).

3. Metabolomics of mesenchymal stem cells (MSCs)

3.1. Metabolic profile variability between tissue sources and donors

MSCs have been widely chosen as promising treatment options for various injuries and diseases, many studies having clearly established that their proliferation, differentiation, and immunological characteristics may differ significantly between source tissue types and donors. A global lipid profiling MS study of cell extracts showed that mouse ear MSCs, 3T3-L1 white preadipocytes, and BAT-C1 brown preadipocytes differed significantly in lipid composition [14]. A more recent MS-based metabolomics study [15] compared human SCs derived from bone marrow (hBMMSCs), adipose tissue (hAMSCs), periodontal ligaments (hPDLSCs) and salivary glands (hSGSCs), harvested from different donors. The levels of endogenous metabolites were significantly dependent on tissue type and MVA revealed significant changes in the levels of lysophosphatidylcholines (lower in hPDLSCs and hSGSCs) and lysophosphatidylethanolamines (higher in hPDLSCs and hSGSCs), such compounds having been suggested as potential biomarkers of source tissue, with possible relation to different differentiation capacities.

Increasing interest has been noted on metabolomics of MSC secretomes, which can comprise both soluble metabolites and extracellular vesicles important to cellular function. Cell supernatants analysed by liquid chromatography (LC)-MS metabolomics were observed to differ between hBMMSCs and hAMSCs, and suggested to relate to different efficacies of these cells in atherosclerosis therapies [16]. Indeed, exometabolome analysis revealed different impacts on the metabolisms of linoleic acid (LA), sugars and their derivatives (*e.g.* galactose, amino sugars and nucleotide sugars), amino acids (proline, glycine serine) and retinol. The authors noted, however, that inter-donor variability might contribute to the observed metabolic differences. More recently [17], it was reported that mouse AMSCs harvested from subcutaneous adipose tissue and visceral adipose tissue (related to the onset of metabolic diseases) exhibited complex ^{1}H NMR spectra (Fig. 3) and different exometabolome characteristics (as observed through MVA scores and loadings analysis, Fig. 4a,b), as well as distinct proliferation/differentiation potentials. Results showed that, although both cell types presented high secretion of lactate and citrate (alterations in glycolysis and mitochondrial metabolism, namely tricarboxylic acid, TCA, cycle), visceral AMSCs used mostly glycolysis. By changing culture media composition, distinct sensitivity to glutamine availability seemed to distinguish the two cell groups, along with different glutaminolysis roles in pyruvate metabolism. The distinct patterns of pyruvate assimilation were suggested to account for the lower ability of visceral AMSCs to differentiate into adipocytes and osteoblasts (Fig. 4c), unveiling the importance of metabolic mechanisms as potential discriminators between AMSCs from different depots [17]. The importance of exometabolome was again highlighted in a comparative study of hMSCs isolated either from umbilical cord stroma (Whärton jelly, UCMSCs) or from dental pulp (DPSCs) [18], both less invasive and ethically accepted sources of MSCs for therapy. The study presented a complete account of NMR secretome profiling and measurement of an extensive set of bioactive factors, in relation to cell characteristics and differentiation performance (in osteogenesis, adipogenesis and chondrogenesis). Secretome composition, characterised by ^{1}H NMR, was seen to vary in relation to levels of pyruvate, lactate, alanine and other metabolites, mostly related

to energy metabolism. The authors indicated, however, that overall profiles were similar for the two cell types over the conditioning period, whereas more significant changes were noted in secreted bioactive factors (although not impacting significantly on cell proliferation, migration, or tube formation capacity).

In relation to donor characteristics, the impact of donor obesity on hAMSCs endo- and exometabolomes was measured by MS metabolomics (LC-MS, gas chromatography(GC)-MS and capillary electrophoresis(CE)-MS) to investigate obesity implications on AMSCs stemness capacity [19]. Results unveiled increased secretion and intracellular accumulation of metabolites associated with glycolysis, TCA cycle, pentose phosphate pathway (PPP) and polyol pathway in obesity-derived hAMSCs compared to controls, in tandem with decreased proliferation, migration and differentiation abilities. In the same study, murine AMSCs also revealed significant changes in obese animals, particularly related to lipid and amino acid catabolism, however such features differed from those obtained for hAMSCs, highlighting that extrapolation of animal data to humans must be handled with care. Other authors have proposed the use of iPSCs-derived MSCs (iMSCs) to circumvent inter-donor variability [20]. The study compared native MSCs (nMSCs) with their corresponding iMSCs, for two different donors and using two different protocols. Cell endometabolome was analyzed by GC-MS, in tandem with other characteristics comprising differentiation capability, transcriptome, immunomodulatory potential and response to the proinflammatory interferon gamma (IFN-γ) cytokine. Unsupervised MVA of GC-MS data indicated enhanced metabolic activity in nMSCs and in some iMSCs, depending on the differentiation protocol employed, which also impacted importantly on immunomodulatory potential, plasticity and proliferation capacities [20]. It was concluded that, although the two differentiation protocols produced iMSCs similar to nMSCs, the few differences noted indicated the need of adequate protocol definition according to the desired clinical end use.

3.2. Differentiation

3.2.1. Adipogenesis

An initial report compared the ^{1}H HRMAS NMR spectra (cell pellets) of mouse BMMSCs before and after adipogenic differentiation [21], with a view to translating results (decreased levels of choline, creatine, glutamate, *m*-inositol, and increase in fatty acids, FAs) into non-invasive *in vivo* magnetic resonance imaging (MRI) measurements. Most subsequent studies targeted lipids, often for comparison of differentiated MSCs with adult adipose tissue cells. NMR of hUCMSCs extracts [22] recognized the need for high cell numbers (*ca.* $7x10^{6}$) and indicated increased methionine and FAs levels (and non-detectable creatine) as characteristic of adipogenesis. Subsequent reports employed MS metabolomics, with hBMMSC lipid extracts unveiling changes in free FAs throughout adipogenesis [23, 24]. MSC-differentiated preadipocytes (days 5-7) and adipocytes (day 21) lacked specific elongases (possibly hindering endogenous polyunsaturated FA, PUFA, synthesis) and differed from primary adipocytes in decreased levels of LA, α-linolenic acid (ALA) and arachidonic acid (AA); and increased

docosahexaenoic acid (DHA). The absence of essential FAs in adipogenic media (suggesting them as potential interesting supplements) was related to the MSCs inability to differentiate into fully functional adipocytes. GC-MS lipid analysis of rat BMMSCs, in tandem with functional studies, confirmed distinct lipid compositions for BMMSC-derived and native adipocytes, consistently with intra-cellular accumulation of autophagic vesicles rather than lipid droplets in the former. A lipidomics MS study [14] of MSCs extracts of different tissue types compared undifferentiated cells with derived adipocytes, and with mouse native adipose tissue cells (perirenal and inguinal white tissues, and interscapular brown adipose tissue). Substantial differences were noted in the levels of triacyl- and diacylglycerols (TAG and DAG), sphingomyelins (SM), glycerophospholipids, cardiolipin and hexosylceramides, results suggesting new lipid biomarkers for tissue-specific adipogenic processes.

More recently, adipogenic differentiation of hAMSCs was followed through GC-MS of volatile organic compounds (VOCs) in the headspace of cell cultures [25]. Adipogenesis was accompanied by changes in some VOCs, including acetaldehyde (related to mitochondrial pyruvate decarboxylation) and other compounds possibly related to increased oxidation processes [25]. The same type of SC was used to reconstruct a 3D adipose tissue model for comparison with native adipose tissue, comparing adipogenesis differentiation characteristics, as well as TAGs (main constituents of intracellular lipid droplets) and phospholipids (PLs) profiles, measured by GC-MS [26]. MVA of GC-MS data and Ingenuity pathway analysis assessed differential pathways between native and reconstructed adipose tissue, determined by distinct gene expression features. Genes regulating lipid metabolism were down-regulated in reconstructed tissue, possibly relating to the noted insufficient levels of essential FAs. Interestingly, culture medium supplementation, for instance with LA, was seen to alter end-point lipid profile in reconstructed tissue, emphasizing the possibility of media supplementation to specifically modulate PL profile. Combination of omics (metabolomics of both non-lipid metabolites and of lipids, or lipidomics, and proteomics) to follow hAMSC adipogenesis involved a LC-MS-based strategy [27] that unveiled reprogramming of more than 100 metabolic pathways in differentiated cells, revealing TAGs, gangliosides, and carnitine as important players in adipogenesis, along with down regulation of specific nucleotide derivatives and amino acids. The influence of different MSCs sources (rabbit adipose tissue or skeletal muscle) on lipidome evolution upon adipogenesis (compared to osteogenesis, as the two processes are found to often compete), assessed by MS of lipid extracts [28], showed that differentiation triggered changes in lipid composition, which however led to end lipid profiles that did not significantly depend on the original source tissue.

Although to a much lesser extent, vibrational spectroscopy has also been used in the study of MSCs adipogenesis. Following an initial Raman spectroscopy study of hAMSCs [29] that showed, through PCA of cell culture spectra, changes in a lipid band at 1438 cm^{-1} during adipogenesis, a synchrotron radiation-based Fourier transform infrared (SR-FTIR) microspectroscopic study of single hMSCs cells [30], as a function of time, reported changes in lipid structure and nucleic acids, as early

as days 1-3 of the differentiation process. This report revealed other important early-stage changes in cell components, which may determine further changes in low molecular weight (M_w) intracellular compounds. SR-FTIR was further applied to investigate the regulating role of the Yes-associated protein (YAP) in hMSC differentiation into fat and bone, having reported unique metabolic fingerprints for cells with different YAP expression levels [31].

3.2.2. Osteogenesis

In relation to 2D MSC cultures, one study monitored dexamethasone (Dex)-induced osteogenesis of mouse BMMSCs using LC-MS metabolomics of culture media [32], having registered a clear metabolic trajectory over time (Fig. 5). MSCs conditioned media exhibited increased levels of metabolites possibly related to cell proliferation, some of which (*e.g.* citrate, *cis*-aconitate, 2-oxoglutarate, succinate, glycerol, orthophosphate) were suggested as potential biomarkers of osteogenesis. Another report [33] considered hUCMSCs cultures and analysed cell extracts by GC-MS, during osteogenesis induced either by Dex or bone morphogenetic protein 2 (BMP-2). Results showed that hUCMSCs go through various metabolic stages before reaching their final, treatment-dependent, metabolic phenotype. Dex induced a global metabolic hyperactivity at day 7 (increased levels of glutamine, cholesterol, one-carbon metabolism intermediates, PLs, and glycerolipid precursors), subsequently reduced at day 14, and continuing to decrease until day 21 (low levels of TCA cycle, glycolysis, and glutaminolysis intermediates). BMP-2-stimulated cells exhibited less metabolite variations and a 7 day-delay, reflecting lower osteogenic efficiency. The end metabolic profile of Dex-treated cells was similar to that of primary osteoblasts, although showing higher levels of glucose, lactate, glutamine, lipid precursors, and TCA cycle intermediates. Some research has been directed to the use of giant plasma membrane vesicles (GPMVs) and MS metabolomics has shown that hBMMSC differentiation leads to lineage-specific lipidomic remodelling in the plasma membrane, resulting in unique membrane phenotypes for osteoblasts or adipocytes [34]. Following supplementation with DHA (a ω-3 FA), an osteoblastic membrane phenotype was promoted, possibly by up-regulating Akt (or protein kinase B, PKB) and unveiling a potential mechanism for dietary lipids promotion of and bone. Later, it has been suggested that GPMVs generated by osteoblasts [35] can direct osteogenic differentiation of hMSCs, possibly depending on size, complexity and lipid composition, as viewed by NMR of GPMV extracts. More recently, the role of YAP in regulating the adipo-osteogenic balance of hUCMSCs was investigated [31]. FTIR microspectroscopy was used to track structural changes of nucleic acids, proteins, and lipids in differentiated cells after manipulation of the YAP expression level. It was clear that YAP promoted osteogenesis (even within an adipogenic medium), whereas the absence of YAP induced adipogenesis. Notably, YAP-targeted differentiated cells and non-manipulated controls all displayed unique metabolic fingerprints. In addition, the role of natural products in SC differentiation has triggered some interest and the effect of astaxanthin (AST), a carotenoid pigment, on osteogenic differentiation of rat BMMSCs endometabolome was studied by LC-MS metabolomics [36]. Depending on the dose, AST was observed to impact on the levels of amino acids, organic acids and other compounds, and metabolic pathway enrichment analysis unveiled that AST mainly regulated phenylala-

nine metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; and pantothenate and coenzyme A (CoA) biosynthesis during the process of osteogenic differentiation.

Regarding MSCs osteogenesis, many studies have explored different 2D nanostructures and 2D/3D cell culture hydrogel-based environments. The physical properties of biomaterials alone have been observed to induce osteogenesis, for example, through nanotopography or mechanical stimuli (mechanotransduction), without the need for chemical or biological differentiation inductors. For instance, NSQ50 surfaces (engineered with 120 nm diameter dots disordered by a 50 nm offset in x/y axes) triggered osteogenesis, contrary to more tightly controlled dot arrangement surfaces (named SQ), which prolonged multipotency and led to modest differentiation degree [37]. With basis on MS data and metabolic pathway analysis, together with small RNA microarray analysis, among other measurements, the authors proposed that the observed increased levels of unsaturated metabolites in undifferentiated MSCs (compared to cells exposed to NSQ50) seemed related to mediation of differentiation through redox status regulation and activation of oxidative pathways. Furthermore, it was suggested that SQ surfaces mimic small adhesion sites in the hBMMSCs niche, whereas in NSQ50, MSCs have significantly larger adhesion complexes and intracellular tension that induce osteogenesis [37]. Adhesion blockage (with integrin antibodies) on SQ surfaces led to up-regulation of amino acid, lipid, nucleotide and carbohydrate metabolism, as active differentiation was initiated and, in the same report, LC-MS of cell fingerprints was recognized as an almost instant indicator of MSCs phenotype (compared to transcriptomics or proteomics) with high sensitivity to nanotopography-induced metabolite reorganizations. Other work revealed a possible association of lipoate, sphingosine, and several amino acids with the osteogenesis of hBMMSCs cultured on titania planar or nanostructured surfaces [38]. MSC osteogenic potential and associated intracellular LC-MS metabolic profiles were also evaluated on polycaprolactone surfaces patterned with bacterial inclusion bodies (IB) [39], used to vary surface topography, stiffness and wettability. A general metabolic up regulation trend was noted, with increased levels of carbohydrates, nucleotides, lipids, and amino acids. In addition, regulation of cell adhesion for the study of MSCs growth and differentiation was carried out by using enzyme-cleavable nanoscale surfaces [40]. A noted switch from low to high adhesion conditions led to significant metabolome changes, as viewed by LC-MS of cell extracts. High adhesion that promoted osteogenesis led to changes in pathways involved in energy, growth, protein, and skeletal development, whereas reduced adhesion was suggested to possibly result in adipogenesis. A very recent report [41] has also shown that microparticles with different architectural features induce distinct osteogenic behaviour of hBMMSCs, accompanied by significant change in the LC-MS metabolic profile of the cells exometabolome. Furthermore, the suitability of oxidized alginate (alginate di-aldehyde, ADA) functionalized with glycine-histidine-lysine (GHK) peptide, was compared to gelatin-based control hydrogels for seeding and enhancing hUCMSCs osteogenic differentiation [42]. As revealed by MVA of GC-MS metabolomics data, hUCMSCs in ADA-GHK hydrogels unveiled metabolic profiles that grouped close to those of primary osteoblasts (although retaining lower levels of lipids, lipid precursors cholesterol and *m*-inositol, and some amino acids: glutamine, threonine, aspartate and tyrosine), confirming improved osteogenic potential. Osteogenesis was accompanied by reduced activity of glycolysis, TCA

cycle, glutaminolysis and urea cycle, following a transition from a glycolytic phenotype in undifferentiated MSCs to significant energy-producing oxidative phosphorylation in differentiated cells. The authors suggested that GHK action in MSCs may relate to integrin ß1 and mediated by integrin linked kinase (ILK). Furthermore, the interaction between hAMSCs and a 3D nanocomposite scaffold composed of polyurethane, nano-hydroxyapatite, and decellularized bone particles was assessed through variations in both transcriptional and metabolite domains, the latter measured by LC-MS [43]. hAMSCs seeded on material scaffolds were compared to standard 2D cell cultures and several metabolites were specifically related to osteogenesis supported by the 3D scaffold, which contributed to an intricate cellular environment, promoting cell attachment and subsequent osteogenesis.

Interestingly, a nanovibrational bioreactor of hBMMSCs in 3D collagen gels was also observed to stimulate metabolic profiles characteristic of osteogenesis (upregulated lipid and carbohydrate pathways), linked to signalling mechanisms *e.g.* associated to adhesion and cytoskeletal tension, while leading to mineralized 3D tissue [44]. The role of mechanoresponsive channels in the osteogenic process was investigated, and transient receptor potential vallanoid sub-family 1 (TRPV) channels were proposed to play an important role in the activation of β-catenin signalling. The use of a nanovibrational bioreactor that converts hBMMSCs into bone-forming osteoblasts was further investigated as to vibrational amplitude conditions and induced cell mechanisms, importantly bringing together LC-MS metabolomics, gene and protein adaptations, in a full interdisciplinary study (Fig. 6) [45]. Increased vibrational amplitudes led to changes, not only in adhesion and ion channel expression, but also in the activation of energetic metabolic pathways, in turn related to low levels of reactive oxygen species (ROS) and inflammation, characteristics resembling those of natural bone-healing processes.

3.2.3. Differentiation into other lineages

NMR of the exometabolome of hUBMSCs during neuroglial differentiation on poly(DL-lactide-ε-caprolactone) (PLC) membranes [46] showed evidence of a metabolic switch from glycolysis-dependent expansion of undifferentiated cells to an oxidative metabolism characteristic of differentiated cells. The presence of more alanine than lactate in the media of neuroglial-like cells was associated with a reduced redox cytosolic state, which may indicate an important role of oxidative stress during the neuroglial differentiation of hUBMSCs. In tandem with *in vivo* testing of both undifferentiated and differentiated hUBMSCs, the authors have stated the promising potential of hUBMSCs, delivered through PLC membranes, for treating trauma to sensory nerves. Two metabolomic studies [47][48] have monitored the dynamic metabolism of hBMMSCs during chondrogenesis in 3D cultures. NMR analysis of cell-containing alginate beads suspensions [47] identified several metabolites as potential markers of chondrogenesis: increased FAs, decreased alanine and leucine, and increased glutamate and GABA. Chondrogenesis was also studied by MS imaging to determine lipids spatial distribution in hBMMSCs 3D micromasses, during the first steps of chondrogenic differentiation [48]. Over time, there were decreases in phosphocholine (PC), phosphatidylcholines (PTCs) and SMs, and increases of ceramides (products of SM metabolism), inositol- and ethanolamine-derived PLs.

3.3. Culture conditions

Several beneficial roles of hypoxia on SC biology have been recognized, including maintenance of undifferentiated status, cell proliferation rate enhancement, senescence inhibition, as well as on directing cell differentiation ability [49]. hMSCs under low oxygen tension were shown, by GC-MS and isotopic tracing of ^{13}C-glucose [50], to undergo increased glucose consumption, reduced pyruvate dehydrogenase (PDH) activity, and some extent of oxidative-phosphorylation, with oxygen tension having more subtle effects on hMSCs, compared to the differentiated cells (osteoblasts). Lipids have been revealed to play unexpected important roles in chondrogenesis, when carried out in hBMMSCs/chondrocytes co-cultures [51], where MS and MVA indicated that, under low oxygen tension, lipids decrease (in particular, cholesterol) seemed to be associated to improved extracellular matrix (ECM) deposition under hypoxia. On their own, hBMMSCs responded to hypoxia in a different way, as shown by up-regulation of TAGs, FAs and DAG, the latter suggested to affect MSC angiogenic potential [52]. Using chemical isotope labelling LC-MS metabolomics, several endo- and exometabolomes metabolites of human placenta-derived MSCs (hPMSCs) were also suggested as potential biomarkers of hypoxia, indicating a perturbation in arginine and proline metabolism, pantothenate and CoA biosynthesis, and alanine, aspartate, glutamate metabolisms [53]. More recently, MSCs exposed to hypoxia and serum deprivation displayed decreased expression of numerous lipid membrane components and significant alterations in glycolysis and TCA cycle intermediates, as viewed by GC-MS and LC-MS metabolomics, suggesting a conversion from aerobic respiration to glycolytic metabolism [54]. In the same study, exosomes (nanovesicles that mediate intercellular signalling) derived from these MSCs were shown to be packed with metabolites linked to the biosynthesis of amino acids, carbohydrates and nucleosides, including metabolites associated with anti-inflammatory and immunoregulatory functions. Hypoxia mimetic agents may be employed to avoid hypoxic culturing, which is difficult and expensive to carry out, and the effectiveness of desferrioxamine (DFO) in that role was investigated by GC-MS and LC-MS metabolomics of hBMMSCs to compared hypoxic and DFO-induced conditions [55]. The observed similar metabolic changes noted (except for purines, pyrimidines, and TCA cycle metabolites) led the authors to suggest low-concentration DFO as a potential substitute for hypoxic culturing.

Other culture conditions have been studied by metabolomics, for instance, ^{1}H NMR was used to compare unconditioned commercial and supplemented media (without cell contact) and during hUCMSCs expansion (conditioned media, considered as a possible alternative to hUCMSCs transplantation procedures) (Fig. 7) [56]. Conditioned media exhibited high concentrations of metabolites and soluble factors with proliferative, chemotactic, and immunomodulatory properties and provided valuable information on hMSCs metabolism *in vitro*. In addition, the MS metabolic profiles of hMSCs expanded under different cellular densities [57] unveiled several differences in glycolysis, oxidative phosphorylation, glutamine metabolism, and in the role of the pentose phosphate pathway, for maintaining cellular redox state. Furthermore, a recent study regarded the possible effect of low-intensity pulsed ultrasound (LIPUS) in promoting the proliferation of hASCs [58], as shown by LC-MS metab-

olomics and transcriptome experiments, which identified changes in 30 metabolites and in 27 genes, respectively.

3.4. Aging, senescence, and autophagy

Regardless of their source, MSCs aging may affect their replicative potential, immunomodulatory properties and differentiation capacity, thus some metabolomic studies have explored *in vitro* aging (or replicative senescence). In an initial work, proteomics was combined with NMR metabolomics to investigate the changes in H_2O_2-induced premature senescent hMSCs [59], showing increases in leucine and choline, and decreased in glycine and proline, probably as the result of higher H_2O_2-induced ROS levels. Subsequently, integration of lipidomics (through MS) with transcriptomics unveiled changes in lipid metabolism during *in vitro* aging, namely up-regulation of the majority of glycerophospholipids (PTCs, phosphatidylethanolamines (PTEs), and phosphatidylglycerols (PTGs)), sphingolipids (SMs, ceramides, cerebrosides, and gangliosides), and glycerolipids (DAG and some TAG) and down-regulation of a few glycerophospholipids and sphingoid bases [60]. These results suggested that aging-related lipid metabolism may play an important role in aberrant differentiations of hMSCs and might, to some extent, relate to an increased potential of adipogenic differentiation in aged BMSCs [60]. Furthermore, *in vitro* aging of hMSCs, as measured by GC-MS and LC-MS metabolomics, was suggested to involve a senescence-associated shift from oxidative metabolism (passages 2-5) to glycolytic pathways (passage 11) [61]. Autophagy capacity is a requirement for stemness and differentiation capacity, however it is known to decline with MSCs aging. This process was investigated for human placenta-derived hPMSCs through chemical isotope labelling LC-MS to target amine/phenol-containing and carboxylic acid-containing metabolites [62] and results showed a significant impact on arginine/proline metabolism, β-alanine metabolism and FA biosynthesis pathway.

4. Metabolomics of pluripotent stem cells (iPSCs and ESCs)

4.1. Metabolic remodelling during pluripotency acquisition

NMR footprinting and fingerprinting, in tandem with proteomics, was able to distinguish iPSCs from parental fibroblasts, in terms of metabolite levels that indicated elevated glucose utilization and production of glycolytic end products [63]. The authors concluded that the energetic metabolism of somatic cells converts into a required glycolytic metabotype, to induce pluripotency. More recently, the metabolomic profiles of rat ESCs, iPSCs and embryonic fibroblasts (EFs), were compared using NMR and LC-MS of cell extracts, as well as NMR of the exometabolomes [64]. Results showed that EFs reprogramming into iPSCs involved significant changes in cell metabolic profiles, while those of ESCs and iPSCs were much less marked, comprising differences in the levels of adenosine, cysteic acid, glucose, glycerophosphoglycerol, guanosine, inositol phosphate, m-inositol, phosphoserine and xanthosine.

Besides the reports above, most studies of the metabolic signature and biological events involving PSCs have been carried out by MS metabolomics, to the best of our knowledge. Sphingolipids, and particularly ceramides, have been analysed for the differentiation of mouse ESCs into

embryoid bodies (EBs) [65], using LC-MS for ceramide identification and transcriptomic profiling for mRNA measurements. Results indicated that conversion of mESCs to EBs is accompanied by changes in gene expression changes as well as in distribution for both sphingolipids and fatty acyl-CoAs. Other metabolomic studies reported that iPSCs exhibited a metabolic shift away from parental cells (and towards ESCs), displaying a clear conversion from somatic oxidative bioenergetics to glycolysis [63, 66] and a dynamic remodelling of PLs [67], along with decreased levels of pyrimidine and purine nucleotides, Krebs cycle intermediates [66] and some amino acids [68]. Despite the metabolic similarity of iPSCs and ESCs, iPSCs were found to have lower levels of unsaturated FAs and higher levels of metabolites involved in the *S*-adenosyl methionine cycle, suggesting that iPSCs adapt to a more ESC-like state the longer they remain in culture [66]. Another study [68] reported other differences between iPSCs and ESCs, namely in terms of PTCs, PTEs, amino acids and polyamine biosynthesis intermediates. Interestingly, partially reprogrammed cells were found to be characterized by an intermediate metabolic profile between those of fully reprogrammed iPSCs and their parental cells [69], as viewed by CE-MS metabolomics. The same technique was employed to help monitor iPSC quality through footprinting [70]. In the same year, a GC-MS metabolomics report addressed hPSCs response to Rho kinase inhibitor (ROCK), a serine-threonine kinase which helps maintaining growth and pluripotency [71]. Importantly, while gene expression and protein levels did not reveal any changes in the physiology of the cells, metabolomics revealed the fluctuating state of the metabolism, namely ROCK exposure leading to changes in glycolysis, glutaminolysis, the TCA cycle as well as the amino acid metabolism. Finally, the role of lipid metabolism has been revisited in a few recent studies, one of which has coupled CE-MS metabolomics with targeted proteomics to characterise the role of FA biosynthesis in the differentiation of hPSCs into hPSC-derived cardiomyocytes (hPSC-CMs) [72], demonstrating the importance of de novo FA synthesis for the survival of undifferentiated hPSCs and suggesting FA synthase inhibition as useful tool in regenerative medicine.

4.2. Differentiation

Untargeted MS metabolomics on mESCs differentiation [73] indicated that mESCs become enriched in highly unsaturated metabolites (including ω-6 and ω-3 FAs, *e.g.* AA, eicosapentaenoic acid, DHA), and depleted in saturated free FAs and acyl-carnitines. Interestingly, supplementation with these depleted compounds enhanced neuronal and cardiomyocyte differentiation, respectively, whereas eicosanoid pathway inhibitors promoted pluripotency. MS metabolomics was also used, in tandem with transcriptomic analysis, to establish that, although vascular endothelial and smooth muscle cells were metabolically distinct from the parental hESCs, their metabolic profiles seemed to match those of primary differentiated cells [74].

Many differentiation studies of PSCs using metabolomics have addressed iPSC differentiation into hepatocytes, mainly measuring lipid moieties by typical MS metabolomics, as a means to understand diseases such as fatty liver disease, atherosclerosis or acute liver failure. When in tandem with gene or transcriptomic profiling, as well as other biochemical measurements, such work has identified pathways involving polyunsaturated PLs and sphingolipids as important during differentiation, as well as

FAs, biliary acids and metabolites involved in glycolysis, TCA cycle, pentose phosphate shuttle and FA oxidation, as shown by a recent development of a microfluidic biochip strategy (coupling metabolomics and transcriptomics) to follow human iPSCs maturation into hepatocytes [75]. Still on an analytical note, other incurrences into chip-MS compared to LC-MS [76], HRMAS NMR compared to cell extract analysis [77], and single-cell Raman microspectroscopy (SCRM) [78] have been reported, aiming at further consolidating metabolite profiles of PSC differentiation. Characterisation of PSCs differentiation into endothelial cells [79] and neurons [77, 78] have also made use of metabolomics.

4.3. Culture conditions

In terms of different culture conditions, a CE-MS metabolomic analysis, in tandem with isotopic tracing studies [80], investigated the impact of media composition on hPSCs and derived cardiomyocytes. Glycolysis and glutamine oxidation were shown to be important pathways for energy generation and general survival of hPSCs, suggesting media tailoring as a strategy to eliminate residual and potentially harmful undifferentiated SCs. Isotopic tracing and GC-MS metabolomics were also used [5], supporting the idea that media importantly influence the metabolic state of hPSCs, being able to determine the interplay between lipogenic behaviour and redox pathways, again confirming a key role played by glutamine. A subsequent report employed NMR metabolomics to establish that ESC pluripotency seems to rely on the available relative levels of ascorbic acid and L-proline, the authors suggesting an important epigenetic role for those metabolites [81].

In a different context, the exometabolomes of iPSC-derived cardiomyocytes exposed to hypoxic conditions were compared with those of equivalent cells from non-human primates, using LC-MS metabolomics and transcriptomic analysis [82], with the aim of investigating treatment of myocardial infarction. The inter-species metabolic similarities and disparities in terms of hypoxia-changed pathways were discussed. Furthermore, another recent and multidisciplinary report [83] coupled LC-MS metabolomics with transcriptomic analysis, as well as epigenetic regulators and specific protein markers, demonstrates that the use of a stirred suspension bioreactor results in more effective maintenance of a hPSCs state of naïve pluripotent state, which may improve hPSC production for therapeutic applications.

4.4. Disease models

An earlier untargeted LC-MS study of cell extracts and media addressed the function of the Huntington locus (htt) gene and associated mutations and encoded proteins by comparing the metabolic profiles of syngeneic mouse ESCs [84]. The deviant metabolite levels thus found indicated metabolic aberrations triggered by htt in mESCs, *e.g.*, involving failure of ATP production, activated glycolysis and ketogenesis, depletion of intracellular nucleotides and deviant purine metabolism. Such results suggested a relationship between htt, mitochondrial function and early embryonic lethality. Other applications have used MS metabolomics to address Pompe disease (PD), a condition originated by an inborn deficiency in acid α-glucosidase, which causes glycogen excess in lysosomes (mainly in skeletal muscle and heart). The associated cellular metabolism was investigated in a PD model, through

LC-MS and CE-MS of cardiomyocytes differentiated from late-onset PD iPSCs, having shown that metabolites related to oxidative stress and mitochondrial disfunction seem particularly important [85]. When PD occurs in early infancy, the patients suffer from serious muscle weakness ad heart failure and iPSCs obtained from patients with infantile-onset PD and differentiated into myocytes were characterized by CE-MS metabolomics, in tandem with transcriptomic analyses [86]. Detected metabolite changes could be related to disturbance of mTORC1 signaling, involving changes in energy status and mitochondrial oxidative function, which may become possible therapeutic targets in PD. In the same year, in order to help investigate the metabolic impact of a genetic variant pinpointed by Genome-Wide-Association-Studies (GWAS) related to cardiometabolic disease, iPSCs obtained by reprogramming of peripheral blood cells were then differentiated into hepatocytes and adipocytes and MS metabolomics was performed on all three cell types, targeting lipid species and nucleotide/nucleoside phosphates [87], in tandem with transcriptomics. In particular, lipid accumulation was observed in differentiated hepatocytes, providing additional knowledge on the effects of GWAS variants related to metabolic disease. In addition, fibroblast-like synoviocyte (FLS) cells and iPSCs derived from patients suffering from rheumatoid arthritis and osteoarthritis were characterized as to their LC-MS metabolomic profiles [88] in search for metabolic biomarkers of such diseases. The metabolic profiles of patients´ FLSs and iPLS cells were clearly distinguished upon PLS-DA analysis, and nicotinamide (NAM) was found to impact importantly on the proliferation of rheumatoid arthritis iPSCs. The NAM inhibitor tannic acid was investigated as a possible therapy for those patients. More recently, Danon disease, a condition related to deficiency of a lysosome membrane protein, which also affects the muscle, heart among other manifestations, was investigated by a metabolomics and transcriptomics (as well as Raman and atomic force microscopy) approach to compare the metabolic profiles of patient-derived cardiac tissue with primary fibroblasts and human iPSCs differentiated into cardiomyocytes [6]. Both undifferentiated iPSCs and iPSC-derived cardiomyocytes were analyzed by LC-MS and isotopic tracing experiments, results having unveiled the importance of glycolysis and tryptophan metabolism, among other characteristics, in determining loss of cardiac biomechanical competence and, hence, Danon disease progression.

5. Metabolomics of neural stem cells (NSCs)

5.1. Differentiation

As noted earlier, NSCs have been studied through metabolomics to a much lower extent, compared to MSCs and PSCs. One study has employed NMR and MVA [89] to unveil metabolic distinguishing features between phenotypically similar SCs, namely rodent fetal, postnatal and adult NSCs (harvested from the subventricular zone (SVZ)) and Olfactory Ensheathing Cells (OECs, obtained from the olfactory bulb). Specific metabolite levels were observed to significantly vary both between NSCs and OECs, and between NSCs of different ages, with the results presenting potential usefulness in monitoring SC properties in different environments or in pathological conditions (*e.g.*, ageing and neurodegeneration). A second recent report was based on GC-MS and direct infusion (DI)-MS [90] to analyze both the polar and lipid metabolomes of rat NSCs (which proliferate in the presence of growth

factors) and differentiated cells (DCs, neurons and glia, win the absence of growth factors). Markedly distinct metabolic profiles were noted between NSCs and DCs, involving six lipids, together with creatinine, lactate, lysine, glutamine, glycine and pyroglutamate, such knowledge potentially aiding in the classification of NSCs and DCs in therapeutics of neurological disorders.

5.2. Culture conditions

As NSCs display tissue trophic and immune modulatory therapeutic roles in central nervous system (CNS) disorders, after transplantation, and it has been suggested that inflammatory cytokines may lead to metabolic reprogramming of NSCs to help regulate their immune modulatory effects [91]. Mouse NSCs harvested from the SVZ of the animals were analysed by GC-MS and LC-MS cell fingerprinting and footprinting (in tandem with other measurements), upon exposure to specific inflammatory cytokines. NSC incubation with ^{13}C-labeled arginine also helped tracking arginine and its downstream metabolites, by LC-MS. Results demonstrating the powerful hypotheses-generating ability of metabolomics to help understand how SC-mediated actions of tissue regeneration are affected by local inflammation effects. Notably, arginase signalling was unveiled as important in the communication between NSCs and the immune system. More recently, untargeted LC-MS metabolomics and pathway analysis supported a more global study of protein and metabolite changes in NSCs, in response to the IFN-γ cytokine, either in solution or immobilized in a biomimetic hydrogel [92]. The aim of IFN-γ immobilization was to provide conditions for prolonged NSCs neurogenesis to achieve more efficient therapeutic results in treating CNS injury. Both soluble and immobilized IFN-γ triggered changes in energy, lipid, and amino acid metabolisms, with the former impacting more significantly on NSC metabolome. Along a similar context, mouse NSCs have been cultured in a 3D graphene foam [93], believed to support cell growth and proliferation, enhancing NSC differentiation into astrocytes and neurons. GC-MS metabolomics clearly distinguished the metabolome of NSCs grown in 3D graphene, from those grown in 2D graphene films or polystyrene surfaces, mainly in relation to increased amino acid incorporation and enhanced glucose metabolism and the authors suggest a possible association of the observed effects with pathways relevant in Parkinson's disease.

6. Metabolomics of other SCs

6.1. Differentiation

A study of perivascular SCs investigated differentiation within hydrogels of different stiffness, specifically following the processed through LC-MS metabolomics of cell extracts [94]. Interestingly, soft gels induced neuronal differentiation, whereas stiff and more rigid gels induced preferential chondrogenic and osteogenic lineage, respectively. Metabolomics identified lysophosphatidic acid and cholesterol sulphate as particularly depleted in the latter two processed, respectively, and their specific needs were confirmed by posterior enrichment of culture media with such lipid moieties. This shows that integration of supramolecular biomaterials and tailored culture media may be effective in conducting SC differentiation. A similar strategy was followed to improve differentiation of human adipose

tissue pericytes (or perivascular SCs) into chondrocytes [95], by using a peptide hydrogel and showing that LC-MS metabolomics detects different metabolic features between such method and conventional chemical induced chondrogenesis methods. The authors highlight the importance of metabolomics to investigate cell crosstalk with the microenvironment and discriminate between different differentiated cell phenotypes.

The idea that metabolite levels in culture media may direct and influence SC fate was pursued in a study comparing mouse haematopoietic SCs (HSCs) to restricted haematopoietic progenitors using LC-MS metabolomics [96]. It was noted that each haematopoietic cell type had a distinct metabolic signature and that both human and mouse HSCs had unusually high levels of ascorbate, which decreased with differentiation. It was suggested that this metabolite may regulate HSC function and help to suppress leukaemogenesis. Untargeted comprehensive LC-MS metabolomics of primary human skeletal muscle cells during myogenic progression showed significant changes in metabolomic profiles in myoblasts undergoing proliferation and differentiation [97]. Pathway analyses revealed an interplay of different metabolic pathways in early and late differentiating cultures, shedding light into the maturation of muscle from progenitor myoblasts, potentially applicable in muscle regeneration and pathophysiology. In addition, lipid metabolism features, as evaluated by MS metabolomics [98] of human epidermal SCs revealed lipid accumulation during differentiation into keratinocytes, identifying ceramides and glucosylceramides as main players in the process.

6.2. Other studies

An initial NMR study of human hepatoblasts (hHBs) and human hepatic stem cells (hHpSCs) footprinting characterized cultures in in hyaluronan gels mixed with specific extra cellular components [99]. Another exometabolome NMR metabolomics study [100] (interestingly employing the projections of 2D ^{1}H J-resolved spectra, instead of the more typical approach of standard ^{1}H spectra) was recently carried out to investigate the effect of simulated microgravity, which promotes a 3D environment on the footprinting of human biliary tree stem/progenitor cells (hBTSCs), as well as of human hepatic cells (HepG2). Both cell types were cultured in a weightless environment obtained through a Rotatory Cell Culture system, compared to conditions under normal gravity. The produced 3D environments induced increased stemness and hindered differentiation of hBTSCs into mature hepatocytes. There were clear exometabolome changes, with microgravity inducing higher usage of glucose and lower glutamate and releasing higher levels of fermentation and ketogenesis product, compared to HepG2 cells. The exploitation of this behaviour of hBTSCs stemness maintenance contrasting with differentiation in hBTSCs-derived liver devices was discussed. A similar system was recently also used to test the effects of microgravity on epidermal SCs (EpSCs) [101], as viewed by LC-MS cell fingerprinting, and demonstrating significant impact on several metabolic pathways (including amino acid, lipids metabolisms).

In relation to MS-based studies, a CE-MS metabolomic analysis of [102] HSCs showed that a different intracellular metabolic profile is exhibited under hypoxic conditions, namely by generating ATP by anaerobic glycolysis involving pyruvate dehydrogenase kinase. This report suggested that such

mechanism may be determinant in HSC quiescence and function. A subsequent untargeted LC-MS study of the endometabolome of spermatogonial stem cells (SSCs) upon deprivation of Glial cell line-derived neurotrophic factor (GdnF), known to be determinant for SSCs proliferation, was reported [103]. Decreases were noted in 11 intracellular metabolites, whereas other 3 were observed to increase. Within the former, glycylglycine was observed to positively affect SC proliferation, although no association with the expression levels of self-renewal genes was found.

Future perspectives

The state of the art of metabolomics in the study of SC properties and function is presented in this text, after a brief introduction to traditional and emerging analytical strategies used in cell metabolomics. Metabolomic strategies employ either MS- or NMR-based data, the former having been the chosen analytical tool in most reports. However, the complementarity of both techniques is made clear in this paper, while uncovering underexplored research analytical niches, for instance employing direct cell analysis (through HRMAS NMR of cell pellets or single-cell microscopy-based metabolomics). As a valuable hypotheses-generation platform, metabolomics requires suitable statistical data handling (including statistical validation strategies), as well as strategies to test biological hypotheses, for instance through bioinformatics metabolic pathway analysis, isotope tracing studies to validate putative up/down-regulation of specific pathways, or correlation with other omics data. In some reports, such interdisciplinary strategies have been reported, clearly indicating the need to strengthen such methodologies, to ensure data robustness and demonstrate putative new biological hypotheses. Furthermore, the standardization standard operating procedures in relation to SC culture, harvesting, storage and handling for metabolomics would be of great value, particularly regarding cell numbers, cell status and passage, harvesting method, storage time/temperature and extraction methods.

Various aspects of MSCs behavior are identified as the most studied by metabolomics so far, followed by PSCs and NSCs, and only a few reports on other SC types. This text unveils, therefore, several additional SC types and research aspects as niches for future metabolomic applications, namely hematopoietic SCs, which hold a strong impact in clinical strategies. Some of the central ideas investigated so far comprise the metabolic impacts of origin tissue and donor, differentiation conditions, and other culture conditions (such as hypoxia) or media enrichment. In the case of PSCs, metabolic remodeling, and their use to build disease models has also been addressed. Generally, studies have addressed either or both endometabolome and exometabolome (including cell secretome), having measured lipid species to a large extent, along with different types of polar metabolites involved in a variety of metabolic pathways. Recent advances draw attention to the importance of 3D culture environments, mechanical cues and/or media enrichment to guide differentiation into optimized particular lineages.

This review demonstrates the enticing potential of metabolomics strategies to significantly contribute to a deeper knowledge of SC behavior and identify cell performance biomarkers, which may, in time, become of particular importance for instance for identifying adequate donors/tissues for particular ends or guiding differentiation into pure specific lineages. The evaluation of *in vivo* post-implantation SC performance is also particularly suited to metabolomic strategies (*e.g.* through bioflu-

ids), an approach which is presently still underexplored. Hence, either *in vitro* or *in vivo,* metabolomics has the undeniable potential of becoming of practical use in personalized clinical applications related to SC-based tissue regeneration strategies or disease characterization and follow-up.

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Authors' Contributions

AMG had the idea for this review article; DSB, CSHJ, IMCM, and KMR performed the literature search and data analysis; AMG and DSB drafted the manuscript; JFM and MBO critically revised the manuscript; all authors read, revised and approved the final version of this work.

Figure captions

Fig. 1. Scheme of a typical metabolomics workflow applicable to cell analysis. LC-MS, liquid chromatography in tandem with mass spectrometry; GC-MS, gas chromatography in tandem with mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; HRMAS NMR, high-resolution-magic-angle-spinning NMR. Some elements of this picture were adapted from Servier Medical Art (https://smart.servier.com/) licensed under a Creative Commons Attribution 3.0 Unported (CC BY 3.0) license.

Fig. 2. Stem cell metabolomics studies to date (excluding cancer stem cell research). (A) Number of research papers published up to 15 January 2021 (*), as a function of year and analytical technique employed: nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS), and/or others (including Fourier-transform infrared spectroscopy and Raman spectroscopy). **(B)** Number of research papers on different types of stem cells (colour code), distributed by main research topics. Web of Science searches using [stem cell*] AND [metabolomic* OR metabonomic* OR (metabol* AND (profil* OR fingerprint))] and [stem cell*] AND [lipidomic*]. These comprise studies of different differentiation conditions in 2D and 3D cultures, and/or nanotopographic surfaces; exposure of stem cell (SCs) to several other conditions (*e.g.* different O_2 availability, presence/absence of clinically relevant compounds); metabolome analysis of one or more SC types, donors and/or tissue sources; studies of SC aging, senescence and autophagy; and several disease models. MSCs: Mesenchymal Stem Cells; PSC: Pluripotent Stem Cells; NSC: Neural Stem Cells.

Fig. 3. Typical ^{1}H NMR spectrum of subcutaneous mouse adipose-derived mesenchymal stem cells (mAMSCs) culture medium 72 h after reaching 75% confluency (600 MHz, 30.0°C). Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) was supplemented with 2 mM L-glutamine-L-alanyl (stable glutamine), 1 mM pyruvate, 100 U/ml penicillin, 100µg/ml streptomycin, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10% fetal calf serum. Major metabolite peak assignments are indicated. Adapted from reference [17], licensed under Creative Commons Attribution 4.0 International (CC BY 4.0) license.

Fig. 4. Multivariate statistical analyses of the differences between the exometabolomes (72 h after 75% confluency) of subcutaneous mAMSCs (S-ASC) and visceral mAMSCs (V-ASC). Culture medium composition as described in Fig. 3. NMR absolute spectra bins after subtraction of the

culture medium signal analysis performed on NMR spectra buckets without normalization to the cell number. (A) Untargeted principal component analysis (PCA) readily evidences the tissue of origin as a major source of the dataset variance. Score plot (PC1 and PC2; n = 11, R^2 = 0.962, and Q^2 = 0.843 on 5 PC). (B and C) Orthogonal partial least squares discriminant analysis (OPLS-DA, supervised multivariate analysis) shows a strong discrimination between subcutaneous mAMSCs (S-ASC) and visceral mAMSCs (V-ASC), characterized by high values of goodness-of-fit model parameters (R^2X=0.796, R^2Y=0.991, and Q^2=0.969). The discrimination robustness was validated by resampling 1000 times the model under the null hypothesis (data not shown), and the analysis of variance (CV-ANOVA) of the model led to a p-value of 1.20×10^{-4}. (B) Score plot discriminating S-ASC (in green) and V-ASC (in blue). (C) Loading plot complemented by color-coded correlation indicating statistically significant signals. (1) leucine, (2) valine, (3) lactate, (4) alanine, (5) acetate, (6) glutamine, (7) citrate, (8) glucose, (9) tyrosine, (10) phenylalanine. (D) Simplified non-quantitative representation of the main metabolic pathways (represented in italics) in actively dividing mAMSCs. Colors identify the metabolic pathways analysed in this study. EAA, essential amino acids; mAMSCs, mouse adipose-derived mesenchymal stem cells; TCA, tricarboxylic acid cycle. Adapted from reference [17], licensed under Creative Commons Attribution 4.0 International (CC BY 4.0) license.

Fig. 5. OPLS-DA scores plot of LC-MS data from mBMMSCs conditioned medium samples (controls and dexamethasone-induced osteogenesis), and their corresponding medium-only blanks, analysed after 5, 10 and 15 days in culture. (A) Combined controls and OS conditioned samples *vs*. their respective medium-only blanks (R^2=0.841 and Q^2 = 0.644). (B and C) Metabolic trajectory over time (arrows) of control samples (B, R^2 = 0.991 and Q^2 = 0.756) and OS (C, R^2 = 0.992 and Q^2 = 0.777). LC-MS, Liquid chromatography mass spectrometry; mBMMSCs, Mouse bone-marrow mesenchymal stem cells; OPLS-DA, Orthogonal partial least squares discriminant analysis; OS, osteogenesis. Adapted from reference [32].

Fig. 6. Untargeted LC-MS metabolomics analysis of hBMMSCs after 1 and 2 weeks of nanostimulation at 30 nm (N30) and 90 nm (N90) amplitudes. (A) Lipid heatmaps of N30 and N90 compared to control conditions. (B) Principal component analysis (PCA) of lipid data, compared to controls. (C) Observed metabolite changes in ROS pathways following 1 week of culture under N30 or N90 conditions. (D) Potential pathways derived from the heatmap data. The data indicate the activation of ROS and redox-balancing pathway occurs in conditions of increasing nanostimulation amplitude. LC-MS, Liquid chromatography mass spectrometry; mBMMSCs, Mouse bone-marrow mesenchymal stem cells; ROS, reactive oxygen species. Adapted from reference [45], licensed under Creative Commons Attribution 4.0 International (CC BY 4.0) license.

Fig. 7. ^{1}H NMR spectra of media samples before (control samples) and after exposure to human umbilical cord mesenchymal stem cells (hUCMSCs) and plasma from umbilical cord blood. (A) Dulbecco's Modified Eagle Medium/Nutrient Mixture (DMEM, Gibco) before SC exposure. (B)

DMEM after 24 h hUCMSCs exposure (exometabolome). (C) Commercial medium (from PromoCell, LabClinics, Promocell, C-28010) after 48 h SC exposure (exometabolome). (D and E) Commercial medium after 24 h hUCMSCs exposure (exometabolome). (F) Commercial medium before hUCMSCs exposure. (G-K) Plasma samples from different donors. Peak assignment: 1, Lipids; 2, Isoleucine/Leucine/Valine; 3, Ethanol; 4, b-hydroxybutyrate; 5, Lipids; 6, Threonine; 7, Lactate; 8, Alanine; 9, Lysine/Arginine; 10, Acetate; 11, Glutamate; 12, Glutamine; 13, Methionine; 14, Pyruvate; 15, Citric acid; 16, Choline; 17, Inositol; 18, a-Glucose; 19, b-Glucose; 20, Tyrosine; 21, Phenylalanine; 22, Histidine; 23, Nicotinamide; 24, Tryptophan; 25, Thiamine; 26, Formate; 27, Urea; 28, Purines. Adapted from reference [56], licensed under Creative Commons Attribution 4.0 International (CC BY 4.0) license.

Tables

Table 1. Selected review papers that emphasize the importance and potential of stem cells (SCs) metabolomics.

Reference	SC type(s)	Analytical technique	Main subject
[13]	PSCs and adult SCs	MS	SC lipidome and its association with genetic and/or pharmacological features for SC regulation
[11]	MSCs	NMR/MS	Elucidation of MSC function (intracellular metabolites and secretome)
[1]	SCs in general	NMR/MS	Targeted and untargeted metabolomics to study stem and progenitor cells
[12]	Adipose SCs	MS	Lipidomics of adipocyte differentiation, physiology and pathophysiology

MS, mass spectrometry; MSC, mesenchymal SCs; NMR, nuclear magnetic resonance spectroscopy; PSC, Pluripotent SCs.

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