

Development of non-destructive methods of the Characterization of Mesenchymal Stem Cell Differentiation in vitro

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II. ABSTRACT

Real time monitoring of stem cells has been a growing area of interest over the past decade because of new regenerative medicine approaches. Also, the effect of culture composition on stem cell metabolic pathways and their regulation of cellular fate are of increasing importance. In our project, non-destructive metabolomic analysis of stem cells and their physiological status during proliferation and differentiation stages will be investigated. This will be achieved through mass spectrometric analysis of released metabolites by LC-MS and GC/MS; therefore stem cells should ideally be cultured in a specialized format to maintain their physiological properties during non-invasive live analysis while being compatible with real time GC/MS. To address this issue, mesenchymal stem cell attachment, proliferation and differentiation were tested in 20ml glass vials required for GC/MS analysis. Environmental conditions in which MSCs can proliferate under sealed condition and at room temperature were also optimized. Our data show the potential of MSCs to attach, proliferate and differentiate in 20ml glass vessels. Furthermore, cells could maintain their metabolic activity for approximately 6hrs not only under hypoxic but also hypothermic conditions. Therefore, live time analysis of their chemical volatiles could be performed in a format compatible for LC-MS and GC/MS which will be performed for undifferentiated MSCs and their differentiated populations (osteogenic and adipogenic lineages) in the next phase of the project. A specific treatment approach has emerged from hMSCs osteogenic study and identified a particular pathway suggested to be involved in chondrogenesis, and linked to the collagen II biosynthesis. The treatment is based on natural products (L-lysine and ascorbic acid) and when tested on

human cell cultures, was observed to significantly increase the morphological and functional markers of chondrogenesis, suggesting its potential to promote de novo cartilage formation in vitro.

III. TABLE OF CONTENTS

I. ACKNOWLEDGMENTS.....	1
II. ABSTRACT.....	2
III. TABLE OF CONTENTS.....	4
IV. LIST OF FIGURES.....	10
V. LIST OF TABLES.....	13
VI. LIST OF ABBREVIATIONS.....	14
1. CHAPTER-1- REVIEW ARTICLE	18
1.1 ABSTRACT.....	17
1.2 INTRODUCTION	17
1.2.1 Direct monitoring of cell populations using in vitro cellular assays	18
1.2.2 Indirect cell monitoring in vitro.....	21
1.3 GENERAL PROJECT AIMS	31
1.3.1 Objectives	32
2 CHAPTER-2- NON-DESTRUCTIVE CHARACTERISATION OF MOUSE MESENCHYMAL STEM CELL DIFFERENTIATION USING LC- MS-BASED METABOLITE FOOTPRINTING.....	33
2.1 ABSTRACT.....	33
2.2 INTRODUCTION	35

2.3	MATERIAL AND METHODS	37
2.3.1	Cell culture and differentiation assay	38
2.3.2	Metabolites extraction	39
2.3.3	Analytical methodologies	39
2.3.4	Metabolites identification process	40
2.4	RESULTS	41
2.4.1	In vitro model of osteogenic differentiation	41
2.4.2	Multivariate analysis of LC-MS profiling data	43
2.4.3	Feature selection: Analysis of MSC culture samples versus blank media without cells.....	46
2.5	DISCUSSION	53
2.5.1	Confirmation of osteogenic differentiation	53
2.5.2	Non-invasive metabolic profiling of MSC osteogenic differentiation	54
2.6	CONCLUSION	60
3	CHAPTER-3- COMBINED METABOLOMICS/VOLATOLOMICS ANALYSIS OF MOUSE MESENCHYMAL STEM CELL DIFFERENTIATION	60
3.1	ABSTRACT:.....	60
3.2	INTRODUCTION	62
3.3	MATERIAL AND METHODS	65
3.3.1	Cell culture and differentiation	63

3.3.2	Metabolic activity assay	65
3.3.3	LC-MS and /GC-MS analyses	65
3.4	RESULTS	67
3.4.1	The potential of MSCs for proliferation into 20ml glass vials and under hypoxic/hypothermic conditions	67
3.4.2	Testing the influence of increased seeding density	74
3.4.3	Metabolomics changes in MSCs under osteogenic/adipogenic treatment:.....	75
3.4.4	Volatolomics compounds detected in MSC cultures undergoing osteogenic/adipogenic differentiation	84
3.5	DISCUSSION	84
3.5.1	The potential of MSCs for proliferation into 20ml glass vials and under hypoxic/hypothermic conditions	84
3.5.2	The optimum seeding density for GC-MS analysis.....	85
3.5.3	Candidates arisen from the metabolomics footprinting.....	90
3.5.4	Candidates arisen from the volatolomics footprinting.....	94
3.6	CONCLUSION	100
4	CHAPTER-4- METABOLOMIC ANALYSIS OF INTRACELLULAR AND EXTRACELLULAR CHANGES DURING HUMAN MESENCHYMAL STEM CELL OSTEOGENESIS.....	103
4.1	ABSTRACT:.....	103
4.2	INTRODUCTION	104

4.3	MATERIALS AND METHODS.....	100
4.3.1	Cell culture and differentiation assay	100
4.3.2	Metabolites extraction	102
4.3.3	Analytical methodologies	103
4.3.4	Metabolites identification process	104
4.4	RESULTS N	105
4.4.1	In vitro model of osteogenic differentiation	105
4.4.2	Multivariate analysis of LC-MS finger and footprinting data: .	108
4.4.3	Metabolic finger of MSC under osteogenic differentiation.....	111
4.4.4	Metabolic footprinting of MSC under osteogenic differentiation	113
4.5	DISCUSSION	115
4.5.1	In vitro model of osteogenic differentiation	115
4.5.2	Metabolic fingerprinting of MSC under osteogenic differentiation	115
4.5.3	Metabolic fingerprinting of MSC under osteogenic differentiation	119
4.6	CONCLUSION	124
5	CHAPTER-5- A NOVEL METABOLIC COMBINATION FOR ENHANCED CHONDROGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS IN VITRO.....	131

5.1	ABSTRACT:.....	131
5.2	INTRODUCTION	132
5.3	MATERIALS AND METHODS.....	139
5.3.1	Cell culture and differentiation in 2D and 3D conditions.....	139
5.3.2	Quantification of GAG production using Alcian Blue	143
5.3.3	Quantification of GAG production using Safranin-O	143
5.3.4	Quantification of GAG production using 1,9-dimethylmethylene blue (DMMB).....	144
5.3.5	Collagen fibres detection by Masson Trichrome.....	145
5.3.6	Pellets histology.....	146
5.3.7	Statistics.....	147
5.4	RESULTS	148
5.4.1	In vitro model of chondrogenic differentiation	148
5.5	DISCUSSION	158
5.6	CONCLUSION.....	162
6	CHAPTER-6- GENERAL CONCLUSIONS AND FUTURE WORKS.....	164
6.1	NON-DESTRUCTIVE CHARACTERISATION OF mMSCs OSTEOGENIC DIFFERENTIATION USING LC-MS-BASED METABOLITE FOOTPRINTING.....	180
6.2	MONITORING OF mMSCs OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION USING COMBINED LC-MS AND GC-MS.....	182

6.3	COMBINED INTRA- AND EXTRACELLULAR METABOLOMICS ANALYSIS OF hMSCs OSTEOGENIC DIFFERENTIATION.....	184
6.4	A NOVEL APPROACH IN CHONDROGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS...	185
	REFERENCES.....	172

IV. LIST OF FIGURES

Figure 1.1 : Free and bound NADH distribution within NPSCs..	24
Figure 1.2: Schematic diagram of the electrochemical detecting system for continuous monitoring of extracellular lactate production from cardiomyocyte following hypoxia.....	27
Figure 2.1: Osteogenic differentiation in MSC cultures after 5, 10 and 15 days	42
Figure 2.2: OPLS-DA scores plots of MSC medium samples from standard and OS-treatment, and their corresponding medium-only blanks, analysed after 5, 10 and 15 days in culture.	45
Figure 2.3: Functional categorisation of the nutrients and metabolites produced by MSCs in their culture medium.....	47
Figure 2.4: MSC-related metabolites found to increase over time in culture medium in the presence of MSCs, both in control medium and OS medium, at day 5, 10 and 15 of culture.	49
Figure 2.5: Metabolite changes identified in the culture medium MSC-related metabolites found to increase over time in culture medium in the presence of MSCs under OS treatment compared to control medium, at day 5, 10 and 15 of treatment..	51
Figure 2.6: Biological pathways highlighted by the compounds differentially detected in MSC-conditioned culture medium.....	57
Figure 3.1: Testing the attachment of MSCs into 20ml glass vials.	68
Figure 3.2: Testing metabolic activities of miMSCs at different sealing times	70
Figure 3.3: Microscopic appearance of miMSCs under hypoxia and hypothermia and their metabolic activities.....	72

Figure 3.4: Testing miMSCs metabolic activities under hypoxia and hypothermia	75
Figure 3.5: Testing seeding density in glass vials.....	75
Figure 3.6: MSCs maintained in standard, adipogenic and osteogenic medium analysed at day 0, 5 and 10.....	76
Figure 3.7: LC-MS analysis of the compounds identified in medium samples from MSCs under standard, OS and AD treatments, and their corresponding medium-only blanks, analysed after 0, 5 and 10 days of treatment	78
Figure 3.8: Metabolite changes identified with LC-MS in MSC cultures under OS/AD conditions compared to control medium, at day 0, 5 and 10 of treatment.	80
Figure 3.9: Volatile compounds identified showing differential detection in samples from standard, OS and AD differentiated MSC cultures.	83
Figure 4.1: Evaluation of osteogenic differentiation in hMSC cultures after 7, 14 and 21 days..	107
Figure 4.2: OPLS-DA scores plots of MSC medium samples from standard and OS-treatment, and their corresponding medium-only blanks, analysed after 0, 14 and 21 days in culture	110
Figure 4.3: Intracellular metabolite changes identified in the culture medium hMSC-related metabolites found to increase over time in culture medium in the presence of MSCs under OS treatment compared to control medium at day 5, 10 and 15 of treatment.	112
Figure 4.4: Extracellular metabolite changes identified in the culture medium hMSC-related metabolites found to increase over time in culture medium in the	

presence of MSCs under OS treatment compared to control medium at day 5, 10 and 15 of treatment..	114
Figure 4.5: Biological pathways highlighted by the compounds differentially detected in MSC finger and footprinting	123
Figure 5.1: Schematic of the chondrogenic differentiation process	134
Figure 5.2: Effect of L-lysine and Ascorbic Acid supplementation on hMSC chondrogenic differentiation. hMSCs micropellet cultures treated for 21 days with different CD media.	150
Figure 5.3: Alcian blue staining of hMSC chondrogenic differentiation for 21 days in 2D.	153
Figure 5.4: Comparison of the hMSC chondrogenic response in 3D and 2D culture after 21 days of treatment Safranin O staining	157
Figure 5.5: Comparison of the hMSC chondrogenic response in 3D and 2D culture after 21 days of treatment Massion Trichome staining	161

V. LIST OF TABLES

Table 2-1: The most significant metabolites that were increased specifically during OS differentiation analysed by OPLS-DA and one-way ANOVA.....	52
Table 3-1: Measuring surface area well plates compared to 20 ml glass vials.	67
Table 4-1: The most significant metabolites that increased specifically during OS differentiation compared with untreated control analysed by OPLS-DA and one-way ANOVA.	125
Table 4-2: The most significant metabolites that increased specifically within untreated control compared with OS treated samples analysed by OPLS-DA and one-way ANOVA.	129
Table 5-1: Chondrogenic differentiation medium and effects of individual components on chondrogenesis. Adapted from (Tang et al, 2015).	137
Table 5-2: The composition of different chondrogenic treatments used in 2D and 3D.....	141

VI. LIST OF ABBREVIATIONS

AB	Alamar blue
AB	Alcian Blue
ACP	Acid phosphatase
AD	adipogenic
ALP	Alkaline phosphatase
ASC	Ascorbic acid
AZ	Alizarin Red
CD	Chondrogenic differentiation
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
FAP	fluorapatite-based glass-ceramic
FCS	Fetal calf serum
FCS	fluorescence-activated cell sorting
FLIM	Fluorescence Lifetime Imaging
FRET	Fluorescence/Förster resonance energy transfer
GC-MS	Gas Chromatography Mass Spectrometry
hHpSC	human hepatic stem cells
HIF-I	hypoxia-inducible transcription factor I

hMSC	human mesenchymal stem cells
hESC	human embryonic stem cells
IBMX	3-isobutyl-1-methylxanthine
LC-MS	Liquid Chromatography Mass Spectrometry
LDH	Lactate dehydrogenase
m/z	mass-to-charge ratio
MMC	mitomycin C
mMSC	Mouse mesenchymal stem cells
MT	Masson Trichome
MTT	Thiazolyl Blue Tetrazolium Bromide
NADH	Nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NSC	neural stem cells
OPLS-DA	orthogonal partial least squares discriminant analysis
OS	osteogenic
PB	PrestoBlue
pHEMA	Poly(2-hydroxyethyl methacrylate)
PTH	Parathyroid hormone

SO	Safranin O
SPME	phase through solid-phase microextraction
SPR	Surface plasmon resonance
TCA cycle	Tricarboxylic cycle
VOC	Volatile organic compounds
β GP	beta-glycerophosphate

1. CHAPTER-1- REVIEW ARTICLE

1.1 ABSTRACT

To establish a process of quality control of the cells before they are used in new regenerative medicine approaches. In this chapter, non-destructive methods for cell monitoring in vitro are reviewed, including recent developments enabling metabolomic monitoring using LC-MS and GC-MS. Finally, the application of metabolomics in the examination of stem cell differentiation is discussed, suggesting the potential of metabolomic profiling as a powerful tool for regenerative medicine approaches.

1.2 INTRODUCTION

The potential of stem cells for self-renewal and differentiation into specialized cell lineages has opened promising therapeutic opportunities such as tissue repair and regeneration. Cell characterizations methods based on polymerase chain reaction (PCR) for detecting specific gene expression [1] and immunoassays for protein detection [2] are sensitive but either destructive or semi quantitative . Furthermore, variation in their metabolic phenotypes has a great impact on each stage of proliferations and specializations. Consequently, the need for developing cell metabolism analysis to explore intracellular activities/metabolic pathways that affected by growth factors, nutrients and cytotoxic agents throughout cultivation process has been concerning scientists for decades.

Therefore, alternative non-invasive techniques are introduced not only to measure molecular substances released into culture medium by viable or dead cells or analyze intracellular fluorophores but also to monitor the metabolites released to the microenvironment. In this section, an overview of some present non-destructive methods for the evaluation of cells viability/toxicity and proliferation will be introduced. Recently, real-time microscopy to analyse growing human embryonic stem cells hESCs has been introduced using Cell-iQ analyser [3]. Though live cell microscopic imaging allows the observation of active changes that provide more insight into a variety of cellular processes, they can be challenging. Maintaining the cells as closely as possible to their physiological condition requires the use of onstage incubator to control the temperature, humidity and gases, and real time laps imaging of dynamic processes requires active observation. Also, quantitative comparisons between optical and biochemical measurements are needed to validate the sensitivity and specificity of optical assessments [4]. Therefore, this review is focusing on the non-microscopy based methods of cell monitoring.

1.2.1 Direct monitoring of cell populations using in vitro cellular assays

1.2.1.1 Non-destructive cell viability/ metabolic activity and toxicity assays

Enzymatic assays for detecting and semi-quantifying viable cells are commonly used in pre-clinical and clinical research. They rely on measuring enzymatic activity through absorbance/emission of colorimetric/ fluometric substrates or metabolites that provide a read-out for the number of viable cells in culture medium. The absorbance or emission is then measured at particular wavelengths using a plate reader (spectrophotometer). Cell toxicity assays such

as the ones described below are also used in pre-clinical study particularly in pharmaceutical research. The main advantage of these techniques is that they can be applied to live cultures in monolayer or cell suspension. Also they are simple, non-destructive, inexpensive and environmentally safe [5].

1.2.1.1.1 Acid phosphatase (ACP)

Acid phosphatase is a lysosomal enzyme that hydrolyzes phosphate under acid condition through the conversion of intracellular *p*-nitrophenyl phosphate into *p*-nitrophenol [6]. Osteoclast activity has been linked to type 5b ACP synthesis and secretion, therefore its serum level has been used to monitor bone resorption conditions [7] ACP is involved in a variety of metabolic activities such as permeability, growth and cell differentiation, protein synthesis, absorption transport of nutrients and bone resorption [8]. Although present in small amount in different cell type, acid phosphatase concentration is higher in endothelial cells [6]. However due to the variation of acid phosphatase levels among different cell types, the use of cell specific controls is a recommended step to correlates enzyme activity in culture with tested cell number [6].

1.2.1.1.2 Thiazolyl Blue Tetrazolium Bromide (MTT)

MTT assay is based on analyzing the absorption of blue formazan crystals as a result of tetrazolium salt conversion by mitochondrial dehydrogenase, which is responsible for electron transport system- such as Nicotinamide adenine dinucleotide NADH and nicotinamide adenine dinucleotide phosphate NADPH [9, 10], therefore its concentration reflects cellular metabolic activity and is thought to be constant in different cell types. However, MTT contains sulfur oxide which could explain its reported cytotoxicity by affecting cellular

membrane permeability [11]. Hamid and coworkers (2004) reported MTT cytotoxic effect leading to loss of adherence and necrosis; therefore, cells cannot be reused after the assay. Moreover, MTT reaction seems to be altered by medium components such as glucose, as elevation of glucose concentration in the culture medium has been reported to increase MTT reaction [12]. Consistent with this finding, alternative techniques sharing the same principle of enzymatic conversion have been introduced as described below.

1.2.1.1.3 Alamar blue AB

For instance, **AB** is an alternative assay based on the reduction of the blue non-fluorescent dye resazurin into pink fluorescent resorufin by diaphorase, which are reductase diaphorases located into mitochondria or cytoplasm such as NAD(P)H:quinoneoxidoreductase and flavin reductase [13]. Due to the solubility of both dyes in culture medium, cultivated cells are not compromised and further cellular investigation can be performed. Alamar blue has been widely used for studies in plant, bacteria and higher organisms. Recently, Hudman and Sargentini [14] reported the potential of alamar blue not only to quantify resazurin concentration but also to visualize colorimetric changes of resazurin in bacteria post exposure to different doses of radiation. However, some cell death has been observed in live HL-60 and “Jurkat” leukemia cells [15]. A possible explanation for this phenomenon is that resazurin may cause some oxidative stress, which impairs mitochondrial function and produces reaction oxygen species (ROS) [16]. Nevertheless, due to its simplicity, ease of use and non-destructive impact on cultivated cells, alamar blue remains a widely used assay for analyzing cellular metabolism across a wide range of cell

types including primary neuronal cell [17], normal lymphocytes [18] and cancer cell lines such as BeWo and JEG-3 choriocarcinoma cells [19].

Similarly, **PrestoBlue (PB)** is the enzyme that converts resazurin into resorufin and consequently measures metabolically active cells. Lall and coworkers have tested the ability of PrestoBlue reagent to detect bacterial growth [20]. In addition, recent study on human mesenchymal stem cell hMSCs proliferation into Porous Tantalum- which is a metallic implant biomaterial- has been validated by PrestoBlue [21]. Furthermore, comparing cell metabolic activity of hMSCs isolated from pelvis, femur, and tibia were assessed by PrestBlue assay [22]. Generally, PrestBlue is a fast, simple and has less cytotoxic affect compared with MTT and Alamar Blue assays [23].

1.2.1.1.4 Lactate dehydrogenase (LDH)

LDH is a cytoplasmic isoenzyme that stimulates the conversion of pyruvate to lactate and binds with NAD⁺ that produces fluorescent signals [24], thus it can be analyzed spectrophotometry. LDH assay is a colorimetric measurement based on the quantification of LDH released from lysed cells due to plasma membrane damage and cell death. Therefore, the colorimetric quantification correlates with the amount of necrotic cells. LDH assay performed with the conditioned medium without affecting cell layer, and it is a quick, easy and nondestructive assay [25]. Therefore, this assay has been used for the identification of cellular cytotoxicity [26] and as a diagnostic tool for some cancers [27].

1.2.1.2 Indirect cell monitoring in vitro

1.2.1.2.1 Optical biosensors

Optical biosensors are based on targeting cellular compounds without altering their natural condition, and quantitatively measure molecular interactions. It involves molecular interaction with a biological material and formation of light reflection changes (transitory wave) that can be measured by close optical biosensor [28]. Therefore, unlike traditional optical reporters such as fluorescent techniques where background signals can interfere and exact fluorescent quantification may not be obtained, optical biosensor could directly identify target molecules. One of these biosensors is fiber based with various diameter and shape but sensitive refractive index (RI) as a result of molecular collaboration [28].

1.2.1.2.2 Fluorescence/Forster resonance energy transfer FRET

Owing to the fact that fluorescent proteins (such as GFP) and non-protein organic fluorophores (such as xanthene, naphthalene) possess the potential to absorb (extinction coefficient) and emit (quantum Yield) the light directed to them, the emission spectrum is directly related to their concentration [29]. This unique characteristic underpins the development of fluorescence biosensors such as FRET. FRET is a technique capable to measure the emission of fluorescence signals as a result of energy transfer from donor fluorophores into recipient fluorophores when they become as close as 10nm [30]. FRET technique has been improved to monitor numerous intracellular biological activities; such improvements include integration of a heated stage for live time analysis [31]. King et al has demonstrated that FRET based fluorescence-

activated cell sorting (FACS) can be used to isolate Oct4-expressing pluripotent (hESCs) transfected with molecular beacons which are oligonucleotide probes that can identify the presence of specific nucleic acid in homogenous samples [32]. However, one of the major limitations of FRET is background spectrum which could contaminate FRET data when the amount of donor and recipient fluorophores are not equal [30]. Another limitation is cross-talk as recipient fluorophores may be stimulated by light that is supposed to activate the donor ones [31].

1.2.1.2.3 Fluorescence Lifetime Imaging (FLIM)

FLIM is a non-invasive measurement of lifetime fluorescence deterioration of endogenous fluorophores which are sensitive to cellular biological environment [33]. Generally, the donor fluorescence is stimulated by internal laser beams followed by its intensity decay measurement on recipient over picosecond or nanosecond. Molecules such as nicotinamide adenine dinucleotide (NAD), flavin adeninedinucleotide (FAD), melanin, FMN and riboflavin retain effective property (auto fluorescence) allowing the identification of fluorophores by imaging.

Stringari and coworkers demonstrated the capability of FLIM to monitor not only the metabolic status of neural stem cells, but also their lineage commitment potential by measuring (NADH) intensity [34]. Since NADH is an autofluorescent coenzyme involved in redox reaction during cellular metabolism, its intensity measurement can be correlated with the cell metabolism (Figure- 1.1). Similarly, a reduction in NADH intensity was previously associated with mesenchymal stem cells (MSCs) following

osteogenic differentiation [35]. Although FLIM demonstrates its capability to identify intracellular biological activities non destructively, this technique is expensive and not widely available, thus limiting its application [30].

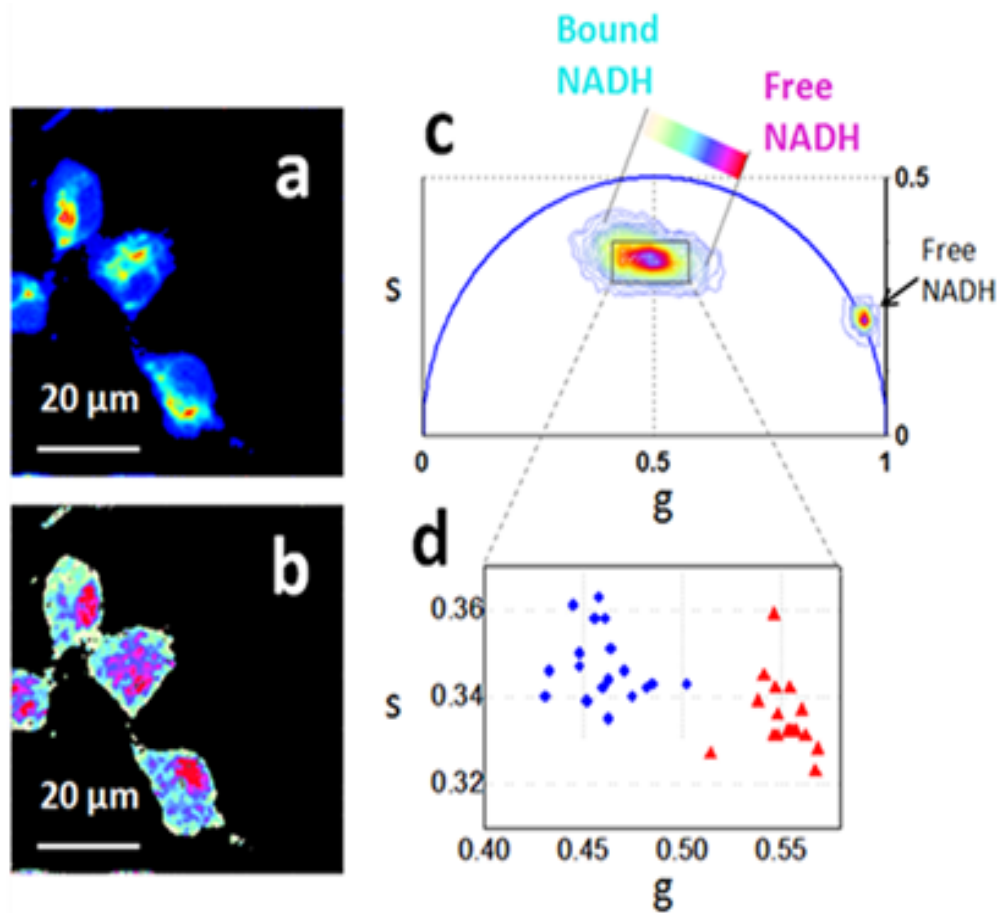


Figure 1.1 : Free and bound NADH distribution within NPSCs. (A) Two-photon fluorescence intensity images. (B) Free/bound NADH FILM maps. (C) The E12 NPSCs excited at 740 nm FILM phasor plot of E12 NPSCs autofluorescence. A linear cluster represents relative concentrations of free NADH (purple) and bound NADH (cyan-white). Red-purple color indicate a high free/bound NADH ration, white violet, cyan and white indicate linearly and progressively decreasing ratios of free/bound NADH. (D) Scatter plot of the cell mitochondria (blue circle) and cell nuclei (red triangle). Nuclei contain a higher concentration of free NADH, white mitochondria contain mainly bound NADH. (Adapted from Stringari et al, 2012).

1.2.1.2.4 Raman Microscopy (RM)

Application of laser beams for single cell analysis has been also demonstrated by Raman Microspectroscopy. It is a technique in which chemical components of a single cell are analyzed by mapping their vibration scattering wavelengths with a laser, which correlate with Raman bands [36]. Cellular applications of Raman microspectroscopy have been developed not only to discriminate normal and malignant cells [37] but also to detect pre-cancerous stages [38]. Moreover, approaches allowing the identification of cell cycle phases of stem cells [39], cell aging assessment [40], and extracellular matrix analysis [41] have been described using Raman technology. Furthermore, prevention of lipid accumulation related cardiovascular disease can be obtained by this Raman imaging [42]. Ghita and coworkers demonstrated the ability of this technique to distinguish neural stem cells (NSCs) from differentiated cells through the measurement of nucleic acid concentration of each cell type [43]. Osteogenic lineage characterization of mouse ESC and human MSCs was also achieved in vitro by detecting spectrum differences at pre mineralized stage and after terminal differentiation. [44-46].

1.2.1.2.5 Surface plasmon resonance (SPR)

The principle of SPR is based on measurement of ultraviolet (UV) light emission when surface plasmon waves interact with sample particles, the light transmission through the metal-coated nanostructure results in a strong electric field that is highly sensitive to the dielectric properties of materials immediately adjacent to the metal [47-49]. Due to its rapid, high sensitivity and specificity, it has been used to detect pathogens, toxins, drug residue, vitamins, hormones, antibodies, chemical contaminants, allergens and proteins [47-49].

Real time assessment of cytokine production by epithelial cells in culture medium has been developed in vitro by SPR [50]. In this technology, cell medium was sampled from each cell culture flask and delivered to the analytical device that connected to optical fibers to deliver and collect the transmitted light, then signals was recorded by the computer. Though, significant differences were detected between control samples and those exposed to tumor necrosis factor alpha and gold nanoparticles, only a limited numbers of cytokines were identified (interleukin 8, interleukin 6 and the monocyte chemotactic protein).

1.2.1.2.6 Electrochemical conductors

In contrast to optical biosensors, the use of electrochemical biosensors does not lead to background signals from endogenous fluorophores [51] as this technique depends on the changes in electrical current that are generated when a chemical interaction occurs between a biological membrane (on working electrode) and the detected molecule. When constant power is applied, current changes are compared with the wide ranges of reference electrodes [52]. Electrochemical conductors with silica proton-conducting membrane have been developed to measure metal deposit from gas phase [53]. Real time monitoring of the effect of trypsin on mouse 3T3 fibroblasts on organic electrochemical electrodes consist the Poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS) polymer was achieved and proofed its biocompatibility [54]. Electrochemical biosensors are the most practically used due to their simplicity and low cost. Moreover, unlike other biosensors; cross contamination is excluded by integration of a purification membrane [51].

Electrochemical biosensors can be fabricated based on property of the tested molecules. For example, a hydrogenase-based electrochemical biosensor was used to monitor extracellular lactate production from rat cardiomyocytes post oxygen reduction [55] (Figure- 1.2). Results indicated the effectiveness of such a biosensor to tolerate high pH environment following hypoxia and its sensitivity to detect such physiological condition. Likewise, catecholamines which controls neurotransmitters release from adrenal chromaffin cells in a microfluidic condition, were detected by Iridium oxide fabricated electrodes [56].

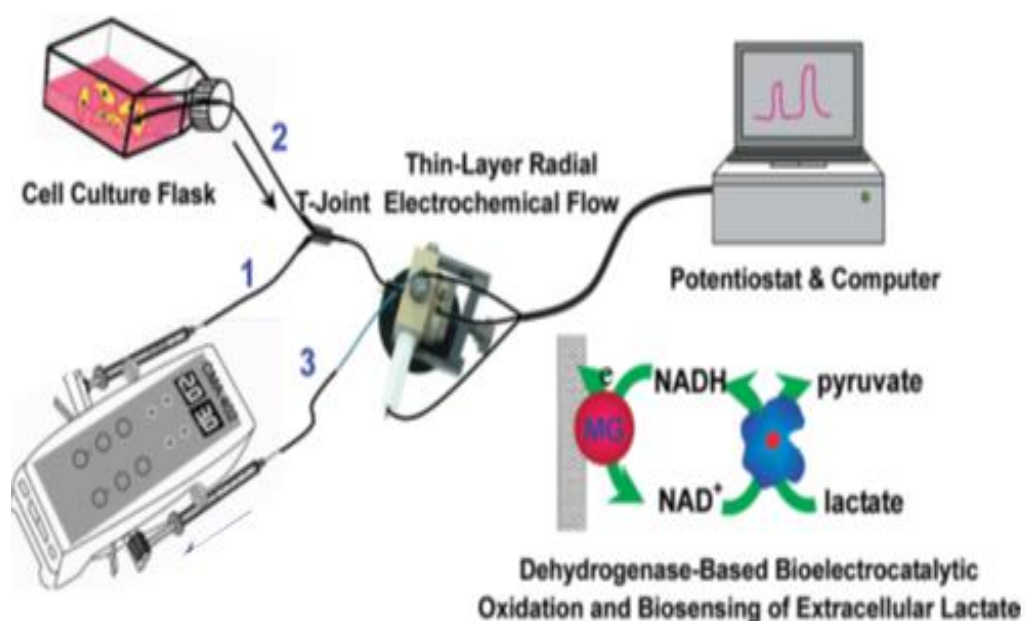


Figure 1.2: Schematic diagram of the electrochemical detecting system for continuous monitoring of extracellular lactate production from cardiomyocyte following hypoxia (Adapted from Li et al, 2012)

1.2.1.3 Metabolomic-based approaches for cell characterization:

Mass spectrometry with chromatographic technique integration has been introduced to the field of cell biology due to its sensitive and rapid metabolic analysis [57]. The principle of MS is to generate multiple ions, then separate them according to their specific mass-to-charge ratio (m/z), and then record the relative abundance of each ion type [58, 59]. The first step in the mass spectrometric analysis of compounds is the ionization of a neutral molecule, which is then analysed by the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. Finally, a mass spectrum of the molecule is produced to display the result in the form of a plot of ion abundance versus mass-to-charge ratio. Ion separation occurs between two phases, a stationary phase and a mobile phase (liquid or gas), and moves in a definite direction to be transmitted to analyzer and detector.

1.2.1.3.1 Liquid Chromatography Mass Spectrometry (LC-MS):

Liquid Chromatography Mass Spectrometry LC-MS is a powerful tool for the quantification of small molecules, as well as the identification of known and unknown metabolites in biological samples. The principal difference of ion separation in LC-MS is a liquid mobile phase delivered under high pressure (up to 400 bar (4×10^7 Pa)) to ensure a constant flow rate, and thus reproducible chromatography, while the stationary phase is packed into a column capable of resisting the high pressures which are necessary [60].

Zhou and coworkers have demonstrated the efficient use of liquid chromatography/ mass spectrometry LC-MS in tracing various concentrations of mitomycin C (MMC) in mouse and human embryonic stem cell feeders

which known to cause genetic aberration [61]. In their study, cells were treated with MMC; followed by different washing steps. MMC levels were detected at each step by LC-MS and a decrease was monitored upon medium dilution. In another study, saturated metabolites such as fatty acids that have considerable effects during mesenchymal stem cell differentiation were detected by LC-MS [62]. Quantitative time-point analysis of key molecular changes occurring during osteogenesis has also recently been demonstrated and linked to energy production and phosphate metabolism [63].

It is therefore reasonable to speculate that MSC differentiation could be characterised by its distinct metabolic footprint, including metabolite secretion and/or excretion and nutrient consumption, which may be unique to the specific metabolic pathways activated during MSC differentiation. The pathways identified by LC-MS-based footprinting can be used to monitor stem cell differentiation through the analysis of metabolite changes not only between various cell fates, but also at different stages within specific lineage maturation.

1.2.1.3.2 Gas Chromatography Mass Spectrometry (GC-MS)

In principle, Gas Chromatography Mass Spectrometry (GC-MS) segregates chemicals into discrete components in a mobile phase through solid-phase microextraction (SPME) in GC-MS [64], then transferred into stationary phase by helium gas (GC-MS). In GC-MS, the stationary phase consists of a capillary column made from various materials (glass, stainless steel or wax), and of different length and width, which is held in an oven at a controlled temperature. Consequently, separation of molecules occurs based on the

interaction between mobile and stationary phase. Components are eluted according to their interface speed as well as boiling rate. During the segregation process, the abundance of each detected compound is measured against elution time; followed by identification of each molecule's ion according to the mass spectral libraries and databases [65].

Volatile organic compounds (VOCs) have been widely used to explore various physiological and pathological conditions in human. For instance, food malabsorptions as well as sleep monitoring have been investigated by measuring VOCs concentration in patient's breath [66]. Also, detection of volatiles emission from skin has been reported to provide a fingerprinting profile of skin cells for applications in dermatology and dermatopathology [67]. Compounds analysed by this method can indicate physiological changes in cells as a result of responses to their microenvironment and changes in their metabolic pathways. Interestingly, some cancers could potentially be diagnosed according to VOCs profiles, for instance high level of 1,1,4,4-tetramethyl-2,5-dimethylene-cyclohexane was found associated with colorectal malignancy by analysing patient's blood [68], elevation of acetone; methyl ethyl ketone; n-propanol in lung cancer patient's breath [69]. Thus, VOCs analysis could potentially be useful in cancer screening and prevention programs to facilitate the detection of precancerous stages.

GC-MS methodology has been used in cell culture to monitor chemical volatiles affected by the presence of growth factors, nutrients and cytotoxic agents during cell culture leading to a range of metabolic entities such as alcohols, aldehydes, furans, ketones, pyrroles, terpenes [65]. Due to its

applicability to the quantification of cellular phenotype [70], GC-MS offers potential for stem cells biology, as illustrated by recent analyses of glucose metabolism in cell extracts from human mesenchymal stem cells (hMSCs) undergoing osteogenic differentiation [71] and adipogenic differentiation [72]. A variety of metabolic profiles were generated in this study; however the GC-MS approach employed relied on the use of cell extracts. Selected ion flow tube mass spectrometry (SIFT-MS) is a direct MS technique that analyses VOCs in milliseconds and eliminates sample preparation and chromatography [73-75]. In real time SIFT-MS analysis, volatiles are directly focused into quadrupole mass filter followed by continuous chemical ionization and detection by mass spectrometry. Though SIFT-MS technology offers a fast real time screening capability, GC-MS provides very good specificity and wide range of compound including volatile and semi volatile VOCs [76]. Therefore, there appears to be scope to explore GC-MS improvements through the implementation of a non-destructive process enabling live analysis of stem cells during their proliferation and differentiation through the sampling of the cellular environment. Recent studies have reported changes in specific metabolites extracted from mesenchymal cultures in response to different microenvironment properties using LC-MS [62].

1.3 GENERAL PROJECT AIMS

The overall aim of this project is to develop a metabolomics/volatolomics approach for the characterization of MSCs and their differentiated populations through the analysis of cellular metabolites using LC-MS and GC-MS technologies. Also, to explore the biological mechanism that can trigger MSCs

differentiation capacities. Our experiments started with mouse MSCs (mMSCs), due to their availability and ease of differentiation in comparison with hMSCs.

1.3.1 Objectives

The objectives of this project were to:

1. To determine whether footprinting technique using LC-MS could identify metabolomics changes during mMSC osteogenic differentiation.
2. To develop and test experimental model to analyze volatiles released during mMSC osteogenic and adipogenic differentiations in real time. This method includes testing direct cell culture into GC-MS vials, and validation of experimental conditions required for analysis.
3. To combine these two analytical techniques (LC-MS and GC-MS) to identify mMSC metabolomics/ volatolomics footprinting.
4. To combine metabolomics foot and fingerprinting analysis of hMSCs during osteogenic differentiation using LC-MS.
5. To investigate the potential pathways linked to the identified candidates that may impact MSC differentiation capabilities.
6. To develop medium components that can support chondrogenic differentiation of hMSC to enhance cartilage repair.

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2 CHAPTER-2-

NON-DESTRUCTIVE CHARACTERISATION OF MOUSE MESENCHYMAL STEM CELL DIFFERENTIATION USING LC-MS-BASED METABOLITE FOOTPRINTING

This chapter was published and chosen as one of the Recent HOT articles in Analyst, 2016. (DOI: 10.1039/C6AN00170J).

2.1 ABSTRACT

Bone regeneration is a complex biological process where major cellular changes take place to support the osteogenic differentiation of mesenchymal bone progenitors. To characterise these biological changes and better understand the pathways regulating the formation of mature bone cells, the metabolic profile of mesenchymal stem cell (MSC) differentiation *in vitro* has been assessed non-invasively during osteogenic (OS) treatment using a footprinting technique. Liquid chromatography (LC)-mass spectrometry (MS)-based metabolite profiling of the culture medium was carried out in parallel to mineral deposition and alkaline phosphatase activity which are two hallmarks of osteogenesis *in vitro*. Metabolic profiles of spent culture media with a combination of univariate and multivariate analyses investigated concentration changes of extracellular metabolites and nutrients linked to the presence of MSCs in culture media. This non-invasive LC-MS-based analytical approach

revealed significant metabolomic changes between the media from control and OS-treated cells showing distinct effects of MSC differentiation on the environmental footprint of the cells in different conditions (control vs OS treatment). A subset of compounds was directly linked to the osteogenic time-course of differentiation, and represent interesting metabolite candidates as non-invasive biomarkers for characterising the differentiation of MSCs in a culture medium.

2.2 INTRODUCTION

During skeletal development or fracture repair, bone is formed by a complex process involving the osteogenic differentiation of mesenchymal progenitors [77]. This process requires a variety of intracellular and extracellular molecular mechanisms orchestrating specialised cell types [77, 78]. Mesenchymal stem cells (MSCs) are multipotent somatic progenitors able to differentiate into osteogenic, chondrogenic and adipogenic lineages [79]. Their capacities to generate new osteoblasts, the bone-forming cell type, and the possibility of harvesting them from bone marrow collections have made them a promising candidate for therapeutic approaches to skeletal repair [80]. Numerous *in vitro* studies have demonstrated the effect of osteoinductive treatment combining dexamethasone (Dex), ascorbic acid (ASC), and beta-glycerophosphate (β GP) on MSC osteogenesis. Dexamethasone induces osteogenesis *in vitro* through activating WNT/ β -catenin signalling-dependent *Runx2* expression [81], whereas ascorbic acid increases collagen type 1 secretion [82] [83]. β GP provides inorganic phosphate (Pi) that facilitates intracellular signalling molecules [84] [85] [86].

Since metabolites provide the phenotypic outcome of gene expression or metabolic activities of a cell, global metabolite profiling can give a rapid snapshot of the cell physiology by monitoring concentration changes of a broad range of metabolites and provide insight into relationships between genotype and phenotype [87] [88]. Therefore, metabolomics has been used widely for understanding underlying molecular processes in applications for human health and disease including biomarker and drug discovery [89]. Recent studies have

reported changes in specific metabolites extracted from mesenchymal cultures in response to different microenvironment properties [62]. Furthermore, parathyroid hormone (PTH) effect on mouse osteogenic lineage was shown to be negatively influenced by suppressing cellular oxidation of glucose through tricarboxylic acid cycle (TCA cycle) [90]. Also, the stimulation of glutamine biosynthesis through WNT signalling boosts the formation of osteoblasts [91]. In addition, studying mesenchymal stem cells in terms of osteogenic metabolomics would not only extend the understanding of bone tissue biology but also provide new approaches for pharmacological and clinical developments.

Metabolite profiling of intracellular metabolites is informative to understanding complex biological processes and systems, but this approach requires complicated sample preparation, including the rapid quenching of metabolism and extraction of intracellular metabolites from cells. Incomplete quenching and extraction methods could lead to biased quantification, due to rapid turnover and loss of metabolites. Therefore, an alternative approach can be the measurement of extracellular metabolites that are secreted and/or excreted from cells into their growth media or nutrients which are consumed by cells. This metabolite footprinting technique requires minimum sample preparation and can provide significant information to understand the microenvironment of a cell of interest [92] [93], as used for human hepatic stem cells (hHpSCs) [94].

Liquid chromatography (LC)-mass spectrometry (MS) is a powerful tool for the quantification of small molecules, as well as the identification of known

and unknown metabolites in biological samples. Recently, LC-MS-based approaches have been employed widely for the quantitative analysis of extracellular metabolites and nutrients in a growth medium [95], it is therefore reasonable to speculate that MSC differentiation could be characterised by its distinct metabolic footprint, including metabolite secretion and/or excretion and nutrient consumption, which may be unique to the specific metabolic pathways activated during MSC differentiation. The pathways identified by LC-MS-based footprinting can be used to monitor stem cell differentiation through the analysis of metabolite changes not only between various cell fates, but also at different stages within specific lineage maturation.

Established cell phenotyping methods including gene expression analysis, protein immunodetection and flow cytometry are widely used to produce high resolution molecular phenotyping at the cellular and population level [96] [97, 98], however these approaches involve invasive and cell destructive steps which limit their application to live cell monitoring. This study proposes a non-invasive analysis for metabolic changes occurring in the culture medium during MSC osteogenic differentiation, and for the first time, demonstrates a quantitative time-point analysis of key molecular changes occurring during MSC osteogenesis.

2.3 MATERIAL AND METHODS

Materials were purchased from Life Technologies (Paisley, UK) unless otherwise stated.

2.3.1 Cell culture and differentiation assay

Mouse mesenchymal cells (D1, ATCC CRL-12424) [99] were seeded in 22.5 mm x 75.5 mm vials (Fisher Scientific, UK) as six replicates at density of 3×10^4 cells/mL in standard MSC medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10 % (v/v) fetal calf serum (FCS), 1 % (v/v) L-glutamine, 1 % (v/v) non-essential amino acids and 1 % (v/v) antibiotics, and incubated at 37 °C in 5 % CO₂. Confluent cells treated with osteogenic medium consisting of standard MSC medium, 100 nm dexamethazone (Sigma-Aldrich, Gillingham, UK), 0.05 mM L-ascorbic acid 2-phosphate (Acros Organics, Geel, Belgium) and 10 mM beta glycerophosphate (βGP, Sigma-Aldrich), and treated for 15 days with medium changes every 2 days. Cell maintained in standard MSC medium were used as a control medium sample. All supplements were prepared according to the manufacturer's guidelines. The same media, reagents and supplements were used throughout the experiment for consistency.

Calcium deposits were analysed using Alizarin Red staining. At the stated time point, cells from 4 replicates were trypsinised and pellets were smeared onto glass slides, air dried, fixed with 100 % methanol and washed with distilled water. Slides were incubated in 1 % (w/v) Alizarin Red staining solution (Sigma-Aldrich) for 10 min at room temperature, and washed with distilled water until all excess stain had been removed. Mineral deposits were then observed using a Nikon Eclipse Ni90 light microscope. In parallel, Alkaline phosphatase (ALP) activity was measured in the remaining two culture replicates using 100 µL SIGMA FAST p-Nitrophenyl Phosphate reagent

(Sigma-Aldrich) for 20 min at 37 °C, 5% CO₂. 100 µL from each sample transferred to a 96 well plate were analysed in triplicate using an Tecan Infinite 200 plate reader and Tecan-i-control 1.10 software to measure absorbance at 405 nm. Quantification of ALP absorbance was not normalized to the cell number present in each vial.

2.3.2 Metabolites extraction

At day 5, 10 and 15, 1 mL of the medium in each culture (MSC-conditioned medium) was collected and centrifuged at 7378 g for 5 min. 250 µL of the collected medium were transferred to a new tube for extraction and protein precipitation by adding 750 µL of cold methanol in a ratio of 1:4, then mixing vigorously and incubating at -20 °C for 20 min. After incubation, samples were centrifuged at 13,200 g at 4 °C for 10 min, transferred to pre-cooled tubes and stored at -80 °C until LC-MS analysis. Samples of fresh untreated control MSC medium and OS medium were processed in parallel as no cells controls. All samples were prepared as six biological replicates, and the experiment was run as two successive independent repeats. An equal mix of all samples was prepared as quality control (QC) for instrument performance assessment [100].

2.3.3 Analytical methodologies

For metabolite footprinting of spent culture media, LC-MS was performed on an Accela system coupled to an Exactive MS (Thermo Fisher Scientific, Hemel Hempstead, UK). Spectral data was acquired in full scan ion mode (m/z 70-1,400, resolution 50,000) in both positive and negative electrospray ionisation modes. The probe temperature and capillary temperature were maintained at 150 and 275 °C, respectively. The calibration mass range was extended to

cover small metabolites by inclusion of low-mass contaminants with the standard Thermo calibration mixture masses (below m/z 1400), $C_2H_6NO_2$ for positive ion electrospray ionisation (PIESI) mode (m/z 76.0393) and $C_3H_5O_3$ for negative ion electrospray ionisation (NIESI) mode (m/z 89.0244). Chromatographic separation was carried out using a ZIC-pHILIC (150 mm $\times$ 4.6 mm, 5 μ m column, Merck Sequant) maintained at 45 °C and a flow rate of 300 μ L/min as previously described in [101]. Briefly, the mobile phase consisted of (A) 20 mM ammonium carbonate in water, (B) 100 % acetonitrile which were eluted with a linear gradient from 80 % B to 5 % B over 15 min, followed by a 2 min linear gradient from 5 % B to 80 % B, and 7 min re-equilibration with 80% B. The injection volume was 10 μ L and samples were maintained at 4 °C.

2.3.4 Metabolites identification process

LC-MS raw data from medium samples (control medium samples and their blanks, OS treated medium samples and their blanks and QC samples and reagent blanks) were processed with XCMS for untargeted peak-picking [102], and peak matching and annotation of related peaks were carried out using mzMatch [103]. IDEOM with the default parameters was used for noise filtering and putative metabolite identification. Level 1 metabolite identification was performed by matching accurate masses and retention times of authentic standards which were run on the same instrument according to the metabolomics standards initiative [104] [105], but Level 2 putative identification was considered when standards were not available and predicted retention times were employed.

Supervised orthogonal partial least squares discriminant analysis (OPLS-DA) [106] was initially performed by SIMCA-P 13.0.2 version (Umetrics AB, Umea, Sweden) (i) for general visualisation of metabolite differences between control medium samples and their controls, and OS treated medium samples and their blanks, (ii) to identify the temporal shift within control and OS treated conditions, and (iii) to observe the differences between control and OS treated medium samples (n=6 in each group). Mass ions for discriminant biomarkers were selected by variable importance in projection values (VIP) where VIP values greater than one were considered as potential biomarkers. In addition to the investigation of VIP scores from OPLS-DA for the discovery of key discriminatory metabolites, univariate analysis was applied as a final feature selection. One-way ANOVA was carried out using Metaboanalyst that is a web server for metabolomic data analysis and interpretation [107], and was used to evaluate levels of significant difference between control and OS treated media.

2.4 RESULTS

2.4.1 *In vitro* model of osteogenic differentiation

In order to model the cellular changes occurring upon osteogenic induction, mMSCs D1 (single donor) were treated *in vitro* to induce osteogenic differentiation (Figure-2.1), and the cultures were analysed at day 5, 10 and 15. No mineralisation was detected with standard medium, whilst OS conditions showed mineral deposits that were positively stained at day 10 and strongly at day 15 (Figure- 2.1A). ALP levels increased at early stages of OS induction with significant elevation toward day 10, followed by a notable decline toward

day 15, when ALP activity returned to comparable levels to standard control medium (Figure 2.1B).

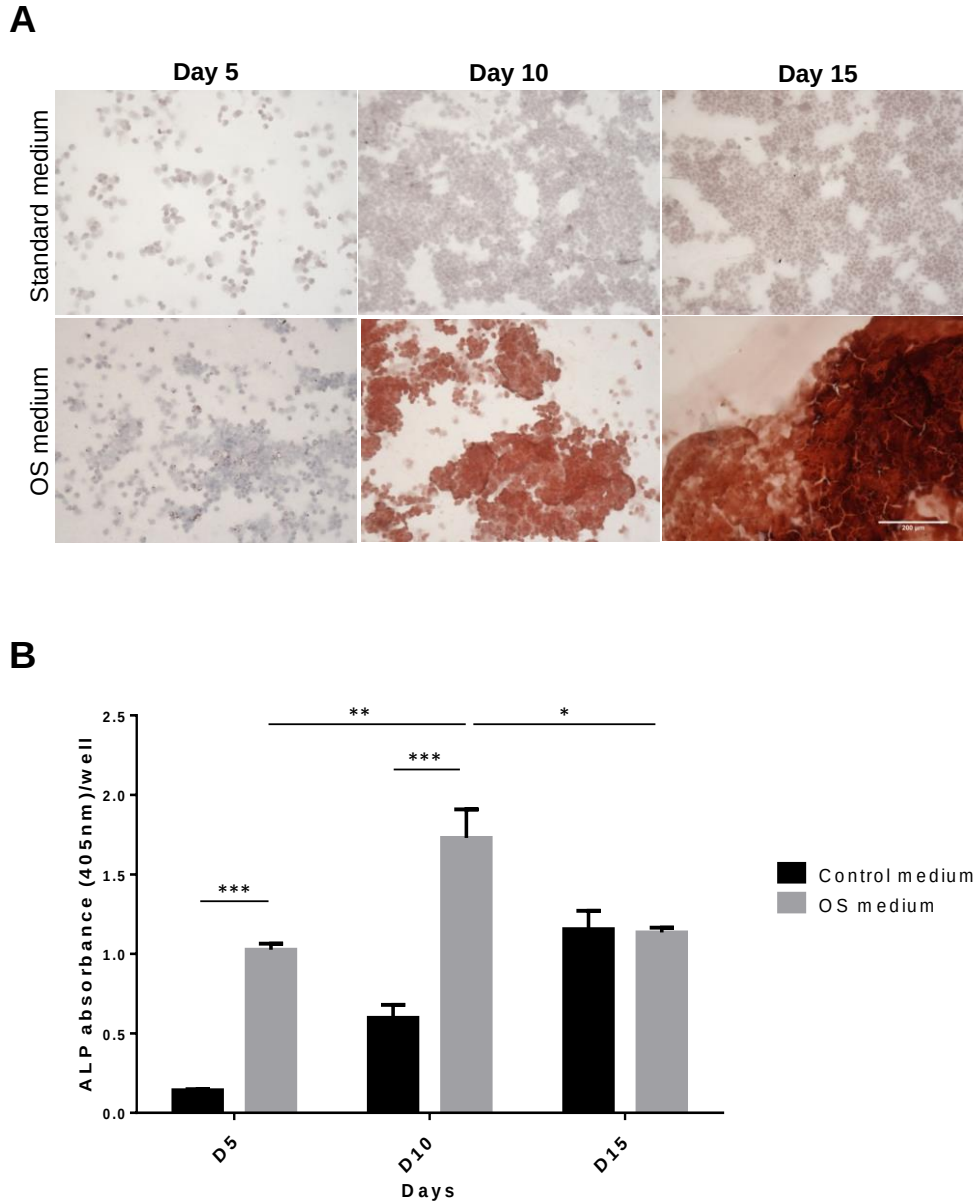


Figure 2.1: Osteogenic differentiation in MSC cultures after 5, 10 and 15 days. (A) Alizarin Red staining of cells treated with either control (top panel) or OS medium (bottom panel) showing no mineralization in control medium, while under OS treatment calcium deposits were identified at D10 and 15. **(B)** ALP activity assay showing an increase in response to OS treatment at day5 and 10. (** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; $n=3$). Scale bar= 200 μ m.

2.4.2 Multivariate analysis of LC-MS profiling data

In order to investigate the metabolic changes occurring in the MSC culture medium over the 15-day treatment period, samples from untreated medium cultures, OS-treated cultures, and their respective blank media (not exposed to cells) were analysed by LC-MS. LC-MS data from these MSC-conditioned or blank media samples were transformed into 2D matrix by mzMatch, to introduce an exact mass and retention time for each mass ion. OPLS-DA was then performed to determine the overall biological variation between all conditions, and to identify noticeable trends upon conditioning with MSCs, and over the course of the OS treatment.

OPLS-DA scores plots showed reproducible clustering of six biological replicates for each sample group, and highlighted clear differences between the different conditions tested (Figure-2.2). The OPLS-DA models were cross-validated by calculating Q^2 which is an estimate of the predictive ability of the model. The non-conditioned fresh media (blanks) analysed in the absence of MSCs showed distinct clusters from each spent culture medium conditioned in the presence of MSCs (untreated control and OS medium samples) with R^2 and Q^2 values of 0.841 and 0.644, respectively (Figure-2.2A). Similarly, samples exposed to osteogenic medium clustered in a different quadrant from the standard control medium samples in the OPLS-DA scores plot. This multivariate analysis clearly showed that LC-MS-based metabolite footprinting was able to non-invasively detect changes of metabolites according to cell differentiation. From separate scores plots of either standard control or OS medium conditioned (Figure-2.2B-C), the scores of all cell-conditioned

samples (at day 5, 10 and 15) showed a significant shift away from their respective fresh media (blanks), indicating a distinctive MSC-related metabolomic signature. The obtained R² and Q² values from OPLS-DA models of each sample were 0.991 and 0.756 for untreated control medium, and 0.992 and 0.777 for OS medium conditioned, respectively, demonstrating an acceptable model. As clearly shown in Fig. 2B and C, the scores of fresh media in the absence of cells are observed in the middle left hand side, and as the length of culture under treatment increased, the cluster spread from left to right (D5 and 10) and then goes to the bottom (D15). These resultant scores plots clearly showed cluster patterns directly related to the length of cell culture treatment. It is therefore reasonable to consider that the metabolite footprinting data from MSC cell culture media analysed using LC-MS contains valuable information regarding metabolite secretion/excretion and nutrient consumption relevant to the metabolic effect of MSC differentiation.

Changes in MSC medium were monitored over the 15-day differentiation time-course. When comparing the three time-points analysed within each treatment group, distinct differences were observed suggesting dynamic changes in the MSC culture medium over time following the same trend in both standard and OS treatment conditions, with datasets at early culture stages (day 5 and 10) showing closer metabolomic proximity than the late time point (day 15).

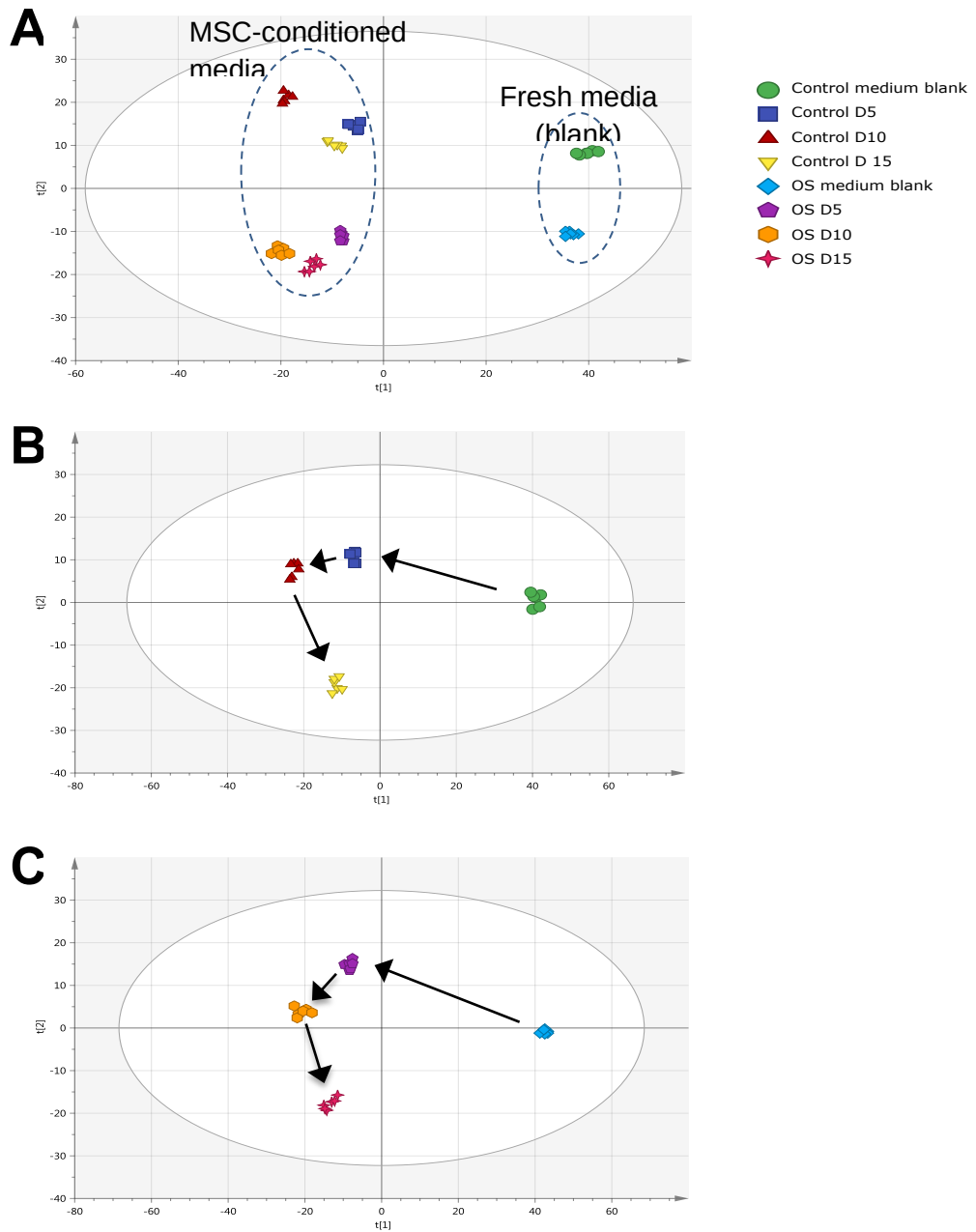
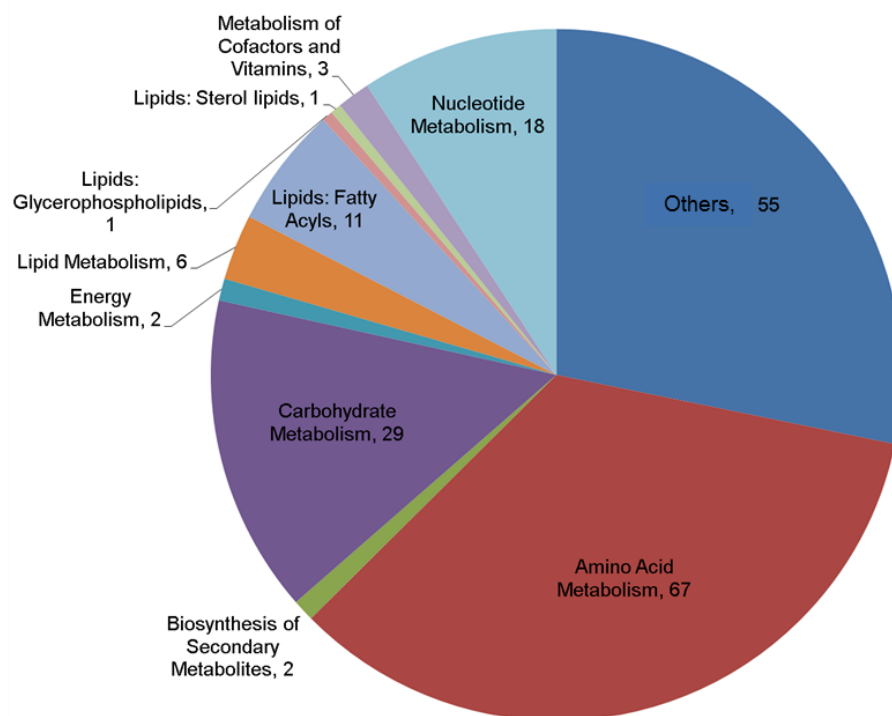


Figure 2.2: OPLS-DA scores plots of MSC medium samples from standard and OS-treatment, and their corresponding medium-only blanks, analysed after 5, 10 and 15 days in culture. (A) Combined standard and OS treated MSC-conditioned samples and their respective medium-only blanks ($R^2 = 0.841$ and $Q^2 = 0.644$) (B) Untreated control medium MSC samples and their corresponding medium-only blanks ($R^2 = 0.991$ and $Q^2 = 0.756$). (C) OS medium-treated MSC samples and their corresponding medium-only blanks ($R^2 = 0.992$ and $Q^2 = 0.777$). Each condition was analysed in 6 biological replicates. Arrows indicate the metabolic profile changes between the sample conditions.

2.4.3 Feature selection: Analysis of MSC culture samples versus blank media without cells

To determine which mass ions contributed to the clusters and trends between MSC cultured media and medium only blank samples, group comparisons were performed between fresh medium (day 0) and day 15 samples within the OPLS-DA models. Key mass ions for discriminant biomarkers were selected by VIP values higher than one were considered as potential key mass ions. In addition to the multivariate analysis, univariate one-way ANOVA testing was also performed to identify significant compounds consumed and excreted by MSCs (both in standard and OS treatments) compared to their medium-only blanks over time. Key mass ions were also selected from the univariate analysis with the false discovery rate $Q \leq 0.05$. The analysis was repeated in two independent experiments, and the combination of multi- and univariate analyses identified 215 metabolites showing variation between the MSC-conditioned medium samples compared to matching medium-only blanks, both under control medium and OS treatment (Appendix- table 1). A pie chart showing the classification of 195 metabolites according to their metabolism was generated by focusing on compounds selectively increased in the presence of MSCs (Fig-2.3), and a heatmap showing their fold changes is presented in Appendix- Figure. 1.

A



B

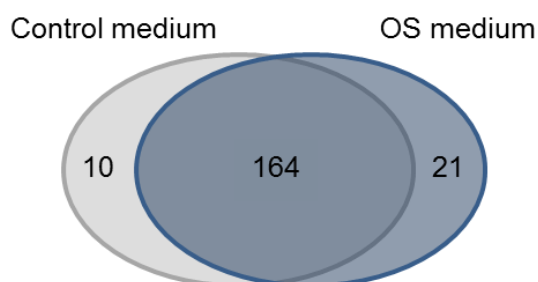


Figure 2.3: Functional categorisation of the nutrients and metabolites produced by MSCs in their culture medium. (A) 195 metabolites identified by LC-MS are categorised based on their biological function. (B) Venn diagram showing that within the total 195 identified metabolites, 10 were selectively increased in untreated control and 21 metabolites in OS treated medium.

This first analysis identified significant metabolomic changes related to MSC conditioning of the culture medium compared with corresponding medium only blanks, such as the increase of deoxyuridine, orotidine, indole-3-acetaldehyde, acronycidine, (S)-3-methyl-2-oxopentanoic acid, 1-methylnicotinamide, 2-hydroxy-4-methylthiobunoate, 3-(4-hydroxyphenyl)pyruvate (Figure-2.4).

Our data identified a significant increase of Deoxyuridine and 3-(4-hydroxyphenyl) pyruvate in ('OS') compared to the untreated control from throughout ('OS') treatment (Figure-2.3.4.2A and H). Orotidine and 1-methylnicotinamide showed no differences at day 5 but it significantly increased at day 10 and 15 with ('OS') treatment compared to the untreated control (Figure-2.4B and F). No significant differences were noted with indole-3-acetaldehyde and Acronycidine at days 5 and 10 but toward day 15 a noticeable reduction of indole-3-acetaldehyde was noted compared with ('OS') treated condition (Figure-2.3.4.2C and D). In contrast, a significant reduction of (S)-3-methyl-2-oxopentanoic acid and 2-hydroxy-4-methylthiobunoate were noted with ('OS') treated conditions compared with the untreated control , though with (S)-3-methyl-2-oxopentanoic acid a significant increase was noted with ('OS') induction only at day 5 (Figure-2.4E and G).

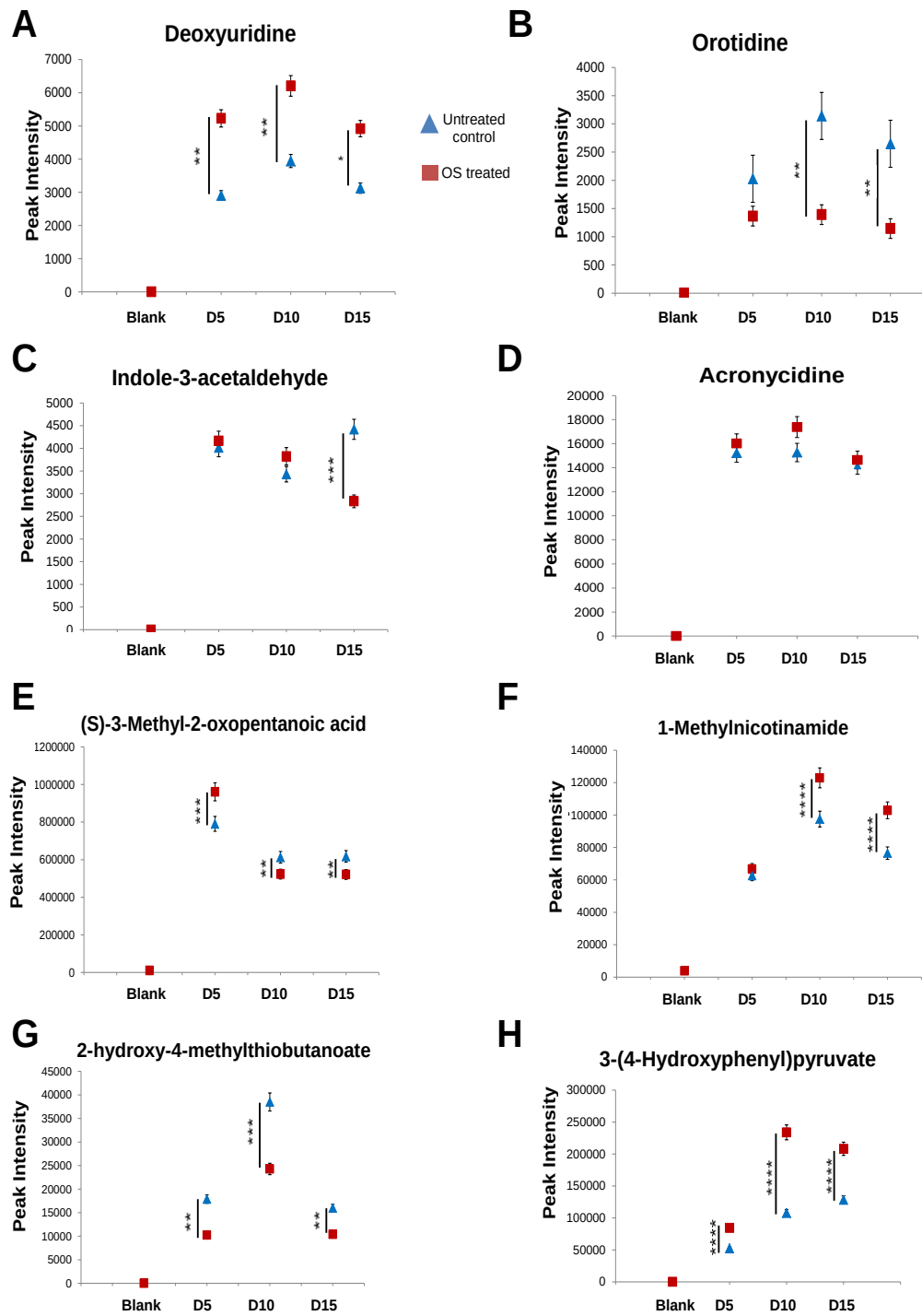


Figure 2.4: MSC-related metabolites found to increase over time in culture medium in the presence of MSCs, both in control medium (blue triangle) and OS medium (red square), at day 5, 10 and 15 of culture. Zero peak intensity was defined by the level detected in medium only blanks. (** $p < 0.0001$ *** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$); $n = 6$.**

Further investigation was carried out to identify metabolomic differences between MSC-conditioned medium produced under standard and ('OS') treatment. Out of 195 compounds initially detected as potential biomarkers, 21 metabolites increased specifically during ('OS') differentiation of MSCs (Table 2.1).

Measurements of these compounds over the culture time-course showed significant upregulation of citrate, cis-aconitate, 2-oxoglutarate (α -ketoglutarate derivative), succinate, glycerol and orthophosphate in MSC medium upon ('OS') exposure (Figure 2.5A, C, D, F and G). Conversely, the concentration of sn-glycerol-3-phosphate was rapidly diminished from the OS-treated medium over time. Higher levels of glycerol and orthophosphate were consistently detected throughout osteogenic differentiation time-course (Figure 2.5F-G). Although orthophosphate was increased with osteogenic treatment, the LC-MS technique detected low concentration of this compound in the standard medium control (Figure 2.5B).

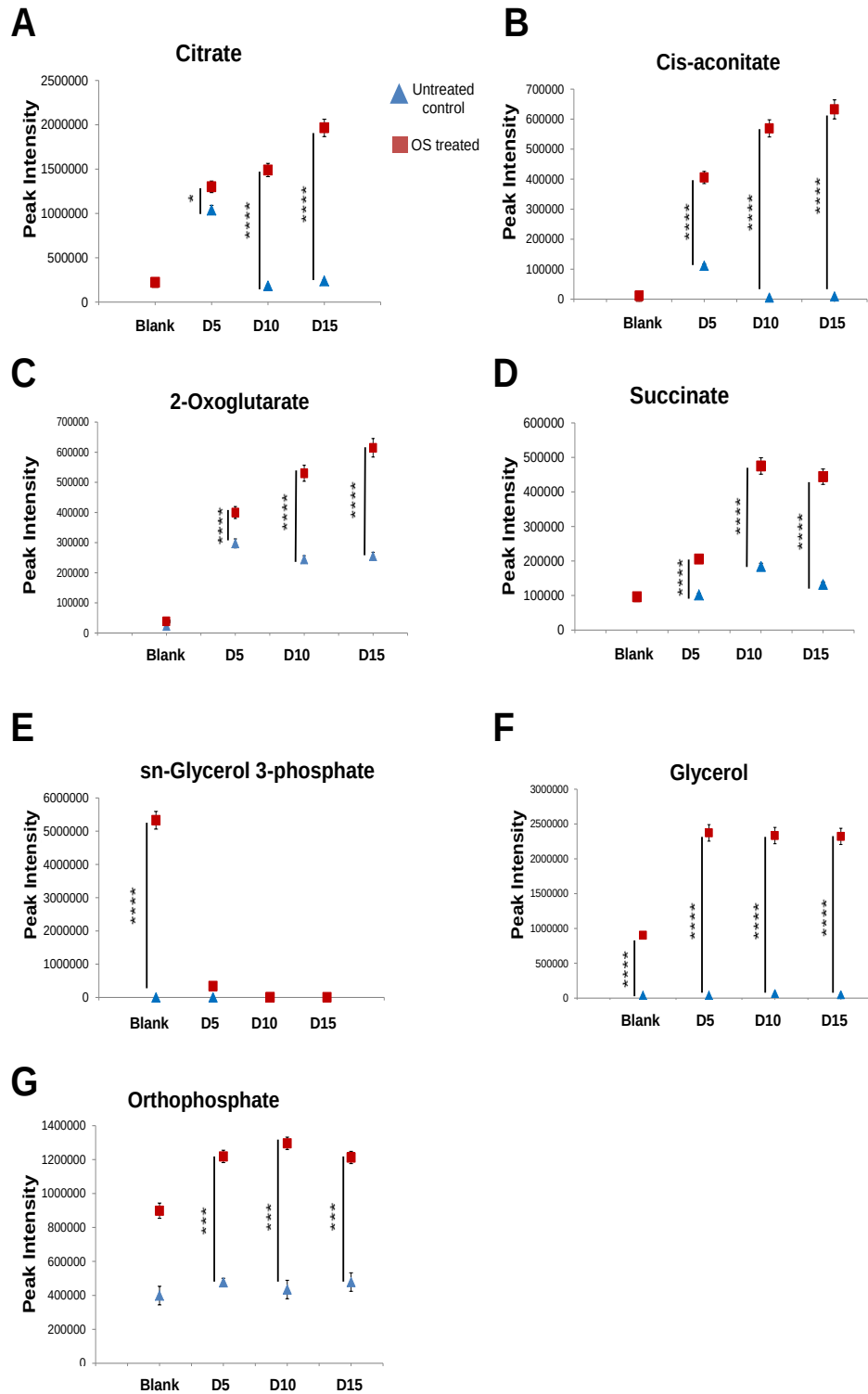


Figure 2.5: Metabolite changes identified in the culture medium MSC-related metabolites found to increase over time in culture medium in the presence of MSCs under OS treatment (red square) compared to control medium (blue triangle), at day 5, 10 and 15 of treatment. (** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; $n = 6$).**

Exact mass	RT (min)	Formula	Putative metabolite	ID confidence	Identifier (PubChem)	↑ OS
214.0244	9.68	C ₅ H ₁₁ O ₇ P	2-Deoxy-D-ribose 5-phosphate	L2	45934311	+
112.0161	11.85	C ₅ H ₄ O ₃	2-Furoate	L2	6919	++
120.0059	11.54	C ₃ H ₄ O ₅	2-Hydroxymalonate	L2	45	+
192.0805	8.73	C ₁₉ H ₂₈ O ₆ S	3b-16a-Dihydroxyandrosthenonesulfate	L2	22833522	+
174.0164	11.70	C ₆ H ₆ O ₆	cis-Aconitate	L2	643757	++
192.0270	11.88	C ₆ H ₈ O ₇	Citrate	L2	311	++
276.0958	10.99	C ₁₀ H ₁₆ N ₂ O ₇	GammaGlutamylglutamicacid	L2	92865	++
92.0474	8.77	C ₃ H ₈ O ₃	Glycerol	L1	753	+
246.0505	8.84	C ₆ H ₁₅ O ₈ P	Glycerophosphoglycerol	L2	439964	+
262.0148	11.26	C ₉ H ₁₀ O ₇ S	Homovanillicacidsulfate	L2	29981063	+
166.0477	9.49	C ₅ H ₁₀ O ₆	L-Arabinonate	L2	5459849	+
120.0423	7.90	C ₄ H ₈ O ₄	L-Erythrulose	L2	5460032	+
72.0210	8.76	C ₃ H ₄ O ₂	Methylglyoxal	L1	880	+
146.0215	11.92	C ₅ H ₆ O ₅	Methyloxaloacetate	L2	440890	+
237.0849	11.88	C ₈ H ₁₅ NO ₇	N-Acetyl-D-glucosamine	L2	16126799	++
217.1314	8.08	C ₁₀ H ₁₉ NO ₄	O-Propanoylcarnitine	L2	107738	++
97.9771	10.61	H ₃ O ₄ P	Orthophosphate	L2	1061	+
102.0681	5.26	C ₅ H ₁₀ O ₂	Pentanoate	L2	114781	++
173.9986	4.89	C ₆ H ₆ O ₄ S	Unknown			+
118.0267	10.09	C ₄ H ₆ O ₄	Succinate	L1	1110	++
139.9780	12.05	C ₂ H ₄ O ₅ S	Sulfoacetate	L2	31257	+

Table 2-1: The most significant metabolites that were increased specifically during OS differentiation analysed by OPLS-DA and one-way ANOVA.

+ indicates the increase during OS treatment only compared to control medium samples, and ++ indicates an increase over time toward terminal differentiation (day 15).

ID confidence: Metabolite identification level according to the metabolomics standards initiative^{29,30}; L1 – Level 1, L2 – Level 2

2.5 DISCUSSION

2.5.1 Confirmation of osteogenic differentiation

Our data confirm osteogenic differentiation *in vitro* by investigating changes in alkaline phosphatase activity (ALP) and mineral content, which are two major characteristics of bone formation [108]. ALP activity returned to comparable levels in line with previous reports [109]. The ALP changes and associated mineral deposits accumulation during osteogenic treatment containing ascorbic acid and beta glycerophosphate confirmed MSC differentiation during the 15-day time course [110] [111].

This first analysis identified significant metabolomic changes related to MSC conditioning of the culture medium, such as the increase of deoxyuridine, orotidine, indole-3-acetaldehyde, acronycidine, (S)-3-methyl-2-oxopentanoic acid, 1-methylnicotinamide, 2-hydroxy-4-methylthiobunoate, 3-(4-hydroxyphenyl)pyruvate. Some of these compounds are linked to cell proliferation. For instance, deoxyuridine is involved in the synthesis of nucleosides, the structural subunits of nucleic acids [112], and are known to fluctuate during DNA synthesis or the S-phase of the cell cycle [113, 114]. Similarly, orotidine is involved in nucleotide synthesis through pyrimidine synthesis (KEGG pathway; MAP00240). In our study, LC-MS-based footprinting detected changes in deoxyuridine and orotidine in culture medium conditioned by MSCs. During OS treatment, deoxyuridine significantly increased while orotidine decreased compared to MSC conditioned medium under control conditions. This finding could suggest epimetabolomic changes during nucleotide synthesis over the osteogenic differentiation time-course.

In addition, L-tryptophan, which is an essential amino acid for protein synthesis [115] and one of the essential component of DMEM, was found at comparable levels in all medium conditions including medium only blanks (data not shown). Indole-3-acetaldehyde, a derivative of L-tryptophan reported during gluconeogenesis [116, 117], was significantly increased in medium conditioned by MSCs, suggesting that indol-3-acetaldehyde might have been formed from the L-tryptophan present in the culture medium (DMEM). Our data also showed a significant elevation in 1-methylnicotinamide upon MSC culture and during osteogenesis compared to the control medium conditions. 1-methylnicotinamide is reported as a nicotinamide metabolite [118], and a reduction in nicotinamide adenine dinucleotide (NADH) intensity has previously been observed in MSCs following osteogenic differentiation [35], suggesting that metabolomic changes in NADH metabolism may reflect different stages of osteogenic maturation.

2.5.2 Non-invasive metabolic profiling of MSC osteogenic differentiation

The measurements of identified compounds over the culture time-course showed significant upregulation of citrate, cis-aconitate, 2-oxoglutarate (α -ketoglutarate derivative), succinate, glycerol and orthophosphate in MSC medium upon ('OS') exposure. Conversely, the concentration of sn-glycerol-3-phosphate was rapidly diminished from the OS-treated medium over time. Interestingly, compounds such as sn-glycerol 3-phosphate, glycerol, orthophosphate, glutamate and citrate are linked to the degradation of β GP added to OS medium, which is hydrolysed by the glycerol 3-phosphatase enzyme to glycerol and orthophosphate during glycerolipid metabolism (KEGG REACTION: R00841) (KEGG ENZYME: EC3.1.3.19). Higher levels

of glycerol and orthophosphate were consistently detected throughout osteogenic differentiation time-course. Although orthophosphate was increased with osteogenic treatment, the LC-MS technique detected low concentration of this compound in the standard medium control, possibly due to other metabolic processes such as glutamine metabolism by L-glutamine synthetase enzyme ($\text{ATP} + \text{L-glutamate} + \text{ammonia} \rightleftharpoons \text{ADP} + \text{orthophosphate} + \text{L-glutamine}$) (KEGG REACTION: R00253) and the Citrate cycle ($\text{ATP} + \text{citrate} + \text{CoA} \rightleftharpoons \text{ADP} + \text{orthophosphate} + \text{acetyl-CoA} + \text{oxaloacetate}$) (KEGG ENZYME: 2.3.3.8). Furthermore, orthophosphate could play a role in ALP inhibition at the late stages of osteogenic differentiation by binding to the enzyme active site and inhibiting its activity [119]. This could indicate a link between the noticeable orthophosphate elevation toward day 15 of differentiation and the reduction in ALP level in day 15 OS-treated cells (Figure 2.5.2B).

A subset of compounds differentially detected in OS culture medium could also be related to the Krebs cycle (Figure 2.5.2A). Citrate, cis-aconitate, α -ketoglutarate, succinate, malonate and malate are component of the Krebs cycle or Tricarboxylic cycle (TCA cycle), which represents an essential energy metabolism pathway in cells as first described by H.A. Krebs [120]. In the typical Krebs cycle, pyruvate produced by glycolysis is converted to acetyl-CoA by the pyruvate dehydrogenase complex. Acetyl-CoA then enters the mitochondria and combines to oxaloacetate to produce citrate (through the citrate synthetase enzyme) which is then utilised to form cis-aconitate. The later becomes isocitrate, is then oxidised to form oxoglutarate (α -ketoglutarate derivative), followed by conversion of succinyl-CoA to succinate with GTP production. Succinate will then converts to fumarate and malate, completing

the cycle to form oxaloacetate. Significant changes in levels of Krebs cycle metabolites were identified in the medium of MSC cultures under both standard and osteogenic conditions. LC-MS analysis indicated a substantial and gradual increase in citrate and cis-aconitate production throughout osteogenic induction (peaking at day 15) in comparison with standard medium control. Interestingly, citric acid has been described as essential for bone organic nanostructure [121] and bone tissue engineering in bone marrow-derived rat MSCs [122]. This suggests that the change in citrate levels may be linked to osteogenic treatment. Cis-aconitate is an intermediate in cell respiration, however there is no report to date describing the possible effect of this compound on osteogenesis.

It was reported that 2-oxoglutarate (α -ketoglutarate derivative) is a precursor of hydroxyproline, which is essential amino acid for bone protection [123]. Interestingly, in our data 2-oxoglutarate shows a significant increase during osteogenic treatment compared to untreated control which could support its importance in bone protection.

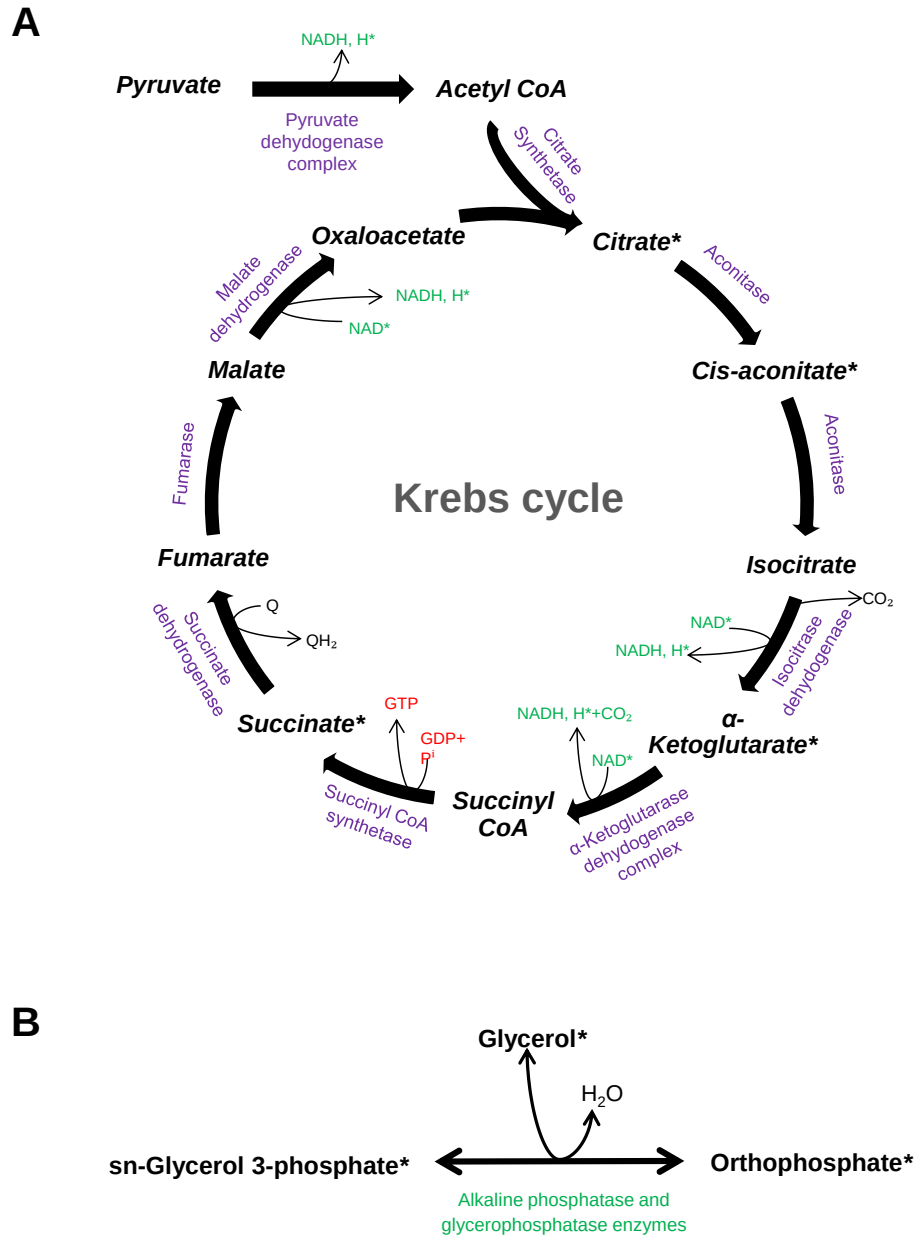


Figure 2.5: Biological pathways highlighted by the compounds differentially detected in MSC-conditioned culture medium (asterisk). (A) Components involved in cell respiration (Krebs cycle) KEGG PATHWAY: MAP00020. (B) Components involved in β GP degradation to glycerol and orthophosphate.

2.6 CONCLUSION

In this study, extracellular metabolites were sampled and analysed at different time points during MSC osteogenic induction *in vitro*, leading to the identification of differentially regulated compounds linked to cellular changes in culture.

The therapeutic potential of mesenchymal stem cells for applications in regenerative medicine and drug discovery has warranted a number of investigations targeting conditions such as osteoporosis and other conditions where skeletal tissue is compromised. Studies on osteogenic induction require the development of in-line, non-destructive characterisation methodologies to be able to phenotype MSCs during differentiation and limit the requirement for sacrificial cultures needed for most cellular and molecular techniques. In our study, for the first time, LC-MS-based footprinting with multi- and univariate analyses has been used for the non-destructive characterisation of MSC differentiation. OPLS-DA analysis from MSCs in undifferentiated and osteogenically-induced states showed significant and reproducible changes demonstrated by OPLS-DA scores plots from metabolic footprints of each condition. The MSC cultures samples analysed showed clear metabolomic changes during osteogenesis, with a particular prominence of compounds linked to cell respiration and the production of orthophosphate, which plays an important role in regulating ALP level during bone differentiation. Further analysis of these metabolomic changes compared to cell proliferation dynamics will be useful to refine the profile of differentiation-related compounds.

Trends of individual OPLS-DA scores plots revealed MSC-related metabolic signatures, and were also able to clearly discriminate between medium samples

from undifferentiated and OS treated cultures. Changes were followed during the 15-day differentiation kinetics and showed gradual shifts in the metabolomic profile of the culture medium. LC-MS spent culture medium analysis enabled the fine characterisation of stem cell environment, and showed that it can generate a chemofingerprint of differentiated cells amenable to real-time analysis of therapeutic cells in culture. Although, these observations confirm the power of LC-MS to screen chemical compounds present in the cellular environment and quantitatively measure cell-based differences non-destructively, they are limited to a single murine cell line. Also, it is worth mentioned that significant phenotype differences were observed within different MSCs species that isolated from the same tissue such as bone marrow [124]. Therefore, to confirm the importance of identified metabolites, future work to compare multiple MSCs donors and species could be useful to identify the possibility of metabolomic differences. Moreover, combined with stable isotope assisted approaches [95, 125], this LC-MS technology could be used for future stem cell metabolite profiling to quantify intracellular metabolites involved in specific stages of differentiation and investigate specific pathways identified by the metabolite footprinting analysis in the current study.

3 CHAPTER-3-

COMBINED METABOLOMICS/VOLATOLOMICS ANALYSIS OF MOUSE MESENCHYMAL STEM CELL DIFFERENTIATION.

3.1 ABSTRACT:

Mesenchymal stem cells (MSCs) represent an established and therapeutically relevant model of cell differentiation, which can be reliably induced in vitro to produce osteogenic and adipogenic cell types. In this chapter, the metabolomics and volatolomics profiles of MSC cultures have been analysed non-invasively during in vitro osteogenic (OS) and adipogenic (AD) treatment using a dual footprinting technique to characterise the biological and biochemical markers associated with mesenchymal stem cell differentiation. Culture medium sampled non-invasively at various time-points of differentiation was analysed using Liquid chromatography-mass spectrometry (LC-MS), as mMSCs were undergoing osteogenic and adipogenic treatment in vitro. Mineral deposits, alkaline phosphatase and lipid accumulation were also assayed and confirmed the efficiency of the MSC differentiation response. In parallel, chemical volatiles released by the differentiating cultures were analysed by Gas chromatography-mass spectrometry (GC-MS) to produce matching volatolomic profiles. Data analysis using a combination of univariate and multivariate analyses uncovered significant changes in extracellular metabolites between the samples collected at different stages of differentiation.

In particular, the non-invasive LC-MS-based analytical approach revealed significant metabolic changes between medium from untreated and differentiated MSC, showing distinct environmental footprints for cells exposed to different conditions (control vs. OS/AD treatment). Some metabolites that link to energy production and lipogenesis were identified during adipogenic induction, while metabolomic changes associated with collagen synthesis were detected during osteogenic treatment. GC-MS identified significant changes in volatile compounds linked to insulin during ('AD') treatment, and others showed a link to proline metabolism. These results provide evidence that metabolomics and volatolomics changes can be non-invasively monitored in differentiating mesenchymal stem cells, providing a new analytical approach to identify biological pathways and non-invasive biomarkers for the characterisation of stem cell populations.

3.2 INTRODUCTION

Metabolomes were first defined as the group of small molecules showing concentration changes due to either gene deletion or overexpression; as a result of physiological or environmental changes [87]. They represent chemical classes such as amino acids, lipids, organic acids, alcohols, ketones, and each compound differs in its physicochemical properties and concentration. Unlike other "omics cascade" such as genomics, transcriptomics and proteomics, no single-instrumental technology is able to analyse all metabolites due to their chemical diversity. Metabolomics approaches currently range from metabolite quantification (metabolic profiling), classification of metabolites in response to environment or disease (metabolic fingerprinting) and extracellular metabolites excreted into the microenvironment (metabolic footprinting) [126]. Applied in

combination with more than one separation techniques (GC or LC) coupled to a MS detector, the sensitivity of metabolic analysis can be comprehensively amplified [127].

Recently, LC-MS-based approaches have been employed widely for the quantitative analysis of extracellular metabolites and nutrients in a growth medium due to their easy sample preparation [95]. Mouse brown adipocytes protein profile that activated in response to insulin stimulation was identified by LC-MS [128]. Additionally, mMSCs characterization during osteogenic differentiation was recently achieved by analysing extracellular metabolites [63]. Based on these recent developments, this project aimed to investigate whether MSC differentiation could be characterised by its distinct metabolic footprint, including metabolite secretion and/or nutrient consumption, which may be unique to the specific metabolic pathways activated during MSC differentiation. The changes identified by LC-MS-based footprinting of the culture medium could thus be used to monitor stem cell differentiation through the analysis of metabolite changes not only between differentiation treatments, but also at different stages within each specific lineage maturation.

GC-MS is comprehensively used in environmental field such as chemical classification of pesticides [129]. Clinically, GC-MS showed powerful quantification of cellular phenotype such as monitoring *Mycobacterium Tuberculosis* growth through analyzing tuberculostearic acid methyl ester [70]. Also, a range of metabolites such as alcohols, aldehydes, furans, ketones, pyrroles, terpenes have been detected in biological fluids such as plasma and urine [65]. In addition, GC-MS has recently been applied to the detection of breath volatile biomarker profiles from breast cancer patients, which were

shown to contain 3-methylhexane, decene, caryophyllene, naphthalene and trichloroethylene and 1-methylnaphthalene [130]. More recently, GC-MS has been applied to cell culture to monitor chemical volatiles affected by cytotoxic agents during cell culture to test the effect of β -carotene (BC) on primary rat hepatocytes and pneumocytes [131]. However, to date the use of GC-MS for cell studies has mostly relied on the destructive extraction of volatiles from cell extracts. This chapter presents a non-destructive application of a combined LC-MS and GC-MS analysis to mesenchymal stem cell cultures, and report on the metabolomics and volatolomics changes measured during the osteogenic and adipogenic differentiation of live stem cell cultures, highlighting specific chemical species as potential markers of cell phenotype.

3.3 MATERIAL AND METHODS

All reagents used were purchased from Thermofisher (UK) unless otherwise stated.

3.3.1 Cell culture and differentiation

Mouse mesenchymal cells (D1, ATCC CRL-12424) [99] were cultured in standard MSC medium consisting of Dulbecco's Modified Eagle's Medium DMEM, 10% (v/v) FCS, 1% (v/v) L-glutamine, 1% (v/v) non-essential amino acids and 1% (v/v) antibiotics, and incubated at 37°C and 5% CO₂.

For GC-MS experiments, MSCs were seeded at cell densities of 3×10^4 cells/ml, as 3 replicates, in 20ml glass vials (X100 Headspace clear glass round vial, Fisherbrand) as well as 12 well plates. In some cases culture vessels were coated as indicated with 10 mg/ml of Poly(2-hydroxyethyl methacrylate) pHEMA (Sigma) to inhibit cell attachment [132].

Confluent cells treated with osteogenic medium ('OS') consisting of standard MSC medium supplemented with 100 nM dexamethasone (Sigma-Aldrich, Gillingham, UK), 0.05 mM L-ascorbic acid 2 phosphate (Acros Organics, Geel, Belgium) and 10 mM beta glycerophosphate (β GP, Sigma-Aldrich); or standard MSC medium supplemented with 1 μ M Dexamethasone, 100 μ M 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), 10 μ g/ml Insulin, 1 μ M Rosiglitazone (Cayman) for adipogenic induction ('AD'), and treated for 15 days with medium changes every 2 days. Cells maintained in standard MSC medium were used as a control medium sample. All supplements were prepared according to the manufacturer's guidelines. The same media, reagents and supplements were used throughout the experiment for consistency.

To test the effect of hypoxia and hypothermia on MSCs, miMSCs were seeded at density of 10^4 /ml into six T12.5 tissue culture flasks and cultured according to standard culture protocol [133] for 2 days. After 24 hours of seeding (day 1), 10 % (v/v) DMSO was added to the negative control flask. To achieve anoxic and no CO₂ culture conditions, flasks that were labelled with (24hrs) were sealed 24hrs before testing cellular metabolic activities supplementary table-3. Also at day 2, flasks labelled with (9, 6 or 3 hour) were sealed at their labelled time point before testing their metabolic activities. Negative and positive controls were incubated under normoxic condition and all flasks were kept at 37°C throughout the experiment. To test the influence of room temperature on cell proliferation, flasks were then incubated at RT according to the required time points (12, 9, 6 and 3hr). Negative and positive controls were incubated under normoxia and all flasks were kept at 37°C throughout the experiment. In addition, sealed positive control was added and kept at 37°C throughout the

experiment. At day 2 and 24hrs after sealing first flask PrestoBlue assay was performed see section 3.3.2

Calcium deposits were analysed using Alizarin Red staining. At the stated time point, cells from 4 replicates were washed by PBS, and incubated in 1 % (w/v) Alizarin Red staining solution (Sigma-Aldrich) for 10 min at room temperature, and washed with distilled water until all excess stain had been removed. To stain lipid droplet, cells were washed by PBS and fixed with PFA for 10 mins; followed by washing with distilled H₂O (DW) three times. 100 µl of Oil Red O solution consists of 3:2 (100 mg of Oil Red O (Sigma-Aldrich) in 20 ml isopropanol) in DW was added into each well for 10 min then washed with DW until clear fluid was observed. Mineral deposits and lipid droplets were then observed using a Nikon Eclipse Ni90 light microscope.

3.3.2 Metabolic activity assay

MSCs were seeded in to 20 ml glass vials (in triplicates) with 3×10^4 , 5×10^4 cells/ml and 7×10^4 cells/ml seeding densities in standard MSC medium (previously mentioned) and incubated at 37°C and 5% CO₂. At day 3, 6 and 9, metabolic activity was measured using PrestoBlue Cell Viability Reagent according to the manufacturer's instructions and fluorescent intensity was measured in 5 replicates using Tecan i-control infinite 200 plate reader set at 560 nm excitation and 590 nm emission wavelengths.

3.3.3 LC-MS and GC-MS analyses

For LC-MS culture, metabolite extraction and measurement techniques were carried out on 6 replicate samples using an Accela system coupled to an Exactive MS (Thermo Fisher Scientific, Hemel Hempstead, UK) as previously

described [63]. Medium extract were performed at three time points (day 0, 5 and 10) of differentiation using day 0 as a base point.

For GC-MS, 3 replicate vials were sealed with crimp caps (Sigma-Aldrich, Poole, UK) and kept at 37°C until analysis. Vials containing media without cells were also analysed to account for medium-related volatile compounds. Considering the difficulties to identify metabolite peaks at day 0 due to low cell numbers, volatiles were analysed at day 5, 10 and 15. Headspace volatiles were sampled with a 50/30 µm DVB/Carboxen/PDMS StableFlex SPME fibre (Sigma-Aldrich) for 30 min at 37°C. The volatiles were transferred in split/splitless injection mode onto a new ZB-WAX column - 30mm x 0.25mm ID, x 1µm film thickness (Phenomenex, UK) and chromatogram with Helium as the carrier gas at 18 psi. The GC starting temperature of 60°C was held for 1 min and ramped to 180°C at 8°C/min. All the compounds were detected with a DSQ Mass spectrometer (Thermo scientific) in scan mode, 20-200 m/z. Using Xcalibur Processing Data; GC-MS chromatograms were normalized to their medium only blank. Then, untargeted peaks were analysed and putative compounds were identified according to the NIST library. Using GraphPad Prism 7, one way AVOVA testing was performed to identify statistically significant differences within different sample conditions and 0.05 was chosen as significant.

3.4 RESULTS

3.4.1 The potential of MSCs for proliferation in 20ml glass vials and under hypoxic/hypothermic conditions

The aim of this experiment was testing the potential of miMSCs to attach into glass vials without the need of supporting anchorage system (coating). Therefore, a polystyrene culture condition was used as a comparable control for cell attachments. Also, due to the ability of pHEMA for cell attachment inhibition, it was used to compare its effect in both conditions. 2D culture system (12 well plates) was used due to its surface area close to those in 3D (20ml glass vials) (Table-3.1). The number of attached cells in glass vials was approximately similar to those in 12 wells plate without pHEMA coating. In addition, attached cell counts due to pHEMA were also similar in both conditions (Figure-3.1).

	Medium volume (ml)	Approx. 2D (CM ²) surface area	Approx. 3D (CM ³)
6 well plate	1.9 – 2.9	9.5	29.281
12 well plate	0.76 – 1.14	3.8	7.4076
24 well plate	0.38 – 0.57	1.9	2.619
20 ml glass vial	0.83	4.15	8.4542

Table 3-1: Measuring surface area well plates compared to 20 ml glass vials.

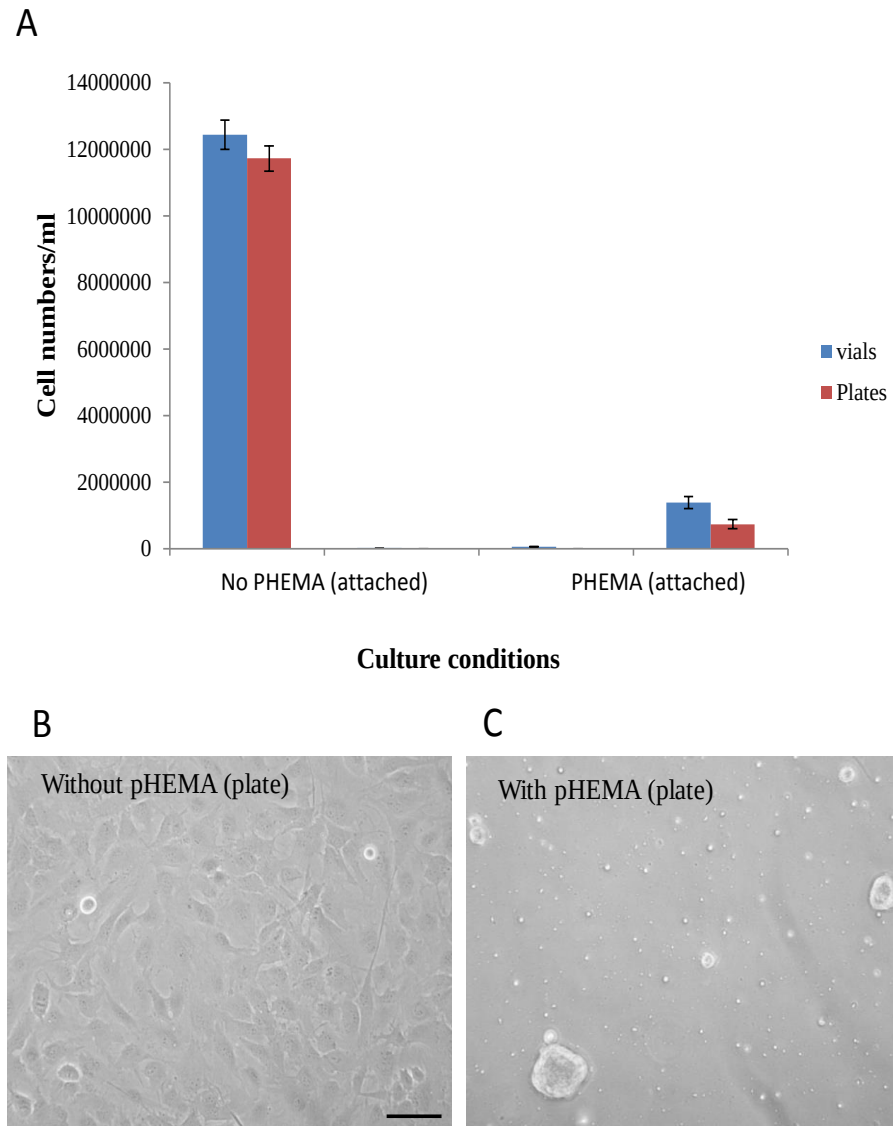


Figure 3.1: Testing the attachment of MSCs into 20ml glass vials. miMSCs were seeded into 20 ml glass vials as well as 12 well plates in duplicates (some wells and vials were coated with 10mg/ml pHEMA before seeding) and incubated for two days. Cell counts were performed on cell suspensions as well as attached cells after trypsinization. Our data show no significant differences between attached cells in uncoated vials and plates (A). This suggests MSCs adhere to glass vials as well as to culture plates. The effect of pHEMA can be noted in plate (C) in which clusters of detached MSCs are observed in comparison with MSCs monolayer (B). Scale bar =100µm. n=4

Under standard culture condition, both O₂ and CO₂ circulation is maintained. However, in our challenging culture condition glass culture vessels should be sealed prior to and throughout the analytical process. Therefore, in this experiment miMSCs were first grown in a standard culture conditions; followed by testing the effect of different sealed culture periods on their metabolic activity. Our data showed that 3hrs after sealing, the cellular metabolism was significantly increased in comparison with positive control; followed by gradual decrease after 6hrs (figure-3.2). However, cellular proliferation was maintained above the standard control before declining from 9hrs of sealing. When the sealed period was extended up to 24hrs; cellular metabolism was dramatically affected.

It is speculated from our experiment that metabolic activity of miMSCs was significantly increased up to 6hrs under lack of O₂ and CO₂ circulations in comparison with cultivated cells under standard culture condition (positive control). However, cells have the potential for maintaining their proliferation up to 9 hours under the same condition.

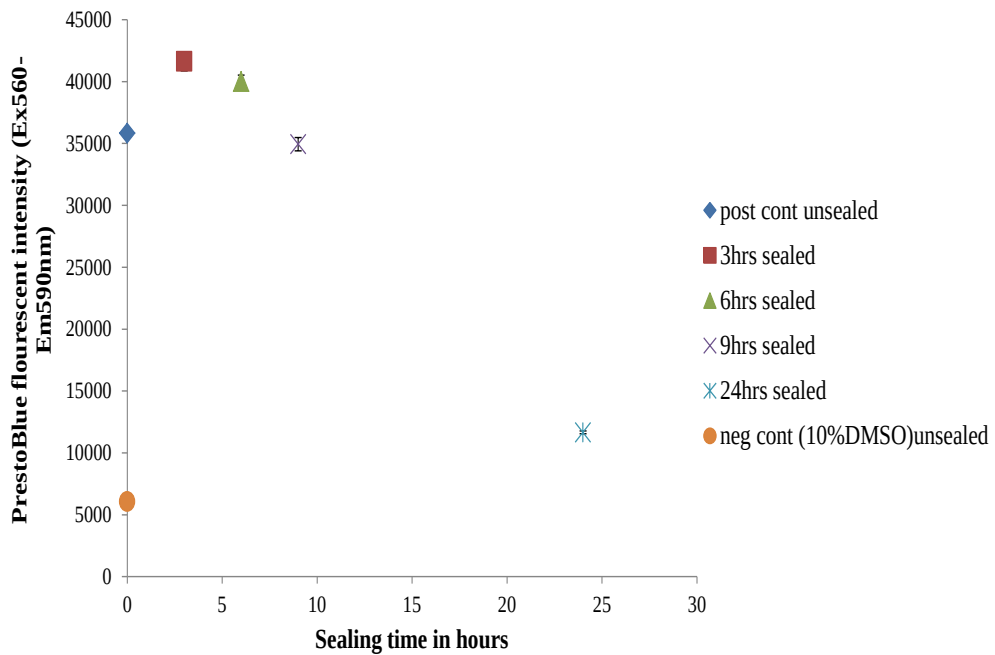


Figure 3.2: Testing metabolic activities of miMSCs at different sealing times: miMSCs were seeded in to six 12.5T flask and cultured for 2 days. At day 2 flasks were sealed according to the required time points (9, 6 and 3 hours). Negative and positive controls were incubated under normoxic condition and all flasks were kept at 37°C throughout the experiment. It seems that cells can maintain their metabolic activity for up to 9hrs under hypoxic condition with no significant differences with positive control. (****P< 0.0001/ **P< 0.05, n=2)

Due to the unusual hypothermic culture condition, which the cells will be experiencing during their metabolic analysis, it is important to investigate the impact of room temperature on cellular metabolic activities. Therefore, in this experiment the cells were cultured under standard conditions before transferring them at room temperature for various period of times. Continuing with the previous experiment, sealed condition was also maintained; then PrstoBlue cell viability assay was performed. In our result, sealing alone (represented by positive sealed control) had a substantial influence on cell proliferation rate in comparison with standard unsealed positive control (figure-3.3). This can be noted by microscopic decrease in cell number (Figure-

3.3A); which explains the significant drop in metabolic activity (Figure-3.3B). After 3hrs of incubation in sealed flask at room temperature, metabolic activity significantly increased in comparison with positive sealed control. This can be noted microscopically (figure-3.3A) and their relative PrestoBlue intensity (figure-3.3B). Cellular metabolism then displayed gradual decreased toward 6hrs, followed by further declined at 9hrs in comparison with positive sealed control. Unexpected increase in cell metabolic activity at 12hrs was noted in PrestoBlue data which contradict the observation of cells; which suggest technical error because cell images were checked twice. A drop in cellular proliferation was also noted at 24hrs qualitatively (figure-3.3A) and quantitatively (figure-3.3B). Comparing metabolic activity of positive control experienced sealing only for 9hrs with those under both sealed and at RT for same length of time (Figure-3.4A) showed that RT has dramatic decline on cell metabolic activity. Also, it is noteworthy that sealing alone for 9hrs has a substantial effect on cell metabolism compared to positive unsealed control. However, cell metabolic activity at RT under sealed condition showed significant increase at 3 and 6hrs compared to sealed positive control (Figure-3.4B). Three independent experiments have been performed to achieve above mentioned data.

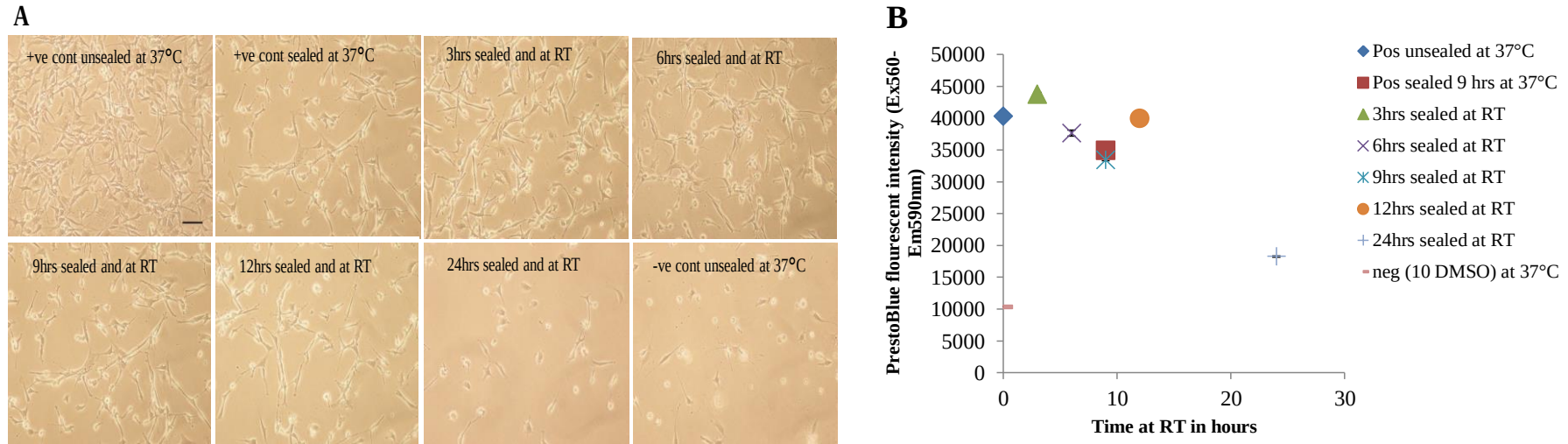


Figure 3.3: Microscopic appearance and metabolic activities of miMSCs under hypoxia and hypothermia: miMSCs seeded in to 12.5T flask and cultured according to standard culture protocol for 2 days. At day 2, flasks were then incubated under hypoxic condition and at RT according to the required time points (24, 12, 9, 6 and 3hr). Negative and positive controls were incubated under normoxia and all flasks were kept at 37°C throughout the experiment. (A) Microscopic observation of cells under hypoxia show a noticeable decrease in cell numbers in comparison with those cultures under standard condition, however cells experienced hypoxic condition and low temperature for up to 6hrs demonstrate slight increase in comparison with those grown under hypoxia only. (B) It is noticed that cells can maintain their proliferation up to 6hours under hypoxic condition and at room temperature followed by a significant decrease at 9hrs and above Scale bar= 200µm. (** $P < 0.0001$ / * $P < 0.05$, $n = 3$)**

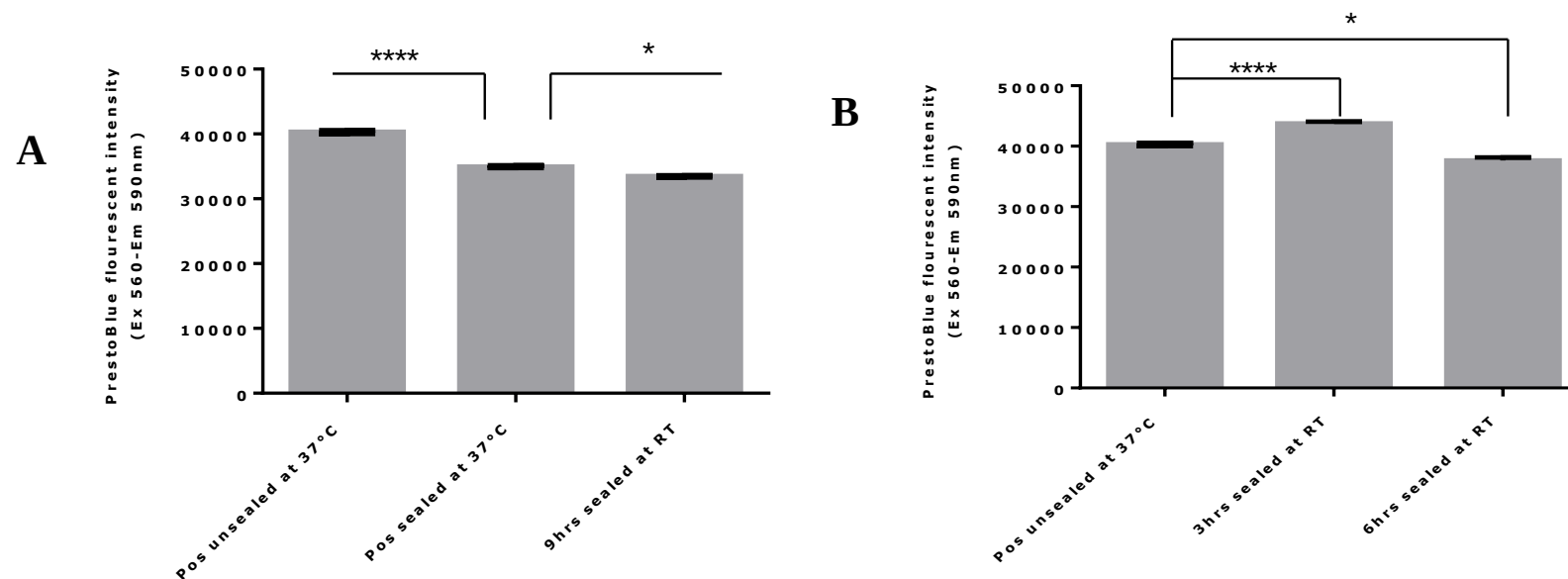


Figure 3.4: Testing miMSCs metabolic activities under hypoxia and hypothermia: miMSCs seeded in to 12.5T flask and cultured according to standard culture protocol for 2 days. At day 2, flasks were then incubated under hypoxic condition and at RT according to the required time points (24, 12, 9, 6 and 3hr). Negative and positive controls were incubated under normoxia and all flasks were kept at 37°C throughout the experiment. (A) A significant drop is noted between +ve sealed control for 9hrs with +ve unsealed, also it seems that 9hrs at RT has a dramatic influence on metabolic activity compared to their +ve sealed control for 9hrs. (B) Cell metabolic activity show significant increase at 3 and 6hrs incubation at RT compared to their +ve sealed control.

3.4.2 Testing the influence of increased seeding density

This test was done to identify the optimum seeding density that can be performed in 20ml glass vials to insure the maintenance of cell metabolic activities. To achieve that, various seeding densities have been tested against different time points and show sharp increase in miMSCs metabolic activities up to day 6; however this proliferation rate was gradually elevated toward day 9 for low cell densities (figure-3.5). On the other hand, increasing the seeding density to 5×10^4 /ml show slow metabolic rate toward day 6; followed by sharp drop at day 9. Similarly, with cells density of 7×10^4 /ml the metabolic activities were slowly decreased after day 3 before they sharply declined toward day 9 in culture.

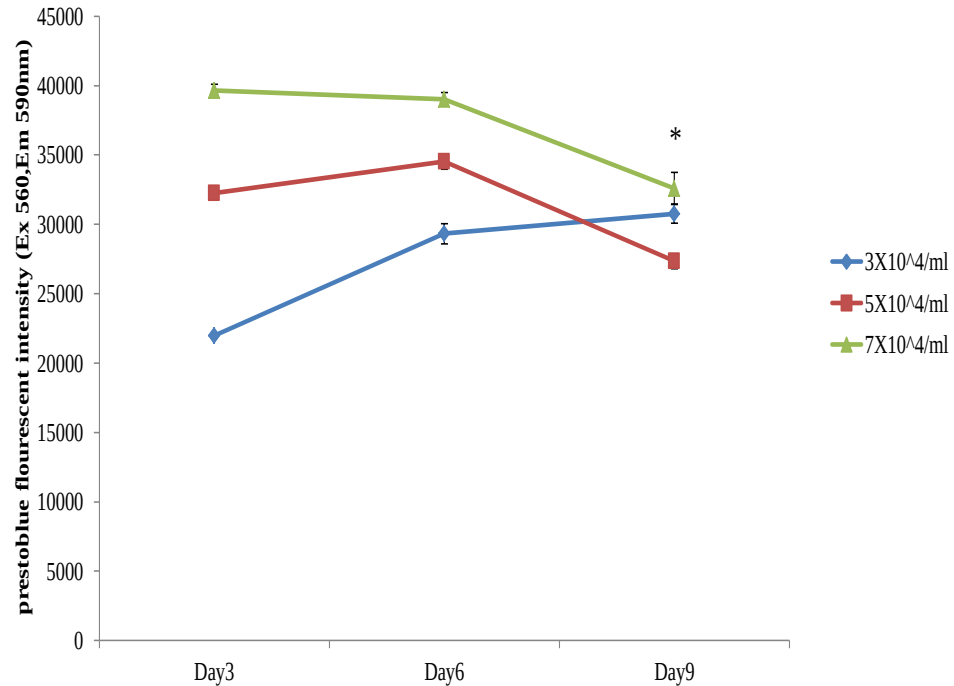


Figure 3.5: Testing seeding density in glass vials. miMSCs were seeded in 20ml glass vials with $3,5$ and 7×10^4 cells/ml seeding and incubated for 9 days. Cell metabolic activity increased up to day 6 with lower seeding densities followed by sharp drop toward day 9 in comparison with day 3. But, with 7×10^4 cells/ml metabolic activity show a dramatic drop with after 3 days. (* $P < 0.05$, $n=3$)

3.4.3 Metabolomic changes in MSCs under osteogenic/adipogenic treatment:

In order to confirm cellular changes triggered upon osteogenic and adipogenic induction treatments, mMSC treated with ('OS') and ('AD') media were analysed for mineral deposition and lipid accumulation, respectively, to confirm changes in phenotype. Under ('AD') conditions, lipid droplets were visible at day 5 and more strongly at day 10, and under OS conditions, Alizarin Red staining seen at day 5 further increased by day 10 (Figure.3.6).

A

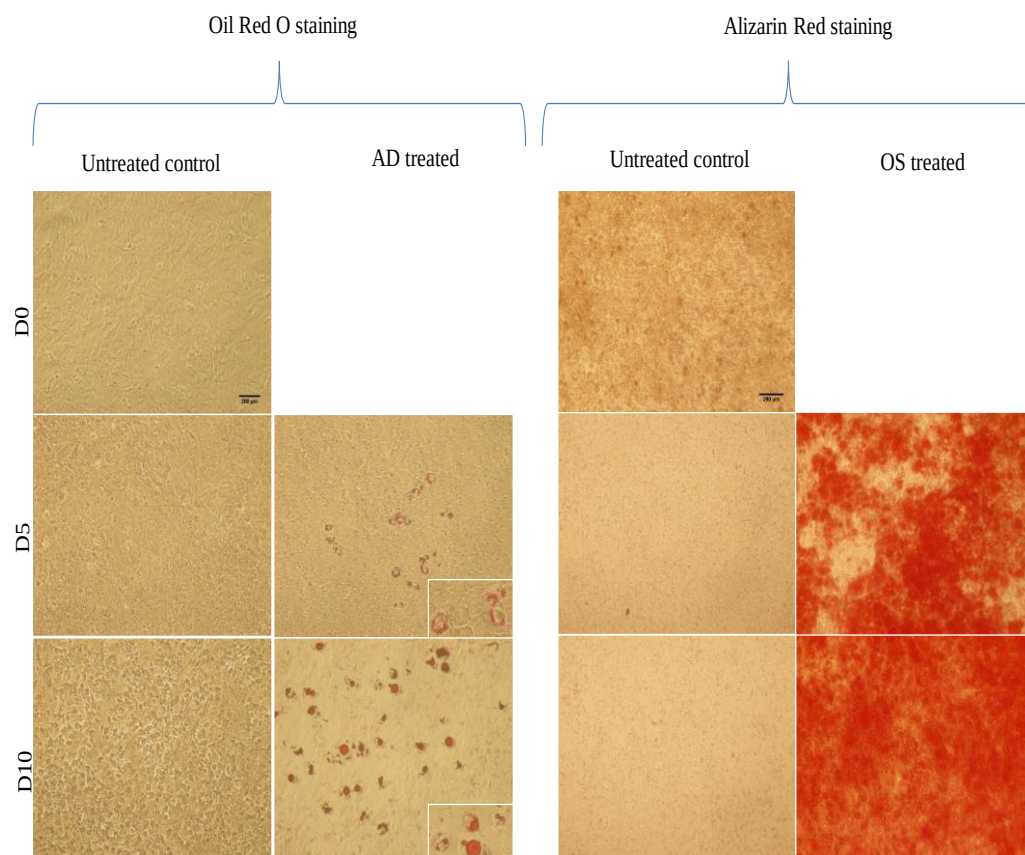


Figure 3.6: MSCs maintained in standard, adipogenic and osteogenic medium analysed at day 0, 5 and 10. Oil Red O was used to stain for the presence of intracellular lipids accumulated under AD treatment, and Oil Red O was used to stain mineral deposits in OS-treated cultures. Scale bar =200μm.

Based on this cellular response, spent medium samples collected at day 0, 5 and 10 of treatment were analysed by LC-MS in order to evaluate changes in molecular footprint upon dual mMSC differentiation. OPLS-DA analysis was performed to identify significant compounds differentially released/consumed from mMSCs under standard, ('OS') and ('AD') culture conditions. The molecular composition of each sample, compared to its corresponding medium only blank, showed discrete clustering of the metabolic profile in different replicates (Figure.3.7A). As shown in Figure. 3.7A, the scores of fresh media

in the absence of cells were observed in the left hand side, and as the length of culture under treatment increased, the cluster spread from left to right (D0) and then went to the top (D5) then moved to the bottom (D10). These resultant scores plots clearly showed cluster patterns directly related to the length of cell culture treatment. It is therefore reasonable to consider that the metabolite footprinting data from MSC cell culture media analysed using LC-MS contains valuable information regarding metabolite secretion/excretion and nutrient consumption relevant to the metabolic effect of mMSC differentiation.

Changes in mMSC medium were monitored over the 15-day differentiation time-course. When comparing the three time-points analysed within each treatment group, distinct differences were observed suggesting dynamic changes in the mMSC culture medium over time following the same trend in both standard and ('OS') treatment conditions, with datasets at early culture stages (day 0) showing closer metabolomic proximity than the late time-point (day 5 and 10).

260 metabolites found during ('OS') or ('AD') differentiations were selected and analysed based on their chemical nature (Figure.3.7B). As shown in Figure.3.7B, the analysis identified different categories classes of metabolites such as vitamins, lipids, nucleotides, as well as metabolites associated with carbohydrates and energy metabolism, and a large proportion associated with amino acid metabolism.

Development of non-destructive methods of the Characterization of Mesenchymal Stem Cell Differentiation in vitro

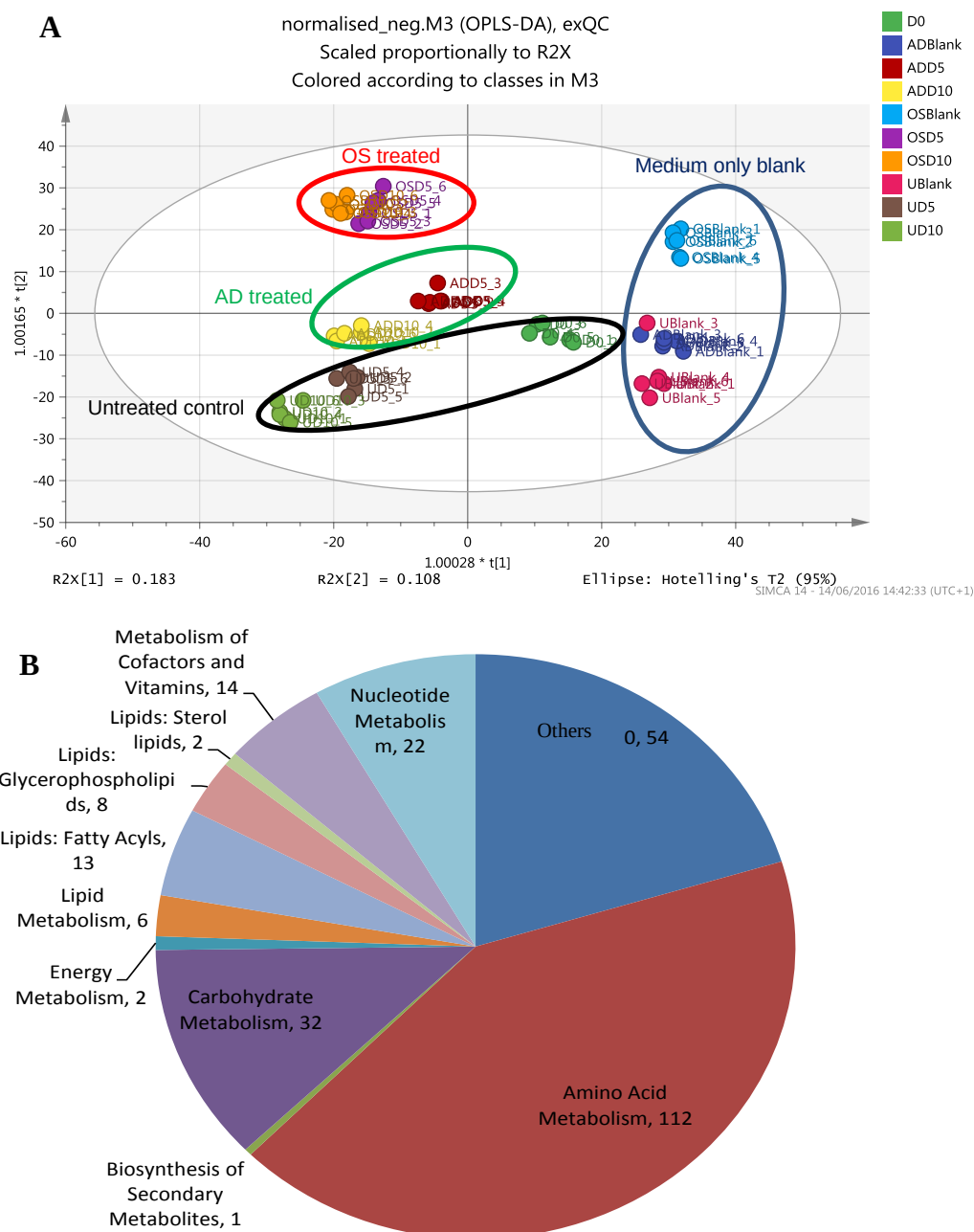


Figure 3.7: LC-MS analysis of the compounds identified in medium samples from MSCs under standard, OS and AD treatments, and their corresponding medium-only blanks, analysed after 0, 5 and 10 days of treatment. (A) OPLS-DA scores plots showing combined standard, OS and AD treated MSC- conditioned samples and their respective medium-only blanks. (B) Chemical composition of the 260 metabolites identified by LC-MS based on their reported biological function.

To determine which mass ions contributed to the clusters and trends between MSC cultured media and medium only blank samples, group comparisons were

performed between fresh medium (day 0) and day 10 samples within the OPLS-DA models. Key mass ions for discriminant biomarkers were selected by VIP values higher than one considered as potential key mass ions. In addition to the multivariate analysis, univariate one-way ANOVA testing was also performed to identify significant compounds consumed and excreted by MSCs (both in standard, ('OS') and ('AD') treatments) compared to their medium-only blanks, over time. Key mass ions were also selected from the univariate analysis with the false discovery rate $Q \leq 0.05$.

When assessing chemical alterations between the differentiations conditions, metabolites that were significantly elevated during either ('OS') or ('AD') differentiation compared to untreated controls were identified and examined (Figure-3.8). A subset of metabolites appeared to be significantly and time-dependently increased in mMSC cultures undergoing ('AD') differentiation (Figure. 4A- D), while another subgroup of compounds were significantly increased during ('OS') differentiation in comparison to untreated control and ('AD') treatment (Figure.3.8F-J). Among these, three metabolites (3b-16a-dihydroxyandrostenonesulfate and xylitol) were specifically detected during ('OS') differentiation and showed strong signal at both day 5 and day 10, while they remained undetected in MSC cultures under standard or ('AD') medium (Figure. 3.8E and G). In addition, metabolite such as norepinephrinesulfate showed no significant difference between ('OS') and untreated control at day 5, while toward day 10 it noticeably upregulated during ('OS') compared to the untreated control (Figure. 3.8H).

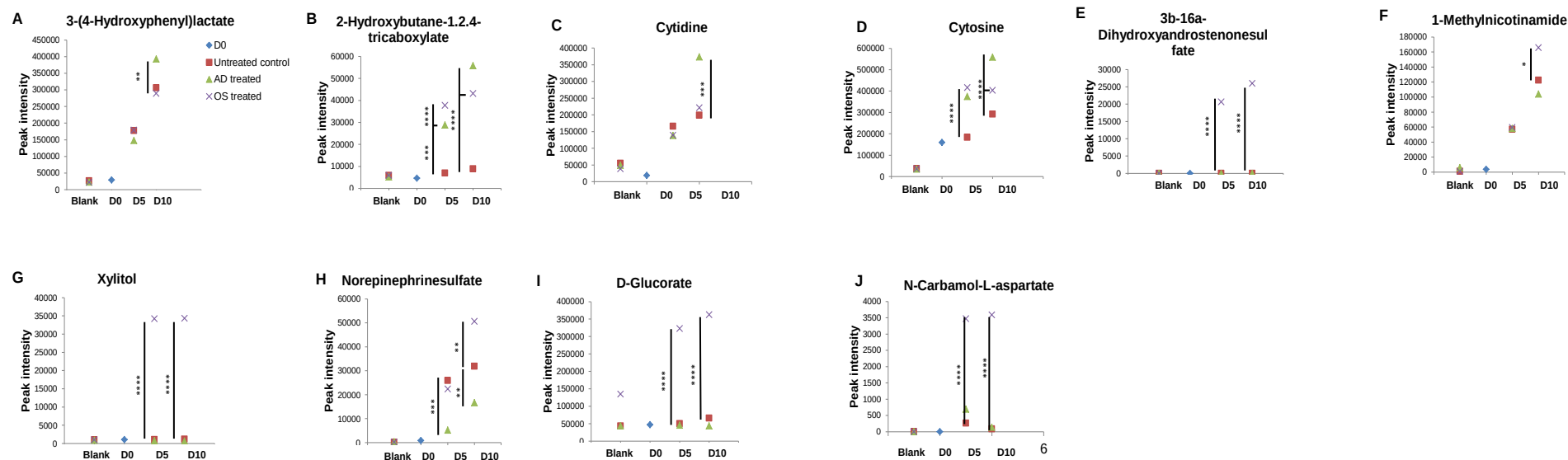


Figure 3.8: Metabolite identified by LC-MS showing change over time in culture medium in the presence of MSCs under OS (✕)/AD (▲) conditions compared to control medium (■), at day 0, 5 and 10 of treatment (** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; $n = 6$).**

3.4.4 Volatolomics compounds detected in MSC cultures undergoing osteogenic/adipogenic differentiation

In parallel, volatile compounds released by mMSC at different time points of ('OS')/('AD') differentiation were analysed through GC-MS-based analysis of culture headspace composition at day 5, 10 and 15 of treatment. Detected chromatogram peaks were then analysed and compounds were identified according to their mass and retention time, which were matched with the integrated Xcalibur library. Out of 40 peaks detected, only 12 compounds could be identified based on the library (Table-3.2). The peak intensity of each metabolite was statistically analysed using one way AVOVA testing to identify statistically significant differences between untreated control, ('OS') and ('AD') conditions at each time point. GC-MS analysis identified significant volatolomics changes in MSC cultures undergoing ('OS')/('AD') differentiation, with most of the identified candidates upregulated during ('AD') treatment compared with OS and untreated control (Figure 3.9).

Ethanol was identified and detected at significantly higher levels in undifferentiated cultures maintained in standard medium, while its level declined in ('OS') and ('AD') treated cultures (Figure. 3.9A). In contrast, a number of aromatic compounds showed significantly increased levels at later stages of ('AD') differentiation compared to cultures treated with standard medium and ('OS') medium (Figure. 3.9B-H and I-L). Three decanes (Tridecane, Tetradecane and Dodecane) showed similar profiles with significant increase during ('AD') treatment compared to the untreated control (Figure.3.8B, C and D). D-arabinose and Azetidine displayed similar profile where significant reduction during ('OS') and ('AD') treatments was noted at

day 5 compared to the untreated control; while toward day 15 a noticeable upregulation was noted during ('AD') and downregulation during ('OS') compared to the untreated control (Figure.3.9 E and F). In contrast, cyclohexanol reported a significant increase in untreated control at day 5 and 10 compared to both ('OS') and ('AD') treatments, although it showed a continuous elevation with untreated toward day 15 sudden elevation during ('AD') was also observed (Figure.3.9 G). A consistent elevation toward day 15 was detected during ('AD') treatment with compounds such as 2-chloroethanol, 4-methylquinoline, 3,6-diphenyl,1,2,4,5-tetrazine, fumarylchloride and dimethylamine and continuous reduction toward day 15 was observed with untreated control (Figure.3.9 H,I, J,K and L).

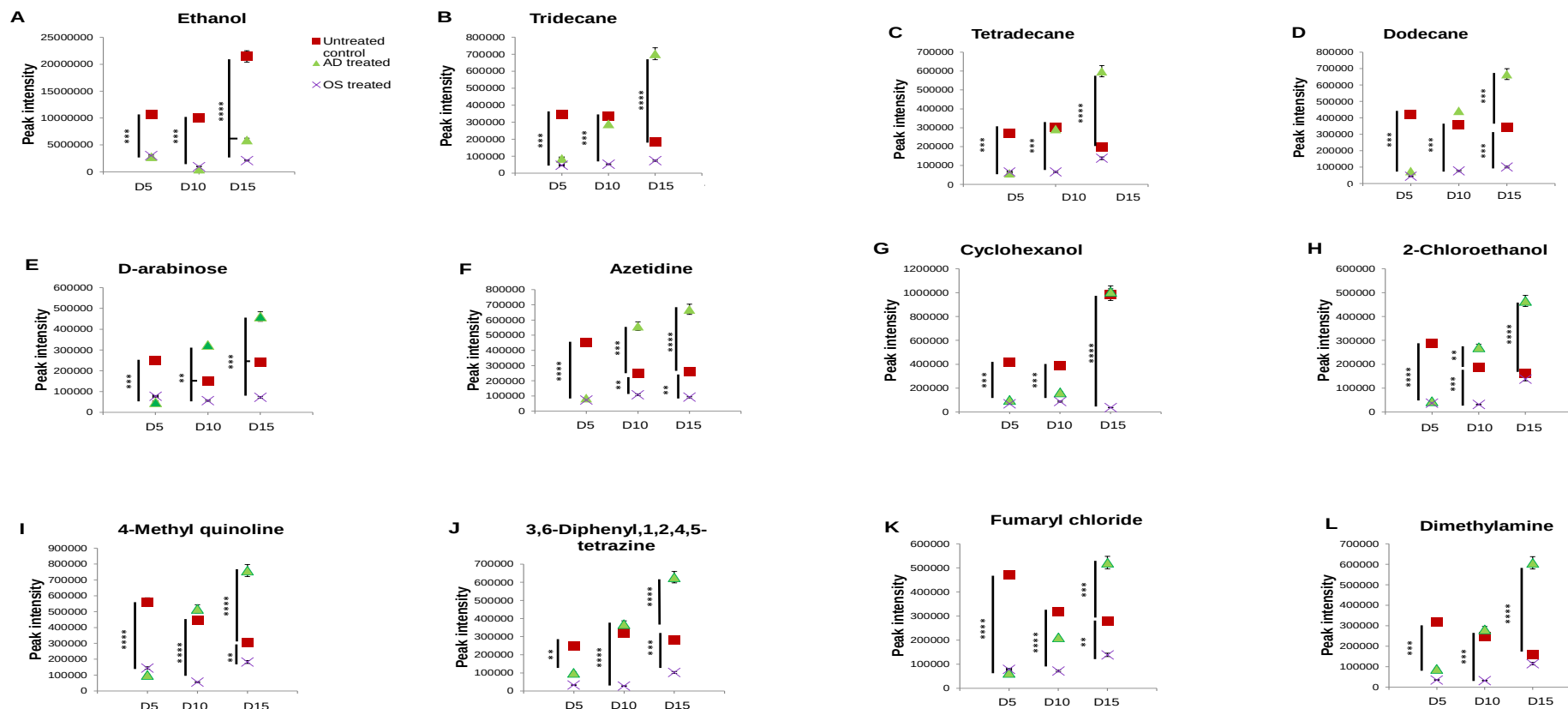


Figure 3.9: Volatile compounds identified showing differential detection in samples from standard, OS and AD differentiated MSC cultures. Related volatiles found to change over time in culture medium in the presence of MSCs under OS (✕)/AD (▲) treatment compared to control medium (■), at day 5, 10 and 15 of treatment (** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; $n = 3$)**

3.5 DISCUSSION

3.5.1 The potential of MSCs for proliferation into 20ml glass vials and under hypoxic/hypothermic conditions

Our data showed the ability of mMSCs to adhere to glass vials with no significant difference to those in tissue culture plates (TCPs). Similar observation was noted previously with human mesenchymal stem cells (hMSCs) responsiveness to a niobium-doped fluorapatite-based glass-ceramic (FAp) [134]. In this study hMSCs showed their ability not only to attach but also to proliferate and differentiate into osteogenic lineage on a fabricated glass surface. Therefore, this is a promising finding as it indicates pre-coating glass vials is not mandatory for mMSCs.

It is speculated from our experiment that metabolic activity of mMSCs was significantly increased up to 6hrs under lack of O₂ and CO₂ circulations in comparison with cultivated cells under standard culture condition (positive control). However, cells have the potential for maintaining their proliferation up to 9 hours under the same condition. This data correlates with previous study which shows the ability of certain cell populations to maintain their proliferation status under hypoxic condition due to the increase in DNA synthesis [135]. In vivo condition could also be correlated with our data when cells sustain their proliferation status under ischemic condition [135]. Owing to the fact that in the absence of oxygen, hypoxia-inducible transcription factor I (HIF-I) activates the expression of numerous genes to regulate their proliferation, immortalisation, migration and death [136].

Also, mMSCs showed a significant increase of their metabolism not only under lack of standard O₂ and CO₂ but also at room temperature for up to six hours in comparison to those under hypoxic condition only (positive control sealed at 37°C). Though, a substantial decline was detected when cells were incubated for 9hrs and more. Although, this may contradict our previous experiment where the cells maintained their proliferation for 9hrs, in this experiment cells not only experienced a hypoxic condition but also a temperature shift from 37°C in to 25°C. The influence of hypothermic condition on protein expression and cell proliferation has been tested before showing reduction of protein synthesis and cell proliferation rate (Kumar et al., 2008).

3.5.2 The optimum seeding density for GC-MS analysis

Our data exhibit an increase in metabolic activity up to day 6 with 3 and 5x10⁴/ml seeding densities. However, a decline in cell proliferation was noted with 7x10⁴/ml density after day 3. This indicates that seeding 20ml glass vials with 3 or 5x10⁴/ml miMSCs can be suggested for up to 6 days culture process. It also indicate that 3x10⁴cells/ml may be capable to maintain their metabolic activity for 9 days.

3.5.3 Candidates arisen from the metabolomics footprinting

Our data identified significant metabolites that have never previously been linked with ('OS')/('AD') differentiation of mesenchymal stem cells. Therefore, speculation into the biological pathways in which the identified metabolites were involved during osteogenesis and adipogenesis of mMSCs may provide a clear understanding on the cellular metabolism. Our data revealed a pool of metabolites that potentially involve in the energy production in fat cells such as (hydroxy-phenyllactate, 2-hydroxybutane-1.2.4-

tricarboxylate) and bone extracellular matrix formation (3b-16a-dihydroxyandrostenedione sulfate, 1-methylnicotinamide, glucuronate 1-phosphate and norepinephrine sulfate). The key compounds identified in this study are discussed below with reference to their reported physiological roles.

Hydroxy-phenyllactate: It is a tyrosine metabolite (KEGG PATHWAY:MAP00350) that previously documented to be up-regulated in classically activated macrophages (CAM) of adipose tissue by fatty acid transport protein 1 overexpression (FATP1-OE) [137]. Also, it is known that hydroxy-phenyllactate is catalyzed to 3-(4-hydroxyphenyl)pyruvate and produce NAD^+ and NADH (KEGG REACTION:R03336) and was reported that energy production from adipose tissue is as a result of fatty acids or conversion of glucose into fatty acids [138]. In our data, LC-MS analysis revealed to an elevation in 3-(4-hydroxyphenyl)lactate at the late stage during ('AD') treatment, not only in comparison with untreated control but also with ('OS') treated, in correlation with above mentioned studies, suggesting a biological link between the identified metabolite and mitochondrial energy production during adipogenesis.

2-Hydroxybutane-1,2,4-tricarboxylate: Another metabolite that showed significant increase during adipogenesis and links to Tricarboxylic acid cycle (TCA) is 2-hydroxybutane-1,2,4-tricarboxylate (KEGG REACTION: R00271) (Figure-4B), however it was also elevated significantly during osteogenic lineage formation. This finding may introduce a new biomarker for mMSCs ('AD')/('OS') differentiations in the field of stem cell biology. In addition, cytidine and cytosine are metabolites involved in pyrimidine metabolism (KEGG REACTION: R02137), they formed cytidine diphosphate

Diacylglycerol (a liponucleotide known for its role during lipid metabolism [139]). Thus, it not surprising that, in our study, cytidine and cytosine were substantially increased upon lipid accumulation during adipogenesis (Figure-3.8 C and D).

3b-16a-Dihydroxyandrostenonesulfate: From the standpoint of bone metabolism, 3b-16a-dihydroxyandrostenonesulfate is a metabolite found in human placenta and breast milk (HMDB00386). In correlation with the fact that adrenal androgens play an essential role during bone metabolism [140], our analysis showed noticeable elevation of this metabolite only during ('OS') differentiation. This finding may suggest that the level of 3b-16a-Dihydroxyandrostenonesulfate is related to osteoblast formation and consequently bone maturation.

1-Methylnicotinamide this compound is a metabolite located in the cytoplasm and extracellular spaces (HMDB00699) and associated with nicotine and nicotinamide metabolism (KEGG PATHWAY: hsa00760). It also seems to be involved in joints and arteries calcification (KEGG DISEASE: H00824). Although the link between this metabolite and bone formation has not been described, our analysis revealed significant increase of this metabolite in all conditions at day 5 and 10 of differentiations compared to day 0 and medium only blanks ($P < 0.0001$) and in particular a considerable rise in ('OS') conditioned medium, which may suggest the compound's association to mineralization (Figure-3.8F). Glucuronate 1-phosphate: It is known that ascorbic acid is an essential compound for collagen synthesis [141], and couples with 2-oxoglutarate to form a cofactor for prolyl 3-hydroxylase and prolyl 4-hydroxylase enzymes to hydrolyse proline (KEGG: EC 1.14.1.2) and

produce hydroxyproline (KEGG: R03219) during collagen synthesis. During ascorbic acid synthesis, free glucuronic acid forms upon UDP-glucuronic acid hydrolysis and conversion to pentose phosphate intermediates, leading to the formation of glucuronate1-phosphate (KEGG PATHWAY: rn00040) [142]. Our analysis indicates a substantial increase in glucuronate 1-phosphate during OS treatment only, which would support its link with ascorbic acid synthesis.

Xylitol is an intermediate product of glucuronate/xylulose cycle (KEGG PATHWAY: rn00040) and it was reported previously that xylitol oral supplements increase bone density in rat [143, 144] and decrease body weight of xylitol fed rats with noticeable elevation of triglycerides and free fatty acids levels in rat's serum as an indication of lipolysis [145]. During our analysis, it appeared only during ('OS') treatment compared to zero peak intensity seen in untreated control and ('AD') treated (Figure-3.8G). Therefore, further investigation on xylitol is recommended to determine not only the link between xylitol and ascorbic acid catabolism during osteogenesis, but also its physiological effect during lipid synthesis.

Norepinephrinesulfate is synthesized upon the hydroxylation of dopamine by dopamine β hydroxylase in the cytosol [146]. It is also known that ascorbic acid inhibits the hydrolysis of norepinephrinesulfate [147], [148] which may explain the noticeable elevation in its level during ('OS') treatment compared to untreated control and ('AD') treated. On the other hand, its reduction during ('AD') treatment might be as a result of the inhibitory effect of insulin on norepinephrine transporter [149]. Moreover, during pyrimidine metabolism, carbamoyl phosphate and L-aspartate are catalysed (KEGG REACTION: R01397) by aspartate carbamoyltransferase (REACTOME: 9152324) to

produce N-Carbamol-L- aspartate and orthophosphate. Our previous data showed an increase in orthophosphate during osteogenic differentiation compared to untreated control and linked to the addition of glycerophosphate [63]. Currently Carbamol-L- aspartate was identified only during osteogenesis (Figure- 3.8J), this may indicate its link to orthophosphate either during glycerolipid metabolism (KEGG REACTION: R00841), glutamine metabolism (KEGG REACTION: R00253) or the citrate cycle (KEGG ENZYME: 2.3.3.8).

3.5.4 Candidates arisen from the volatolomics footprinting

Our analysis has identified a set of compounds detectable in the headspace of live mMSC cultures under differentiation conditions. Although not much is known about the association between ethanol, decanes and cyclohexanol during mesenchymal stem cells differentiation, a link between alcohol and osteoporosis has been reported before [150]. Alcohol is also reported to reduce human osteoblast proliferation in vitro [151]. However, testing the effect of ethanol consumption on osteogenic markers showed no significant effect on osteocalcin level after 24 days of human mesenchymal stem cell osteogenic differentiation [152]. In our study, **ethanol** production seemed to be reduced during osteogenic/adipogenic treatment compared to the non-treated condition.

Cyclohexanol on the other hand was reduced only during ('OS') treatment compared to untreated and ('AD') treated conditions. However, the clear understanding of the biological link between this compound and MSC has not reported yet.

Decanes: Similar reduction during ('OS') treatment was noted with tridecane, tetradecane and dodecane, however with decanes an interesting gradual elevation toward terminal ('AD') differentiation was identified. Interestingly,

alkanes were previously extracted from lipid components in fresh water [153]. As decane is an alkane hydrocarbon, this observation correlates with our finding and may explain the elevation of alkane during adipogenic differentiation of mMSCs.

Azetidine: Our GC-MS analysis highlighted compounds that may play an important role in bone metabolism. For instance, (AZ) is an organic compound that does not occur naturally in nature, however, its derivative L-Azetidine-2-carboxylic acid (AZC) is a naturally occurring proline analogue. Li and coworkers reported that AZC binding to proline increased uncoupled 2-Oxoglutarate and consequently affected proline hydroxylation [154] observed in reduction of collagen synthesis in rat with liver cirrhosis [155]. In our analysis, AZ was significantly decreased during OS differentiation which might be due to the presence of ascorbic acid that catalyzes an uncoupled 2-oxoglutarate decarboxylation to increase proline hydroxylation, and substantially increased during ('AD') differentiation where no proline hydroxylation occurs. Yet, the mechanism by which AZ was produced in cell culture has not yet been reported.

D-arabinose: It is known that during carbohydrates metabolism D-arabinose generates some intermediate metabolites entering the pathway in mammals [156]. Through reverse reactions, D-arabinose is isomerized to form D-Ribulose (KEGG REACTION: R01577), that catalysed to D-Arabitol (KEGG REACTION: R07134) then to D-Xylulose (KEGG REACTION: R05604). The latter is catalysed to Xylitol (KGG REACTION: R01896). During our analysis and in contrast with ('OS') treated condition, D-arabinose showed remarkable increase at day 10 and 15 during adipogenic treatment compared to the

untreated samples (Figure- 3.9E). This observation can be linked to the report that this compound showed little or no effect on bone healing of guinea pigs on the absence of ascorbic acid [157]. Although insulin facilitates the cellular penetration of some sugars, it dramatically reduced the cellular absorption of others such as D-arabinose [158, 159]. This could explain the significant elevation of this sugar during ('AD') treatment that contains insulin.

2-Chloroethanol (CE) is generated during Chloroalkane and chloroalkene metabolism where degradation of 1,2-Dichloroethane (DCE) proceeds via 2-chloroethanol, chloroacetaldehyde and chloroacetate to glycolate (KEGG PATHWAY: map00625). Although the exact source of DCE is still unclear, interaction of amino acids (Glycine, L-alanine, L-valine, L-serine, and L-proline) with CE has been reported before [160]. In our experiments, cell culture medium was provided with the previously mentioned amino acids, which may interact with CE. Our GC-MS analysis showed a significant reduction in CE during ('OS') treatment, which may correspond with the hydroxylation of proline during collagen synthesis; however an increase of CE throughout ('AD') differentiation was detected. Interestingly, the peak intensity of L-alanine (LC-MS data) during ('OS') differentiation was reduced compared to untreated control ($P < 0.0001$) while no significant changes was observed between ('AD') treated and untreated control data not shown.

Although not much is known about the association of 4-Methylquinoline with cell culture, our data reported its notable increase during ('AD') treatment. This might point to a link with insulin in ('AD') medium, since Methylquinoline (or Lepidine) has been shown to have hypoglycaemic ability

potential through activation pancreatic insulin secretion in diabetic rat [161], therefore warranting further investigation.

1,2,4,5-Tetrazines a gradual increase in 1,2,4,5 Tetrazine derivative was noticed during ('AD') treatment, with significant increase toward day 15 compared to untreated control and OS treated. Thus, the biological understanding of its association with MSCs and their differentiated forms need to be investigated.

Dimethylamine (DMA) is an organic compound found in animals and plants [162], however inhibition of DMA formation by ascorbic acid has been reported before as attempt to use ascorbic acid in the curing of meat to reduce carcinogenic nitrosamines in food [163]. This correlates with our observation where DMA was reduced during OS treatment, which contains ascorbic acid, compared with untreated and ('AD') conditions at day 5 and 10. Also, during ('OS') treatment a slight increase was noted at day 10 ($P < 0.05$), which could be due to its production during arginine and proline metabolism as shown below:

(NG,NG-Dimethyl-L-arginine + H₂O $\rightleftharpoons$ Dimethylamine + L-Citrulline)
(KEGG REACTION:R02513).

The parallel analysis of metabolic and volatolomic footprints of mMSCs non destructively and comprehensively enhanced the sensitivity of metabolic analysis. LC-MS is a powerful tool for the quantification of small molecules, as well as the identification of known and unknown metabolites in biological samples. Recently, LC-MS-based approaches have been widely used for the quantitative analysis of extracellular metabolites and nutrients [95] [128], though only rare study has demonstrated its power in characterization of

mMSC footprinting during ('OS') differentiation non destructively [63]. In this study, metabolites increased during ('OS') induction have been identified and linked to TCA cycle and orthophosphate pathways. Moreover, Munoz and coworkers have demonstrated the potential of GC-MS to explore glucose metabolism in human mesenchymal stem cells (hMSCs) treated for osteogenic differentiation [71]. Also, clinical identification of bone metabolic markers by GC-MS has been also reported to identify metabolomic changes in serum and showed an increase in lipid profile associated with osteoporosis [164]. Recently, volatolomic analysis to evaluate the effects of dexamethasone and BMP-2 on human Umbilical cord blood (UCB) MSCs during osteogenesis was achieved using GC-MS [165]. Although metabolic profiles have been described in these earlier studies, they relied on the analysis of cellular extracts, which is a destructive endpoint method. Therefore, the real time analysis of stem cells during their proliferation and differentiation could be investigated as a non-destructive improvement. Our analysis demonstrated the non-invasive metabolomic/volatolomic profiling of mMSC osteogenic and adipogenic treatment, and revealed significant changes at different time points of differentiation. However, real time volatolomic analysis was challenging and need further optimization due to the difficulties in including internal controls between the vials during the analysis and maintaining metabolic activity time point. Lee has demonstrated a non invasive method to analyse hMSCs volatile during adipogenic differentiation by insertion SPME fibres into the culture flasks and incubate them for days before the analysis [166]. Though his method revealed to the detection of fatty acid methyl esters (FAMES), it consumed two SPME fibres to analyse one flask headspace.

3.6 CONCLUSION

For the first time, extracellular metabolites were sampled and analysed at different time points during live MSC osteogenic and adipogenic induction in vitro, leading to the identification of differentially detected compounds during cellular changes in culture. Our study combined two different analytical methods (LC-MS and GC-MS) used non-destructively with multi- and univariate analyses for the chemical characterisation of MSC differentiation. The mMSC culture samples analysed showed clear metabolomic changes during osteogenesis and adipogenesis, with a particular prominence of volatile and non-volatile compounds linked to polysaccharide and orthophosphate degradation which play an important role in bone extracellular matrix synthesis. Also, compounds link to insulin and lipid accumulation has been identified in samples exposed to the adipogenic treatment. These observations confirm the power of combining LC-MS and GC-MS to screen chemical compounds present in the cellular environment and quantitatively measure cell-based differences non-destructively. Moreover, it is worth mentioned that both techniques are known for their high level of detection (at picomole) [167, 168]. Though, metabolomic differences between two lineages were analysed, our observations are limited to the use of a single cell line as mentioned before in section 2.6. Therefore, metabolomic analysis of multiple donors including mouse and human could confirm our findings and may introduce significant differences. Further targeted analysis of these metabolites will be a key to better deciphering the specific pathways involved in mMSC differentiation. Also, LC-MS and GC-MS technology could be used for future stem cell metabolite profiling, as our results demonstrate the ability to perform these

analyses non-destructively over a differentiation time-course. Finally, these footprinting methods could be combined with standard destructive methods to quantify intracellular metabolites, in order to identify the full range of biochemical pathways involved in processes of stem cell differentiation.

m/z	RT	Formula	PubChem	Putative compounds	Category	U C	O S	A D
80	4.51	C ₂ H ₅ ClO	34	2-Chloroethanol	Alcohol	↓	↓	↑
103	17	C ₁₄ H ₁₀ N ₄	23263	3,6-diphenyl- 1,2,4,5-Tetrazine	Benzene	↓	↓	↑
57	5.90	C ₃ H ₇ N	10422	Azetidine	saturated heterocyclic organic compound	↓	↓	↑
57/ 82	15	C ₆ H ₁₁ OH	7966	Cyclohexanol	Alcohol	↑	↓	↑
73	0.3	C ₅ H ₁₀ O ₅	66308	D-arabibose	Monosaccharide	↑	↓	↑
45	16.7	(CH ₃) ₂ NH	674	Dimethylamine	organic compound	↓	↓	↑
57	11.3	C ₁₂ H ₂₆	8182	Dodecane	Alkane	↓	↓	↑
31/ 45	5.39	C ₂ H ₆ O	702	Ethanol	Alcohol	↑	↓	↓
89	20.8	C ₄ H ₂ Cl ₂ O ₂	5325504	Fumarylchloride	organic compound	↓	↓	↑
143	20.6	C ₁₁ H ₁₁ NS	86082290	4-Methylquinoline	heterocyclic aromatic organic compound	↓	↓	↑
57	10.6	C ₁₄ H ₃₀	12389	Tetradecane	Alkane	↓	↓	↑
57	9.83	C ₁₃ H ₂₈	12388	Tridecane	Alkane	↓	↓	↑

Table 3.2: The significant volatiles that were increased ↑ or decreased ↓ specifically toward day 15 during untreated control ('UC'), ('OS') and ('AD') treatments analysed by one-way ANOVA.

4 CHAPTER-4-

METABOLOMIC ANALYSIS OF INTRACELLULAR AND EXTRACELLULAR CHANGES DURING HUMAN MESENCHYMAL STEM CELL OSTEOGENESIS

4.1 ABSTRACT:

The skeleton is a metabolically active organ undergoing continuous remodelling initiated by mesenchymal progenitor cells present in bone and bone marrow. Under certain conditions this metabolic balance shifts to increase resorption and consequently disrupts bone microarchitecture. While a number of genes and molecular pathways have been identified as key regulators for the osteogenic process, metabolomic approaches are still relatively new in this field and could develop our understanding of the biochemical factors influencing bone formation. In this study, combined intra and extracellular metabolic profiling was applied to the analysis of human mesenchymal stem cells (hMSCs) undergoing osteogenic differentiation in vitro. Using a combination of univariate and multivariate analyses, concentration changes in metabolites and nutrients were monitored over 10 days of osteogenic treatment. A subset of compounds was found to be differentially detected in treated cells, suggesting essential link to metabolic pathways involved in bone formation, and highlighting interesting metabolite candidates as potential biomarkers for bone formation.

4.2 INTRODUCTION

The health of our bones is involved to our health in general and failure to protect our bone can result in severe bone loss with the result being osteoporosis and consequently bone fracture. As bone consists of minerals and organic component (collagen), bone strength products contain variety of ingredients to support its strength [169]. Bone strength is determined by integrating bone mass (BM) that has been used to predict fractures [170] and bone quality. The quality of bone is characterized by its microarchitecture, turnover rate, micro-damage accumulation, degree of calcification and bone matrix protein properties [171, 172]. Clinically, measurement of metabolic markers is the most successful element for the evaluation of bone turnover and bone matrix proteins and consequently understanding the pathological conditions associated with bone [173]. Established bone monitoring systems are invasive and localized such as bone biopsy [174] and failed to accurately identify metabolic status of the bone such as measurement of bone mineral density (BMD) [175]. Thus, bone metabolic markers have become a crucial clinical test for osteoporosis management [176, 177]. Metabolic footprinting is a comprehensive profiling system of chemical molecules secreted or excreted from living cells into their microenvironment that can give a rapid snapshot of the cell physiology [87, 88]. Therefore, it presents an attractive tool for monitoring fluctuations in cell metabolism. The metabolomics approaches in osteoporosis research have been instigated for prediction, diagnosis, and therapeutic applications [178]. Recent clinical bone metabolic assays particularly focus on the measurement of urine/blood levels of the compounds affecting bone metabolism such as calcium [179], however calcium deficiency

is not always associated with bone loss [180]. Since biochemical synthesis of bone is a complex mechanism [181], metabolomic approach analysing the possible pathways associated with bone formation is extremely important.

LC-MS is a powerful tool for the quantification of small molecules, as well as the identification of known and unknown metabolites in biological samples. Recently, LC-MS-based approaches have been widely used for the quantitative analysis of extracellular metabolites and nutrients [95]. Clinical identification of bone metabolic markers by GC-MS has been also reported to identify metabolomic changes in serum and showed an increase in lipid profile associated with osteoporosis [164].

The maintenance of bone mass and quality is regulated by osteoblasts; this cell type is replenished by the osteogenic differentiation of mesenchymal progenitors [77, 78]. Mesenchymal stem cells (MSCs) are multipotent somatic progenitors able to differentiate into osteogenic, chondrogenic and adipogenic lineages [79]. Their capacities to generate new osteoblasts, the bone-forming cell type, and the possibility of harvesting them from bone marrow collections have made them a promising candidate for therapeutic approaches to skeletal repair [80]. Numerous *in vitro* studies have demonstrated the effect of osteoinductive treatment combining dexamethasone, ascorbic acid, and beta-glycerophosphate (β GP) on MSC osteogenesis. Dexamethasone induces osteogenesis *in vitro* through activating WNT/ β -catenin signalling-dependent *Runx2* expression [81], whereas ascorbic acid increases collagen type 1 secretion [82], [83]. β GP provides inorganic phosphate (Pi) that facilitates intracellular signalling molecules [84], [85], [86].

In chapter 2 and 3, characterization of mMSC during osteogenic differentiation was successfully achieved by analysing not only the metabolites released into the culture medium by the cells but also their accumulated volatiles in surrounded headspace. Pools of metabolites were identified and linked to particular biological pathways that may play a crucial role during the formation of bone extracellular matrix. In order to analyse whether osteogenic differentiation of human mesenchymal stem cells could be characterised by distinct changes in metabolic profiles, relating to specific metabolic pathways involved in MSC differentiation, this chapter applied combined fingerprinting and footprinting analyses using LC-MS, in order to identify intracellular and extracellular metabolic changes during osteogenic treatment. The compounds identified by LC-MS-based finger/footprinting were evaluated for their potential biological relevance. This study highlighted the potential of finger- and footprinting approaches for the quantitative time-point analysis of key molecular changes occurring during MSC osteogenesis.

4.3 MATERIALS AND METHODS

Materials were purchased from Thermo Fisher Scientific (Paisley, UK) unless otherwise stated.

4.3.1 Cell culture and differentiation assay

Immortalized human bone marrow-derived mesenchymal stem cells (MSCs) [182] were seeded at density of 3×10^4 cells/ml in standard MSC medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10 % (v/v) FCS, 1 % (v/v) L-glutamine, 1 % (v/v) non-essential amino acids and 1 % (v/v) antibiotics, and incubated at 37 °C in 5 % CO₂. Confluent cells treated with

osteogenic medium consisting of standard MSC medium, 100 nM dexamethazone (Sigma-Aldrich, Gillingham, UK), 0.05 mM L-ascorbic acid 2-phosphate (Acros Organics, Geel, Belgium) and 10 mM beta glycerophosphate (β GP, Sigma-Aldrich), and treated for 21 days with medium changes every 2 days. Cells maintained in standard MSC medium were used as a control medium sample.

Cells metabolic activity was tested at days 7, 14 and 21 using Presto Blue assay kit (Invitrogen PrestoBlue Cell Viability). Medium were aspirated and 100 μ l of 10 % (v/v) presto blue reagent in HBSS solution was added into each well and incubated for 50 mins at 37 °C, 5 % CO₂. Presto Blue were then transferred into a new 96 well plate and fluorescent excitation and emission were measured at 560 nm and 590 nm wavelength respectively using infinite 200 plate reader and Tecan-i-control software. Then, cells were washed with PBS three times; followed by the addition of sterile distilled water (SDW). A DNA standard curve generated with DNA (Quant-iT™ PicoGreen® ds DNA assay Kit, Life Technologies) was used to determine the concentration of DNA in each sample. Therefore, freezing and thawing cells was performed three times to insure nuclear rupture and DNA release. 100 μ l of λ DNA standard in buffer was added into each well and incubated for five mins at RT protected from light. Reagent was then transferred into 96 wells plate flat bottom and fluorescent intensity was measured at 480nm excitation and 520 nm emission. Calcium deposits were analysed using Alizarin Red staining. At the stated time point, cells from 4 replicates were trypsinised and pellets were smeared onto glass slides, air dried, fixed with 100 % methanol and washed with distilled water. Slides were incubated in 1 % (w/v) Alizarin Red staining solution

(Sigma-Aldrich) for 10 min at room temperature, and washed with distilled water until all excess stain had been removed. Mineral deposits were then observed using a Nikon Eclipse Ni90 light microscope. In parallel, Alkaline phosphatase (ALP) activity was measured in the remaining two culture replicates using 100 µl SIGMA FAST p-Nitrophenyl Phosphate reagent (Sigma-Aldrich) for 20 min at 37 °C, 5% CO₂. 100 µl from each sample transferred to a 96 well plate were analysed in triplicate using an Infinite 200 plate reader and Tecan-i-control software to measure absorbance at 405 nm.

4.3.2 Metabolites extraction

4.3.2.1 Intracellular metabolites

At day 0, 14 and 21, medium of the 3 replicates was aspirated and cells were washed by pre-warmed at 37 °C and quenched by 1 ml of precooled methanol at -48°C. While flasks on ice, cells were removed from using a cell scraper and cell solution was transferred into pre-cooled fresh tubes. Extraction was then performed by freeze/thaw (flash freeze in LN₂ for 1min and briefly thaw on ice - vortex for 30s) × 4 times at least. The solution was then centrifuged at max speed at 4 °C for 10 min, transferred to pre-cooled tubes and stored at -80 °C until LC-MS analysis [88].

4.3.2.2 Extracellular metabolites

At day 0, 14 and 21, 1 ml of the medium of 6 replicates in each culture (MSC-conditioned medium) was collected and centrifuged at 10,000 rpm for 5 min. 250 µl of the collected medium were transferred to a new tube for extraction and protein precipitation by adding 750 µl of cold methanol in a ratio of 4:1, then mixing vigorously and incubating at -20 °C for 20 min. After incubation,

extracts were centrifuged at 17,000 rpm at 4 °C for 10 min, transferred to pre-cooled tubes and stored at -80 °C until LC-MS analysis. Samples of fresh standard MSC medium and ('OS') medium were processed in parallel as controls with no cells. All samples were prepared as six biological replicates, and the experiment was run as two successive independent repeats. An equal mixture of all samples was prepared as quality control (QC) for instrument performance assessment [63, 100].

4.3.3 Analytical methodologies

For metabolite footprinting of spent culture media, LC-MS was performed on an Accela system coupled to an Exactive MS (as described in chapter-3). Spectral data was acquired in full scan ion mode (m/z 70-1,400, resolution 50,000) in both positive and negative electrospray ionisation modes. The probe temperature and capillary temperature were maintained at 150 and 275 °C, respectively. The calibration mass range was extended to cover small metabolites by inclusion of low-mass contaminants with the standard Thermo calibration mixture masses (below m/z 1400), $C_2H_6NO_2$ for positive ion electrospray ionisation (PIESI) mode (m/z 76.0393) and $C_3H_5O_3$ for negative ion electrospray ionisation (NIESI) mode (m/z 89.0244). Chromatographic separation was carried out using a ZIC-pHILIC (150 mm $\times$ 4.6 mm, 5 μ m column, Merck Sequant) maintained at 45 °C and a flow rate of 300 μ l/min as previously described in [101]. Briefly, the mobile phase consisted of (A) 20 mM ammonium carbonate in water, (B) 100 % acetonitrile which were eluted with a linear gradient from 80 % B to 5 % B over 15 min, followed by a 2 min linear gradient from 5 % B to 80 % B, and 7 min re-equilibration with 80% B. The injection volume was 10 μ l and samples were maintained at 4 °C.

4.3.4 Metabolites identification process

LC-MS raw data from sample extracts (control medium samples and their blank, OS treated medium samples and their blank and samples prepared QC) were processed with XCMS for untargeted peak-picking [102], and peak matching and annotation of related peaks were carried out using mzMatch [103]. IDEOM with the default parameters was used for noise filtering and putative metabolite identification. Level 1 metabolite identification was performed by matching accurate masses and retention times of authentic standards according to the metabolomics standards initiative [104] [105], but Level 2 putative identification was considered when standards were not available and predicted retention times were employed.

Supervised orthogonal partial least squares discriminant analysis (OPLS-DA) [106] was initially performed by SIMCA-P 13.0.2 version (Umetrics AB, Umea, Sweden) (i) for general visualisation of metabolite differences between control medium samples and their control, and OS treated medium samples and their blanks, (ii) to identify the temporal shift within control and OS treated conditions, and (iii) to observe the differences between control and OS treated medium samples (n=6 in each group). Mass ions for discriminant biomarkers were selected by variable importance in projection values (VIP) where VIP values greater than one were considered as potential biomarkers. In addition to the investigation of VIP scores from OPLS-DA for the discovery of key discriminatory metabolites, univariate analysis was applied as a final feature selection. One-way ANOVA was carried out using Metaboanalyst that is a web server for metabolomic data analysis and interpretation [107], and was used to

evaluate levels of significant difference between control and ('OS') treated media.

To determine which mass ions contributed to the clusters and trends between MSC cultured media and medium only blank samples, group comparisons were performed between day 0, 14 and 21 samples within the OPLS-DA models. Key mass ions for discriminant biomarkers were selected by VIP values higher than one were considered as potential key mass ions. In addition to the multivariate analysis, univariate one-way ANOVA testing was also performed to identify significant compounds consumed and excreted by MSCs (both in untreated control and ('OS') treatments) compared to their medium-only blanks over time. Key mass ions were also selected from the univariate analysis with the false discovery rate $Q \leq 0.05$.

4.4 RESULTS

4.4.1 *In vitro* model of osteogenic differentiation

Osteogenic differentiation of human mesenchymal stem cells (hMSC) were treated with an established osteogenic medium *in vitro* to model the cellular changes occurring upon osteogenic induction (Figure- 4.1), and the cultures were analysed at day 7, 14 and 21 to investigate changes in proliferation capacity, alkaline phosphatase activity (ALP) and mineral content, which are the major hallmarks of osteogenic differentiation [108]. The metabolic capacity/ μg of DNA did not show significant differences within ('OS') treated conditions in comparison with untreated controls, however throughout 21 days the metabolic activity of treated cells significantly decreased (Figure-4.1B). ALP levels increased at early stages of ('OS') induction with significant

elevation toward day 14, followed by a notable decline toward day 21 (Figure-4.1C), in line with previous reports [109] [83]. No mineralisation was detected with standard medium, whilst OS conditions showed mineral deposits that were positively stained at day 14 and strongly at day 21 (Figure-4.1A and D). The proliferation capacity, ALP changes and associated mineral deposits accumulation during osteogenic treatment confirmed hMSC differentiation during the 21-day time course [110] [111].

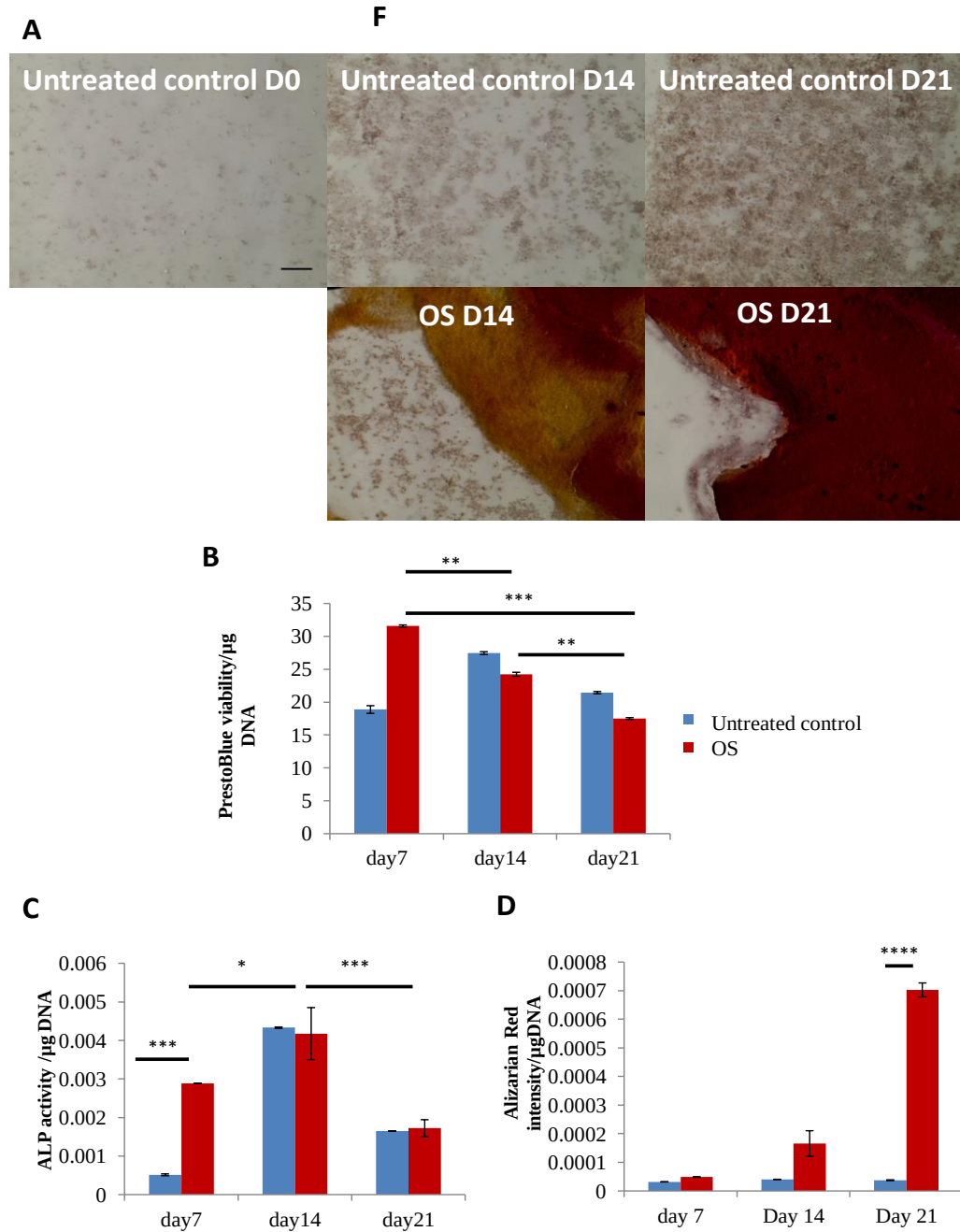


Figure 4.1: Evaluation of osteogenic differentiation in hMSC cultures after 7, 14 and 21 days. (A) Alizarin Red staining of cells treated with either MSC medium untreated control ('UC') (top panel) or ('OS') medium (bottom panel) showing no mineralization in control medium, while under ('OS') treatment calcium deposits were identified at D14 and 21. **(B)** Proliferation capacity of hMSC toward ('OS') was reduced compared to untreated control. **(C)** ALP level showing an increase in response to ('OS') treatment at day7 and 14 followed by significant reduction at day21. **(D)** Quantification of calcium deposit was significantly increased toward day 21 of OS differentiation. **(D)** (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; $n=3$). Scale bar= 200 μ m. All data were normalized to DNA content.

4.4.2 Multivariate analysis of LC-MS finger and footprinting data:

In order to investigate the changes occurring in the hMSC culture medium over the 21 day treatment period, samples from standard medium cultures, ('OS')-treated cultures, and their respective blank media (not exposed to cells) were analysed by LC-MS. LC-MS data from these MSC-conditioned or blank media extracts were transformed into 2D matrix by mzMatch, to introduce an exact mass and retention time for each identified compound. OPLS-DA was then performed to determine the overall biological variation between all conditions, and to identify noticeable trends upon conditioning with MSCs, and over the course of the ('OS') treatment.

OPLS-DA scores plots showed reproducible clustering of biological replicates for each sample group, and highlighted clear differences between the different conditions tested (Figure 4.4.2). For intracellular metabolites analysis, distinct clusters from untreated control D0, untreated control D14 and D21 from ('OS') treated hMSC with R^2 and Q^2 values of 0.841 and 0.644, respectively (Figure- 4.2A). For extracellular metabolites, the non-conditioned fresh media (blanks) analysed in the absence of MSCs showed distinct clusters from each spent culture medium conditioned in the presence of hMSCs (untreated control and ('OS') medium samples) (Figure. 4.2B). Similarly, samples exposed to osteogenic medium clustered in a different quadrant from the untreated control medium samples in the OPLS-DA scores plot. This multivariate analysis clearly showed that LC-MS-based metabolite footprinting was able to non-invasively detect changes of metabolites according to cell differentiation. Changes in hMSC medium monitored over the 15-day differentiation time-course, showed distinct differences suggesting dynamic changes in the hMSC

culture medium over time in both standard and ('OS') treatment conditions, with datasets at early culture stages (day 0 and 14) showing closer metabolomic proximity than the late time-point (day21).

These resultant scores plots showed cluster patterns directly related to the length of cell culture treatment, suggesting that metabolic footprinting data from hMSC cell culture media contains valuable information regarding changes in extracellular compounds relevant to MSC differentiation.

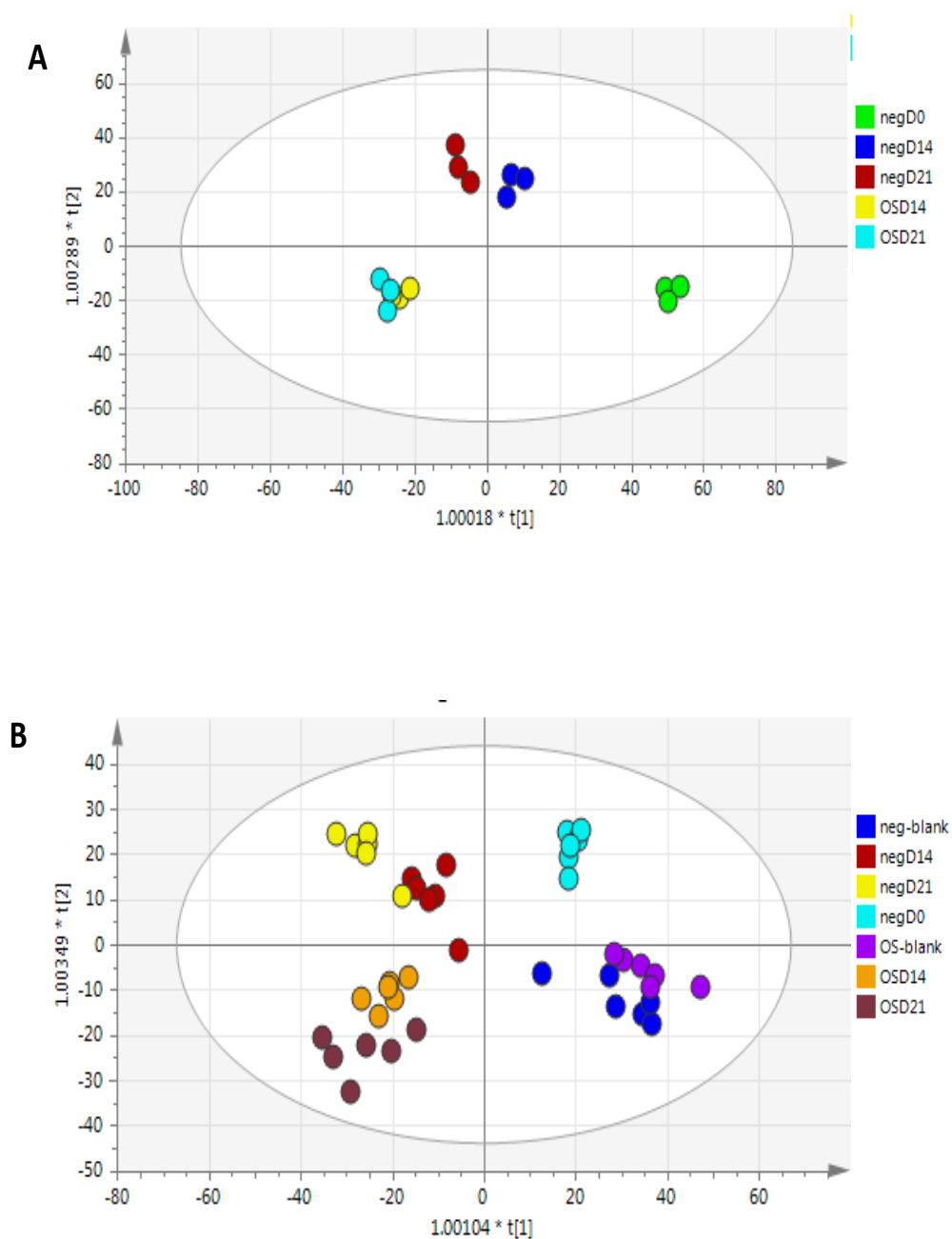


Figure 4.2: OPLS-DA scores plots of MSC medium and cell extract samples from standard and OS-treatment, and their corresponding medium-only blanks, analysed after 0, 14 and 21 days in culture. (A) OPLS-DA scores plot from extracellular metabolites. (B) OPLS-DA scores plot from intracellular metabolites

4.4.3 Metabolic fingerprinting of MSC under osteogenic differentiation

Further investigation was carried out to identify metabolomic differences between intra/extracellular metabolites produced under standard and ('OS') treatment. Out of 308 intracellular compounds initially detected, 31 intracellular metabolites increased specifically during ('OS') differentiation of hMSCs (Table-4.1 and 4.2). A pool of metabolites involved in amino acid metabolism was identified and showed significant increase during ('OS') differentiation compared to the untreated control (Figure.4.3).

L-lysine for instance, showed an increase at D0 followed by significant upregulation during ('OS') treatment compared with untreated control toward (Figure.4.3A). Metabolites such as N6-(L-1,3-dicarboxypropyl)-L-lysine, L-pipecolate, L-2-aminoadipate showed consistent increase during ('OS') treatment compared to the untreated control and around 0 peak intensity at D0 (Figure.4.3B,C and D). Interestingly, L-proline and L-glutamate 5-semialdehyde, N-acetylputrescine and L-threonine showed a sharp increase at D14 during ('OS') treatment followed by slight reduction toward D21 though they were significantly increased compared with the untreated control at both days (Figure.4.3E, F, J, and P). Other amino acids such as L-citrulline, S-adenocyl L-methionine, 4-hydroxy-2-oxoglutarate, L-tyrosine, L-histidine, L-methionine, L-methionine S-oxide and N(pi)-methyl-L-histidine maintained their noticeable increase throughout the treatment time points compared with untreated control (Figure.4.3G, I, L, M, N, O and Q). On the other hand, metabolite such as S-adenocyl L-methionine and L-adrenaline were observed to increase gradually toward D21 compared with untreated control (Figure.3H andK).

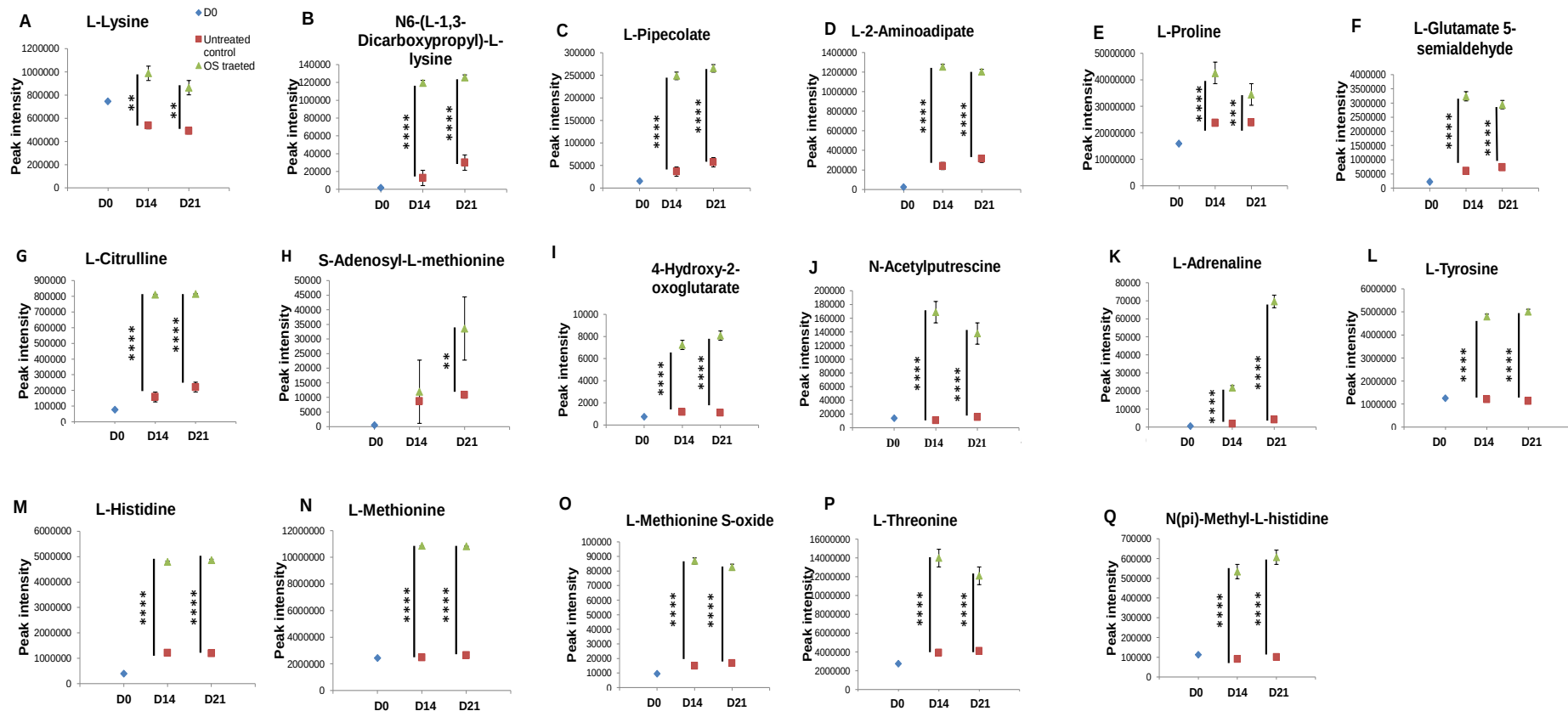


Figure 4.3: Intracellular metabolite changes identified in the culture medium MSC-related metabolites found to increase over time in culture medium in the presence of MSCs under OS treatment (▲) compared to control medium (■), at day 5, 10 and 15 of treatment. (** p<0.0001, *** p<0.001, ** p<0.01; n=3)**

4.4.4 Metabolic footprinting of MSC under osteogenic differentiation

Further investigation was carried out to identify metabolomic differences between extracellular metabolites produced under standard and ('OS') treatment. Out of 312 extracellular compounds initially detected, only 7 extracellular metabolites showed significant increase under ('OS') treatment (Table-4.1 and 4.2). A pool of metabolites was identified and showed significant changes between the treatment conditions (Figure-4.4).

Metabolites such as (R,R)-tartaric acid and vinylacetylglycine were present in medium only blank of both untreated and ('OS') treated conditions with no significant differences within D0, 14 and 21 of untreated control, while a significant increase was detected in the conditioned medium during ('OS') treatment (Figure-4.4A and E). Glycerol on the other hand, was present only during ('OS') treatment but was absent in medium only blanks, D0, 14 and 21 on untreated control. However, a reduction toward D21 of ('OS') was also observed (Figure-4.4B). Other metabolites that presented only during ('OS') treatment and sharply increased toward D21 were hydantoin-5-propionate, D-glucurate and gammaglutamylglutamic acid (Figure-4.4., G and J). 4-imidazole-5-propanoate, 3-(4-hydroxyphenyl)lactate and L-2-aminoadipate and N6-(L-1,3-Dicarboxypropyl)-L-lysine were absent in the medium only blanks and at D0, but gradual increase with untreated control was shown at D14 and continued toward D21. Interestingly, with ('OS') condition a significant increase was detected throughout the treatment time point (Figure-4.4D,F,HandI).

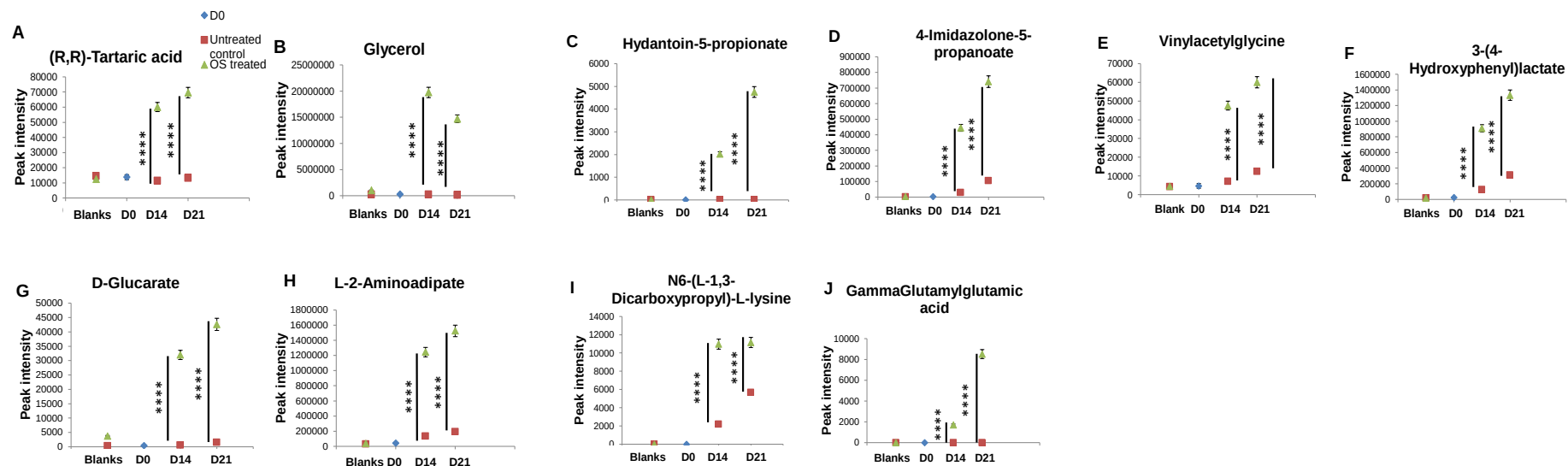


Figure 4.4: Extracellular metabolite changes identified in the culture medium MSC-related metabolites found to increase over time in culture medium in the presence of MSCs under OS treatment (▲) compared to control medium (■), at day 5, 10 and 15 of treatment. (** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$; $n = 6$).**

4.5 DISCUSSION

4.5.1 In vitro model of osteogenic differentiation

Mineralization and ALP level were analysed to confirm osteogenic differentiation of hMSC. Interestingly, our data contradict with the previous study on mouse osteoblast [183] that showed the effect of other medium composition such as ascorbic acid and Dex in addition to β GP on cell proliferation at early days of differentiation. Therefore, our data may indicate that upon the formation of multilayer cell culture over 21 days, cell metabolic activities may be negatively influenced.

4.5.2 Metabolite profiling of intracellular metabolite of MSC under osteogenic differentiation

As mentioned in section 4.2, current clinical tools for monitoring of bone pathology are insufficient to understand the complex mechanism associated with bone metabolism. Therefore, analysis of intra and extracellular chemical molecules secreted from bone forming cells into their microenvironment could introduce new approaches for prediction, diagnosis, and therapeutic potential of osteoporosis.

Interestingly, compounds such as N6-(L-1,3-dicarboxypropyl)-L-lysine (also known as saccharopine), L-pipecolate and L-2-amino adipate are linked to **lysine degradation** (KEGG PATHWAY: MAP00310) but their interaction with stem cell proliferations and differentiations has not been reported before. Lysine is an essential amino acid that cannot be synthesised by mammalian cells and known as a precursor for protein synthesis [184]. Although, Lysine is one of the standard MSC and OS medium constituents, some of the identified

degradation pathway metabolites increased significantly during OS differentiation compared to the untreated MSCs. In mammals, it is known that lysine degradation has two main routes: First, it is combining with α -ketoglutarate followed by the conversion to N6-(L-1,3-dicarboxypropyl)-L-lysine (also known as Saccharopine) (KEGG REACTION: R00715) in liver and kidneys [185] and second is the conversion to L-pipecolate (KEGG REACTION: R00459) in brain contributing to not only the general nitrogen balance but also the controlled conversion of lysine into ketone bodies [186] [187]. Also, aforementioned, that saccharopinuria - excessive amount of saccharopine in urine - is a disease caused by impaired lysine degradation [188]. In addition, L-2-aminoadipate is another intermediate metabolite of biochemical pathway of lysine and its role during skin collagen formation has been reported before [189]. Our observation revealed that intracellular and extracellular elevation of N6-(L-1,3-dicarboxypropyl)-L-lysine and L-2-aminoadipate occurred during OS treatment which might confirm an intracellular role of lysine not only through energy production [190] but also by regulating OS differentiation through collagen synthesis [191]. Therefore, such metabolites could retain the potential for bone metabolism biomarkers.

Another remarkable intracellular metabolite elevation was identified during OS treatment and link to **Arginine and Proline metabolism** such as L-glutamate-5-semialdehyde, L-citrulline, S-adenosyl-L-methionine, 4-hydroxy-2-oxoglutarate and N-acetylputrescine (KEGG PATHWAY: MAP00330). It is known that proline is a non-essential amino acid that is synthesized from glutamic acid and is crucial component of collagen [192] [141]. Arginine plays an important role during bone healing through the production of nitric

oxide (NO) and conversion into L-citrulline [193]. Also, L-citrulline and orthophosphate are formed by catalysing carbamoyl phosphate and ornithine (KEGG REACTION: R01398). Orthophosphate and L-glutamate-5-semialdehyde are formed during proline biosynthesis (KEGG REACTION: R03313), and L-Glutamate-5-semialdehyde is the metabolic precursor for proline biosynthesis that can be converted to or be formed from L-proline (KEGG ENZYME: EC 1.5.99.8 and EC 1.5.1.2). Interestingly, our previous observation (Also refer to Chapter 2) on mouse MSCs OS differentiation revealed to an increase in orthophosphate extracellular level during OS differentiation [63] which may indicate the biological interaction of these amino acids during bone formation. S-Adenosyl-L-methionine is a methyl donor that possesses an anti-inflammatory effect [194] [195] and N-Acetylputrescine is arginine downstream metabolites that upregulated in neural stem cells [196], although their association with bone formation has not been reported before. In contrast, when extracellular metabolites were analysed L-glutamate-5-semialdehyde, L-citrulline, 4-hydroxy-2-oxoglutarate and N-acetylputrescine showed no significant differences with untreated control (data not shown). These findings may suggest that some metabolites which are produced during proline and arginine metabolism play an intracellular role during bone formation. It is also interesting that N-acetylputrescine and S-adenosyl-L-methionine were not identified extracellularly which might be either due to technical limitation (eg. limit of detection) or may indicate their complete degradation inside the cells, further investigation is needed.

LC-MS also identified metabolic profile changes during **amino acid metabolism**, for instance L-adrenaline, L-tyrosine, L-histidine, L-methionine,

L-methionine S-oxide, L-threonine, and N(pi)-methyl-L-histidine were significantly elevated intracellularly and extracellularly during OS treatment compared to the untreated condition. L-adrenaline is a catecholamine derived from the amino acids phenylalanine and tyrosine (KEGG PATHWAY: MAP00350). While, its role in regulating mesenchymal stem cells proliferation and migration toward rat lung injury was reported before [197], our data may indicate an additional intracellular role during bone generation. It also links to the previously mentioned S-adenosyl-L-methionine (KEGG REACTION: R02920) during tyrosine metabolism. L-tyrosine and L-histidine are non-essential amino acid that rapidly metabolized during protein synthesis and vitamin C is a cofactor for their metabolism [198], additionally they react with 2-oxoglutarate (KEGG REACTION: R00734) for L-tyrosine and (KEGG REACTION: R01161) for L-histidine. Also, generate orthophosphate for L-tyrosine (KEGG REACTION: R04730) and for L-histidine (KEGG REACTION: R01991). Interestingly, the breakdown of histidine produce N(pi)-Methyl-L-histidine (KEGG REACTION: R01159) that further breakdown to Orthophosphate (KEGG REACTION: R03286). It is aforementioned that ascorbic acid is an essential compound for collagen synthesis [141], it couples with 2-oxoglutarate to form a cofactor for Proly 3-hydroxylase and Proly 4-hydroxylase enzymes to hydrolyse proline (KEGG: EC 1.14.1.2) and produce hydroxyproline (KEGG: R03219) during collagen synthesis. Therefore, the dramatic increase of L-tyrosine, L-histidine and N(pi)-methyl-L-histidine during OS induction might be explained by the addition of ascorbic acid and β GP in OS medium. L-methionine is a substrate for protein synthesis and an intermediate in transmethylation reactions, serving as the

major methyl group donor in vivo [199]. Also, it regulates human embryonic stem cells iPSC/ESCs differentiation [200]. This may indicate that L-methionine and its oxidation product L-methionine S-oxide (KEGG REACTION: R02025) could be crucial biomarkers for hMSCs OS differentiation. L-threonine is another amino acid regulating human and mouse ESC proliferation [201] and regulates the G1/S phase conversion [202]. Our data may indicate not only its role in hiMSC proliferation but also during bone formation.

4.5.3 Metabolic footprinting of MSC under osteogenic differentiation

Further investigation was carried out to identify metabolomics differences between extracellular metabolites produced under standard and OS treatment. Our LC-MS data identified metabolites that have never, to our knowledge, been linked with OS differentiation of mesenchymal stem cells in the literature. The multidimensional analysis obtained from LC-MS revealed to sustainable elevation of (R,R)-tartaric acid, D-glucarate, glycerol, hydantoin-5-propionate, vinylacetyl glycine, hydroxy-phenyllactate and 4-Imidazolone-5-propanoate in OS spent medium compared to untreated condition.

(R,R)-tartaric acid is an organic acid that involves in glyoxylate and dicarboxylate metabolism (KEGG PATHWAY: map00630), which links to ascorbate and aldarate metabolism (KEGG PATHWAY: map00053) showed a significant increase during osteogenic treatment spent medium compared to the untreated control medium. It was also reported that Tartrate Resistant Acid Phosphatase –isoform 5b (TRAP5b), the enzyme involved in osteopontin/bone sialoprotein dephosphorylation is present in osteoclasts borders to cleave type I collagen into fragments [203].

Interestingly, compounds such as **Glycerol** is linked to the degradation of β GP added to OS medium, which is hydrolysed by the glycerol 3-phosphatase enzyme to glycerol and orthophosphate during glycerolipid metabolism (KEGG REACTION: R00841) (KEGG ENZYME: EC3.1.3.19). In correlation with our previous observation on mouse in chapter 2 [63], higher level of glycerol was consistently detected throughout osteogenic differentiation time-course which may also link to ALP enzyme activity during osteogenic differentiation.

Hydantoin-5-propionate is a metabolite of histidine (KEGG PATHWAY: map00340) was significantly increased extracellularly during osteogenic treatment. It was mentioned previously that histidine was one of the amino acids that upregulated intracellularly, which may lead to the production of hydantoin-5-propionate, this may advocates its biochemical marker potential.

4-Imidazolone-5-propanoate is another histidine metabolite (KEGG PATHWAY: map00340) that increased during OS induction. It is produced from urocanic acid by the enzyme urocanate hydratase [EC:4.2.1.49]. It is also converted to N-forminimo-L-glutamate by the enzyme imidazolonepropionase [EC:3.5.2.7]. N-forminimo-L-glutamate is then converted to Glutamate which links to Proline metabolism through L-1-Pyrroline-5-carboxylate (KEGG PATHWAY: map00330). Intracellular increase of L-Glutamate-5-semialdehyde was noted during our fingerprint analysis. This possibly illustrate the role of histidine during proline metabolism which starts with extracellular conversion of Histidine into 4-Imidazolone-5-propanoate then the conversion of Glutamate into L-Glutamate-5-semialdehyde intracellularly to start proline metabolism.

Vinylacetylglycine is one of acyl glycines which are minor metabolite of fatty acids (HMDB00894). However, its link to bone formation has not been reported yet, further investigation is needed to identify its role during osteogenesis.

Hydroxy-phenyllactate, is a metabolite involved in Phenylalanine and Tyrosine metabolism. It was identified earlier in our metabolomic/volatolomic analysis of mMSC (See section 3.3.5). In our data, LC-MS analysis revealed to an elevation in 3-(4-Hydroxyphenyl)lactate at the late stage during OS treatment in comparison with untreated control, in correlation with above mentioned data this suggests a biological link between the identified metabolite and mitochondrial energy production not only during adipogenesis but also osteogenesis.

In this chapter, for the first time in our knowledge a comprehensive analysis of hMSC intra and extracellular metabolites during ('OS') induction was analysed using LC-MS and show significant changes occurred intra and extracellularly in response to osteogenic inducers. Recently, volatolomic analysis to evaluate the effects of dexamethasone and BMP-2 on human Umbilical cord blood (UCB) MSCs during osteogenesis was achieved using GC-MS [165]. In this study, concentration of serine, threonine, and methionine was significantly increased at early time point D7; while TCA cycle metabolites were significantly reduced with both Dexamethasone and BMP-2 treatments. Similarly, our LC-MS analysis of finger and foot printing identified a pool of amino acids that significantly elevated during ('OS') induction while TCA cycle metabolites were reduced.

Our biological analysis of each identified metabolite highlighted a promising link to pathways that could enhance MSC differentiation such as lysine degradation, proline metabolism and other amino acids that may play a crucial role in skeletal development (figure-4.5). It is also important to analyse the concentration of identified metabolites and test their effect on the formation of skeletal tissue *in vitro* and *in vivo*. Since recent bone markers are mostly focusing on the measurement of calcium level [179], bone loss is not always associated with calcium deficiency [180]. Since bone is a complex organ [181], the metabolomic approach described in this chapter could add new candidates for monitoring bone metabolism clinically.

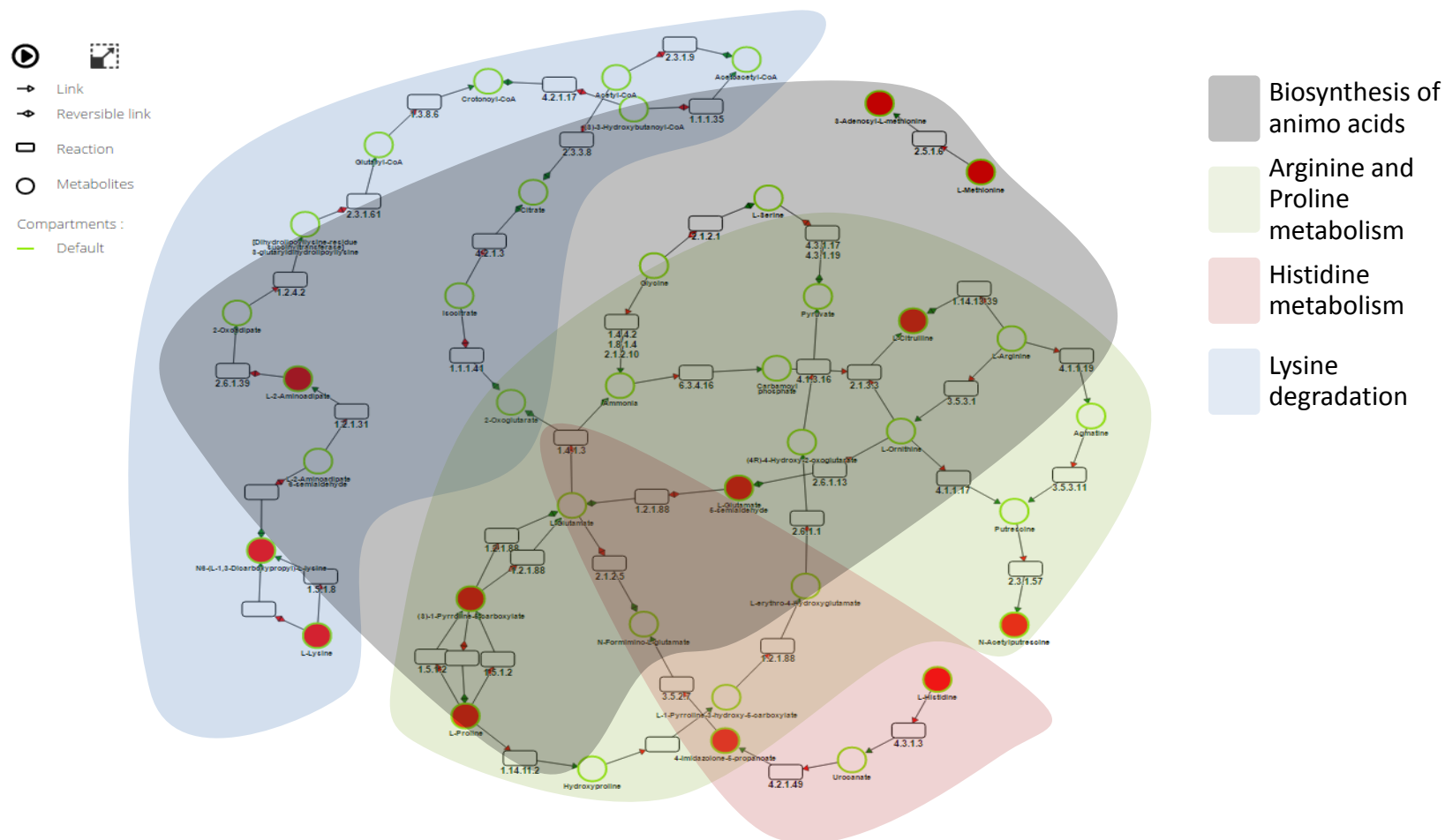


Figure 4.5: Biological pathways highlighted by the compounds differentially detected in MSC finger and footprinting (asterisk). (A) Compounds link to Lysine degradation, Arginine and Proline metabolism and their pathway impact (B).

4.6 CONCLUSION

The therapeutic potential of mesenchymal stem cells for applications in regenerative medicine and drug discovery has warranted a number of investigations targeting conditions such as osteoporosis and other conditions where skeletal tissue is compromised. In our study, and for the first time, LC-MS-based finger and footprinting with multi- and univariate analyses have been used in conjunction for the characterisation of MSC differentiation. OPLS-DA analysis of MSCs in undifferentiated and osteogenically-induced states showed significant and reproducible metabolomic changes, with a particular prominence of compounds linked to lysine degradation and proline metabolism known to play an important role in collagen type I synthesis. However, due to the time limitation only one cell line has been analysed. It is important to consider the variation in differentiation potential within hMSCs sources, for instance bone marrow MSCs yield high osteogenic potential that adipose tissue derived MSCs [204]. Therefore, repeat on other hMSCs sources need to be done to confirm our findings.

Such intra and extracellular chemosignals released by stem cells could enable to identify biological pathways stimulating osteogenesis. Future applications of this study include the possible use of these compounds as of biomarkers, leading to better understanding of the mechanisms of cell differentiation, and possibly screening and diagnostics of bone disorders and drug development and discovery. Furthermore, application of metabolic biomarkers could provide an effective way of developing new means of diagnosis and treatment for bone pathology.

Table 4-1: The most significant metabolites that increased specifically during OS differentiation compared with untreated control analysed by OPLS-DA and one-way ANOVA.

Mass	RT	Formula	Putative compound	PubChem CID	Intracellular FDR	Extracellular FDR
208.06	9.192	C ₇ H ₁₂ O ₇	1-O-methyl-β-D-glucuronate	Not Found	1.71E-04	Not found
182.06	7.768	C ₉ H ₁₀ O ₄	3-(4-Hydroxyphenyl)lactate	9378	6.75E-05	1.63E-06
184.07	10.21	C ₉ H ₁₂ O ₄	3-Methoxy-4-hydroxyphenylethyleneglycol	10805	3.08E-05	Not found
102.07	5.023	C ₅ H ₁₀ O ₂	3-Methylbutanoic acid	10430	2.32E-04	2.42E-05
162.02	9.108	C ₅ H ₆ O ₆	4-Hydroxy-2-oxoglutarate	599	8.92E-05	6.54E-06
156.05	8.743	C ₆ H ₈ N ₂ O ₃	4-Imidazolone-5-propanoate	128	1.44E-05	2.24E-06
230.09	10.42	C ₉ H ₁₄ N ₂ O ₅	Aspartyl-L-proline	170612	1.23E-04	Not found
117.08	9.085	C ₅ H ₁₁ NO ₂	Betaine	247	1.02E-04	Not found

Mass	RT	Formula	Putative compound	PubChem CID	Intracellular FDR	Extracellular FDR
210.04	11.40	C ₆ H ₁₀ O ₈	D-Glucarate	33037	Not found	9.55E-05
92.047	8.939	C ₃ H ₈ O ₃	Glycerol	753	1.90E-04	1.49E-5
285.13	9.681	C ₁₂ H ₁₉ N ₃ O ₅	Glycylprolylhydroxyproline	21252274	4.21E-05	7.66E-04
522.99	11.92	C ₁₀ H ₁₆ N ₅ O ₁₄ P ₃	GTP	6830	3.01E-04	Not found
168.04	8.876	C ₈ H ₈ O ₄	Homogentisate	780	8.22E-05	2.55E-05
161.07	10.36	C ₆ H ₁₁ NO ₄	L-2-Aminoadipate	59066632	1.11E-04	1.38E-05
183.09	8.333	C ₉ H ₁₃ NO ₃	L-Adrenaline	5816	3.41E-05	2.63E-05
175.1	11.17	C ₆ H ₁₃ N ₃ O ₃	L-Citrulline	6992098	2.34E-04	9.65E-04
131.06	10.59	C ₅ H ₉ NO ₃	L-Glutamate 5-semialdehyde	193305	1.15E-06	Low
155.07	11.22	C ₆ H ₉ N ₃ O ₂	L-Histidine	6274	8.91E-05	2.21E-04

Mass	RT	Formula	Putative compound	PubChem CID	Intracellular FDR	Extracellular FDR
146.11	15.67	C ₆ H ₁₄ N ₂ O ₂	L-Lysine	5962	1.21E-03	Low
149.05	9.237	C ₅ H ₁₁ NO ₂ S	L-Methionine	6137	2.01E-04	3.47E-7
165.05	9.773	C ₅ H ₁₁ NO ₃ S	L-Methionine S-oxide	1548907	7.52E-07	1.35E-04
129.08	9.619	C ₆ H ₁₁ NO ₂	L-Pipecolate	439227	1.4E-06	Not found
115.06	9.871	C ₅ H ₉ NO ₂	L-Proline	145742	5.31E-04	Low
119.06	10.53	C ₄ H ₉ NO ₃	L-Threonine	6288	1.4E-04	4.94E-05
204.09	9.581	C ₁₁ H ₁₂ N ₂ O ₂	L-Tryptophan	6305	1.93E-04	6.57E-05
181.07	10.09	C ₉ H ₁₁ NO ₃	L-Tyrosine	6057	1.44E-04	8.50E-06
169.09	9.75	C ₇ H ₁₁ N ₃ O ₂	N(pi)-Methyl-L-histidine	64969	4.67E-05	2.19E-05
335.13	12	C ₁₂ H ₂₁ N ₃ O ₈	N4-(Acetyl-beta-D-glucosaminy)asparagine	123826	3.4E-05	Not found

Mass	RT	Formula	Putative compound	PubChem CID	Intracellular FDR	Extracellular FDR
276.13	10.49	C ₁₁ H ₂₀ N ₂ O ₆	N6-(L-1,3-Dicarboxypropyl)-L-lysine	160556	6.14E04	4.61E-05
150.02	11.22	C ₄ H ₆ O ₆	(R,R)-Tartaric acid	444305	2.24E-03	8.06E-06
398.14	11.19	C ₁₅ H ₂₂ N ₆ O ₅ S	S-Adenosyl-L-methionine	34755	1.72E-04	Not found
113.05	7.701	C ₅ H ₇ NO ₂	(S)-1-Pyrroline-5-carboxylate	440162	1.70E-04	6.23E-05
228.11	10.17	C ₁₀ H ₁₆ N ₂ O ₄	(S)-ATPA	447407	5.44E-5	5.95E-04
143.06	7.696	C ₆ H ₉ NO ₃	Vinylacetylglycine	53477718	Not found	3.99E-06

Table 4-2: The most significant metabolites that increased specifically within untreated control compared with OS treated samples analysed by OPLS-DA and one-way ANOVA.

Mass	RT	Formula	Putative compound	PubChem CID	Intracellular FDR	Extracellular FDR
366.35	4.045	C ₂₄ H ₄₆ O ₂	[FA (24:0)] 15Z-tetracosenoic acid	Not Found	1.33E-04	Not found
647.62	4.26	C ₄₂ H ₈₁ NO ₃	[SP (24:0)] N-(15Z-tetracosenoyl)-sphing-4-enine	Not Found	1.10E-04	Not found
252.09	8.29	C ₁₀ H ₁₂ N ₄ O ₄	Deoxyinosine	65058	1.62E-04	8.01E-06
229.09	10.95	C ₉ H ₁₅ N ₃ O ₂ S	Ergothioneine	5351619	5.10E-04	Not found
283.09	10.02	C ₁₀ H ₁₃ N ₅ O ₅	Guanosine	6802	2.74E-04	Not found
132.05	10.90	C ₄ H ₈ N ₂ O ₃	L-Asparagine	6267	1.01E-04	No difference
146.11	15.67	C ₆ H ₁₄ N ₂ O ₂	L-Lysine	5962	1.15E-06	No difference
115.06	9.871	C ₅ H ₉ NO ₂	L-Proline	145742	1.42E-02	No difference
368.37	4.045	C ₂₄ H ₄₈ O ₂	Tetracosanoic acid	11197	3.71E-04	Not found

Mass	RT	Formula	Putative compound	PubChem CID	Intracellular FDR	Extracellular FDR
242.09	7.36	C ₁₀ H ₁₄ N ₂ O ₅	Thymidine	5789	2.30E-04	7.78E-07
126.04	7.38	C ₅ H ₆ N ₂ O ₂	Thymine	1135	2.03E-03	No difference
152.03	9.24	C ₅ H ₄ N ₄ O ₂	Xanthine	1188	Not found	5.93E-03
208.08	9.07	C ₁₀ H ₁₂ N ₂ O ₃	L-Kynurenine	161166	Not found	3.53E-06
188.12	9.69	C ₈ H ₁₆ N ₂ O ₃	N6-Acetyl-L-lysine	6991978	No difference	1.64E-7
229.04	11.31	C ₁₂ H ₇ NO ₄	Resazurin	11077	Not found	1.45E-02
125.01	11.02	C ₂ H ₇ NO ₃ S	Taurine	1123	9.93E-04	1.18E-06
244.07	8.67	C ₉ H ₁₂ N ₂ O ₆	Uridine	6029	2.68E-04	8.07E-07
131.06	10.59	C ₅ H ₉ NO ₃	L-Glutamate 5-semialdehyde	193305	Low	2.73E-04

5 CHAPTER-5-

A NOVEL METABOLIC COMBINATION FOR ENHANCED CHONDROGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS IN VITRO

5.1 ABSTRACT:

Chondrogenesis and endochondral ossification occur as a result of mesenchymal cell condensation, chondroprogenitor cell differentiation and chondrocyte hypertrophy that lead to skeletal formation and growth in the developing vertebrate. The challenge in developing mesenchymal stem cell-based therapy for cartilage repair is the ability to generate an appropriate cartilage matrix resembling native cartilage tissue. Existing protocols enable the formation of chondrogenic organoids in vitro, however the efficiency of this process is relatively low and matches the limited regeneration observed in vivo, suggesting that additional steps might be required to boost the chondrogenic capacity of MSCs for therapeutic use. Lysyl hydroxylase is involved in a central pathway during collagen II formation, which is regulated by the presence of ascorbic acid, ascorbic acid and 2-oxoglutarate play as a cofactor for lysyl hydroxylase to hydrolyse lysine into hydroxylysine during

collagen synthesis. Aiming to enhance the efficiency of chondrogenic differentiation, the effect of different media supplemented with ascorbic acid and the addition of lysine was investigated using MSCs treated in 2D and 3D culture formats. Addition of lysine with a mild supplementation of ASC increased the accumulation of GAG and collagen fibres formation in both 2D and 3D MSC cultures. The ability of this novel treatment to promote GAG formation from MSCs opens the possibility of testing it in vivo to evaluate its potential for cartilage repair as a new possible therapeutic option.

5.2 INTRODUCTION

Cartilage is a smooth tissue consisting of specialized cells called chondrocytes imbedded into extracellular matrix (ECM). It presents low vascularization and low repair capacity, making cartilage damage due to sudden injury or gradual degeneration a serious health issue [205]. However, the matrix acts as a barrier to isolate the joint from the immune system and its disruption has been linked to osteoarthritis (OA) [206]. Cartilage composed of cellular components (chondrocytes) and non-cellular components (Collagen, proteoglycan, water and other proteins). The organisation and composition of cellular and non-cellular components vary according to the type of cartilage providing the tissue its unique biochemical and mechanical properties [205].

During skeletal development, complex molecular events regulate chondrogenesis including mesenchymal cell condensation with the expression of collagen I, III, V and fibronectin, chondroprogenitor cell differentiation with elevation of collagen II, IX, XI, Sox5, Sox6 and Sox9 expressions, chondrocyte proliferation that express collagen VI followed by terminal differentiation

[207] (Figure -5.1). In some tissue, chondrocytes undergo hypertrophy where collagen X is expressed followed by endochondral ossification where tissue is replaced by bone [208-210]. Chondrocytes shape and size vary depend on zonal position. Generally, chondrocytes range from spheroids at transitional and radial zones to flattened ellipsoid shaped at superficial zone and columnar at calcified zone [205, 211] (Figure5.2) positive control mouse tail. Though the number of chondrocytes is low, they are metabolically active to synthesise the tissue extracellular matrix ECM macromolecules responding to environmental stimuli and soluble mediators, also releasing proteolytic enzymes (metalloproteinases) include collagenase, gelatinase, aggrecanases and stromelysin responsible for matrix degradation. The balance between synthesis and degradation of the matrix is heavily influenced by mechanical loading on the joint [205].

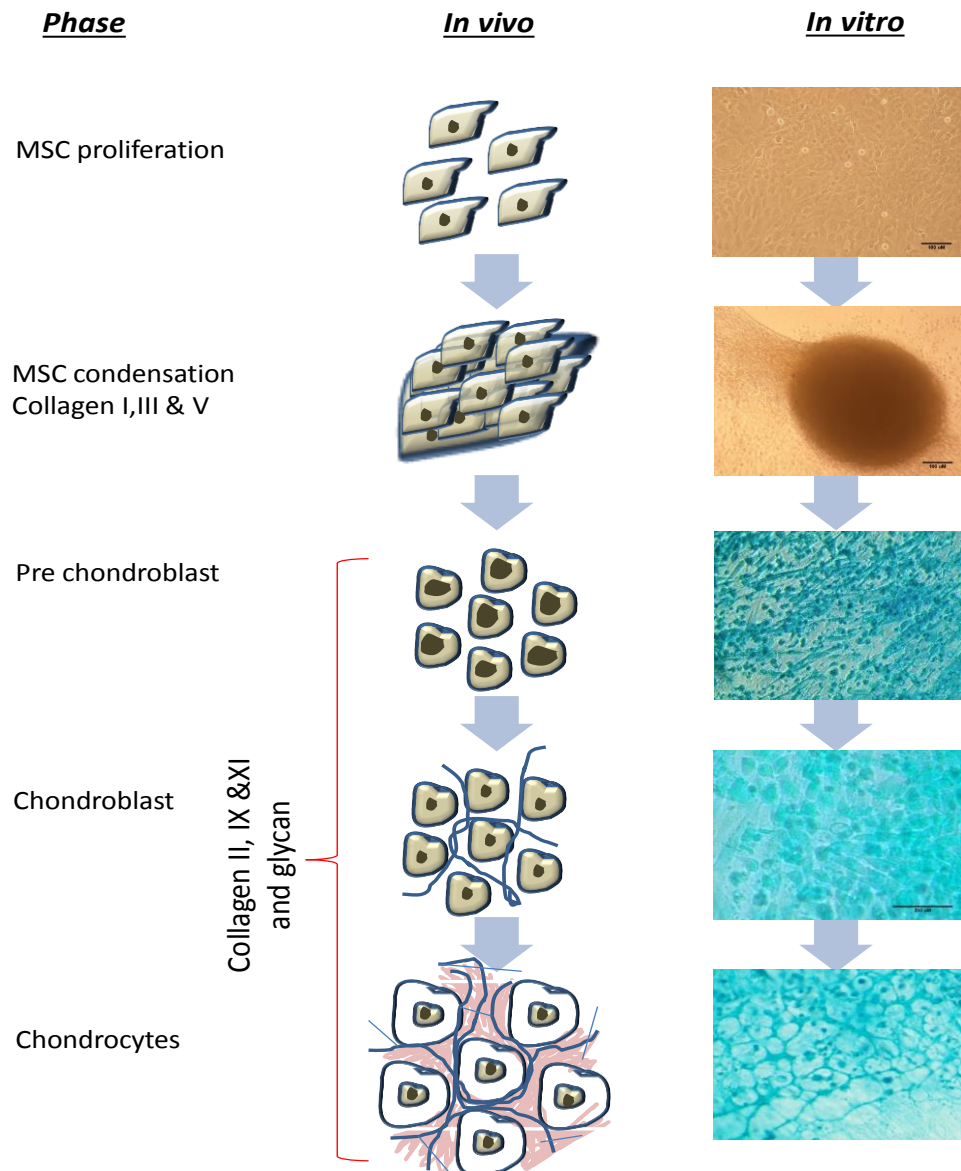


Figure 5.1: Schematic of the chondrogenic differentiation process. The *in vivo* chondrogenesis initiated by MSC condensation, followed by the formation of pre-chondroblast then chondroblast and finally terminal differentiation and the production of glycan and collagen fibers. In 2D cultures, the onset of hMSC condensation was observed after the first week of chondrogenic treatment, followed by the appearance of small round to polyhedral cells, which at the later stage embedded into Alcian blue-positive ECM.

The integrity and stability of cartilage ECM are maintained by collagen fibrils [212]. Although several collagens were identified in cartilage ECM, collagen II, IX and XI remain the most abundant types [212-214]. Collagen consists of triple helical molecules arranged head to tail and side by side in linear arrays. While type II and XI collagen arise in the same fibrils; non fibril collagen type IX resides on the surface of the fibrils to facilitate interaction between collagenous framework and proteoglycan [215, 216]. Our previous data during osteogenic differentiation of human mesenchymal stem cells (hMSC) showed intra/extracellular metabolic changes during proline and lysine degradation (chapter 4). This observation might suggest that the structural units of collagen are synthesised intracellularly, however aggregation into fibres occurs extracellularly.

Proteoglycan (PG) is a protein bounds with glycosaminoglycan chains (GAG) which are composed of disaccharide non sulfated hyaluronan and sulfated units; chondroitin sulfate, dermatan sulfate, heparin sulfate, and keratin sulfate [217]. The crucial functions of proteoglycan in cartilage are providing swelling pressure to absorb mechanical stress and a tissue regulator for soluble molecules [218, 219]. Stockwell, Grassel and Flik described cartilage development, biomechanical and metabolism with details [220-222].

It is known that mesenchymal stem cells (MSCs) are multipotent progenitors present in the bone marrow and other connective tissues that can give rise to new chondrocytes [79]. Existing protocols enable the formation of chondrogenic organoids in vitro with the presence of different chondrogenic inducers (Table- 5.1) [223]. However the efficiency of this process is relatively low and matches limited regeneration observed in vivo [224, 225], suggesting

that additional steps might be required to boost the chondrogenic capacity of MSCs for therapeutic use. Therefore, this study focused on the development of chemically defined culture conditions that can stimulate chondrogenic differentiation of human MSCs in vitro. Based on a recently established approach exploiting metabolomics for stem cell characterisation [63], a pathway that appears central to the MSC transition to chondrocyte have been identified. Owing to the fact that ascorbic acid is an essential compound for collagen synthesis [141], when couples with 2-oxoglutarate to form a cofactor for lysyl hydroxylase [141] enzymes to hydrolyse lysine (KEGG: EC 1.14.11.4) and produce hydroxylysine (KEGG: R03376). Also, in correlation with the previous study that showed maximum hydroxylation of lysine occurs in cartilage [226-228], we hypothesise that addition of Lysine can trigger the cells to produce more cartilage tissue, and thus exert a positive effect on intra-articular cartilage. In this study, a proof-of-concept carried and has confirmed the ability of this candidate to enhance chondrogenesis in cell cultures.

Table 5-1: Chondrogenic differentiation medium and effects of individual components on chondrogenesis. Adapted from (Tang et al, 2015).

	Factors	Functions	Signalling	Dose	Synergy	Remarks
Components of the standard Chondrogenic medium	TGF-β	Promotes chondrogenesis, enhances extracellular matrix production and downregulates collagen I expression	MAPK, Wnt, β -catenin, Smad	10 ng/mL	IGF-I, BMPs, PHTrP	TGF- β 1, β 2 and β 3, have similar unctions
	Insulin	Stimulates collagen synthesis, inhibits hypertrophy	Enhances tyrosine kinase activity of the insulin receptor	1-10 μ g/mL	Transferrin selenium	
	Transferin	Iron-binding		1-30 μ g/mL	Insulin, selenium	
	Selenium	Anti-oxidant and enhances proliferation		5 μ g/mL	Insulin, transferrin	
	Dex	Supports cell viability and delays the appearance of type X collagen	GRs, Scrapie Responsive Gene 1	10^{-7} M	TGF- β	
	Glucose	Major energy source, precursor for the synthesis of glycosaminoglycans	PKC α , p38MAPK, ERK	4.5 g/L		
	Ascorbic acid	Proliferation, DNA synthesis, collagen biosynthesis and reductan	Reduction of iron to its ferrous state	50 μ M	Proline	
	Pyruvate	Energy source, against hydrogen peroxide-induced cytotoxicit		1 mM		
	Proline	Stabilisation of the collagen triple helix , promotes ES cell differentiation	mTOR signalling pathway	40 μ g/mL	Ascorbic acid	

	Factors	Functions	Signalling	Dose	Synergy	Remarks
Selected pro-chondrogenic growth factors according to the literature	BMPs	Enhances cartilage formation	MAPK, Wnt, Smad	10-500 ng/mL	TGF- β	BMP-2, enhances proliferation and ECM, and downregulates Col I expression. BMP-4, accelerates the progression of cartilage differentiation to maturation BMP-6, enhances chondrogenesis in special subpopulation of MSCs, increases proteoglycans BMP-7, inhibits cell proliferation, induces chondrogenic differentiation BMP-9, enhances Sox9, Col2A1 and aggrecan, overcome the inhibitory effect of IL-1. GDF-6(BMP-13), inhibits hypertrophic chondrocyte specific marker, upregulate of proteoglycan
	GDF-5 (BMP-14)	Enhances cell condensation, chondrogenesis, and ECM production	Gap junction intercellular communication, p38MAPK	150 ng/mL	TGF- β , BMP	
	PHTrP	Inhibits the TGF- β -induced hypertrophic differentiation	PTHrP and IHH feedback loop	10 μ M	TGF- β	PHTrP1-34
	FGF	Regulates proliferation, and increases cartilaginous ECM production	ERK	10 ng/mL FGF-2 10 ⁻⁹ M FGF-18	TGF- β	FGF-2, increases proliferation, and proteoglycan production FGF-18, inhibits cell proliferation and induces chondrogenic differentiation

5.3 MATERIALS AND METHODS

Materials were purchased from Life Technologies (Paisley, UK) unless otherwise stated.

5.3.1 Cell culture and differentiation in 2D and 3D conditions

For 2D culture condition, immortalized human bone marrow-derived mesenchymal stem cells (hMSCs) [182] were seeded at density of 3×10^4 cells/ml in 24 wells plates with coverslips initially aimed to allow for immunostaining by mounting the coverslips on slides in standard MSC medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10 % (v/v) FCS, 1% (v/v) L-glutamine, 1 % (v/v) non-essential amino acids and 1 % (v/v) antibiotics, and incubated at 37 °C in 5 % CO₂. Confluent cells treated with different chondrogenic medium (Table-5.2). Standard chondrogenic medium containing (4.5 g/L) DMEM medium without serum whilst media containing serum has been shown to be detrimental to chondrogenic differentiation of hMSCs [229] and containing 1 % (v/v) L-glutamine, 1 % (v/v) non-essential amino acids and 1 % (v/v) antibiotics, dexamethasone (0.1 µM), ascorbic acid phosphate (ASC) (50 µM, and 1mM), sodium pyruvate (1 mM), L-proline (40 µg/ml), TGF-β1 (10 ng/ml) and Insulin-Transferrin-Selenium Supplement (ITS+) was also included. Cell maintained in standard MSC medium were used as a control medium. For 3D culture condition, cell suspension containing 1×10^6 cells /ml was made in previously mentioned CM compositions (except controls which were seeded in SC), and 1 ml was added to each tube, and centrifuged at 500 g in to form a cell pellet. After 24 hours the medium was replaced with 1 ml of each treatment condition. Culture

medium was replaced three times a week throughout the differentiation period a similar protocol to that previously described [230].

Table 5-2: The composition of different chondrogenic treatments used in 2D and 3D.

Medium	Composition
ASC/-PRO/+LYS	4.5 g/L DMEM medium without serum containing 1 mM L-glutamine, 1 % non-essential amino acids and 10 % antibiotics, dexamethasone (0.1 μ M), ascorbic acid phosphate (50 μM, 1mM, 10mM and 50mM) , sodium pyruvate (1 mM), L-lysine (40 μg/ml) , TGF- β 1 (10 ng/ml) and Insulin-Transferrin-Selenium Supplement (ITS+).
ASC/-PRO/-LYS	4.5 g/L DMEM medium without serum containing 1 mM L-glutamine, 1 % non-essential amino acids and 10 % antibiotics, dexamethasone (0.1 μ M), ascorbic acid phosphate (50 μM, 1mM, 10,mM and 50mM) , sodium pyruvate (1 mM), TGF- β 1 (10 ng/ml) and Insulin-Transferrin-Selenium Supplement (ITS+).

Medium	Composition
1mM ASC/+PRO/+LYS	4.5 g/L DMEM medium without serum containing 1 mM L-glutamine, 1 % non-essential amino acids and 10 % antibiotics, dexamethasone (0.1 μ M), ascorbic acid phosphate (50 mM), sodium pyruvate (1 mM), L-lysine (40 μg/ml), L-Proline (40 μg/ml) , TGF- β 1 (10 ng/ml) and Insulin-Transferrin-Selenium Supplement (ITS+).
1mM ASC/+PRO/-LYS	4.5 g/L DMEM medium without serum containing 1 mM L-glutamine, 1 % non-essential amino acids and 10 % antibiotics, dexamethasone (0.1 μ M), ascorbic acid phosphate (50 mM), sodium pyruvate (1 mM), L-Proline (40 μg/ml) , TGF- β 1 (10 ng/ml) and Insulin-Transferrin-Selenium Supplement (ITS+).

5.3.2 Quantification of GAG production using Alcian Blue

Alcian Blue (AB) is a dye that binds to glycosaminoglycans and can be used to quantify chondrogenic differentiation [231]. Adapting the protocol previously described by [231], a method to quantify GAG production in the well, without the need for blotting into a membrane, has been developed. The protocol is the same for monolayer cultures and tissue sections (although no quantifications were made for tissue sections). Briefly, cultures were rinsed twice with PBS and fixed in 4% (w/v) paraformaldehyde for at least 12 hours at 4°C. Each well was incubated with 500 µl of 1% (w/v) Alcian Blue 8-GX in 3% HCl (pH 1.0) (SIGMA ALDRICH) at room temperature for 30 minutes, wells were washed with PBS until all unbound dye had been removed (at least four washes). A final 500µl volume of PBS was added to each well to allow cell monolayers to be imaged using EVOS XL Core microscope. The Alcian Blue dye was extracted using 500µl 6 M guanidine HCl for 2 hours on a plate shaker at room temperature. Quantification was achieved using an Infinite 200 micro-plate reader at 650 nm absorption.

5.3.3 Quantification of GAG production using Safranin-O

Safranin-O (SO) is a commonly used histological stain to detect proteoglycans in cartilage sections and has recently been developed into a quantitative assay using a dot blot method [232]. Based on the method described by Penick [232] a method to stain cells and quantify the stain directly in the well, without blotting onto a membrane, has been developed. The protocol is the same for monolayer cultures and tissue sections (although no quantifications were made for tissue sections). Briefly, cultures were rinsed twice with PBS and fixed in

4% (w/v) paraformaldehyde for at least 12 hours at 4°C. 500 µl of Safranin-O staining solution (Merck Millipore) was added to each well and incubated at room temperature for 20 minutes. Wells were washed with PBS until all unbound dye had been removed (at least three washes) to allow cell monolayers to be imaged using EVOX XL Core microscope. The Safranin-O dye was extracted using 10% cetylpyridinium chloride for 20 min on a plate shaker at 37°C. Quantification was achieved by reading the absorption of the extracted dye at 530 nm using an Infinite 200 micro-plate reader.

5.3.4 Quantification of GAG production using 1,9-dimethylmethylene blue (DMMB)

DMMB is a cationic dye which binds to sulphate and carboxylate groups within GAGs, producing a concentration dependent metachromatic change [233]. The protocol described states the volumes for 96 well plates and is applicable to both monolayer culture and 3D pellet culture. After the culture period, wells were washed twice with PBS and 500 µl of SDW was added to each well. Samples were then freeze thawed three times and sulphated GAGs were dissociated from other glycoproteins using a papain digest. 500 µl of 1% (w/v) papain solution in papain buffer (sodium phosphate (0.1 M), cysteine hydrochloride (0.005 M), and EDTA (0.005 M) in 200 ml SDW, pH 6.5) was added to each well and incubated at 60°C for 24 hours. Triplicate 50 µl aliquots of each sample were transferred to a clean 96 well plate and combined with 50 µl of DMMB solution (sodium formate (0.03 M), DMMB (0.046 mM), 5 ml of ethanol and 2 ml of formic acid in 1 litre of SDW). Each well was mixed using a multichannel pipette to re-suspend any precipitate in the solution, before being read at 540 nm absorption using an Infinite 200 micro-plate reader. For

analysis of pellets cultured in 2 ml tubes, the protocol remains the same but the volumes are adjusted to 1 ml and triplicate 100 µl samples were analysed for each pellet.

5.3.5 Collagen fibres detection by Masson Trichrome

Masson Trichrome (MT) is three dyes that are employed selectively staining muscle, collagen fibres, fibrin, and erythrocytes. The principle of trichrome stain is that the smallest molecular size dye stains the less permeable tissues. However, if a large dye molecule is capable in penetrating the tissue, staining will take place in the rate of the smallest molecule. The tissue is initially stained with Biebrich Scarlet (acid dye), which binds to the acidophilic components of the tissue. Then, when differentiated in phosphomolybdic acid, the less porous elements hold on to red colour, as the red colour is drawn out of the collagen and together providing a link for the collagen to be stained by aniline blue. Collagen fibres will be stained blue, nuclei stained black and cytoplasm, muscle and erythrocytes stained red [234, 235].

Sigma Aldrich Trichrome stains (Masson) procedure number HT15 [236] was used in which sections were first treated with Bouin's solution at 56°C for 15 minutes to intensify the final coloration. The reason for Bouin's and acidified staining solutions (aniline blue) is because the acid pH is essential to increase the selectivity for the collagen fibres. Then nuclei stained in iron hematoxylin working solution for 10 min. The sections were then rinsed in running tap water for 5 minutes followed by distilled water. Next, the cytoplasm was stained in Biebrich scarlet-acid fuchsin solution for 5 minutes. The sections were washed again in distilled water and further treated in phosphomolybdic-phosphotungstic acid solution for 5 minutes or until collagen was not red. Both

the sections then were transferred directly (without rinse) to aniline blue solution and stain for 5-10 minutes. All sections were further differentiated in 1% acetic acid solution for 1 minute and then washed in distilled water. Following that, the slides were dehydrated very quickly (gently dipped in and out for few seconds) through 95% methanol, absolute methanol then cleared in xylene and mounted in DPX mounting medium. Finally all sections were examined under the microscope.

5.3.6 Pellets histology

5.3.6.1 Sample Preparation and Wax Embedding

After the culture period, pellets were washed twice with PBS and fixed in 4% (w/v) paraformaldehyde. Prior to wax embedding, only 3D pellets were encased in the centre of agarose plugs (2% agarose in PBS) to ensure samples were retained in the cassette during tissue processing. A Leica TP1020 automatic tissue processor was used to dehydrate and embed samples through the following stages; PBS, 25% (v/v) ethanol, 50% (v/v) ethanol, 75% (v/v) ethanol, 96% (v/v) ethanol, 100% ethanol, two xylene treatments and two treatments in paraffin wax at 63°C. After processing the samples were fully embedded into paraffin wax blocks using a Leica EG1160 tissue embedding station and allowed to harden at room temperature. Sectioning was performed using a Leica RM2165 microtome to produce 10 micron thick sections.

5.3.6.2 Dewaxing and Staining Slides

Sections were dewaxed using two 10 minute histoclear treatments and rehydrated through a series of 2 minute washes as follows: two 100% (v/v) methanol washes, 75% (v/v) methanol, 50% (v/v) methanol, 25% (v/v)

methanol and finally two 5 minute H₂O washes. Slides were then ready for staining with Alcian Blue, Safranin O and Masson Trichrome. Firstly, slides were rinsed under running tap water then immersed into either 1% (w/v) Alcian blue for 30 minutes, Safranin-O acidified or Masson Trichrome as described in the previous section. Slides were then dehydrated in alcohol, cleared with xylene and mounted with DPX. Finally, slides were imaged immediately after staining using an EVOS XL Core microscope.

5.3.7 Statistics

Data are presented as a mean $\pm$ standard deviation. Data were analysed statistically using Two-way Anova test and $p < 0.05$ was considered significant. Data analysis was performed with GraphPad Prism 7.

5.4 RESULTS

5.4.1 In vitro model of chondrogenic differentiation

In order to refine culture conditions enhancing chondrogenesis; a range of treatments involved in the ascorbate/hydroxylysine pathway were tested on human MSCs, and analysed cultures, setting up a comparison between 2D monolayer cultures in plates and conventional 3D micropellet cultures. Samples were collected at days 14, 21 and 30 of treatment to investigate changes in morphology, proteoglycan production and collagen fibre formation using Safranin-O (SO), Alcian Blue (AB), DMMB (1,9-demethylmethylene blue) and Masson Trichrome (MT) staining, respectively.

5.4.1.1 Effect of ASC supplementation on chondrogenic response in 3D micropellets

3D micropellet cultures were initially set up to test the effect of increased ASC concentration (up to 1mM) on hMSC chondrogenesis, in the presence or absence of (40 µg/ml) L-lysine. After 21 days, pellets were sectioned and analysed using AB and SO. A noticeable increase in colour (Red with SO) was observed between standard CD conditions and untreated control (Figure-5.2).

The effect of ASC on chondrogenic differentiation of hMSC showed a potential enhancement of GAGs colour intensity (Figure-5.2) of pellets treated with 1 mM ASC/+PROL/-LYS, 1 mM ASC/-PROL/+LYS and 1 mM ASC/+PROL/+LYS compared to those treated standard CD medium.

With 1mM ASC/-PROL/-LYS, the staining intensity was relatively reduced compared with the other CD treatments, however a sheet of small polyhedral chondrocytes surrounded by lacunae (pericellular halo) was observed attached

to the pellet but not within the pellet itself, similar to those observed in native cartilage (positive control) (Figure-5.4.1.1.). This suggested that chondrogenic differentiation may have happened outside of the aggregates, in cells attached to it and effectively grown in 2D. Consequently, a differentiation experiment was set up to test the chondrogenic response to these media in 2D, with different ASC concentrations with and without L-lysine.

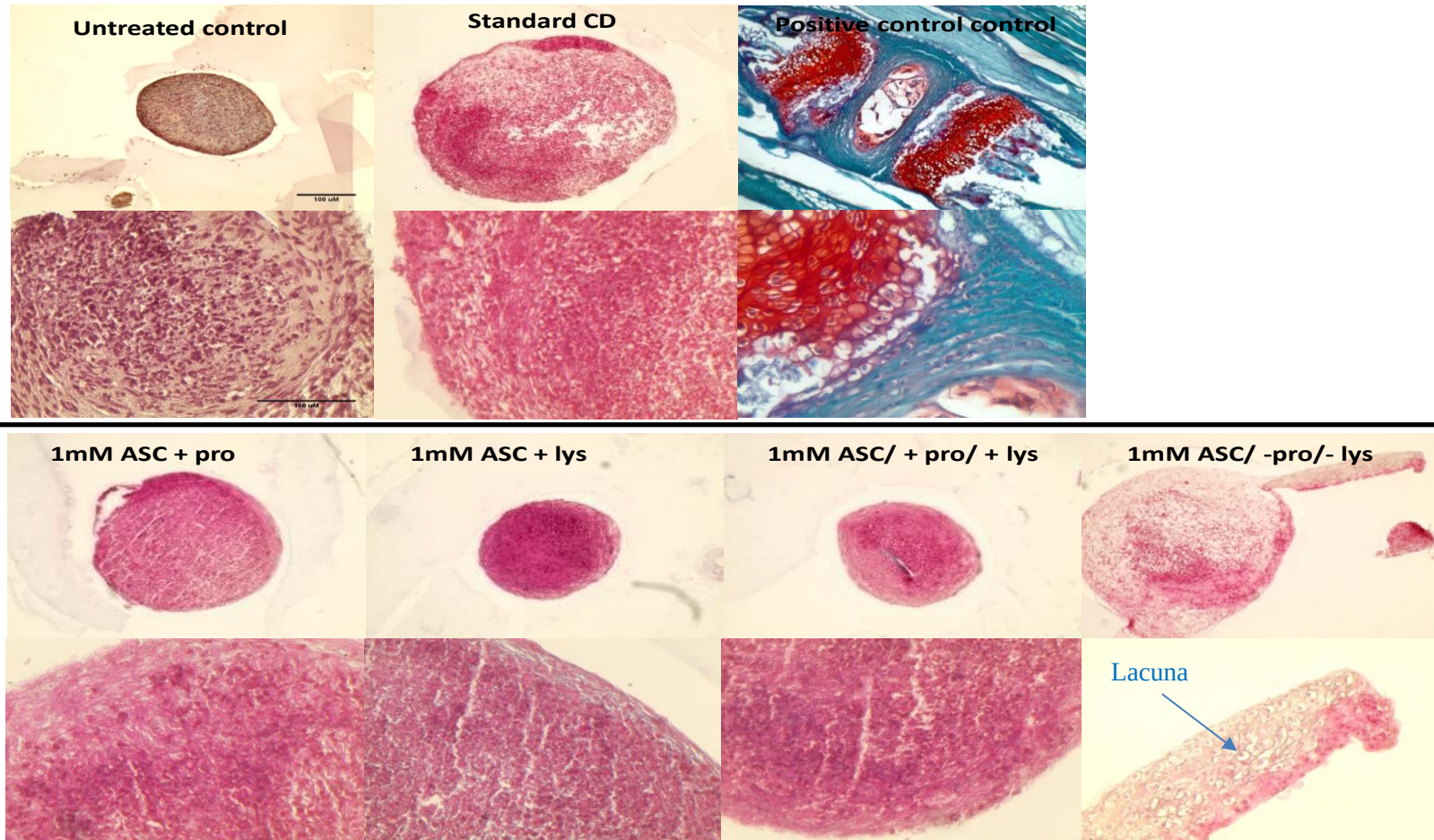


Figure 5.2: Effect of L-lysine and Ascorbic Acid supplementation on hMSC chondrogenic differentiation. hMSCs micropellet cultures treated for 21 days with different CD media. Safranin-O staining of pellet sections for control medium, standard CD, and the positive tissue control (mouse tail section) stained with AB and SO. (B) Safranin-O staining of pellet sections following different treatments with ASC (1mM) with and without the addition of lysine (40µg/ml) and/or proline (40µg/ml). (*p < 0.05; n = 3). Scale bar 100µm.

5.4.1.2 Effect of ASC supplementation on chondrogenic response under 2D conditions

The effect of different concentrations of ASC and the addition of L-lysine were tested under 2D culture and analysed after 21 days using AB. Under these conditions, standard chondrogenic medium did not increase AB staining compared to the untreated control (Figure-5.3B). With 50 μ M ASC/-PROL/+LYS, 10 mM ASC/-PRO/+LYS, 50 μ M ASC/-PROL/-LYS and 10 mM ASC/-PRO/-LYS thin and thick Alcian Blue-stained granular material was noted, however no chondrocytes were identified. Interestingly, small polyhedral chondrocytes surrounded by their lacunae (perinuclear halo) were again observed embedded and surrounded by thin and thick Alcian Blue-stained granular material (GAG) with 1 mM ASC/-PROL/+LYS (see Figure-5.3A). AB absorbance in this medium was found significantly higher compared to all other conditions (Figure-5.3C). Also, upon the increase of ASC concentration to 50mM, cells necrosis was observed, with and without the addition of L-lysine, suggesting a negative effect of higher ASC concentrations. In addition, it was noted that GAG was produced in different layers above and underneath the coverslips in 2D condition.

The second observation in 2D condition was the spontaneous formation of pellets alongside with cell sheets; in all chondrogenic treatments conditions (Figure – 5.3). After a week of culture, pellets produced by this method were seen to have condensed to form almost spherical pellets that did not substantially alter their size throughout the rest of the culture period. In addition, sheets of cells was formed and attached to the coverslip from both sides giving space for extra production of GAG above and underneath the

coverslips. Pellets produced by the tube method also form almost spherical pellets in a few days, but these pellets continue to increase in size throughout the first two weeks of culture before reaching the stage where no further net increase in size is observed. This suggests that the initial stage of culture for plate cultured pellets may consist of more cell differentiation than the tube method. Unexpectedly, our data from 2D condition showed blue cytoplasmic staining in mesenchymal stem cells maintained as untreated control (Figure-5.3B), suggesting some intracellular trapping of the stain in MSCs.

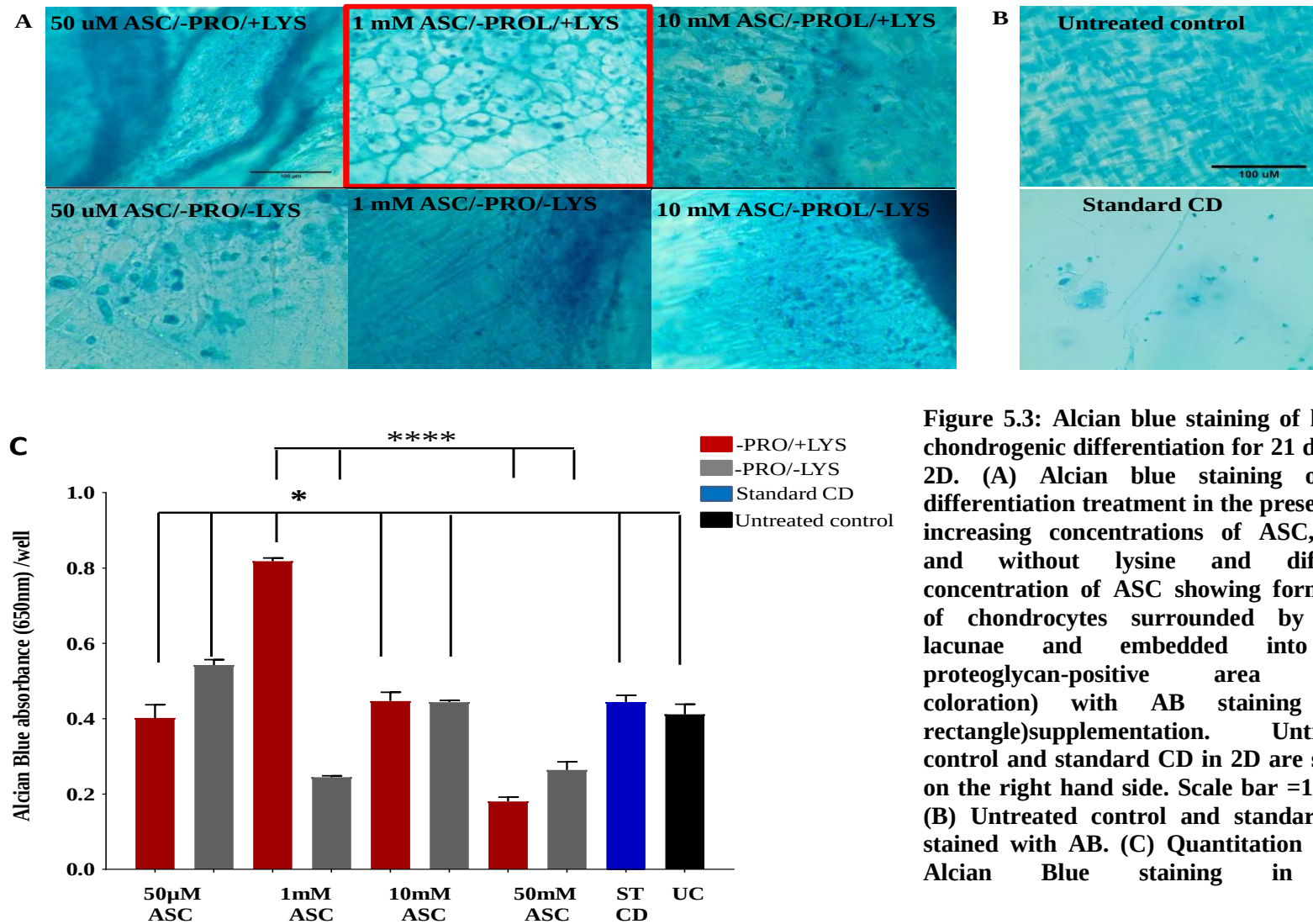


Figure 5.3: Alcian blue staining of hMSC chondrogenic differentiation for 21 days in 2D. (A) Alcian blue staining of 2D differentiation treatment in the presence of increasing concentrations of ASC, with and without lysine and different concentration of ASC showing formation of chondrocytes surrounded by their lacunae and embedded into the proteoglycan-positive area (blue coloration) with AB staining (red rectangle) supplementation. Untreated control and standard CD in 2D are shown on the right hand side. Scale bar =100 μ m. (B) Untreated control and standard CD stained with AB. (C) Quantitation of the Alcian Blue staining in 2D.

5.4.1.3 Histological comparison of chondrogenic aggregates formed in 2D and 3D cultures

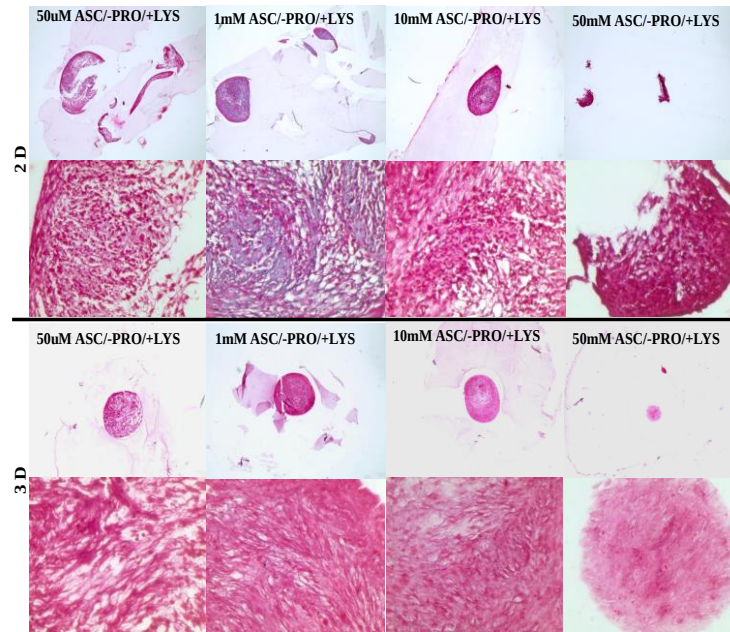
Based on the previous observation, pellets formed after 21 days in 2D and 3D cultures were sectioned and stained using SO and MT to investigate glycan production and collagen fibres generation (Figure 5.4 and 5.6). Pellets formed in 2D were larger in size and more irregular in shape compared to 3D pellets, also they formed associated structures appearing as flat cell sheets. Although the pellets formed spontaneously in 2D conformation showed SO-positive GAG production (Figure- 5.4. A and B) and the formation of collagen fibres shown by MT (Figure- 5.6 A), pellet size was macroscopically diminished when ASC concentration was increased to 50mM with L-lysine, and these disaggregated completely without L-lysine, suggesting a negative effect of high ASC concentration.

Although MT staining is not specific for collagen II, IV or VI, it was observed that with 1mM ASC/-PROL/+LYS collagen fibres appeared darker than in other conditions, in both 2D and 3D cultures. MT-positive fibers seem to be present throughout the pellet with 1 mM and 10 mM ASC with and without L-lysine (Figure-5.6A) compared with the standard CD condition (Figure-5.6B). Moreover, with 1 mM ASC/-PRO/-LYS, multiple well-defined nodules were identified within the pellet and showed strong collagen staining (Figure-5.6A).

Generally, our data showed the potential of increase the concentration of ASC to enhance GAGs production in 2D and 3D. Also, it was observed that under 2D culture condition chondrogenic pellets can be generated. Furthermore, our

data supported our hypothesis in which the addition of Lysine would stimulate the cells to produce cartilage tissue.

A



B

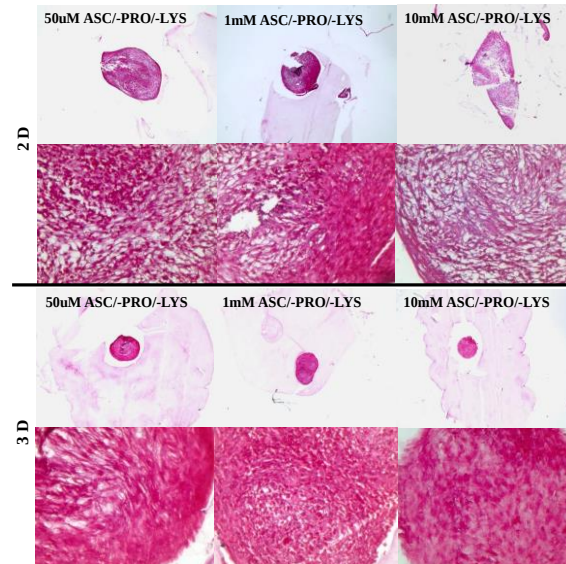


Figure 5.4: Comparison of the hMSC chondrogenic response in 3D and 2D culture after 21 days of treatment with increasing ASC concentrations, in the presence or absence of Lysine supplementation. (A) and (B) Safranin-O staining of pellets formed under 2D and 3D.. Scale bars = 100μM.

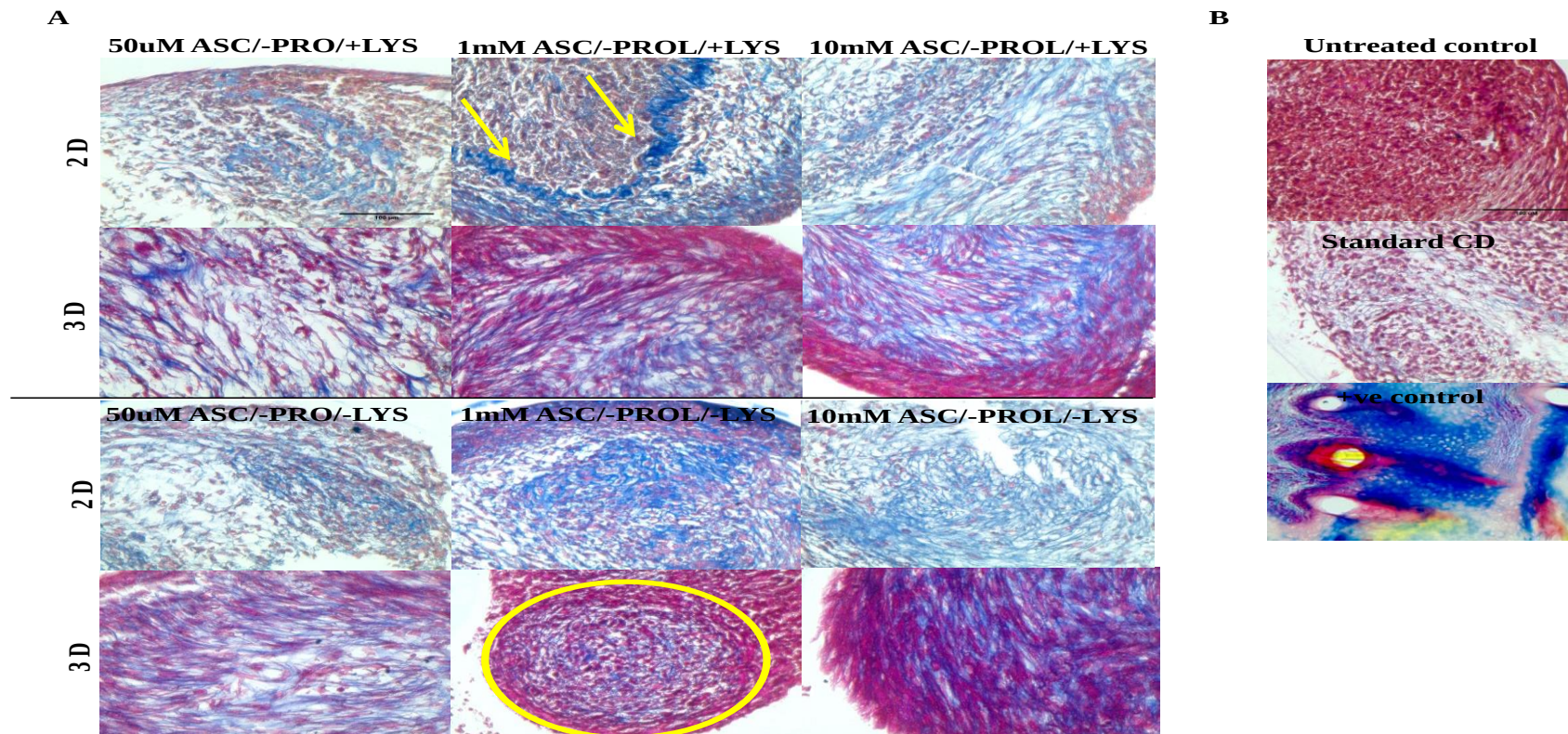


Figure 5.6: Comparison of the hMSC chondrogenic response in 3D and 2D culture after 21 days of treatment with increasing ASC concentrations, in the presence or absence of Lysine supplementation. (A) Masson trichrome staining of pellets formed under 2D and 3D. (B) Masson trichrome staining of untreated control, standard CD and positive control (mouse tail). Scale bars = 100µM. Arrows indicating dense collagen fibres with 1mM ASC + Lysine compared to the other conditions, and circle indicates the presence of nodule.

5.5 DISCUSSION

To enhance hMSC chondrogenic differentiation, a specific treatment combination based on the addition of 2 natural products (L-lysine and ascorbic acid) has been tested and evaluated in 2D and 3D culture conditions. This treatment approach emerged from a previous study (section 4.4.3) which identified a particular pathway suggested to be involved in chondrogenesis and linked to the lysyl hydroxylase pathway. Our previous data showed an increase in L-Lysine during hMSCs ('OS') treatment that contains 0.05 mM ASC compared with the untreated control (Figure- 4.4A), therefore it was hypothesis that supplementing chondrogenic medium with L- Lysine may enhance the formation of cartilage cells.

Two main differences have been identified between the 2D and 3D methods; i) high production of GAG in 2D observed microscopically, however quantification dilemma due to multilayers formation should be optimized to overcome GAG undervaluing in 2D condition ii) large pellets size formed in 2D compared to 3D condition, although the seeding number has varied between 2D and 3D culture system. Pellets produced with 3D (the tubes) method are initially seeded with 1 million cells per pellet (in 1ml of media) whilst pellets produced with 2D (the plate) method were seeded with 30,000 cells per 1ml of media.

Compared to the cell seeding concentrations used in other studies, the tube method seeding density is at the top of the range described and the plate method is in the lower range. The original method described by Johnstone

[230] contained 200,000 cells per pellet (in 1 ml of media) and this concentration has been used in subsequent studies [237], although others have used higher concentrations such as 400,000 cells per pellet (in 1 ml of media) [238]. A study into the optimum pellet size for chondrogenesis with bone marrow derived MSCs [239], determined the optimum initial seeding number of cells was 800,000. However, the next concentration that was tested was 1.6 million cells per pellet and so 800,000 is only an indication of the optimum number [239].

The effect of ASC was discussed before (sections 2.5.1, 3.5.3 and 5.5.2). Interestingly, when the concentration of ASC was increased to 1mM, GAG production in 2D culture format was enhanced with the addition of L-lysine (see Figure. 5.3). The role of ASC in lysine hydroxylation that was mentioned earlier in this paper might explain the enhancement of GAG production upon the addition of lysine but only when ASC concentration was increased to 1mM. But, when ASC concentration was increased to 50mM, cells were negatively affected. Recently, it has been reported that the tumour cells contain large amount of ASC, though the role is not known yet [240]. Therefore, further study on the effect of higher concentration of ASC on mesenchymal stem cells should be addressed.

Though, comparative analyses between 2D and 3D conditions to support chondrogenic differentiation are hardly available, conventional 2D culture condition considered unsuitable for human articular chondrocytes in terms of COL2A and ACAN expression and GAG contents [241]. Therefore, the high seeding density 3D micropellets method is suggested to be the gold standard

for chondrogenic differentiation and favour the maintenance of the chondrocyte phenotype [242, 243].

Our data suggested the addition of L-lysine would significantly enhance chondrogenesis in 2D culture. It was noted that GAG was produced in different layers above and underneath the coverslips in 2D monolayer. This observation may indicate that the use of coverslips in culture wells may represents in vivo condition where low O₂ and nutrients supplements are provided. The second observation in 2D condition was the spontaneous formation of pellets alongside with cell sheets; in all CD treatments conditions.

The effect of L-Lysine has discussed before (Section 4.5.2). In general, our data suggested that the 3D condition is suitable for pellet formation when considering not supplementing the medium with L-proline and L-lysine over 21 days, while 2D is more efficient for chondrogenic differentiation of hMSCs when additional L-lysine is applied.

Moreover, the addition of L-lysine with 1 mM ASC may have enhanced lysine hydroxylation, which has been reported to be relatively high in cartilage tissue and consequently promoted collagen synthesis [226-228, 244, 245]. Also, lysine was reported that it stimulated N-cadherin mediated cell adhesion during chondrogenic differentiation of chick limb mesenchymal stem cells [246]. This may explain the enhancement of chondrogenesis in 2D and 3D through GAG production and collagen fibres production when supplementary lysine was added.

Furthermore, the addition of 1mM ASC with no lysine may enhance proline hydroxylation (that present in MSC medium) (KEGG REACTION: R03219)

during collagen synthesis (Procollagen L-proline + 2-Oxoglutarate + Oxygen
 $\rightleftharpoons$ Procollagen trans-4-hydroxy-L-proline + Succinate + CO₂).

It is well-established that type I collagen also contains 3-hydroxyproline residues [247], and the addition of 1mM ascorbic acid without lysine may enhance collagen I and consequently bone extracellular matrix. Further investigation on the effect of extra L-proline with 1mM ASC on osteogenic differentiation of MSCs is required. Expression of extracellular matrix genes (COL IA1) and mineralization-related genes such as osteocalcin (OC) and osteopontin (OPN), runt-related transcription factor 2 (RUNX2) and bone sialoprotein (IBSP) can be tested [248]. Also, flowcytometer analysis of osteogenic surface markers CD49e, CD140b, CD146 and CD147 can be performed [249].

Unexpectedly, blue cytoplasmic staining of mesenchymal stem cells was seen in untreated control cultures stained with AB suggesting some intracellular trapping of the stain in hMSCs. Interestingly, our metabolomic analysis of hMSCs osteogenic fingerprinting (Chapter 4- Appendices table-1) identified two compounds upregulated with no significant differences between mesenchymal stem cells and ('OS') treated cells and linked to GAG synthesis; N-Acetyl-D-glucosamine and D-Glucuronate, which are two basic units of Chondroitin sulfate (KEGG PATHWAY: map 00532) and heparan sulfate (HS) [250]. These intracellular metabolite upregulations in the untreated control may explain the cytoplasmic absorbance of AB by hMSCs. This may be because Alcian Blue binds to a variety of sulphated glycans with the same sensitivity, despite differences in degree of sulphation [231]. However, histological analysis of GAG contents within the pellets that formed under 2D

and 3D was clearly identified with SO. Both AB and SO are used to determine the expression of cartilage matrix proteoglycan in histological samples [231, 232]. However, application of AB for our 2D was less challenging due to less washing steps required compared to SO.

Therefore, for higher throughput experiments should be considered to confirm the influence of our combined condition on chondrogenic differentiation. These include testing chondrogenic specific surface marker CD10 by flow cytometry and qPCR analysis of extracellular matrix gene expression AGCAN and COL2A [249]. Furthermore, analyse the new treatment's efficacy in vitro, using progenitor cultures as well as tissue explants exposed to increasing doses of the compound, and compared to standard conditions [25]. This will provide efficacy and kinetic information to identify the optimal treatment to be used when in vivo application is considered for cartilage repair.

5.6 CONCLUSION

As part of a previous project analysing (chapter-2, 3 and 4) the differentiation of bone marrow stem cells, we have surveyed changes in metabolites occurring during the differentiation process. This approach identified a particular pathway suggested to be involved in chondrogenesis, and linked to collagen biosynthesis through ASC and lysine interaction within lysyl hydroxylase pathway. The aim of the present study was to exploit the effect of ASC, lysine interaction and evaluate their potential for cartilage tissue formation ex vivo, in order to determine its translational potential as a new therapeutic lead. It can be concluded that a treatment designed to promote this pathway was observed to significantly increase the histological markers of chondrogenesis, suggesting

potential to promote de novo cartilage formation. Two different culture systems were compared; the traditional 3D micropellets method and 2D culture. Both methods were shown to be sensitive to enhancements to chondrogenesis after 21 days; however 2D culture monolayer was proven to be a superior method to 3D pellet chondrogenesis in the production of GAGs and collagen fibres formation at earlier time point D21. Future analysis of the effect of this new treatment on cells, on cartilage tissue, and on joint repair, using complementary in vitro and in vivo models, would now be needed to determine its potential application for cartilage repair.

6 CHAPTER-6-

GENERAL CONCLUSIONS AND PERSPECTIVES.

The work presented here investigated the development of novel characterisation approaches applied to mesenchymal stem cell differentiation using LC-MS and GC-MS based technology. It started with the application of LC-MS metabolomics footprinting to the analysis of mMSCs during osteogenic differentiation in vitro, followed by the parallel identification of significant metabolomics & volatolomics footprinting shifts during the dual differentiation of mMSCs down osteogenic and adipogenic lineages, highlighting potential new biomarkers. Then, concomitant finger- and footprinting analysis of hMSCs were performed during osteogenic differentiation. During the analysis, a compound linked to a pathway associated with chondrogenic differentiation was highlighted and subsequently tested for its potential to enhance hMSCs chondrogenesis in vitro. Therefore, several conclusions can be drawn from this study, not only for use of novel characterisation methods for MSCs differentiation, but also for their potential therapeutic applications, as discussed below.

6.1 NON-DESTRUCTIVE CHARACTERISATION OF mMSCs OSTEOGENIC DIFFERENTIATION USING LC-MS-BASED METABOLITE FOOTPRINTING

In this study, extracellular metabolites were sampled and analysed at different time points during mMSC osteogenic induction in vitro, leading to the

identification of differentially regulated compounds linked to cellular changes in culture.

The therapeutic potential of mesenchymal stem cells for applications in regenerative medicine and drug discovery has warranted a number of investigations targeting conditions such as osteoporosis and other conditions where skeletal tissue is compromised. Studies on osteogenic induction require the development of in-line, non-destructive characterisation methodologies to be able to phenotype MSCs during differentiation and limit the requirement for sacrificial cultures needed for standard cellular and molecular techniques. In this study, for the first time, LC-MS-based footprinting with multi- and univariate analyses have been applied for the non-destructive characterisation of mMSC differentiation through the analysis of culture medium. OPLS-DA analysis from MSCs in undifferentiated and osteogenically-induced states showed significant and reproducible changes demonstrated by OPLS-DA scores plots from metabolic footprints of each condition. The mMSC culture samples analysed showed clear metabolomic changes during osteogenesis, with a particular prominence of compounds linked to cell respiration and the production of orthophosphate, which plays an important role in ALP level during bone differentiation [108]. Unlike recent destructive methods that demonstrated the ability of LC-MS to identify metabolite changes during mesenchymal stem cell osteogenic differentiation [61], our results confirm the power of LC-MS to screen chemical compounds present in the cellular environment and to quantitatively measure cell-based differences in a non-destructive manner. Similar non-destructive approach of LC-MS has been applied to identify Alzheimer biomarkers from culture medium of induced

pluripotent stem cells iPSC during neuron differentiation [251]. Further analysis of these metabolomic changes will be useful to refine the profile of differentiation-related compounds. These techniques may include intracellular metabolite analysis [252], though inappropriate metabolism quenching may lead metabolites loss and biased quantification. To avoid metabolites loss, rapid quenching with cold solvent such as cold methanol prior to quenching with liquid N₂ has been applied for mammalian cells [253].

This LC-MS technology could be combined to stable isotope assisted approaches [254], for future stem cell metabolite profiling to quantify intracellular metabolites involved in specific stages of differentiation and investigate specific pathways identified by the metabolite footprinting analysis in the current study [255-257].

6.2 MONITORING OF mMSCs OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION USING COMBINED LC-MS AND GC-MS.

In this study, extracellular metabolites were sampled and analysed at different time points during MSC osteogenic and adipogenic induction in vitro. Culture medium and headspace volatiles were monitored in parallel with multi- and univariate analyses for the characterisation of MSC differentiation at different time-points. The mMSC culture samples analysed showed clear metabolomic changes during both osteogenesis and adipogenesis, with a particular prominence of volatile and non-volatile compounds linked to polysaccharide and orthophosphate degradation and proline metabolism during osteogenesis, which play an important role in bone extracellular matrix synthesis. Also,

compounds linked to insulin and lipid accumulation were been detected during adipogenesis. To date, volatile analysis applied to MSCs during osteogenic differentiation has relied only on the analysis of cell extracts [159] rather than live cell cultures, whereas volatile analysis of hMSCs during adipogenic differentiation has been reported before [160]. In this study, two SPME fibres inserted in to one flask headspace and kept throughout the differentiation time then analysed by GC-MS which revealed to the identification of some fatty acids. Our approach could provide a promising protocol for analysing MSC volatiles non destructively using only one SPME fibre throughout the analysis. However, GC-MS technical quality control remains a challenge and should be optimized by testing and integration of internal control between the samples.

Future investigation of the volatile and non volatile compounds should be addressed to identify their role in MSCs lineages promotion (please refer to the details in sections-3.5.3 and 3.5.4). For instance, a volatile compound such as Azetidine could possess a particular role in collagen synthesis if injected in vivo that because its derivative L-Azetidine-2-carboxylic acid (AZC) binds to proline and increases uncoupled 2-Oxoglutarate and consequently affected proline hydroxylation. Xylitol is other interesting metabolites that might be tested on both bone and lipid forming cells. Compound such as 4-methylquinoline highlighted a promising effect of insulin of lipid accumulation and should be tested.

6.3 COMBINED INTRA- AND EXTRACELLULAR METABOLOMICS ANALYSIS OF hMSCs OSTEOGENIC DIFFERENTIATION

A combined metabolomics approach was performed to analyse both dynamic intracellular and extracellular metabolites changes occurring in hMSC cells undergoing osteogenic differentiation to capture the broad metabolomics changes and identify differentiation-related compounds. To our knowledge, this study was the first to identify new candidates using this approach. The identification of intracellular and extracellular metabolites detected during bone extracellular matrix formation could enable a more comprehensive understanding the key regulatory pathways, such as those involved in lysine and proline metabolisms as highlighted in our results (see sections 3.5.3 and 4.4.3). Moreover, amino acids that linked to orthophosphate synthesis were identified by this approach as linked to the differentiation treatment (see section 4.5.2 and 4.5.3).

However, our protocols relied on mineralization and ALKP levels to confirm the osteogenic differentiation in MSC cultures, and it will be useful to also monitor the expression of extracellular matrix proteins collagen I and osteocalcin [249] to refine the phenotyping during the differentiation time-course. Furthermore, since some of the identified metabolites could represent new candidate markers alongside the current bone markers for monitoring bone metabolism clinically [171], it will be important to integrate isotope labelling to broad metabolic profiling approach [254] (see more details in sections-4.5.3 and 4.5.4). For instance, (R,R)-Tartaric acid that linked to ascorbate

metabolism, Glycerol is associated with β GP degradation and Hydantoin-5-propionate and 4-Imidazolone-5-propanoate which are histidine metabolites.

6.4 A NOVEL APPROACH IN CHONDROGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

As part of a previous project analysing foot and fingerprinting of the osteogenic differentiation of bone marrow stem cells (see chapters 4), we have surveyed changes in metabolites occurring in MSC cultures during the differentiation process. This approach identified a particular pathway that showed the effect of ASC on proline metabolism and consequently collagen synthesis (see sections 2.5.1, 3.5.3 and 4.5.2) suggested to be involved in chondrogenesis, and linked to collagen biosynthesis through ASC and lysine interaction within lysyl hydroxylase pathway [141]. To evaluate the hypothesis that the combination of ASC with lysine could enhance chondrogenic differentiation of hMSCs, treatments involving these compounds were evaluated in vitro. From the results gathered, it was observed that a treatment designed to promote lysine metabolism pathway could increase the morphological and functional markers of chondrogenesis in vitro, suggesting potential to promote de novo cartilage formation. Different concentrations of ASC were tested on 3D micropellets which has been recommended for chondrogenic differentiation [242, 243], the result from this data suggested chondrogenic potential in 2D monolayer. Therefore, two different culture systems were compared; the traditional 3D micropellets method and 2D monolayer format. Contradicting previous studies that failed to promote

hMSCs in monolayer culture [235, our both methods were shown to be sensitive to enhancements to chondrogenesis after 21 days.

Due to the time limitation, analysis of chondrogenic marker expression could not be added to confirm lineage commitment in addition to the histological stains performed. Therefore, future analysis should be applied to confirm the effect of new treatment combination by immunostaining and qPCR analysis of chondrogenic markers such as AGCAN and COL2A [249].

Future analysis of the new treatment's efficacy in vitro should be extended using other progenitor cultures such as adipose derived mesenchymal stem cells [258] which revealed similar phenotype and differentiated potentials to human bone marrow mesenchymal stem cells. Also, to improve in vitro cartilage tissue expansion and avoid dedifferentiation dilemma [259], our treatment mixture could be tested on cartilage cells in vitro and in vivo using 3D and 2D culture methods.

FUTURE PERSPECTIVES:

It is proposed that the non targeted metabolomics profile that has been identified in this project can be emerged with proteomics and genomics approaches for large scale metabolic understanding that orchestrating biological pathways. Also, future investigations on the metabolites that consumed by MSCs differentiation forms should be performed to identify their role in lineages promoting pathways. Furthermore, extreme analysis of pathways involved in MSC differentiations could bring promising tool to promote tissue regeneration strategies. Finally, repeat the metabolomic analysis

that addressed on this study with other MSCs donor and species need to be done to confirm our findings.

Though LC/GC-MS managed to detect changes that were induced by chemical compounds added to the culture medium, further analysis should be addressed to identify the potential of these technologies to detect minor spontaneous differentiation.

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APPENDECIS: (presented in the attached CD)

- Tables

- Table-1: Metabolites identified during mMSCs osteogenic differentiation using LC-MS (Chapter-2)
- Table-2: Metabolites identified during mMSCs osteogenic/adipogenic differentiations using LC-MS (Chapter-3)
- Table-3: Intracellular metabolites identified during hMSCs osteogenic differentiation using LC-MS (Chapter-4)
- Table-4: Extracellular metabolites identified during hMSCs osteogenic differentiation using LC-MS (Chapter-4)

- Figures

- Heatmap-1: Fold changes of the metabolites identified during mMSCs osteogenic differentiation using LC-MS (Chapter-2)
- Heatmap-2: Fold changes of the metabolites identified during mMSCs osteogenic/adipogenic differentiations using LC-MS (Chapter-3)

- Heatmap-3: Fold changes of the intracellular metabolites identified during hMSCs osteogenic differentiation using LC-MS (Chapter-4)
- Heatmap-1: Fold changes of the extracellular metabolites identified during hMSCs osteogenic differentiation using LC-MS (Chapter-4)