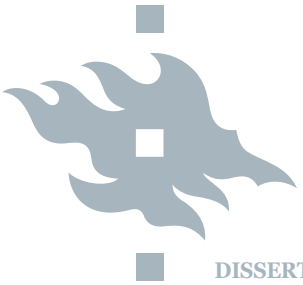

DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

32/2017

FEVEN TIGISTU-SAHLE

Metabolism of Polyunsaturated Fatty Acids in Human Bone Marrow Derived Mesenchymal Stromal Cells

PHYSIOLOGY AND NEUROSCIENCE
DEPARTMENT OF BIOSCIENCES
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI

METABOLISM OF POLYUNSATURATED FATTY ACIDS IN HUMAN BONE MARROW DERIVED MESENCHYMAL STROMAL CELLS

FEVEN TIGISTU-SAHLE

Faculty of Biological and Environmental Sciences
Department of Biosciences
Division of Physiology and Neuroscience

Doctoral School of Health Sciences
Doctoral Program in Integrative Life Sciences
University of Helsinki

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences, University of Helsinki

Auditorium 1041, Biocenter 2, Viikinkaari 5, on 15th May 2017 at 12:00 o'clock.

Helsinki 2017

Supervisors
Docent Reijo Käkelä
Department of Biosciences, University of Helsinki

Ph.D. Saara Laitinen
Research and Development, Finnish Red Cross Blood Service, Helsinki

Thesis Advisory Committee
Docent Eeva-Liisa Eskelinen
Department of Biosciences, University of Helsinki

Professor Jyrki Kukkonen
Department of Veterinary Biosciences, University of Helsinki

Professor Petri Lehenkari
Institute of Biomedicine and Institute of Clinical Medicine, University of Oulu

Reviewed by
Docent Tomas Blom
Department of Anatomy, University of Helsinki, and Minerva Foundation Institute for Medical Research, Helsinki

Associate Professor Susanna Miettinen
BioMediTech, University of Tampere

Opponent
Docent Thomas Nyholm
Department of Biosciences, Åbo Akademi University

Custodian
Professor Juha Voipio,
Department of Biosciences, University of Helsinki

ISBN 978-951-51-3133-1 (paperback)
ISBN 978-951-51-3134-8 (PDF)
Painosalama Oy. 2017

I can do all things in Christ who strengthens me
Philippians 4:13

Table of Contents

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to by their Roman numerals in the text.

I. Kilpinen, L. *, **Tigistu-Sahle, F. ***, Oja, S., Greco, D., Parmar, A., Saavalainen, P., Nikkilä, J., Korhonen, M., Lehenkari, P., Käkelä, R. and Laitinen, S., 2013. Aging bone marrow mesenchymal stromal cells have altered membrane glycerophospholipid composition and functionality. *Journal of Lipid Research*, *54*(3), pp.622-635.

II. **Tigistu-Sahle, F.,** Lampinen, M., Kilpinen, L., Holopainen, M., Lehenkari, P., Laitinen, S. and Käkelä, R., 2017. Metabolism and phospholipid assembly of polyunsaturated fatty acids in human bone marrow mesenchymal stromal cells. *Journal of Lipid Research*, *58*(1), pp.92-110.

III. **Tigistu-Sahle F.** *, Holopainen, M.*, Skirdenko, V., Hyvärinen, Kerkelä, E., Lehenkari, P., Laitinen, S. and Käkelä, R., 2017. Dynamics of incorporation of n-6 and n-3 polyunsaturated fatty acids into the glycerophospholipids of human bone marrow derived mesenchymal stromal cells. (manuscript)

*Equal contribution

Author contribution

I. **Tigistu-Sahle contributed in:** Data collection, ESI-MS/MS lipid and GC-FID fatty acid analysis and interpretation, drafting, revision and approval of the article.

II. **Tigistu-Sahle contributed in:** Conception or design of the work, data collection, analysis and interpretation; drafting, revision and approval of the article to be published.

III. **Tigistu-Sahle contributed in:** Conception or design of the work, data collection, GC-FID fatty acid analysis and interpretation; revision and approval of the final manuscript.

LIST OF ABBREVIATIONS

Δ5	desaturase FADS1
Δ6	desaturase FADS2
ACS	acyl CoA-synthetase
AMPK	AMP-activated protein kinase
cAMP	cyclic adenosine monophosphate
CoA-IT	CoA-independent transacylases
COX	cyclooxygenases
DAG	diacylglycerols
ERK	extracellular signal–regulated kinases
ES	embryonic stem cell
ESI-MS/MS	Electrospray Ionization Mass Spectrometry
FA	fatty acids
FADS	fatty acid desaturases
FBS	fetal bovine serum
GPL	glycerophospholipid
hBMSC	human bone marrow derived MSC
HLA-G5	soluble human leukocyte antigen G
HSC	hematopoietic stem cell
hUCB-MSC	human umbilical cord blood-derived MSC
IDO	indoleamine 2, 3-dioxygenase
IFN-γ	interferon gamma
IL	interleukin
LOX	lipoxygenases
LPAAT	lysophosphatidic acid acyl transferase
LPCAT	lysophosphatidylcholine acyl transferases
LT	leukotrienes
LX	lipoxins

MAPK	mitogen-activated protein kinase
MSC	mesenchymal stem cell
MUFA	monounsaturated fatty acids
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	natural killer cell
NSC	neural stem cell
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEp	PE plasmalogens
PG	prostaglandins
PGD2	prostaglandin D2
PGE2	prostaglandin E2
PI	phosphatidylinositol
PKC	protein kinase C
PLA2	phospholipase A2
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
S1P	sphingosine-1-phosphate
SFA	saturated fatty acids
SM	sphingomyelin
TAG	triacylglycerol
TGF	transforming growth factor
TLR	toll-like receptor
TNFα	tumor necrosis factor α
TSG-6	TNF-α stimulated gene 6
TX	thromboxane
VEGF	vascular endothelial growth factor

SUMMARY

The application of human bone marrow derived mesenchymal stromal cells (hBMSCs) for regenerative or immunomodulatory therapies, *e.g.* treatment of the graft-versus-host disease, requires *in vitro* expansion of the cells. The hBMSCs undergo subtle changes during expansion which may compromise their functionality. In order to evaluate these changes lipidomics techniques were applied and the fatty acid (FA) and glycerophospholipid (GPL) profiles of hBMSCs were determined. During the cell passaging, arachidonic acid (20:4n-6) -containing species of phosphatidylcholine (PC) and phosphatidyl-ethanolamine (PE) accumulated while the species containing monounsaturated fatty acids (MUFA) or n-3 polyunsaturated fatty acids (n-3 PUFAs) decreased. The accumulation of 20:4n-6 and deficiency of n-3 PUFAs correlated with the decreased immunosuppressive capacity of the hBMSCs, which suggests that extensive expansion of hBMSCs harmfully modulates membrane GPLs profiles, affects lipid signaling and eventually impairs the functionality of the cells. Experiments, in which hBMSCs were cultured with different PUFA supplements revealed that the cells may limit the proinflammatory 20:4n-6 signaling by elongating this precursor with high biological activity to the less active precursor, 22:4n-6. It was also found that the ability of hBMSCs to produce long chain highly unsaturated fatty acids from C18 PUFA precursors was limited apparently due to the low desaturase activity of the cells. Thus, when the n-3 PUFA precursor, 18:3n-3, had little potency to reduce the GPL 20:4n-6 content, the eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acid supplements efficiently displaced the 20:4n-6 acyls, allowing attenuation of inflammatory signaling. These findings call for specifically designed optimal PUFA supplements for the cultures with sufficiently 20:5n-3 and 22:6n-3 but moderately 20:4n-6. Studies on the dynamics of PUFA incorporation into the major GPL classes revealed that the PUFAs in PC are remodeled at first, then those of the PEs and phosphatidylserines (PS). These results demonstrate that not only the type of PUFA administered but also the treatment time largely determines the resulting composition of the membrane GPL species, which serve as PUFA donors for the synthesis of lipid mediators. This thesis work highlights the importance of using lipidomics data to complement genomics or proteomics approaches when aiming at understanding of the therapeutic mechanisms of stem/stromal cells. The work provides tools to develop the protocols of hBMSCs culture and manipulate the functionality of the cells.

ማጠቃለያ

በዚህ የመመረቂያ ጽሁፍ ውስጥ ሶስት ሳይንሳዊ ጥናቶችን ተካተዋል። ዋና ትኩረት ያደረጉትም በሰውነታችን የአጥንት መቅኒ ውስጥ የሚገኙ **ሜዘንካይማል ግንደ - ሕዋሳት** (ሜ.ግ.ሕ) ላይ ሲሆን የምንመገበውን ስብ በምን ዓይነት ሁኔታ እንደሚያብላሉት ለማወቅ የተደረጉ ጥናቶች ናቸው። እነዚህ ሕዋሳት በአሁኑ ጊዜ በአጥንት መቅኒ ንቀለ-ተከላ በሰፊው ጥቅም ላይ መዋል በመጀመራቸው የአብዛኛውን ተመራማሪ ትኩረት ስበዋል። ለዚህም ደረጃ ያበቃቸው ሁለት አበይት ጥቅሞች አሏቸው፥ በመጀመሪያ ነጭ የደም ሕዋሳት በተለይም ቲ-ሕዋሳት (T-cell) የተባሉትን ተግባር መቆጣጠር መቻላቸው ሲሆን ሌላው በላቦራቶሪ ሙከራ ወደ የተለያዩ ሕብረ - ሕዋሳት (tissue) የመቀየር ክህሎታቸው በዚህም የተጎዱ ወይም ያረጁ የሰውነታችን ሕዋሳትን መተካት መቻላቸው ነው።

ነገር ግን ሜ.ግ.ሕ ከለጋሽ አካል ወደ ተቀባይ ከመዘዋወራቸው (ከመተከላቸው) በፊት በበቂ መጠን እንዲገኙ በቤተ-ሙከራ ውስጥ ማስፋፋት ግድ ይላል። ለዚህም ምርምር የሚሆኑ የአጥንት መቅኒ ናሙናዎች ከአምስት አረጋዊይን (አማካኝ ዕድሜ 74.6 ዓመት) እና ከአምስት ወጣት (አማካኝ ዕድሜ 22.2 ዓመት) ለጋሾች የተገኙ ሲሆን በውሳጣቸው ያሉትን ሜ.ግ.ሕ በመለይት ለዘጠኝ ቀናት በቤተ ሙከራ ውስጥ ወደ ተፈለገው ቁጥር የሕዋሳቱን መጠን ማባዛት ተችሏል። በዚህ ወቅት ሊፈጠሩ የሚችሉ ረቂቅ ለውጦች ለመከታተል የስብ መጠን/ዓይነት ልኬት የሚያጠናው **ሊፒዶሚክስ** (lipidomics) የሳይንስ ስነዘዴ በመጠቀም የተለያዩ ውጤቶችን አግኝተናል። ከነዚህም ውስጥ በዋናነት የተመዘገቡት የሚከተሉት ናቸው፥ የስብ አሲድ ዓይነቶች እንደ 20:4n-6 (AA) አናጻራዊ መጠን መጨመር በተቃራኒው ደግሞ የ20:5n-3 (EPA) እና 22:6n-3 (DHA) አናጻራዊ መጠን መቀነስ ሲሆኑ በተጨማሪም የሜ.ግ.ሕ የነጭ ደም ሕዋሳትን መራባት የመቆጣጠር አቅም መቅነስ ይጠቀሳሉ። እንደ 20:4n-6 (ኤ.ኤ) ያሉ የስብ አሲዶች በሰውነታችን ሕዋስት ውስጥ ከመጠን በላይ ሲከማቹ አላስፈላጊ ለሆነ ብግነት (inflammation) ያግልጡናል። በተቃራኒው የሕዋሳት የ20:5n-3 እና 22:6n-3 መጠን በበቂ መጠን ሲገኝ ይሄንን የብግነት ሂደት እንደሚገታው ጥናቶች አረጋግጠዋል። ስለዚህ የሕዋሳት የኦሜጋ 6 ለ ኦሜጋ 3 የአሲድ ንፃሬ (ratio) በተቻለ መጠን 1:1 በመሆነ መልኩ ቢስተካከል የሕዋሳትን ጤናማ ተግባር እንዲያከናውኑ ይረዳቸዋል።

በመሆኑም ከነዚህ ውጤቶች ለመደምደም የምንችላቸው አበይት ነጥቦች አሉ። የምንመገበው የስብ ዓይነት ተመጣጣኝ የ20:5n-3 እና 22:6n-3 መጠን ሊኖረው ይገባል። ይህንንም ለማድረግ በእነዚህ ዓይነት የስብ አሲዶች የበለጸጉ የቅባት አህሎችን በተለይም የተልባ፣ የወይራ፣ የኑግ እንዲሁም የሱፍ ዘይቶችን ለምግብነት መጠቅም ይረዳል። በተያያዘም ከእንሥሣት የሚገኙ የስብ ዓይነቶችን እንደ ቅቤ፣ ወተት እና ጮማ ከፍተኛ የ20:4n-6 መጠን ስላላቸው የሰውነታችን ሕዋሳትን ለአለተፈለገ ብግነት ያጋልጣሉ። በማጠቃለል ሜ.ግ.ሕ እና ተመሳሳይ ተግባር ያላቸውን የሰውነት ሕዋሳትን (ነጭ የደም ሕዋሳት) በብቃት አገልግሎታቸውን እንዲያከናውኑ ተገቢውን የስብ ዓይነት (ማለትም በኦሜጋ 3 የበለጸጉ) ማግኘት ይኖርባቸዋል።

INTRODUCTION

1. MESENCHYMAL STROMAL CELLS

1.1. History of MSCs

The present day knowledge of mesenchymal stromal/stem cells (MSCs) is largely based on the works of Alexander Friedenstein and Arnold Caplan. They were able to successfully isolate adherent, fibroblast-like, clonogenic cells from subpopulations of guinea pig-derived bone marrow (Friedenstein, 1990). Their works also showed the high replicative capacity and multilineage differentiation potential of the isolated MSCs including their ability to constitute the hematopoietic microenvironment (Friedenstein et al., 1968, 1970, 1974).

Following these pioneering works, Caplan and others were able to describe optimal seeding density and differentiation conditions for inducing chondrogenic development in both animal as well as human bone marrow derived MSCs (hBMSCs) (Caplan, 1981, 1984; Johnston et al., 1998; Yoo et al., 1998). These experiments together with his own initial findings made Caplan conclude that such inducible progenitor cells that formed cartilage and/or bone can be called as adult MSCs and he proposed a simplistic scheme for depicting their lineage termed the Mesengenic process (**Fig. 1**).

1.2. Origin of MSCs

The most common source for human MSCs is bone marrow aspirate from the iliac crest (Pittenger et al., 1999). Bone marrow stroma is found within the central cavities of axial and long bones, houses yellow marrow (mostly of adipose cells) and red marrow (consisting mainly hematopoietic tissue) and is the major site of hematopoiesis. The stromal microenvironment which occupies approximately 85% of the bone cavity is made up of complex network of cells including endothelial, reticular, adipocyte cells and stromal fibroblasts which are loosely supported by extracellular matrix (Clark and Keating, 1995; Vande et al., 1998; Muraglia et al., 2000). It is then from within this mesh of stroma system that the mesenchymal progenitor cells or MSCs are believed to originate; although the exact anatomical localization remains controversial. Selected immunophenotypic markers and infusion of genetically marked cultured cells are techniques used to ascertain the natural distribution of MSCs in vivo (Bianco et al., 2001; Shi and Gronthos, 2003). The ability of MSCs to engraft nonspecifically in different injured areas and the lack of definitive markers for these cells make the systematic isolation of MSCs from different organs and tissues a complicated task. Nonetheless, MSCs have been successfully isolated and

characterized from the following sources; adipose tissue and umbilical cord blood (Erices A. et al., 2000; Zuk et al., 2001; Panepucci et al., 2004; Laitinen et al., 2016). MSCs have also been found in various other organs and tissues such as liver, spleen, pancreas, kidney, aorta, vena cava, brain and muscle, periodontal ligament, synovial membrane and lungs (de Bari et al., 2001; da Silva et al., 2006; Seo et al., 2004; Sabatini et al. 2005; Meirelles et al., 2006).

1.3. Characterization of MSCs

The term MSCs refers to a set of self-renewing, non-hematopoietic, adult multipotent progenitor cells capable of differentiating into multiple mesodermal lineages including osteocytes, chondrocytes and adipocytes (Caplan, 2009). After the initial characterization of MSCs by Friedenstein and colleagues in the late 1960s as plastic-adherent, spindle shaped stromal cells, the precise morphological and phenotypical definition remained divisive. In order to address this issue the International Society for Cell Therapy (ISCT) described a minimal criteria to characterize human MSCs (Dominici et al., 2006). Accordingly, human MSCs can be identified by their; i) adherence to plastic in a standard culture condition, ii) expression of specific surface antigen markers, and iii) differentiation potential into multiple mesodermal lineages (**Fig. 1**). It should be noted that these criteria apply solely to human MSCs since other animal sources have been shown to differ in marker expression and phenotype (Peister et al., 2004). As the majority of plastic adherent bone marrow-derived MSCs lack the ability to self-renew (Horwitz et al., 2005), with the exception of a subset of cells that express CD146 (melanoma cell adhesion molecule, MCAM) (Sacchetti et al., 2007), the nomenclature of these cells has been in favor of the term stromal instead of stem cells. In this study we will use the designation MSCs to mean Mesenchymal Stromal Cells.

1.4. Multilineage potential of MSCs

One of the defining features of MSCs is their multipotency i.e. their ability to differentiate into several mesenchymal lineages both *in vitro* and *in vivo* (**Fig. 1**). The expression of phenotypic markers for osteocytes, adipocytes, myocytes and chondrocytes has been demonstrated in several studies, by supplementing MSCs growth media with general differentiation-inducing agents e.g. dexamethasone, ascorbic acid, β-glycerophosphate and transforming growth factor (TGF) β-3 or bone morphogenetic protein 2 (Rogers et al., 1995; Wakitani et al., 1994; Pittenger et al., 1999; Jaiswal et al., 1997; Johnstone et al., 1998; Jiang et al., 2002; Yuasa et al., 2015). MSCs have also been shown to differentiate into non-

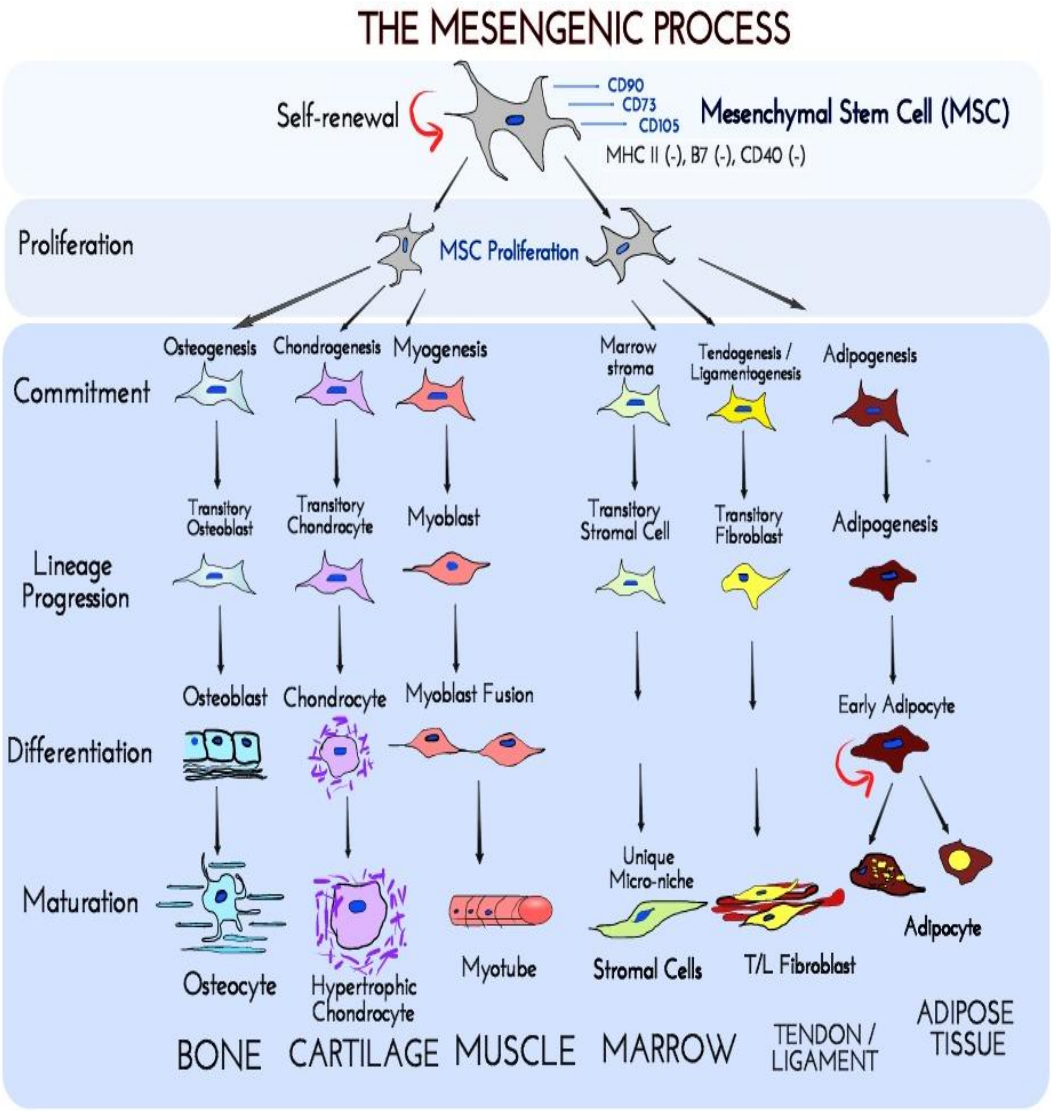

Fig. 1. Mesodermal tissues serve as the source of bone, cartilage, muscle, marrow stroma, tendon, fat, dermis in developing embryos and adult organisms. The continual processes through which these tissues get replenished is referred to as the Mesengenic Process. This involves proliferative and commitment steps from the progenitors **mesenchymal stromal cells (MSCs)**. Their progeny get committed to a specialized lineage and subsequent differentiation steps produces definitive phenotypes such as **osteoblasts**, **chondrocytes** and **myoblasts**. This differentiation multipotency together with ability to plastic adherence in standard culture and cellular surface markers **(CD90, CD73)** help to characterize and isolate MSCs from several sources. Modified from Caplan, A.I., 1994. The mesengenic process. Clinics in plastic surgery, 21(3), pp.429-435, copyright 1994.

mesodermal lineages such as neural cells and hepatocytes (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Lee et al., 2004; Sato et al., 2005). The results from the aforementioned experiments demonstrate the multipotency of MSCs and thereby their importance for tissue engineering and therapeutic applications.

1.5. Immunology of MSCs

MSCs express very low levels of major histocompatibility complex class I but completely lack expression of costimulatory molecules CD80 and CD86 (di Nicola et al., 2002; Le Blanc et al., 2003; Majumdar et al., 2003). These characteristics of MSCs aids to evade recognition by host immune systems during allogenic co-transplantation along with hematopoietic stem cells (HSCs). However, a prominent immunological feature of MSCs is their ability to inhibit allogeneic T cell and natural killer cell (NK cell) proliferation through a suppressive mechanism which does not involve alloantigen or costimulatory molecules such as CD80, CD86 and CD40 (di Nicola et al., 2002; William et al. 2003; Klyushnenkova et al., 2005). Consequently, MSCs have been shown to alleviate graft-versus-host diseases, promote cardiac repair and modulate both innate and adaptive immune responses (**Fig. 2**) (Tomita et al., 1999; Le Blanc et al., 2004; Pittenger et al., 2004; Aggarwal and Pittenger, 2005). It is believed that MSCs exert these immune modulatory functions either through direct cellular contacts or via secretion of soluble factors such as nitric oxide, indoleamine 2, 3-dioxygenase (IDO), prostaglandin E2 (PGE2) and soluble human leukocyte antigen G (HLA-G5) (**Fig. 2**) (Meisel et al., 2004; Krampera et al., 2006; Ryan et al., 2007; Spaggiari et al., 2008; Selmani et al., 2008). For instance, expression of Jagged-1 by hBMSCs inhibits T cell proliferation via the Notch signaling pathway. However, ligation of toll-like receptor (TLR) 3 or 4 on hBMSCs, with poly (I:C) and lipopolysaccharide (LPS) respectively, downregulates expression of Jagged-1 at both mRNA and protein level and subsequently inhibits hBMSCs suppressive effect on T cell proliferation (Liotta et al., 2008). Recent investigation underlined adenosinergic signaling as possible immunosuppressive mechanism of MSCs (Kerkelä et al., 2016). Human MSCs use AMP produced by activated T cells to synthesize adenosine (Ado), a highly immunosuppressive compound (Saldanha-Araujo et al., 2011; Sattler et al., 2011). Subsequent blocking of adenosinergic signaling raised T cell proliferation, providing evidence that Ado may partially be involved in the immunosuppressive functionality of MSCs.

Another valuable aspect of MSCs is their anti-inflammatory property which puts a rein on excessive inflammatory responses. Inflammation is a beneficial process in which the immune system successfully removes offending factors and repairs damaged tissue before eventually physiological homeostasis is re-

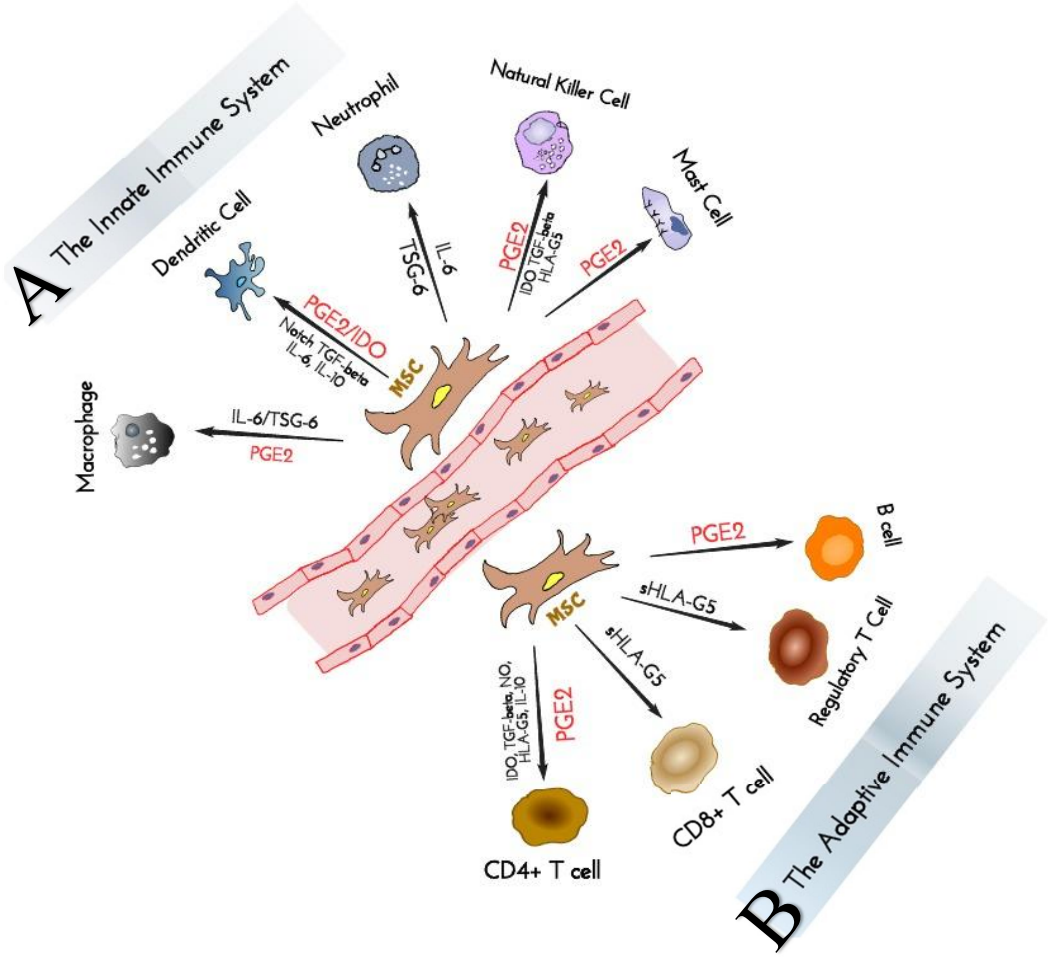

Fig. 2. Immunomodulatory characteristics of mesenchymal stem cells (MSCs). MSCs utilize direct cell-to-cell contact and paracrine factors to modulate immune responses**. A |** MSCs induce regulatory M2 **macrophages** and suppress their polarization to M1 type. The differentiation of monocytes to immature myeloid **dendritic cells** and subsequent maturation is hindered by MSCs. Furthermore, MSCs induce regulatory type of DC cells that promote T_{Reg} cell induction. Constitutive production and release of IL-6 by MSCs dampens the respiratory burst of **neutrophils** and prolong their life span. MSCs repress the cytotoxicity and proliferation of **NK cells**. Mast cell migration, and IgE-mediated degranulation is inhibited by MSCs. **B |** The main effector cells of the adaptive immunity are T cells which proliferation and cytotoxicity of especially **$CD4^+$** and **$CD8^+$ T cells** is inhibited by MSCs. MSCs maintain the survival and suppressive phenotype of **T_{Reg} cells**. Culturing **B cells** together with MSCs induces cell cycle arrest, reduces immunoglobulin production and halts differentiation of B cells. Such regulatory and immunosuppressive characteristics of MSCs is mediated by bioactive lipids i.e. PGE2 and several other soluble factors such as IDO, HLA-G5, TGF β-1, TSG-6, NO, IL-10 and IL-6. (Abbreviations described in the abbreviation and acronyms list).

stored. However, if this fragile process is not properly phased it may lead to pathological inflammation involving tissue damage and auto-immune diseases. It is believed that maintenance of the cell's normal homeostasis requires active balancing between pro- and anti- inflammatory molecules rather than sustaining a passive state that entails absence of proper inflammatory stimuli (Nathan, 2002). Systemically administered MSCs aid this active process by migrating and, homing to sites of injured tissue and secreting anti-inflammatory factors that assist in recovery. Expression of adhesion molecules, P-selectin and vascular cell adhesion molecule 1 enable MSCs to infiltrate the basement membrane and access injured sites (Rüster et al., 2006). During acute inflammation, the body produces both pro- and anti- inflammatory cytokines; interleukin (IL) 1β, IL6 and tumor necrosis factor α (TNFα) in an attempt to defend itself (Serhan et al., 2008). Expression of such molecular signals by resident macrophages at sites of injured tissues activates MSCs to secrete anti-inflammatory factors; TNF-α stimulated gene 6 (TSG-6) and PGE2 (Haynesworth et al., 1996; Aggarwal and Pittenger, 2005; Caplan and Dennis, 2006; Németh et al., 2009; Prockop et al., 2012).Both these factors create a negative feedback loop in resident macrophages. MSCs secretion of TSG-6 reduces nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling to reduce the cascade of pro-inflammatory cytokines and PGE_2 signaling alters the macrophage type to that which produces IL-10 i.e. an anti-inflammatory cytokine involved in the downregulation of T cell proliferation (Kim et al., 2009; Gordon and Martinez., 2010; Medzhitov, 2010).

1.6. Therapeutic use of MSCs

The immunoregulatory characteristics of MSCs in addition to their multipotency has made them the primary choice in cellular therapeutic applications. At present, the international clinical trial registry has over 500 clinical trials conducted using MSCs in variety of cases related either to their tissue regeneration or immunomodulatory capacities. Furthermore, there has been a major shift in the knowledge of the mechanism involved during MSCs-mediated tissue regeneration from an initial view that MSCs replaced damaged tissue to the idea that a wide range of bioactive molecules secreted by MSCs instead conveyed this therapeutic effect via cellular reprogramming and paracrine mechanisms (Caplan and Dennis, 2006; Rose et al., 2008; Ranganath et al., 2012). Such observations highlighted the need to further investigate the soluble factors produced by cells and how this signaling can be manipulated to enhance MSCs functionality. These wide ranges of secretomes from MSCs leads the way in the cellular crosstalk within the microenvironment and mediate physiological functions.

The efficacy of MSCs based cellular therapy will likely be influenced by the micro-environmental cues encountered either during *in vitro* expansion or subsequent *in vivo* administration. A number of strategies

have been implemented thus far in order to modify this micro-environment such as preconditioning of MSCs using physiological, molecular or pharmacological factors *in vitro* or even by cell-to-cell interaction during co-culturing experiments. For instance, growing MSCs in hypoxic/anoxic conditions resulted in the upregulated expression of vascular endothelial growth factor (VEGF), insulin-like growth factor 1, hepatocyte growth factor and angiopoietins that promote cell survival (Kinnaird et al., 2004; Rehman et al., 2004; Tögel et al., 2007; Uccelli et al., 2008; Ranganath et al. 2012; Tran and Damaser, 2015). In another work, growing hBMSCs in 3D spheroids boosted the secretion of PGE2 that changed activated macrophages to an anti-inflammatory M2 phenotype via prostaglandin E2 receptor 4 signaling (Ylöstalo et al., 2012; Bartosh et al., 2013). However, several issues remain unsolved with respect to the safety, tolerability and efficacy of MSCs therapies. In order to develop safer and more widely applicable cellular therapies, it is crucial to elucidate the mechanisms as well as the secretomes involved in the immunomodulatory effects of the MSCs.

2. CELLULAR LIPIDS

2.1. Lipidomics

The view of lipids has come a long way from the early 1950's when they were recognized for their role as energy stores and building blocks of cell membranes, to the current view giving them roles as signaling molecules or their precursors. Currently, lipids have been implicated in several human metabolic diseases such as cancer, diabetes, neurodegenerative and infectious disorders. The pathology of these ailments commonly involve the disruption of lipid metabolic enzymes and pathways. These findings are a result of recent advancements in Lipidomics; the systems-level analysis of lipids and their interacting partners using novel analytical approaches such as Electrospray Ionization Mass Spectrometry (ESI-MS). The lipidomics approach involves the quantification of local concentrations of lipids, the characterization of enzymes involved in their metabolism and the identification of lipid-lipid or lipid-protein interactions, which elucidate some of the major factors that regulate lipid functionality. Thus, lipidomic analysis deals with the quantitative as well as qualitative features of lipids in cells that underwent certain physiological or pathological changes. Due to the advanced ESI-MS methodologies, cellular lipidomics has now been able to supplement biomedical genomics and proteomics studies aiming for drug and biomarker discoveries.

2.2. Classification of lipids

The internationally adopted classification system based on chemical structure of hydrophobic and hydrophilic elements that make up lipids categorizes these molecules into eight groups; fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides (Fahy et al., 2005). Eukaryotic membranes are primarily composed of glycerophospholipids (GPLs), the prominent types being phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI). Simple glycerolipids such as triacylglycerol (TAG) and diacylglycerols (DAG) are mainly found in cytosolic lipid droplets while cholesterol, a major sterol lipid, is an important constituent of plasma membrane (van Meer et al., 2008).

2.3. Structure and origin of lipids

Membrane GPLs consist of two fatty acyl molecules esterified at the *sn-1* and *sn-2* positions of glycerol backbone, and contain a head group linked by a phosphate residue at the *sn-3* position. The functional head groups (choline, ethanolamine, serine and inositol) are linked to the glycerol by a phosphodiester bond. Sphingolipids have a single fatty acid joined to a sphingoid base; and sterols contain rigid molecule of four fused hydrocarbon rings. The hydrophilic moieties in these amphipathic compounds may be either -OH group at one end of the sterol ring system or they may be charged alcohols and phosphate groups. The most common lipid class in nature, the TAG consist of a glycerol backbone with three hydroxyl groups esterified to a fatty acid. Various combinations of the fatty acyl chains and polar head groups result in a huge variety of lipids.

The building blocks of lipids, fatty acids (FA) are synthesized via malonyl coenzyme A condensation reaction catalyzed by fatty acid synthase. Complex lipids such as GPLs are mainly synthesized in the endoplasmic reticulum alongside ceramide, galactosylceramide, cholesterol and ergosterol (Vance, 1990; Henneberry et al., 2002). The Golgi apparatus is another lipid biosynthetic organelle where ceramide containing sphingolipids such as sphingomyelin (SM) and glycosphingolipids are synthesized (Futerman and Riezman, 2005). Non-structural lipids, TAG and cholesteryl esters are synthesized in the endoplasmic reticulum (Bell et al., 1981).

2.4. Cellular distribution of lipids

Despite the localized synthesis of lipids in certain organelles, the resulting composition is varied among different membranes in different cell types (van Meer, 1989). The lipid classes present in different organelles are the same but the different lipid transport and sorting mechanisms contribute to create organelle and membrane specific lipid ratios *i.e.* compositions. In addition to this organelle specificity, membranes also maintain lipid asymmetry by differential enrichment of certain lipid classes in either the cytosolic or exoplasmic leaflet of membranes assisted by ATP-dependent flippases (Martin and Pagano, 1987). Lipid rafts are an example of heterogeneous distribution/composition of lipids in plasma membrane preferentially enriched with sphingolipids and cholesterol. These dynamic clusters serve as docking sites for transmembrane proteins, as relay stations in intracellular signaling and as sorting factors for lipids and proteins involved in the secretory pathway (Simons and Ikonen, 1997; Brown and London, 1998; Simons and Toomre, 2000).

2.5. Synthesis of Lipids

Cellular metabolism of lipids involves the uptake, synthesis, and degradation of these compounds in a tightly regulated process. Liver, the main site of de novo lipogenesis, has been the model tissue for numerous studies dealing with the pathways and regulatory mechanisms of lipid metabolism in mammals. This focus in liver stems from the fact that it is the main site of cholesterol synthesis, can convert free fatty acids to ketone bodies and supports the absorption of dietary lipids by secreting bile acids (Van Golde and Van den Bergh, 1977).

Lipid metabolism in mammalian cells starts with hydrolysis of the glycerophospholipid to produce glycerol and fatty acids. The sequential acylation of fatty acyl-CoA to glycerol-3-phosphate at the *sn*-1 and *sn*-2 positions by acyl-CoA: glycerol-3-phosphate acyltransferase and lysophosphatidic acid acyl transferase (LPAAT) marks the initial steps of *de novo* phospholipid synthesis. This process is the main biosynthetic route for the formation of the most common precursor of all GPLs; phosphatidic acid (PA) (Smith et al., 1957). The biosynthetic path for GPLs separates into two branches that both employ PA as substrate. The first route involves the hydrolysis of PA by cytosolic phosphatidic acid phosphatase in the endoplasmic reticulum to yield DAG. In addition to being the precursor for subsequent biosynthetic pathways of PC and PE, DAG is utilized in the synthesis of TAG. The second route involves the replacement of the phosphate group of PA by other phosphate functional groups to form additional phospholipids; PI, PG or cardiolipin. This step is catalyzed by CDP-diacylglycerol synthase and involves

the condensation of PA and cytidine trisphosphate to create CDP-diacylglycerol molecule which is utilized for synthesis of other lipids (van Meer et al., 2008; van den Bosch and de Vet, 1997).

2.6. Remodeling of lipids

The distinct lipid composition of cellular membranes is a result of the diverse acyl chain as well as polar head group arrangements in each tissue. This diversity is achieved by the concerted actions of several lysophospholipid acyltransferases and CoA-dependent and CoA-independent transacylases (CoA-IT**)** involved in the remodeling pathway first proposed by Lands in 1958 (Lands, 1958). After the initial *de novo* biosynthesis of GPLs from glycerol-3-phosphate and acyl-CoAs via the Kennedy pathway, subsequent cycles of deacylation and reacylation follows by the actions of phospholipase A2 (PLA2s) and lysophospholipid acyltransferases. These enzymes exhibit different specificities as is shown by acyl-CoA:1-acyl-2-lysophopholipid acyltransferase preference for polyunsaturated fatty acyl-CoAs and by acyl-CoA:2-acyl-1-lysophospholipid acyltransferase preference for saturated ones. Moreover, CoA-independent transacylase specifically catalyze the transfer of C20 and C22 polyunsaturated fatty acids (PUFA) to ether-containing lysophospholipids while CoA-dependent transacylase have preferences for specific FA; 18:0, 18:2 and 20:4 (Yamashita et. al., 1997).

The fatty acyl composition of GPLs is strictly regulated and the distribution of FA follows a non-random route. Consequently, the *sn-1* position of phospholipids has a SFA residue while the *sn-2* position is commonly esterified to unsaturated FA such as 20:4n-6. In the *de novo* biosynthesis of PA, the human LPAAT1 is shown to have preference for 14:0-, 16:0-, and 18:2-CoAs but LPAAT2 had higher activity for 20:4-CoA over 16:0- or 18:0-CoA. Since the rest of GPLs are a consequence of the dephosphorylation or activation to CDP-diacylglycerol of PA; the fatty acyl chain compositions of newly synthesized phospholipids is expected to mirror that of PA. However, the molecular species of phospholipids is independent of PA profile because remodeling actions are performed only after *de novo* synthesis is completed. So far five lysophosphatidylcholine acyl transferases (LPCAT) have been identified and of these the Ca^{2+}-independent LPCAT1 have been shown to catalyze the synthesis of disaturated- PC and -PG in alveolar cells and Ca^{2+}-dependent LPCAT2 catalyzes PC and platelet-activating factor production in inflammatory cells. The third enzyme LPCAT3 is expressed ubiquitously and synthesizes PUFA containing PC, PE and PS at the *sn-2* position (Nakanishi et al., 2006; Shindou et al., 2007; Shindou et al., 2009). A lyso-phosphatidylinositol acyltransferase catalyzes 20:4-CoA incorporation into PI (Yamashita et al., 1997; Lee et al., 2008). Such specificities, are however maintained only at physiological concentrations of substrate and the nature of acyltransferases can be influenced by other factors. For

instance, when oleoyl-CoA and arachidonoyl-CoA are present in saturating amounts 20:4n-6 was assimilated at higher proportion leading to the conclusion that acyl-CoA:1-acyl-GPC acyltransferase has high affinity for the low-level acceptor 1-acyl-GPC (Okuyama et al., 1975). Furthermore, during abundant supply of acyl-CoAs, cells have been shown to make phospholipids with two-identical acyl chains (diPUFA) opting for the high-capacity and low-affinity pathway for PUFA incorporation rather than the default low-capacity and high-affinity route (Okuyama et al., 1972; Sundler et al., 1974; Chilton and Murphy, 1986).

Some of the fatty acids, in particular 18:1 and 20:4n-6, are introduced into phospholipids primarily via the remodeling pathway rather than through *de novo* biosynthesis. The level of free 20:4n-6 is tightly regulated in cells and is directly related to the activity of acyl-CoA: lysophospholipid acyltransferase. Enhanced PLA2 activity leads to the release of PUFAs such as 20:4n-6 from the *sn-2* residue of GPLs and the free FA is used for the production of various eicosanoids. The excess FA is reacylated back to the lysophospholipid by the action of acyl-CoA synthetase (ACS) and acyl-CoA: lysophospholipid acyltransferases. Several PLA2s including cytosolic PLA2 (cPLA2) and Ca^{2+} independent PLA2 (iPLA2), have been shown to have lysophospholipase/transacylase activity since inhibition of these enzymes prevents the incorporation of 20:4n-6 into phospholipids (Reynolds et al., 1993; Balsinde et al., 1995). Exogenous 20:4n-6 and 22:6n-3 are both similarly incorporated first into diacyl PC by the actions of ACS and acyl-CoA:1-acyl-GPC acyltransferases. Sequential assimilation of these FAs to alkyl-acyl PC and alkenyl-acyl PE occurs in the presence of CoA-IT (Colard et al., 1984; Sugiura and Waku, 1985; Shikano et al., 1993). Thus both 20:4n-6 and 22:6n-3 are competing for the same remodeling enzyme, with CoA-IT having slightly higher affinity for the latter. The function of acyltransferases and transacylases extends beyond the remodeling system and they are also involved in the biosynthesis of lipid mediators, such as N-acylethanolamines and anandamides (Reddy et al., 1983; Devane and Axelrod, 1994; Kruszka and Gross, 1994; Sugiura et al., 1996).

2.7. Biological function of lipids

Eukaryotic cells invest a relatively small percentage of their genes to synthesize extremely diversified and complex repertoire of lipid molecules (Cotter et al., 2006; van Meer et al., 2008). The wide variety of lipid structures speaks for the diverse functional roles of lipids in mammalian cells. First, being small molecules with reduced carbon, lipids are used as energy storage units in the form of cytosolic droplets.

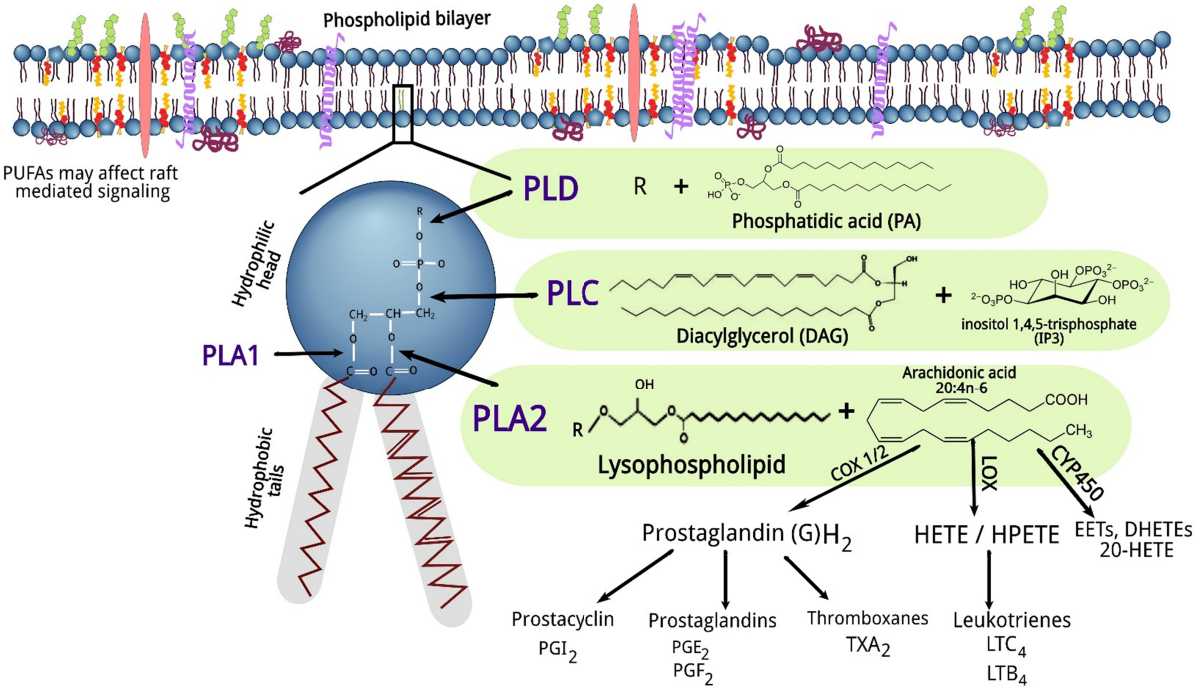

Fig. 3. Phospholipid bilayer and related signaling compounds. A plasma membrane bilayer is mainly made up of glycero-phospholipids, sphingolipids and cholesterol. The raft domains, which mediate important signaling cascades are affected by polyunsaturated fatty acids (PUFAs) proportions. The hydrophobic head of the glycerophospholipids, upon a stimuli are acted upon by different lipases. Phospholipase D (PLD) cleaves the bond after the phosphate moiety to produce phosphatidic acid (PA) and an alcohol (R). Phospholipase C (PLC) hydrolyzes the phosphodiester bond to yield diacylglycerol (DAG) and phosphoryl head group. Phospholipase A (PLA1 / PLA2) attack the fatty acyl ester bond releasing a lysophospholipid and fatty acid moiety (e.g. arachidonic acid, 20:4n-6). The 20:4n-6 serves as the precursor for cyclooxygenases (COX 1/2), lipoxygenases (LOX) and cytochrome P450 enzymes which catalyze the synthesis of several lipid mediators such as prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs). The n-3 PUFAs such as 20:5n-3 and 22:6n-3 also serve as precursors for important lipid mediators with roles mainly in anti-inflammatory actions.

These droplets are made up of neutral lipids; TAGs and steryl esters. Cells can utilize these stores in their aerobic metabolism to fulfill energy needs or carry out membrane biogenesis using the FA stored in the droplet lipids. Second, the amphipathic nature of lipids forms the biophysical platform that is the basis for the spontaneous formation of biological membranes (**Fig. 3**). The hydrophobic moieties of lipids are repelled from the internal aqueous cytosolic environment, while the hydrophilic segment readily associate with this milieu. Such interactions of these two opposing moieties of lipids enables the compartmentalization of the internal cellular milieu and the consequent formation of discrete organelles. Finally, current research is highlighting the signaling role of lipids in pathophysiological as well as metabolic processes.

2.8. Signaling role of lipids

The degradation of membrane lipids by numerous enzymes produces a variety of effectors and second messengers collectively termed as bioactive lipids. Lysophosphatidic acid, sphingosine-1-phosphate (S1P) and phosphoinositides are some of the specialized lipids whose signaling functions have been studied in great detail (Edwards et al., 2002; Wenk and de Camilli, 2004; zu Heringdorf and Jakobs, 2007; Adams et al., 2016). Different types of PLAs act on GPLs to produce lysoPC, lysophosphatidic acid, PA, and DAG that bind membrane receptors to propagate important signaling pathways (Fernandis and Wenk, 2007; zu Heringdorf and Jakobs, 2007). Sphingomyelinase-catalyzed hydrolysis of SM produces Cer, a precursor for a type of lipid mediator, S1P (Perry and Hannun, 1998).

In addition, kinases and phosphatases reversibly phosphorylate the head group of PI to produce unique set of second messengers called phosphoinositides that have roles in a variety of cellular processes (Berridge and Irvine, 1984; Hawkins et al., 2006). These lipid mediators employ their signaling action via specific membrane receptors such as G-protein-coupled receptors, TLR or by recruiting cytosolic proteins (Fernandis and Wenk, 2007).

Metabolism of lipids to generate bioactive mediators is not limited to the head groups of these molecules. Hydrolysis of the hydrocarbon tail of phospholipids by PLAs generates FA in addition to a variety of lysophospholipids (**Fig.3**). The *sn-2* position in the fatty acyl branch of membrane lipids is preferentially made up of PUFAs. PLA2 cleaves the *sn-2* acyl bond of phospholipids releasing different PUFAs mainly 20:4n-6, 20:5n-3, and 22:6n-3. Downstream modifications of 20:4n-6 by cyclooxygenases (COX) and lipoxygenases (LOX) produces potent bioactive compounds termed prostaglandins (PG) and leukotrienes (LT) which are for the most part proinflammatory and lipoxins (LX) with anti-inflammatory

characteristics (**Fig.3**). These lipid mediators are collectively termed as Eicosanoids. The 20:5n-3 is also a substrate for COX and LOX pathways that lead to the synthesis of anti-inflammatory mediators such as PGE3 and leukotriene B5 respectively (Goldman and Goetzl, 1983; Hawkes et al., 1991). The most unsaturated of the n-3PUFAs, 22:6n-3 is metabolized by COX-2 and LOX to produce novel anti-inflammatory mediators termed D-series resolvins, protectins and maresins (Serhan et al., 2002; Serhan et al., 2015).

Classic lipid mediators such as PG and LT serve to magnify the fundamental signs of inflammation (Samuelsson, 1983; Flower, 2006). However, during the resolution phase of inflammation PGE2 and prostaglandin D2 (PGD2) initiate the synthesis of pro-resolving non-immunosuppressive lipid mediators such as resolvins and protectins, to advance tissue homeostasis (Serhan et al., 2000; Hong et al., 2003). In other cases, PGE2 has been shown to promote inflammation when other lipid or protein mediators are present (LTB4 or C5a) (Williams, 1983). PGD2 on the other hand has been noted to exert anti-inflammatory actions by raising cyclic adenosine monophosphate (cAMP) levels and pro-resolving effects by suppression of NF-κB activation in certain cell types (Pons et al., 1994; Haworth and Levy, 2007). LX can act as pro-resolving/anti-inflammatory mediator by inhibiting polymorphonuclear neutrophil chemotaxis and adhesion as well as preventing neutrophil diapedesis into tissues (Colgan et al., 1993; Gewirtz et al., 1998; McMahon et al., 2000). The more readily recognized anti-inflammatory lipid mediators are the resolvins and protectins. The E-series resolvins that stem from 20:5n-3 bind to GPCR, chemokine-like receptor 1 and leukotriene B4 receptor 1, subsequently attenuating NF-κB activation and pro-inflammatory signaling (Arita et al., 2005; Arita et al., 2007). The 22:6n-3 serves as substrate for the D-series resolvins that regulate neutrophil activity and suppress the production of inflammatory cytokines such as IL-1β (Hong et al., 2003). Another class of lipid mediators stemming from 22:6n-3 are the protectins. These mediators inhibit T cell migration while promoting their apoptosis and attenuate the secretion of pro-inflammatory cytokines (Ariel et al., 2005).

2.9. Functional Lipidomics

It is now evident that bioactive lipids such as phosphoinositides, S1P, eicosanoids, docosanoids and FAs play an important role in the regulation of numerous cellular processes. These mediators can exert their biological responses via different mechanisms. For instance, in hMSCs ligation of EP2 receptor by PGE2 employs β-arrestin-1/JNK signaling pathways promoting proliferation and migration of the cells (Yun et al., 2011). Another mechanism by which lipid mediators can influence cell metabolism and growth is that they serve as ligands or coactivators for nuclear transcription factors, such as peroxisome

proliferator-activated receptors, Sterol regulatory element-binding proteins and NF-κB, which affect *e.g.* the expression of key proteins of lipid metabolism, energy utilization, growth and differentiation (Rajasingh and Bright, 2006; Liu et al., 2007; Chapkin et al., 2009). It is therefore, plausible to assume that PUFAs and their metabolites will have profound effects on hBMSCs' characteristics and fate. However, the role of lipids in MSCs multilineage and anti-inflammatory functions have not been investigated. Currently, only few studies exist on the significance of FAs, lipid rafts and bioactive lipids focusing on embryonic stem cell (ES) and neural stem cell (NSC) functions (Rajasingh and Bright, 2006; Yamazaki et al., 2006; Kim and Hermatti, 2009; Lee et al., 2010; Bieberich, 2012).

In general, n-6 and n-3 PUFAs and their metabolites can promote stem cell survival and differentiation. For instance, PGE2 can enhance HSCs homing and proliferation by upregulating the C-X-C chemokine receptor type 4 and increase their survival by stabilizing the β-catenin protein through cAMP/PKA signaling (Goessling et al., 2009; Hoggatt et al., 2009). In mouse ES cells, PGE2 is noted to inhibit apoptosis and stimulate proliferation by inducing phosphorylation of mitogen-activated protein kinase (MAPK) through signaling pathways involving EP1 receptor-dependent protein kinase C (PKC) and epidermal growth factor receptor-dependent PI3K/Akt (Yun et al., 2009). Human umbilical cord blood-derived MSCs (hUCB-MSCs) proliferation can be stimulated by PGE2 through β-catenin-mediated c-Myc and VEGF expression via Epac/Rap1/Akt and PKA cooperation (Jang et al., 2012). In addition, inhibition of COX-2 resulted in suppressed neural progenitor cells proliferation in adult mice showing the important role of PGE2 on NSC (Sasaki et al., 2003; Goncalves et al., 2010). NSC proliferation can also be regulated by a metabolite of PGD2 via epidermal growth factor-dependent pathways (Katura et al., 2010; Sakayori et al., 2011). Other 20:4n-6 derived lipid mediators including LTB4, leukotriene D4 and thromboxane A2 have all been shown to promote proliferation in different types of stem cells. Signaling mechanisms involving extracellular signal–regulated kinases (ERK) and p38 MAPK are employed by thromboxane A2 to modulate the migration and proliferation of adipose tissue-derived MSCs (Yun et al., 2009). In addition, leukotriene D4 is noted to increase mouse ES cell proliferation and migration via the PI3K/Akt, Ca2+-calcineurin, and glycogen synthase kinase 3β/β-catenin pathways (Kim et al., 2010).

While the role of n-6 PUFAs and their metabolites have mainly been involved in stem cell proliferation, the n-3 PUFAs are associated mostly with the stem cell differentiation aspect. The highly unsaturated 22:6n-3 can upregulate dendritic spine-related genes F-actin, growth associated protein 43, glutamate receptor 1 synapsin-1 and postsynaptic density protein 95 to initiate the differentiation of ES into neurons. Evidence indicated that 20:5n-3 and 22:6n-3 can induce neuronal differentiation by reducing hairy and enhancer of split-1 expression while increasing that of cyclin-dependent kinase inhibitor 1B to enable cell cycle arrest (Katakura et al., 2009 and 2013). Furthermore, activation of the AMP-activated protein kinase (AMPK) reinforced by the binding of

PGE2 to prostaglandin E2 receptor 4, leads to endothelial differentiation from bone marrow derived-cells (Zhu et al., 2011). There is evidence suggesting that 22:6n-3 can modulate the canonical pathway to influence the pluripotency of ES cells (Massaro et al., 2015). These findings all suggest that n-6 and n-3 PUFAs together with their metabolites are capable of influencing stem cell fate and have significant roles in regulating their proliferation as well as differentiation characteristics.

In the case of hBMSCs, the role of PUFAs and lipid mediators in their multipotency or immune regulation, is still an under researched topic and important information on the basic lipidome of these therapeutic cells is lacking to the most part. So far we know that bioactive lipids such as PGE2 can be induced in hBMSCs since they do express the COX-2 enzyme constitutively and PGE2 has been implicated as one of the mechanisms by which MSCs exert their immunomodulatory effects (Aggarwal and Pittenger, 2005; Chen et al., 2010; Yanez et al., 2010). Studies found that MSCs from different sources when co-cultured with T cells, can produce high amounts of PGE2 in the presence of interferon gamma (IFN-γ) and TNF-α, IL-1α, or IL-1α. All these inflammatory cytokines together with cell-to-cell contact are crucial to elicit the immunosuppressive characteristics of MSCs (Hegyi et al., 2012).

In light of the available evidence, n-6 and n-3 PUFAs and their metabolites, present a practical opportunity to influence hBMSCs' characteristics. A key issue in using such therapeutic cells for clinical purposes is the control of their fate *in vivo* for which safe and practical methods are limited. Characterization of lipids in hBMSCs is essential to further elucidate PUFAs' role in regulating gene expressions and signaling pathways related to proliferation as well as immunregulation by hBMSCs. Manipulation of the FA and lipid mediator profiles of hBMSCs may present a safe technique which creates a favorable environment to determine their cellular fate in clinical applications.

AIMS OF THE CURRENT STUDY

The therapeutic use of MSCs is dependent on their regenerative and immunoregulatory capacities. The available evidence suggests that n-6 and n-3 PUFA, which have an integral role in mammalian cell structure and function, can influence the proliferation as well as differentiation characteristics of stem/stromal cells. Therefore, in this thesis work we planned to investigate the lipidome profile of hBMSCs. More specifically, we aimed to:

I. Establish the FA and GPL profile of hBMSCs

II. Study the effect of long-term expansion on hBMSCs' lipidome and functionality

III. Examine PUFA metabolism in hBMSCs

IV. Study the dynamics of PUFA incorporation into hBMSCs' GPL

V. Investigate the correlation between the lipidome and functionality of hBMSCs

MATERIALS AND METHODS

The research work entailed in this dissertation were made possible by using methods listed in **Table 1**. The detailed description of these methods can be found in the original publications (I – III). However, concise descriptions of the methods used and the experimental materials are mentioned below for the reader's convenience.

Table 1 *Methods applied in the experiments performed for the publications I-II and manuscript III.*

METHOD	PUBLICATION
Cell culture	I, II, and III (manuscript)
Co-culture assay	I
Microarrays	I, II
Flow cytometry	I, II
ELISA	II, III
Folch – total lipid extraction	I, II and III (manuscript)
Fatty acid methyl ester preparation	I, II and III (manuscript)
Mass spectrometry (ESI-MS/MS)	I, II and III (manuscript)
Gas chromatography (GC-FID and GC-MS)	I, II and III (manuscript)

1. EXPERIMENTAL MATERIALS

The mesenchymal stromal cells derived from human bone marrow aspirates were provided by Professor Petri Lehenkari, University of Oulu (**Table 2**). Sample collections were done after written informed consent were attained according to patient protocols approved by the Ethical Committee of Northern Ostrobothnia Hospital District of Helsinki and Uusimaa and the Declaration of Helsinki accords were followed concerning the use of human material. For comparative analysis purposes FA metabolism were studied in HepG2 (liver hepatocellular carcinoma) cells purchased from BioNordika Oy. Peripheral blood mononuclear cells were obtained from the Finnish Red Cross Blood Service. Unconjugated FA were 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3 and 22:6n-3 purchased from Nu-Chek-Prep, Inc.

Table 2 *List of hBMSCs samples used in the experiments performed for publications I-II and manuscript III.*

Donor (code)	**Age**	**Gender**	**Publication**
081	20	male	I
088	21	male	I
089	23	female	I
091	23	female	I
092	23	male	I
164	75	male	I
172	72	female	I
194	82	male	I
271	75	male	I
437	16	male	III
471	19	male	III
481	70	male	I
492	75	female	I
500	37	female	I, II, III
517	52	male	I, II, III
RE1	63	female	I, II, III
RE7	74	female	I
RE8	42	female	I, II, III
RE9	54	male	I

2. METHODS

2.1. Cell Culture and co-culture assays

The hBMSCs and HepG2 cells were cultured in the appropriate media detailed in the articles I-III. Briefly, the growth media were supplemented with fetal bovine serum (FBS) and maintained in 37ºC prior to harvesting at 70 – 80% confluency. Cells were washed with PBS and centrifuged down and stored at -70°C for experimentation. Peripheral blood mononuclear cells labeled with CFSE (5(6)-Carboxyfluorescein diacetate N-succinimidyl ester) were cultured in a 48-well plate together with

hBMSCs at a 1:10 ratio. Flow cytometry was used to record the proliferation rate of T-cell as well as ascertain the expression of certain surface antigens (FACSAria Becton Dickinson, San Jose, CA USA and FlowJo 7.6.1 software Treestar, Asland, OR, USA).

2.2. Gas chromatography of fatty acids

The total FA from the cells were first converted to their respective methyl esters as detailed in Christie (1993). The transmethylated fatty acids were dissolved in hexane and analyzed using Shimadzu GC-2010 Plus. The initial identification and quantification of the fatty acids methyl esters were based on the retention time and flame ionization detection (FID) responses of standard mixtures. The identifications were confirmed by parallel runs by GC-MS (Agilent 6490 Triple Quad LC/MS and GCMS-QP2010 Ultra, Shimadzu). The results were presented as molar percentages (mol%) and the FA were marked by using the abbreviations: [carbon number]:[number of double bonds] n-[position of the first double bond calculated from the methyl end] (e.g. 22:6n-3).

2.3. Mass spectrometry of glycerophospholipids and triacylglycerols

The Folch et al. (1957) protocol for total lipid extraction was applied to prepare samples for GPL and TAG analysis. Lipids dissolved in chloroform/methanol (1:2 v/v) were spiked with a mixture of internal standards, and 1% NH_4OH was added just prior to the analysis. The sample aliquot was then infused into an electrospray ionization (ESI) chamber of a triple quadrupole mass spectrometer (Waters Quattro Micro triple quadrupole mass spectrometer Micromass, Manchester, UK) and a LC-MS/MS (Agilent 5490 Triple Quad LC/MS with iFunnel technology, California, USA). The instrument settings are described in detail in the articles I – III. Detection of different lipid classes was carried out using head group specific precursor or neutral loss scanning modes. Acid hydrolyzed samples were prepared for the analysis of PE plasmalogens (PEp) that were detected in the MS/MS using specific fragments of the vinyl ether chain at the *sn-1* position of the lipid. The triple quadruple MS/MS also served to establish the acyl chain constituents of each lipid species by setting it to negative ion mode and scanning for the product ion of the anion fragments for all common fatty acids. Specific multiple reaction monitoring method was employed for the detection and quantification of various PUFA metabolites (Le Faouder et al., 2013). The mass spectra data produced from the above analysis were processed using their associated software namely, MassLynx (Micromass, Manchester, UK), and MassHunter Workstation Qualitative Analysis

(Agilent Technologies, Inc. California, USA). Identification and quantification of the found lipid species were carried out by using the LIMSA software (Haimi et al., 2006).

2.4. Microarrays and gene expression analysis

The Qiagen AllPrep DNA/RNA mini kit was used to extract RNA and an accompanying protocol was implemented for the purification of the relative miRNA and DNA. SurePrint G3 Human GE 8 × 60 K from Agilent was used to assess the hybridized RNAs and the raw data files (.txt files) were imported into the R v. 2.13 software and preprocessed by the BioConductor package limma v.3.4.5 before matching the probes with the same Entrez Genes or lincRNAs from public data repository; Gene Expression Omnibus and GeneSapiens/IST online.

2.5. ELISA assay

A selection of PUFAs conjugated to BSA were supplemented to hBMSCs as described above. The cells, after 24h incubation period, were transferred to a serum-free starvation α-MEM medium and cultured for an additional 48h. The growth medium was ultra-centrifuged, and the resulting supernatant was analyzed for PGE2 levels using a PGE2 ELISA Kit (Monoclonal Item No 514010 Cayman Chemical, Ann Arbor, USA).

RESULTS AND DISCUSSION

1. FATTY ACID PROFILE OF CULTURED hBMSCs DIFFERS FROM THAT OF FBS AND MARROW

Research on stem cell biology has mainly focused on the genomics and proteomics aspects while corresponding knowledge in lipidomes is lacking. Recent studies have highlighted a lipogenesis-dependent regulation of stem cell fate advancing the paradigm of a metabolic cell fate control in these types of cells. For instance, the quiescent state of NSC is maintained by the high expression of *Spot14* gene which limits the malonyl-CoA available for fatty acid synthase-dependent lipogenesis resulting in the impairment of proliferation (Knobloch et al., 2013). In another case, the promyelocytic leukemia regulation of peroxisome proliferator-activated receptor δ signaling and fatty-acid oxidation has been identified as a metabolic switch that maintains the asymmetric division of HSC (Ito et al., 2012). Lipid mediators such as PGE2 have be shown to promote the migration and proliferation of hBMSCs and are involved in the regulation of vertebrate HSC homeostasis (North et al., 2007; Yun et al., 2011). Therefore, in our work we determined the lipidome profile of hBMSCs as an initial step to investigate the role of lipids and lipid-derived mediators in the functionality that could contribute for advancement of understanding the mechanism of action of these therapeutically practical cells.

The micro-environment of cells influences their characteristics through biochemical soluble factors or cellular contacts. In the case of hBMSCs this micro-environment is either the bone marrow *in vivo* or the ingredients of the culture medium *in vitro*, including FBS as the main supply of nutrients. In order to understand the role of such micro-environments, we analyzed the FA profiles of the marrow, hBMSCs and FBS. The hBMSCs were cultured in a 5% FBS supplement and the total FA content studied by GC analysis. Comparative study of the FA profiles between hBMSCs, FBS and bone marrow samples revealed clear differences. The total amount of monounsaturated fatty acids (MUFA) in marrow samples was higher by 53% compared to the hBMSCs and by 48% from FBS samples mainly due to the relatively high level of 18:1n-9 in the marrow samples (**Fig. 4A, B**). The levels of 16:0 and 18:0 were higher in FBS samples than in the marrow samples (**Fig. 4B**). Consequently, the ratio of MUFA to saturated fatty acids (SFA) was low in hBMSCs and FBS samples (**Fig. 4B** inserts). Prolonged exposure to elevated levels of SFA such as 16:0 have previously been reported to decrease proliferation and induce apoptosis in various cell types including hBMSCs (Lu et al., 2012). By functioning as a substrate in the synthesis of Cer, a potent cell death mediator, 16:0 may help to propagate this process. The 16:0 activates ERK1/2 and p38 MAPK to induce endoplasmic reticulum stress in hBMSCs but this effect was rescued by administering physiologically relevant levels of 18:1n-9 in studies carried out by Fillmore et al. (2015). Deacylation of

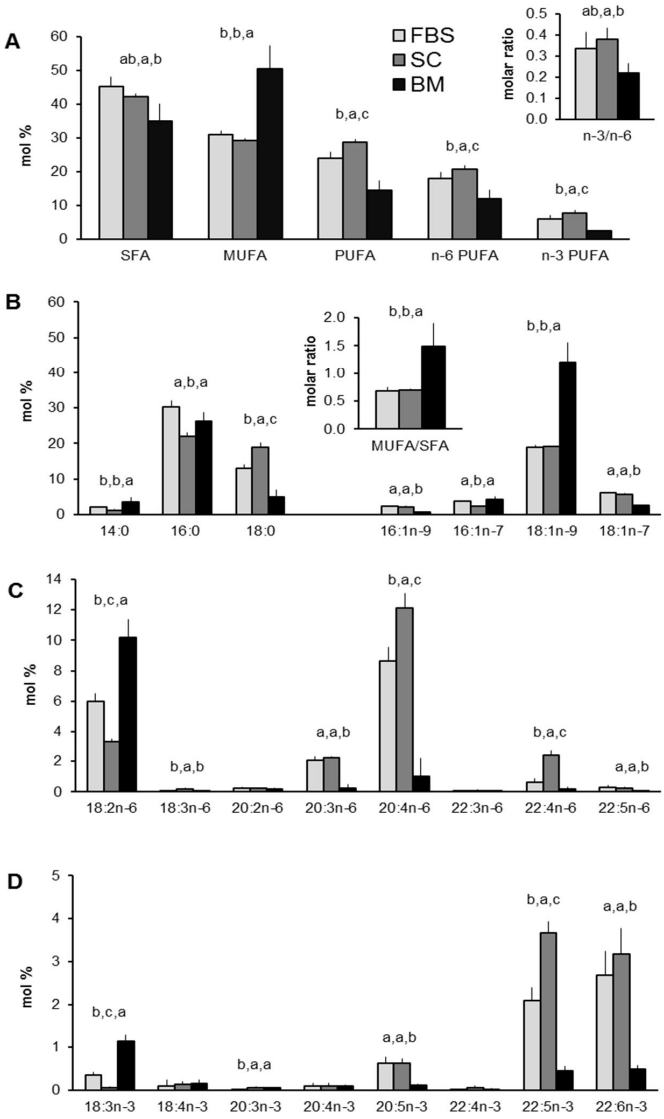

Fig. 4. The FA profiles from hBMSCs (SC, in control medium, n = 4), FBS (n = 5) and human bone marrow (BM, n = 4) samples. Values calculated in mol % of total fatty acids, mean ± SD. A: The total proportions of SFA, MUFA, PUFA, and n-6 PUFAs or n-3 PUFAs. The ratio of n-3 to n-6 PUFAs (n-3/n-6) as an insert. B: Individual SFAs and MUFAs. The ratio of MUFA total to SFA total as an insert. C: The C18, C20 and C22 n-6 PUFAs. D The C18, C20 and C22 n-3 PUFAs. The Kruskal–Wallis non-parametric one-way analysis of variance was used for statistical analysis. In addition post hoc Mann-Whitney test for the means were used after initial Kruskal Wallis testing. The means with no common letter differed at $P < 0.05$ level. **Adapted from Tigistu-Sahle et al., 2017 (Article II)**

16:0, which is an essential component of Lipid A moiety, results in the loss of LPS endotoxic activity (Raetz, 1990). One of the LPS-induced signaling pathways is TLR4 which when coupled to CD14 activates NF-κB and consequent expression of mRNA for several proinflammatory cytokines including COX-2 (Chow et al., 1999). Studies done using RAW 264.7 cells reported that SFA namely 16:0 and 18:0 were potent in inducing COX-2 expression via TLR4/ NF-κB pathway whereas unsaturated fatty acids inhibited this induction through a common signaling pathway derived from TLR4 (Lee et al., 2001; Suganami et al., 2007; Håversen et al., 2009). Taken together, these observations indicate that SFA can be potent endogenous ligands of TLR4/ NF-κB at physiologically high levels to induce inflammatory signals. Therefore our results showing the relatively high ratios of SFA/MUFA in FBS as compared to marrow samples, may put the hBMSCs in a microenvironment that favors the induction of inflammatory processes in these cells.

The PUFA profile showed higher levels of 18:2n-6 and 18:3n-3 in both FBS and marrow samples as opposed to hBMSCs (**Fig. 4C** and **D**). A prominent difference was apparent when comparing the proportions of 20:4n-6 which were found to be 1.4-fold higher in hBMSCs compared to FBS (**Fig. 4C**). Additionally, 20:5n-3 and 22:6n-3 levels were on average 4 fold higher in hBMSCs and FBS but marrow samples were deficient in these n-3 PUFA bioactive precursors (**Fig. 4D**). Consequently, the ratio of n-3/n-6 PUFA was 1.8fold lower in marrow samples when compared to the levels of both FBS and hBMSCs (**Fig. 4A** inserts).

Studies on Paleolithic nutrition state that humans developed on a diet that was much lower in SFA and containing roughly equal amounts of n-6 and n-3 PUFA (Nutrition, 1985; Simopoulos, 1991). However, current data on Western diets indicate that this balance has been offset in favor of n-6 PUFAs resulting in a large increase of the n-6/n-3 ratio, from ancient 1:1 to modern time's 20:1 (Nutrition, 1985; Simopoulos, 2016). The n-6 and n-3 PUFAs have been designated as essential FA that must be derived from the diet due to the lack of endogenous enzymes for n-3 desaturation in mammalian cells (Simopoulos, 2001; Kang, 2004). These two classes of PUFAs have distinct functionality and opposing physiological roles in mammalian cells and their balance is important for homeostasis and normal development. Research has revealed that increased ratio of n-6 to n-3 PUFAs elicit pathophysiological responses by disrupting lipid homeostasis and most importantly inducing systemic inflammation (Banni and Di Marzo, 2010). For instance, hyperactivity of endocannabinoid signaling has been associated to high ratio of n-6/n-3 PUFA in the diet, which results in increased inflammatory state of peripheral tissues (Banni and Di Marzo, 2010). However, this inflammatory response was lowered in Fat-1 mice, a genetically modified mouse capable of converting n-6 PUFA to n-3 PUFA, which increased its n-3 PUFA tissue content compared to wild type (Delpech et al., 2015). In contrast, the gradual decrease of n-6 to n-3

PUFA ratio in the diets of rats fed for six weeks resulted in suppression of spleen lymphocyte proliferation and NK cell activity in response to T cell activation and consequent decline in graft versus host response (Jeffery et al., 1996; Simopoulos, 2016). Our results from marrow samples, representative of the natural microenvironment of these stromal cells, resemble the modern western diet with a high n-6 to n-3 ratio of 1:0.21. The FBS, the primary source of FA to cultured hBMSCs, had similarly high ratio of 1:0.33. These results imply that the ideal ratio of n-6 to n-3 PUFAs (i.e. 1:1) has not been achieved by the artificial supplementation of FBS which should be optimized to increase the proportion of n-3 PUFAs in these sources for enhanced performance of hBMSCs.

2. CHARACTERIZATION OF hBMSCs FROM DIFFERENT DONORS

The relationship between donor age and the biological activity of hBMSCs have been studied. The results reported are inconclusive by far regarding the effect of donor age on the number of colony-forming units-fibroblastics (CFU-Fs), multilineage plasticity as well as proliferative capacity of MSCs from different sources. Initial human studies found that donor age had no effect on the characteristics of hBMSCs (Stenderup et al., 2001) while others reported a negative correlation between age and proliferative capacity and differentiation potential (Quarto et al., 1995; Choumerianou et al., 2010; Alves et al., 2012; Siegel et al., 2013). Other research showed that there is huge variation on the ability of hBMSCs to suppress T cell proliferation and an overall loss of MSC fitness with age (Fehrer et al., 2005; Stolzing et al., 2008; François et al., 2012). In light of such inconsistencies, we studied the role of donor age as well as cumulative population doublings (passage number) on overall characteristics of hBMSCs and tried to correlate the found results with the changes in lipidome profile.

2.1. Proliferation potential and gene expression

We examined the proliferation and immunosuppressive capacity of hBMSCs isolated from five young adults (from 20 to 24 years, mean 22.2 years) and five elderly donors (from 62 to 82 years, mean 74.6 years). Calculations of the cumulative population doublings of the cells starting from passage 4 showed that hBMSCs from the old donors had greater individual variation compared to those from young donors (**I, Fig. 1B**). Since the seeding density of 1000cells/cm^2 were applied for both sources of hBMSCs and the method of isolation was also similar, this difference in growth kinetics can only be attributed to the difference in donor age and/or other physiological disparities. For instance, impaired proliferative

capacity was observed for MSCs from myelodysplastic syndromes and amyotrophic lateral sclerosis patients (Ferrero et al., 2008; Ferrer et al., 2013; Pavlaki et al., 2014).

In order to identify potential differentially expressed markers and to check the variation among different donors we made gene expression analysis of hBMSCs from both young and old donors and compared them between early (p8-p4) and late (p8-p14) passages. In the earliest passage 4, altogether 707 genes were found to be differentially expressed between the young and old donor samples (**I, Fig. 6A**). Further look into the functional role of these genes revealed that gene ontologies (GO) related to lipid metabolism, immunological processes, differentiation and developmental functions were downregulated in the hBMSCs from old donors (**I, Fig. 6C**). Changes in gene expression were the highest for these samples after an extended period of cellular expansion. Moreover, gene ontologies related to apoptosis and signal transduction were upregulated in the samples from old donors (**I, Fig. 6D**). These differences highlight the fact that hBMSCs from various donors do in fact differ in their expression of various housekeeping genes important for biosynthetic as well as developmental processes.

Long-term expansion of hBMCs from both donor groups however, resulted in increased expression of cell-cycle components cyclin-dependent kinase inhibitor 2A and cyclin-dependent kinase inhibitor 1 in addition to shortening of telomere length in passage 11 cells compared to passage 4 (**I, Figs. 1C** and **D**). It has been reported that cyclin-dependent kinase inhibitor 2A is enriched in senescent fibroblasts and a similar increment in the expression of cyclin-dependent kinase inhibitor 1 in HeLa cells resulted in G_2 cell cycle arrests hindering proliferation (Hara et al., 1996; Sato et al., 2002). Both these proteins represent a type of cyclin-dependent kinase inhibitors which are involved in the regulatory process in cellular proliferation phases, and are in general overexpressed in senescent cells. The loss in telomere length from hBMSCs of late passages is in line with previously reported effects of *in vitro* expansion of these cells and signifies a gradual cellular senescence (Bonab et al., 2006). Our results indicating the increased expression of these proteins in hBMSCs from late passages, can therefore signify a decline in their proliferative capacity as a consequence of long-term *in vitro* expansion.

2.2. Immunosuppressive capacity of hBMSCs

In contrast, donor age did not produce significant effect in the immunosuppressive capacity of hBMSCs, despite what has previously been reported (Stolzing et al., 2008; François et al., 2012). Inflammatory activation of hBMSCs using TNF-α and IFN-γ upregulates IDO activity which is a known negative regulator of T cell proliferation (Meisel et al., 2004; François et al., 2012; Laranjeira et al.,

2015). This inducible enzyme was shown to have significantly higher activity in nonagenarians compared to young individuals (Pertovaara et al., 2006). Thus, a possible interpretation of our result is that the immunosuppressive ability of hBMSCs is governed mainly by the amount and type of inflammatory stimuli rather than the age of the donor. The mechanism of immune regulation by hBMSCs is a research area that still needs further investigation. However, these discrepancies in reporting may also be the consequence of variations in the intrinsic differences in; the immune plasticity of MSCs from diverse population groups, culturing conditions, tissue sources and isolation techniques.

On the other hand, the number of population doublings/passage numbers of hBMSCs had significant effect not only on their proliferative capacity but also their immunosuppressive potential. We cultured PBMC together with hBMSCs in ratio of 1:10 for four days. Flow-cytometry results indicated that hBMSCs ability to suppress T cell proliferation was the highest for those from early-passage (p4-p8) than the late-passage cells (**I, Fig. 1E**). Furthermore, this lose in immune regulation of hBMSCs negatively correlated with the level of 20:4n-6 in the cells but had a positive correlation coefficient with 22:6n-3 (**I, Table 2**). As mentioned above the immunosuppressive ability of hBMSCs is governed by factors such as IDO activity which increases with augmented inflammatory stimuli. One of the first reports examining the role of FA on IDO activity demonstrated that 20:4n-6 inhibited the signal transducer and activator of transcription 1 phosphorylation on tyrosine 701 resulting in deterrence of IDO induction in THP-1 cells and monocytes (Bassal et al., 2012). Consequent work by Bassal and coworkers (2016) successfully identified PGD2 as the potent suppressor of IDO mRNA and protein levels via the DP1/cAMP/PKA/CREB pathway. Taken together, our results suggest that the larger population doublings result in the accumulation of 20:4n-6 and its subsequent metabolism via COX1/2 pathway produces PGD2 that contributes to the loss of hBMSCs immune regulatory activity via downregulation of IDO activity.

The immunomodulatory activity of hBMSCs is affected by several genes among which the suppressors of cytokine signaling (*SOCS1 and SOCS2*), and ASB family (Ankyrin repeat and *SOCS* box-containing proteins) had lowered expression levels in the samples from old donors (I, Table 3). The *PTGS1* gene that encodes for COX1 enzyme, and catalyzes the synthesis of PGE2, was markedly suppressed in the late-passage cells unlike the genes of inflammatory cues *TGF β receptor 1* and *IL6* which were both upregulated. The expression level of enzymes involved in Lands remodeling pathway; *LPCAT2* and *LPCAT3* were significantly lower in the hBMSCs from old donors perhaps indicative of an impaired shuffling of 20:4n-6 to different GPL classes. Impaired synthesis of GPLs classes with 20:4n-6 acyl residues, marks the increased availability of this FA for eicosanoid production in the cells from old donors. The increased expression of inflammation associated proteins TGF β receptor 1 and IL6 may

increase IDO activity but the inhibitory effect of 20:4n-6 on IDO may compromise the immune suppression capabilities of hBMSCs from late passages.

3. *In vitro* EXPANSION OF hBMSCs ALTERS THEIR LIPIDOME PROFILE

One of the main aims of this study was to establish the FA and GPLs profile of the therapeutically well-endowed hBMSCs. GC-FID and ESI-MS/MS methodologies were employed to constitute the lipid profiles of hBMSCs from five young and five old donors. Due to lack of published data on the lipid profile of hBMSCs, the results from these analyses were compared to the well outlined lipidome of human primary fibroblasts which had similar immunosuppressive functionality (Haniffa et al., 2007). The average values for GPL class totals for hBMSCs were: PC 43%, PE 36 %, PI 4-5%, and PS 5-8% (**I, Table 1**). In general, the values for GPL class totals in hBMSCs from both the young and old donors resembled that of the fibroblasts as well as other mammalian cells (Blom et al., 2001; Vance and Steenbergen, 2005). However, the relative amount of PE in the GPL class totals was higher for hBMSCs than the human fibroblasts. A closer look into the GPL molecular species profile identified PE38:4 (18:0/20:4n-6) as the main component but in the profiles of human fibroblasts the most abundant species were PE36:1, 36:2 and 38:4 (Blom et al., 2001).

Mammalian tissues maintain a constant composition of phospholipids under normal physiological conditions thus enabling the comparative analysis of lipidome profiles from different cell types. Therefore, PE is the second most abundant structural lipid in mammalian cells and can be synthesized via three main biochemical pathways; de novo production of CDP-ethanolamine (Kennedy) pathway, decarboxylation of PS in mitochondria (decarboxylation pathway) and acylation to lysoPE pathway (Gibellini and Smith, 2010; Vance and Tasseva, 2013). In cultured cells, the PS decarboxylation pathway is taken to be the predominant biosynthesis route of PE and the molecular species formed differ from that made through the Kennedy pathway in that the former pathway creates species with a larger proportion of PUFA at *sn-2* position (Bleijerveld et al., 2004; Vance and Tasseva, 2013). Although the regulation of PE synthesis is not well known, reports have shown that the amount of cellular PE is to a certain extent regulated by degrading to plasmalogens (Baburina and Jackowski, 1999; Dorninger et al., 2015). The data from the GPL class totals of hBMSCs from young and old donors is in line with this report, since a decrease in PE totals is accompanied by a relative increase in the PE alkenyl totals. In view of these facts, the relative abundance of PE totals in hBMSCs compared to fibroblasts might reflect i) a difference in the biosynthetic pathway which in hBMSCs is possibly the PS decarboxylation one i.e. increased PE species

with PUFA residues in their *sn-2* positions ii) since hBMSCs FA pool has an augmented 20:4n-6 content this may contribute to the enrichment of the PE predominant species 18:0/20:4n-6.

3.1. Expansion of hBMSCs increases membrane PI content in relation to PS

The lipid composition is in a state of dynamic and rapid turnover in the case of cultured cells. Pulse labeling experiments using BHK-21 cells demonstrated the half-lives of the major phospholipids to be only 2.5 – 4h with PC, DAG and TAG having the fastest and PS the slowest turnover (Gallaher et al., 1973). Under serum-supplemented culture conditions where adequate supplies of lipids exist, *de novo* lipid biosynthesis is inhibited while a lipid-rich culture medium increased the accumulation of TAG and cholesteryl esters but the membrane phospholipids remained intact (Spector et al., 1980). Mammalian cells regulate their PI synthesis in a number of ways including inositol concentrations and *in vitro* by the local concentrations of other phospholipids (Fischl et al., 1986; Nuwayhid et al., 2006).

During long term culturing of hBMSCs, it was observed that the relative ratio of PI/PS was increased especially in the late passage samples (**I, Fig. 3**). This ratio was also significantly high in the hBMSCs from old donors as opposed to young ones in passage 4. Several factors could be behind such changes in phospholipid composition of hBMSCs *in vitro*. The predominant molecular species in PI is a moiety containing 20:4n-6 in the *sn-2* position while PS had a substrate preference for 22:6n-3 in its principal species (Chilton and Murphy, 1986; Tanaka et al., 2001; Kim et al., 2004). Since the long term expansion of hBMSCs resulted in enrichment of 20:4n-6 at the expense of n-3 PUFAs such as 22:6n-3 in the total FA pool, a corresponding increase in the PI totals coupled with a decline in PS levels seem logical. This notion is further supported by reports which showed that high concentrations of activated 20:4n-6 raise the rate of PI synthesis (Tanaka et al., 2001; Nuwayhid et al., 2006). Another explanation for the increase in PI totals may be the consequence of balancing the membrane net charge under conditions of reduced PS synthesis (Becker and Lester, 1977). According to some studies, increased PI:PS ratio might signify a stationary growth phase (Homann et al., 1987) and this might be the case of hBMSCs from late passages, that naturally exhibit a higher proportion of cellular apoptosis.

The significance of an increasing PI:PS ratio in long term expanded hBMSCs is manifold. Anionic lipids such as PI and PS are known to stimulate PLA2 activity, which results in the production of repertoires of lipid mediators with both inflammatory as well as anti-inflammatory roles (Leslie and Channon, 1990). The intermediate products of PLA2 action, lysophospholipids and PUFA are potent regulators of PKC which has a central role in several cellular processes including proliferation, differentiation and secretion

(Kochs et al., 1993; Fadeel and Xue, 2009). This family of kinases is also stimulated by lipid classes; initially by DAG coupled with an increased intracellular Ca^{2+} levels leading to the translocation of PKC to plasma/nuclear membrane whereby PS, PI or even PIP2 serve as cofactors to complete the activation of this signaling pathway (Kochs et al., 1993). Therefore, an imbalance in the ratio of PI:PS may disrupt the regulation and activities of kinases, and phospholipases with resounding consequences.

3.2. Increase in 20:4n-6 containing GPL species at the expense of n-3 PUFA containing ones

Data obtained from our initial studies illustrate the type of FA modifications that result from long term culturing of hBMSCs. GPL profiles showed that 20:4n-6 containing PC and PE species, specifically 36:4 and 38:4, were enriched in the hBMSCs samples from late passages (**I, Figs. 4A** and **B**). Parallel to this, a decline in shorter and MUFA containing GPL species was found coupled with a decrease in n-3 PUFA containing species. The FA profiles from GC-FID analysis revealed a decline in the relative proportions of MUFAs as well as n-3 PUFAs including 22:5n-3 and 22:6n-3 in the hBMSCs samples from late passages (**I, Fig. 5A**). This decline was coupled with an increase in 16:0, 18:0, and 20:4n-6 and a subsequent raise in the ratio of total n-6 to the total n-3 PUFA. As discussed earlier, increased ratio of n-6 to n-3 PUFAs as well as physiologically elevated levels of SFA elicit pathophysiological responses in several types of cells including macrophages and monocytes and that such increase can be correlated to the loss of hBMSCs functionality (Raetz, 1990; Jeffery et al., 1996; Lee et al., 2001; Suganami et al., 2007; Håversen et al., 2009; Lu et al., 2012; Simopoulos, 2016). It is therefore, crucial to regulate the level of these FAs in cultured hBMSCs in such a way that promotes the healthy expansion of these cells.

Stem cell research suggests the role of PUFA to be mainly in proliferation and differentiation with the main focus being on eicosanoids. These lipid mediators arise from the action of COX, LOX and cytochrome P450 enzymes on 20:4n-6. Therefore, increased availability of 20:4n-6 in hBMSCs from late passages augments production of PGs, thromboxanes (TX), LT and LX. The most abundant eicosanoid PGE2 has a stimulatory effect on the proliferation of HSCs, but may also dose-dependently inhibit growth of macrophages *in vitro* (Feher and Gidáli, 1974; Pelus et al., 1979). In addition, it has been demonstrated that PGE2 increases the expression of VEGF and c-Myc through the cooperative signaling pathway of cAMP/Ras-related protein 1 (Rap1)/Akt plus PKA resulting in the stimulation of hUCB-MSC proliferation (North et al., 2007; Yun et al., 2011; Jang et al., 2012). Other classes of eicosanoids such as LT have been shown to, at physiologically relevant levels promote proliferation but excessive amounts inhibited this growth in NSC (Wada et al., 2006). The n-6 COX pathway also produces TX which facilitate platelet aggregation and also mediate the migration and proliferation of adipose tissue-derived

MSCs via ERK and p38 MAPK signaling routes (Yun et al., 2009). The n-3 PUFAs 20:5n-3 and 22:6n-3 are also primary sources of lipid mediators with mainly anti-inflammatory roles such as the 3-series prostaglandins, resolvins, and protectins (Serhan et al., 2008). Supplementation of 20:5n-3 and 22:6n-3 decreased production of inflammatory cytokines in macrophages, lymphocytes and peripheral mononuclear cells (Khalfoun et al, 1996; Verlengia et al., 2004; Weldon et al., 2007; Vedin et al., 2008). Treating Jurkat T cell line with 20:5n-3 resulted in the displacement of linker for activation of T cells protein and 16:0-labeled Src family kinase Lck from lipid rafts and subsequent inhibition of T lymphocyte activation (Stulnig et al., 2001; Horejsi, 2003; Shaikh and Edidin, 2006). During the resolution phase of inflammation, 22:6n-3 derived maresins stop the infiltration of neutrophils and induce their subsequent clearance by phagocytosis (Serhan et al., 2009).

The majority of these lipid mediators are synthesized from free 20:4n-6 which level is increased by enhanced activity of PLA2 enzymes either under inflammatory stimuli or even under normal physiological conditions. Lysophospholipid acyltransferases are enzymes that regulate the freely available PUFA content of resting cells, by constant reacylation of the cleaved PUFAs (Pérez-Chacón et al., 2009). The accumulation of 20:4n-6 containing GPL in the long term cultured hBMSCs may therefore tip this sensitive balance in favor of synthesis of potent inflammatory lipid mediators by increasing the precursor 20:4n-6 containing lipid moieties which is then readily available for cleavage by PLA2. Consequently, the hBMSCs from late passages are more likely to exhibit pro-inflammatory characteristics and this is negatively correlated to the loss of immunoregulatory functions of these cells.

4. METABOLISM OF EXOGENOUS PUFA BY hBMSCs DIFFERS FROM HepG2 CELLS

Since n-6 and n-3 PUFAs are natural compounds that are readily incorporated into tissues and their metabolites can be altered with drugs, this presents a practical approach to manipulate the lipidome of stem/stromal cells. By doing so, we can better understand the importance of PUFAs and their metabolites on stem cell biology as well functionality.

In the second phase of our project, we were interested to see what effect FA 18:3n-3 and 18:2n-6 might have on hBMSCs lipidome profile. The contents of both SFA as well as MUFA (18:1n-9 and 18:1n-7) and 22:5n-3 and 22:6n-3 all had decreased significantly after 18:2n-6 supplementation (**II, Figs. 2A–D**). The rate limiting enzyme in MUFA synthesis is stearoyl-CoA desaturase, which activity has been shown to be suppressed by dietary PUFA (Ntambi, 1995). The decrease in MUFA content might reflect the hBMSCs' effort to maintain proper lipid viscosity at the impeding high PUFA content, and by doing so

may decrease the rate of cellular metabolism and division that are characteristic of cancer cells (Ntambi, 1995).

Supplementation of 18:2n-6 resulted in a 10-fold increase of its relative amount and also raised the immediate elongation product 20:2n-6 (**II, Fig. 2C**). However, its desaturation product 20:4n-6 showed a 60% decrease as compared to the hBMSCs grown in control medium. On the other hand, exogenous 18:3n-3 proved effective to raise the content of its desaturation product 20:5n-3 albeit slightly, but resulted in the decline of the longer PUFAs 22:5n-3 and 22:6n-3 by 40% (**II, Fig. 2D**). Similar supplementation regime in HepG2 cells effectively metabolized the C18 precursors into their respective long chain PUFA counterparts. As a result, the content of 20:4n-6, 20:5n-3 and 22:6n-3 in HepG2 showed significant increase (**II, Fig. 2C** and **D**).

The disparity in the desaturation capability between the hBMSCs and the neoplastic HepG2 cells lies in the differential expression level of the fatty acid desaturases (FADS) FADS1 (Δ5 desaturase) or FADS2 (Δ6 desaturase) in these cells. The conversion of C18 precursors to their respective C20 PUFA products proceeds in three successive reactions; an initial Δ6-desaturation, elongation and a final Δ5-desaturation. The Δ6-desaturase catalyzes conversion of 18:2n-6 to 18:3n-6 and 18:3n-3 to 18:4n-3. Most diploid human cells have an active Δ6-desaturase and are therefore able to desaturate C18 precursors successfully unlike transformed cells. For example, mouse fibrosarcoma cells and transformed mouse fibroblasts cannot desaturate 18:2n-6 due to loss of Δ6-desaturase activity (Mathers and Bailey, 1975; Spector et al., 1980). The expression of Δ6-desaturase in hBMSCs was 1.2 times to HepG2; thus they had the ability to carry out the initial steps of desaturation (**Fig. 5**). Despite the ability of most studied cell lines to convert 20:3n-6 to 20:4n-6 and 20:4n-3 to 20:5n-3 using Δ5-desaturase; the protein expression data revealed that the expression level of this enzyme was significantly lower (0.6 times) in hBMSCs than those found in HepG2 cells (**Fig. 5**). This result indicates the hBMSCs have an impaired Δ5-desaturase activity compared to HepG2 cells, thus cannot convert C18 precursors to their C20 counterparts in efficient amount.

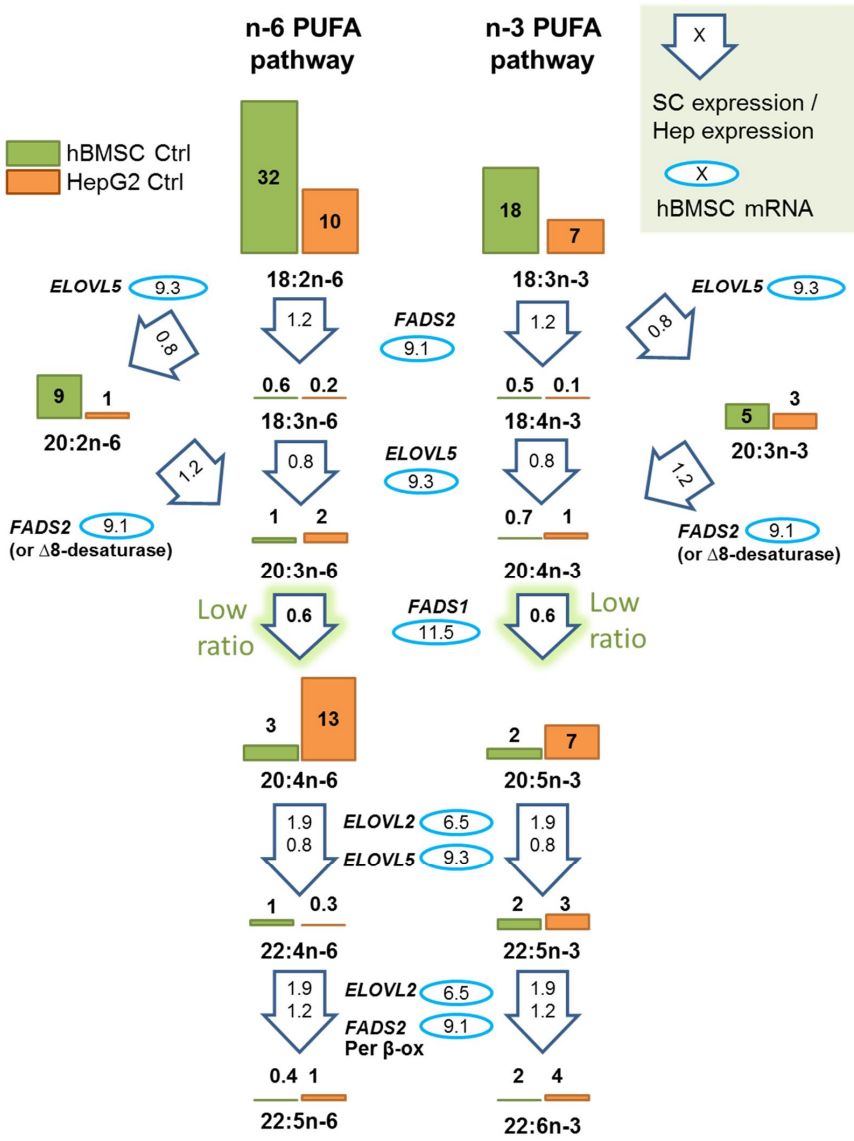

Fig. 5. Schematic representation the biosynthetic route together with catalyzing enzymes of n-6 and n-3 PUFAs in the hBMSCs and HepG2. Bars represent the average levels (mol%) of specific PUFAs in hBMSCs and HepG2 grown in non-supplemented control (Ctrl) media. Blue ovals indicate the mRNA levels of the elongases (ELOVL5 and ELOVL2) and desaturases (FADS2, FADS1). The values from average expression levels of the enzymes are marked inside the arrows and were obtained for mesenchymal stem cells (SC) from a public data base ISTonline and normalized against hepatocyte (Hep) values of the data base. **Adapted from Tigistu-Sahle et al., 2017 (Article II)**

5. INCORPORATION OF EXOGENOUS FATTY ACIDS IN hBMSCs IS GPL CLASS SPECIFIC

The effects of longer, highly unsaturated FA were also assessed by supplementing the hBMSCs culture with 20:4n-6, 20:5n-3 or 22:6n-3 for a 9-day period. The supplementation resulted in decreased contents of SFA and MUFA similar to the effect observed with C18 PUFA addition but albeit to a lesser extent (**II, Figs. 4A–C**). In GPL molecular species profile, the percentages of PC 36:4, 38:4, 38:5 and 42:8 were raised due to 20:4n-6 supplementation (**II, Figs. 5–7**). Highly unsaturated PE species (42:7, 42:8, 42:9) also emerged after n-6 PUFA supplements. In a similar trend, the percentage of 20:4n-6 containing PS 38:4 doubled compared with the control values but the percentages of PS 40:6 (18:0/22:6n-3) decreased after 20:4n-6 addition. The general trend observed as a consequence of exogenous 20:4n-6 supplementation, is therefore the increased proportion of GPL molecular species with 20:4n-6 in their acyl residues coupled with the percentage decrease of n-3 PUFA containing molecular species. In contrast, adding either 20:5n-3 or 22:6n-3 had a lowering effect on the 20:4n-6 levels and overall n-6 PUFA content (**II, Fig. 4C**). From these results it is evident that hBMSCs can effectively incorporate exogenous PUFA and preferentially place them in different GPL classes. Furthermore, the added n-3 PUFAs ability to replace 20:4n-6 containing GPL molecular species is beneficial to limit the PUFA availability that serve as precursors for eicosanoid production. The emergence of diPUFA GPL molecular species is a consequence of the high-capacity/low-affinity pathway for PUFA remodeling that is opted for by cultured cells in times of abundant FA supply (Okuyama and Lands, 1972; Chilton and Murphy, 1986). Moreover, studies have shown that diPUFA species made up of 20:4n-6 are preferentially hydrolyzed by cPLA2IV over 22:6n-3/22:6n-3 species. (Batchu, 2016). The C42 species of the hBMSCs containing 20:4n-6 coupled with 22:4n-6 or 22:5 (n-6 or n-3) resulting from 20:4n-6 addition have beneficial consequences. The COX-2 enzyme synthesizes 1a,1b-dihomo PGE2 from 22:4n-6, which has a much lower biological activity as compared to PGE2, thus deterring possible proinflammatory signaling (Zou et al., 2012; Dong et al., 2016). These results taken together imply that hBMSCs employ different biosynthetic and remodeling routes to regulate the level of free 20:4n-6 that may serve as precursor for the production of inflammatory linked eicosanoids.

The addition of exogenous PUFA to hBMSCs culture revealed the distribution patterns of these FA among the different GPL classes. Data from the mol% sums of all molecular species with specific PUFA species in each GPL class, indicated that in general n-3 PUFA supplements efficiently displaced 20:4n-6 in PE and PS in addition to halving the PC 20:4n-6 content (from 14% to 7%) (**Fig. 6B** and **C**). Exceptionally, PI class which had strict preferences for 20:4n-6, was replaced with 20:5n-3. In contrast, the addition of 20:4n-6 doubled its content in PC and PS with PE and PEp 20:4n-6 content also exhibiting

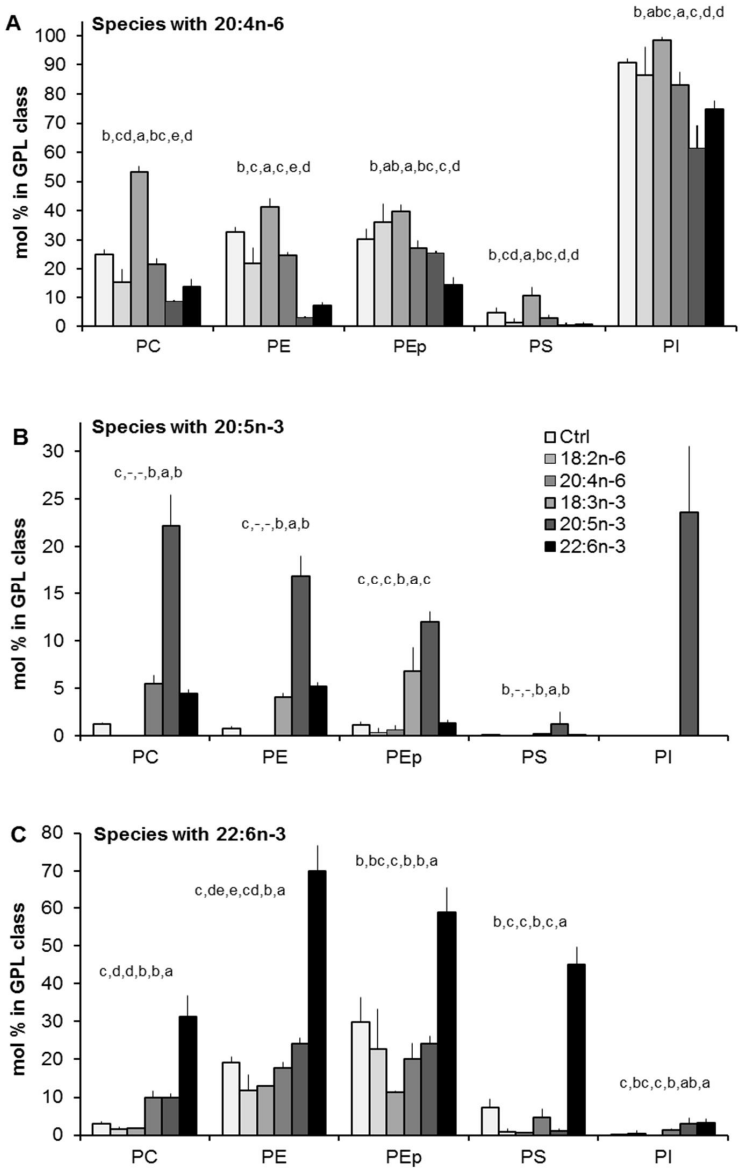

Fig. 6. Distribution of lipid species, having either 20:4n-6, 20:5n-3 or 22:6n-3 as their acyl residues, between different glycerophospholipid (GPL) classes in hBMSCs. Cells were grown in conditioned medium supplemented with either 18:2n-6, 20:4n-6, 18:3n-3, 20:5n-3 or 22:6n-3 and in the control (Ctrl) medium (mol % sums of all species with the specific PUFA in each GPL class). Distribution of GPL molecular species containing A: 20:4n-6 B: 20:5n-3 C: 22:6n-3 between the different classes. Statistics as in Fig. 3. **Adapted from Tigistu-Sahle et al., 2017 (Article II)**

a significant raise (**Fig. 6A**). The n-6 PUFA supplements in general decreased the 22:6n-3 content of the GPL classes.

The actions of ACS and acyl-CoA: lysophospholipid acyltransferases makes possible the reacylation and incorporation of 20:4n-6 into different GPL classes. In the remodeling route, exogenous 20:4n-6 and 22:6n-3 are incorporated into diacyl PC by these enzymes**.** The fact that 22:6n-3 supplementation successfully replaced 20:4n-6 content in hBMSCs GPL classes, is therefore logical because these two PUFAs are incorporated through the same remodeling system. Both the CoA-IT and CoA-dependent transacylases which catalyze the transfer PUFAs to different GPL classes exhibit preferences for specific FA (Yamashita et al., 1997). However, this specificity is only maintained at physiological concentrations and that other factors such as substrate/acceptor concentrations also determine PUFA distribution (Okuyama et al., 1975).

Basing the hBMSCs inefficiency to desaturate C18 PUFA precursors in the classic n-6 and n-3 PUFA biosynthetic pathway; we evaluated the incorporation dynamics of the C20 and C22 PUFA products instead. In this experiment we supplemented hBMSCs with either 20:4n-6, 20:5n-3 or 22:6n-3 for 2h, 6h and 24h (**III, Figs. 1-3**). Results from this unpublished data showed that the fastest relative incorporation rate (fold change) was observed for 20:5n-3 (2h mark) unlike 20:4n-6 and 22:6n-3 which had a steady rate of assimilation. Considering the GPL class profile changes, PC exhibited the initial remodeling modifications already after 2h supplementation (**Fig. 7**). A gradual and steady change in the PUFA acyl chains of PE molecular species was observed at 6h (**Fig. 7**). However, PS with predominantly 22:6n-3 containing molecular species exhibited remodeling effects between the 6h and 24h supplementation time (**Fig. 7**). The rate at which the major GPL class profiles were modified due to exogenous PUFA supplementation is in line with previous reports. Pulse-labeling studies with radioactive FAs indicated that turnover kinetics of PC to be the fastest, an intermediate rate for PE and much slower turnover rate for PS and SM (Gallaher et al., 1973; Spector et al., 1980).

Although the desaturation steps required to produce C20 and C22 PUFAs from their C18 precursors was limited in hBMSCs, the elongation steps however progressed successfully to produce respective long chain FA (**Fig. 7**). The profiles from FA analysis showed that the promptly incorporated exogenous 20:5n-3 increased its mol% by 7-fold in 2h time, compared to the levels of non-supplemented hBMSCs (**III, Fig. 1B**). However, the mol% of its elongation product 22:5n-3 changed considerably in between 6h and 24h time points. Similarly, 20:4n-6 supplementation increased the mol% of its elongation product 22:4n-6 only after the 24h time point (**III, Fig. 1A**). The GPL molecular species containing the elongated products, 22:5n-3 and 22:4n-6, increased their fold change values towards the 24h time point (**III, Fig. 2**).

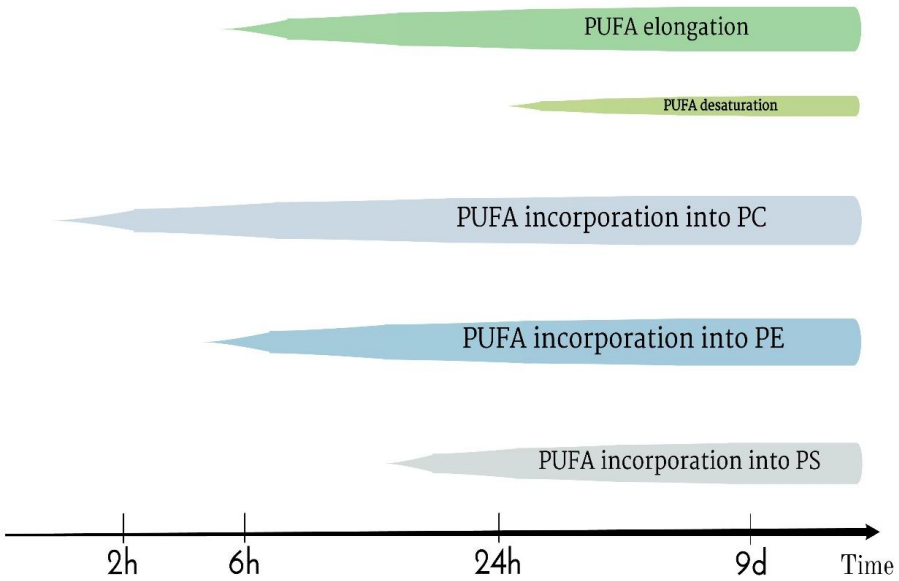

Fig. 7. The time scales of structural modifications and incorporations of PUFA into PC, PE and PS diacyl species by hBMSCs. The incorporation of exogenous PUFA into PC already happens in a 2h period followed by assimilation into PE and finally into PS just before the 24h mark. The elongation process starts after 2h of supplementation and proceeds steadily while PUFA desaturation, which is limited in hBMSCs as compared to HepG2 cells, occurs only after the 24h mark. The size of the arrows corresponds to the proportion of these cellular processes.

This results show that in hBMSCs chain elongation of the C20 PUFAs occur at a relatively slower rate than their incorporation and may require the initial accumulation of these shorter chain species in different GPL classes. The elongation products, 22:4n-6 and 22:5n-3 have inhibitory effects on COX-1 and COX-2 activities and consequently hinder the synthesis of eicosanoids (Akiba et al., 2000; Zou et al., 2012, Dong et al., 2016). For that reason, the gradual increase of 22:4n-6 and 22:5n-3 containing GPL molecular species may suggest a protective mechanism in hBMSCs to limit the availability of C20 eicosanoid precursors especially 20:4n-6, in order to avoid pro-inflammatory signaling. Therefore, the specific type of PUFA as well as the incubation time used to culture hBMSCs in such conditioned medium may determine the type of modifications at both GPL molecular species and FA profile levels. This in turn will have a ripple effect for instance in raft mediated signaling. This is because increased incorporation of either 20:5n-3 or 22:6n-3 into the plasma membrane results in alteration of raft domain size and composition, which in turn suppresses raft mediated cell function such as activation of T cells and transcriptional factors and secretion of cytokines (Turk and Chapkin, 2013).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Prior to the commencement of this study information regarding the lipidome profile of hBMSCs was missing. Attaining this type of data opens the opportunity to investigate the role of lipids in the maintenance of hBMSCs characteristics during long-term expansion that is required prior to clinical application. The work done in this thesis also aimed to contribute to the mounting evidence which suggests n-6 and n-3 PUFAs and their metabolites influence stem/stromal cell characteristics.

In this thesis work we were able to establish, for the first time the FA and GPL profile of hBMSCs. Comparative studies of the lipidome profile of hBMSCs from early and late passages indicated that long term *in vitro* expansion altered their FA and GPL profiles. The main findings included the increase in the relative mol% of GPL molecular species containing 20:4n-6 as acyl residues (PC38:4 and PE36:4) in the hBMSCs from late passages (p8 – p14). In addition, the long term expansion increased the ratio of PI/PS and n-6/n-3 PUFA. The FA profiles from GC-FID analysis showed that the enrichment in 20:4n-6 came at the expense of n-3 PUFAs such as 20:5n-3 and 22:6-3 which decreased considerably. Co-culture assays indicated that the ability of hBMSCs to suppress T cell proliferation was diminished because of long term expansion and this loss in functionality strongly correlated with the increased 20:4n-6 content of these cells. Expansion related lipidome changes in hBMSCs may have tipped the sensitive balance between n-6 PUFAs and n-3 PUFAs in the total FA pool of these cells, in such a way that may advance the production of pro-inflammatory mediators thereby diminishing hBMSCs' immunosuppressive functionality.

Evidence suggest that MSCs are influenced by the cues they get from their extracellular microenvironment. Therefore, we compared the FA profiles of hBMSCs to that of marrow and FBS (representative of the natural vs artificial microenvironment). The proportions of n-6 PUFAs were considerably higher especially in hBMSCs and FBS than the totals of n-3 PUFAs. Elevated ratios of n-6/n-3 PUFAs are associated with increased inflammatory activities in different types of cells and therefore signify the *in vitro* culturing conditions to be sub-optimal for hBMSCs features.

However, with the proceeding experiments we were able to show that supplementing the culture medium with 20:4n-6, 20:5n-3 or 22:6n-3 can successfully alter this important marker i.e. ratio of n-6/n-3 PUFAs in hBMSCs. Specifically, supplementing culture medium with 20:5n-3 or 22:6n-3 increased the proportions of n-3 PUFAs totals in hBMSCs in such a way that the initial

prevalence of n-6 PUFAs was diminished. Furthermore, abundant supply of hBMSCs medium with either 20:5n-3 or 22:6n-3 resulted in the replacement of 20:4n-6 from the acyl residues of GPL classes. Manipulation of hBMSCs' PUFA profile in such a way will subsequently modify the bioactive lipids which are generated from the membrane GPLs by the action of various phospholipases.

In conclusion, the expansion of hBMSCs is a crucial step in their therapeutic application and cannot be avoided. Our results suggest that the expansion induced changes in hBMSCs' lipidome negatively correlates with loss of immunomodulatory functions in these cells. PUFA-based modifications however, present an opportunity to create a favorable environment for hBMSCs. Regulating the cellular level of 20:4n-6 in hBMSCs is crucial to limit the production of various eicosanoids and subsequent induction of proinflammatory signals.

Future studies should focus on elucidating the specific effects of n-6 and n-3 PUFA derived metabolites on hBMSCs functionality as well as proliferative and differentiation capacity. In addition, direct effects of different PUFAs on membrane micro domain structure (size and composition of rafts) and the consequences of the altered lateral organization of the membrane on cellular functions should be studied. Furthermore, characterization of the specific effects of n-6 and n-3 PUFAs on hBMSCs' metabolism of soluble factors such as IDO, PGD2 and HLA-G5 that have a role in their functionality should also be considered. Finally, the formulation, dosage and timing of PUFA supplementation should be taken into consideration in order to successfully manipulate the lipidome profile of hBMSCs and create a favorable *in vitro* environment which advances their functionality.

ACKNOWLEDGMENTS

The project work for this thesis was carried out at the division of Physiology and Neuroscience in the department of Biosciences, Faculty of Environmental and Biological Sciences at the University of Helsinki. I would like to thank TEKES – the Finnish Funding Agency for Innovation (Intelligent Monitoring of Human Health and Well-being –research programme managed by SalWe Oy), Finnish Red Cross Blood Service and Helsinki University Lipidomics Unit for funding this thesis work. Special thanks to Professor Juha Voipio, Professor Kurt Fagerstedt, and Professor Jukka Partanen for providing the excellent resources and support during this research period.

Foremost I would like to express my sincere gratitude to my supervisor Docent Reijo Käkelä, for providing the opportunity to be part of the research group and for introducing me to the fascinating world of lipids. I appreciate all your contributions in knowledge, time and experience as well as the immense patience with which you have adorned my research journey of these past eight years. Thank you for being the buffer between the ever stressful research environment and for teaching me to always prioritize quality of work above everything else.

A well-deserved thanks to my other supervisor Ph.D. Saara Laitinen for providing the opportunity to work on such an interesting project. Thank you for diligently guiding my research work with your knowledge and experience on stem cell biology all throughout these years. I deeply appreciate the dedication with which you provided the resources and support that made my research journey worthwhile.

My heartfelt gratitude to the thesis follow up committee members Docent Eeva-Liisa Eskelinen, Professor Jyrki Kukkonen and Professor Petri Lehenkari for their support during this research journey. I especially thank Petri for his thoughtful opinions all throughout my thesis work, thank you for lending an understanding and genuine support whenever I asked for it. Pre-examiners Docent Tomas Blom and Associate Professor Susanna Miettinen have helped in improving the thesis summary to its final stage, thank you for your constructive comments and for delivering the task in short time. I would also like to thank the co-authors in my scientific publications especially Lotta Kilpinen and Minna Holopainen for their significant contributions.

I thank all the long-term members of our lipidomics unit, Hanna Ruhanen, Malin Tverin, Melissa Westberg, Patrik Tang and Alexander Abrahamsson, and all other past members of our group here in Viikki; you all have been such a delightful addition to my life, am grateful to have worked alongside such bright minds. My academic parallel Maarja Laos you have been a ray of happiness and positivity in the toughest last months of this work, thank you for being you. In addition, I will also like to thank Birgitta Rantala and Sanna Sihvo for their efficient cooperation during these projects.

A good support system is important for surviving the hardships of a PhD life and for that I was blessed with the friendships of Heaven Kelka, Feven Teshome and Bethlehem Adugna, thank you girls for being my sounding board and for lending a tireless shoulder to lean on.

Last and by no means least is my gratitude to my loving family. My parents, Col. Tigistu Sahle and Workienesh Mebrate, you were and will always be my strength in life the reason for my being. Thank you for giving me the best in life, and most of all for believing in me. My amazing siblings Adiam, Lidya and Messay, because of you all I made it this far without giving up, I thank you from the bottom of my heart. I will forever be thankful for my soul sisters, Ph.D. Addishiwot Girma and Fiona Teshome for keeping me reassured, motivated and for being my pillars of strength since childhood. Love you all.

In loving memory of Emaye, my life!

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Helsinki 2017 ISSN 2342-3161 ISBN 978-951-51-3133-1