Lipidomics for Human Bone Marrow Mesenchymal Stem Cells

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ABSTRACT

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Tiivistelmä — Referat — Abstract

In addition to being structural components of biological membranes and energy storage of cells, lipids have recently been found to participate as essential players in cell signaling, subcellular transport mechanisms, adjusting functions of integral proteins, and regulation of cell growth and apoptosis. In this study electrospray ionization mass spectrometry (ESI-MS) techniques were used to analyze the phospholipid composition of human bone marrow derived mesenchymal stem cells (BMSC). Numerous chemically distinct lipid species were quantified and the changes in their relative amounts *i.e.* in the cell's lipid profile after sequential passaging were followed until senescence (usually from passage 4 up to passage 10, in some cases until p14). Subsequently, the total lipids extracted from the cell pellets were analyzed by triple quadrupole ESI-MS equipment and using lipid-class specific scanning modes. The BMSC lines studied originated from ten donors, five of which were young and five elderly individuals.

In culture, the BMSC from both young and aged donors showed time-dependent changes in their phospholipid profiles. The clearest marker findings among individual lipid species were that in phosphatidylcholines (PC) and phosphatidylethanolamines (PE), the species 38:4 (acyl chain pair 18:0/20:4n-6) largely increased towards the late passages, which was seen in the BMSC derived from both the young or aged donors. Thus the reserves of 20:4n-6, the precursor of the eicosanoids having antiproliferative, apoptotic and inflammatory cellular reactions, were increased towards late passages. At phospholipid class level, lysophosphatidylcholine (LysoPC) and phosphatidylinositol (PI) totals, and the ratio of total PI to total phosphatidylserine (PI:PS) were increased from early to latest passages. The results provide new lipid biomarkers to be used for stem cell quality control. The accumulation of polyunsaturated lipid species containing 20:4n-6 or the increase of PI: PS ratio could be potential markers for cell aging and the cells' poor viability and functionality. The results can be used to develop efficient stem cell therapies and improve patient safety.

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ABBREVIATIONS

BMSC = human bone marrow mesenchymal stem cell

ESI-MS = electrospray ionization-mass spectrometry

LysoPC = lysophosphatidylcholine

PC = phosphatidylcholine

PC1, PC2 = principal component 1, principal component 2

PCA = principal component analysis

PCe = phosphatidylcholine ether lipid

PE = phosphatidylethanolamine

PEe = phosphatidylethanolamine ether lipid

PI = phosphatidylinositol

PLA_2 = phospholipase A_2

PS = phosphatidylserine

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1 Introduction

1.1 Lipids

Definitions

Lipids can be defined based on either ***A)*** their **functionality** as ‘‘an important class of compounds that have a wide variety of key cellular functions including compartmentalization, energy storage, cell signaling, protein trafficking and membrane anchoring’’ (Orešič *et al.* 2008, van Meer 2005, Vance and Vance 2004), or ***B)*** their **solubility** into organic solvents (subdivision into amphiphatic or hydrophobic lipids) and **origin** (hydrophobic chains synthesized either via condensation of malonyl or isoprene units) (Fahy *et al.* 2005).

1.2 Composition & Classifications

Though lipids as a class of biological molecules, constitute a highly heterogeneous group of substances with a wide variety of structures, nevertheless a common ground exists in that all contain a major domain of hydrophobic molecule. Lipids typically contain either long hydrocarbon chains, *i.e.* fatty acids and isoprenes, or multiple linked rings, as in steroids (Alberts *et al.* 2008). The predominant types of interactions common to lipids are the van der Waals and hydrophobic effects in addition to their amphipathic nature which allows hydrogen bonding and electrostatic interactions. The broad variation in structure and biological function of lipids makes the task of classification arbitrary. Regardless of this difference, various attempts have been made. For instance, Fahy *et al.* (2005) has broadly classified lipids based on their hydrolysis end-product as either ‘‘simple’’ lipids for those yielding at most two types of compounds, and ‘‘complex’’ lipids which give three or more different products. This classification scheme can further be categorized into subclasses (Fig.1) based on the chemical nature and distinct hydrophilic elements (*i.e.* head groups) of lipids.

Triacylglycerols and waxes are representatives of simple neutral lipids formed when fatty acid chains esterify hydroxyl groups of glycerol or longer chain alcohols, respectively. The triacylglycerols are the major energy reserves enclosed in fat droplets

in the cytosol of adipose and other cells, in addition to serving as excellent insulators. On the other hand polar lipids such as phospholipids and glycolipids are important constituents of cell membranes. The glycerophospholipids' structure consists of a glycerol backbone, two fatty acid chains and a polar alcohol group, such as choline and ethanolamine, linked via a phosphate group (Murphy & Axelsen 2010). Sphingolipids constitute a class of complex lipids which have sphingosine alcohol for a back bone instead of glycerol. This lipid class plays an important role in structural and regulatory actions of cell membranes (Ozbayraktar *et al.* 2009). Last in this classification, the glycolipids constitute those complex lipids that are derivatives of ceramides just like the sphingomyelines but differ in their hydrophilic headgroup which is composed of saccharides. Glycolipids, situated in the outer surface of the plasma membrane function as antigens and receptors, assist cell-cell adhesion, modulate signal transduction along with performing the essential task of organizing and sorting membrane proteins (Hakomori 2008, Sprong *et al.*2001).

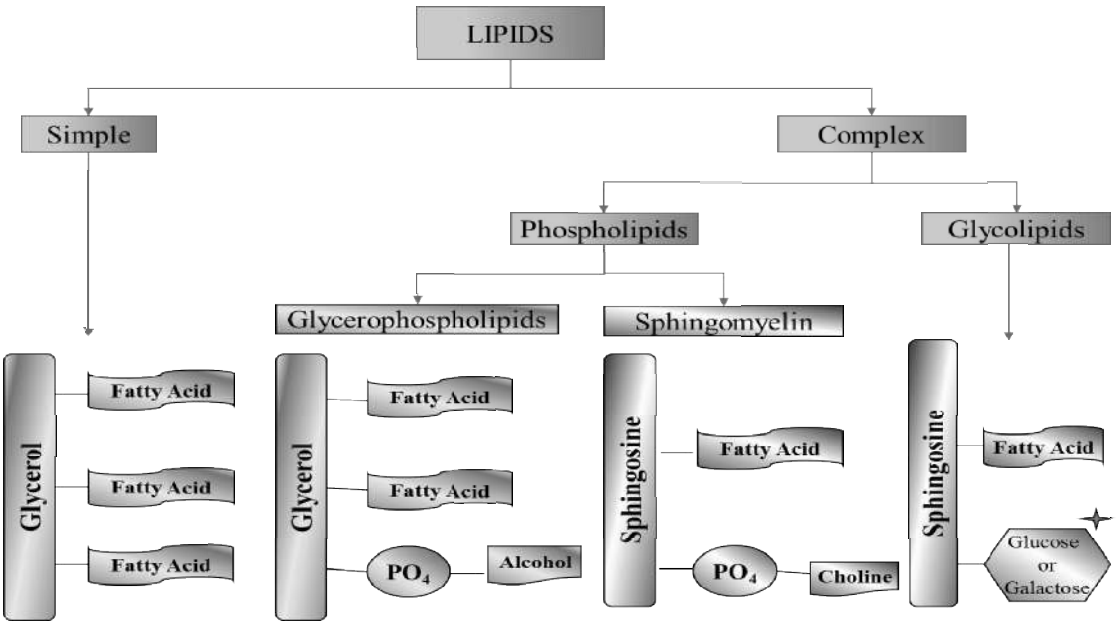


Figure 1 Schematics of Classification of the Most Abundant Cellular Lipids (Adapted from Pursell, D. P. 2009) ✦ **One or more saccharide groups**

The glycerophospholipids, of special interest to this study, contain various types of glycerol-alkyl chain linkages such as ester, ether and vinyl ether bonds which account for the additional diversity among its class (Yetukuri *et al*. 2010). Their amphiphilic nature comes as a consequence of replacing one of the fatty acids in a triacylglycerol with a polar group (Lindblom 2005). Glycerophospholipids are further diversified by the alteration of their polar head group into the following:

Phosphatidylcholines (PC) – the most abundant phospholipid and the key membrane building block. Approximately 50% of the total phospholipid present in membranes of most animal tissues is PC, a neutral zwitterionic phospholipid, which makes up the majority of the outer leaflet of the plasma membrane (Deenen 2006). PC also serves as the biosynthetic precursor for sphingomyelin, phosphatidic acid, lysophosphatidylcholine and platelet-activating factor (Vance & Vance, 2004).

Phosphatidylethanolamine (PE) – the second most abundant phospholipid in a mammalian cell constituting approximately 15% – 25% of the total (Meer 2005). It is also a neutral zwitterionic molecule with a conical geometry due to its small sized polar headgroup (Dowhan 1997). Membrane fusion and maintenance of the functional structure of integral membrane proteins is mainly attributed to non-bilayer forming lipids like PE (Dowhan 1997). PE contributes to the lateral pressure by introducing some stress or strain in the membrane bilayer as a direct consequence of its small head group and conical shape. Consequently this influences membrane proteins' conformation and their functionality as well (Kruijff 1997, Landau & Rosenbusch 1996, Marsh 2007).

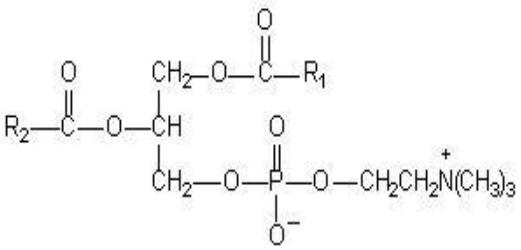
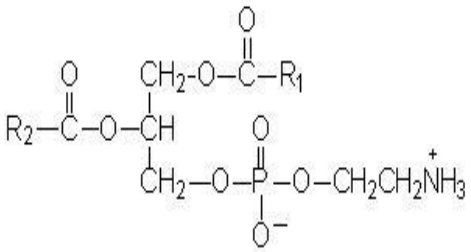
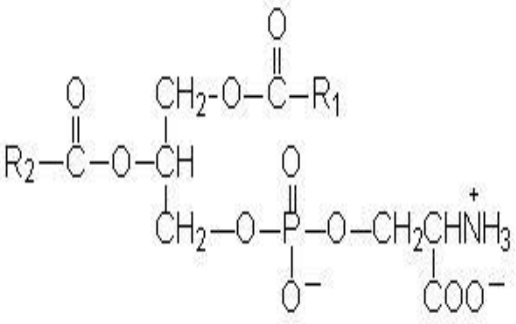
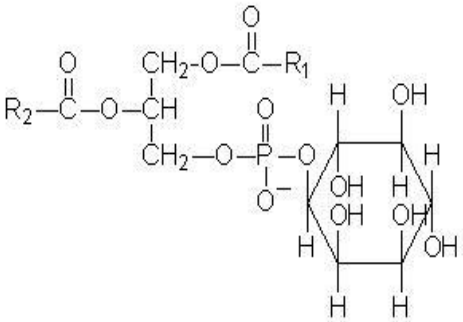
Phosphatidylserine (PS) – quantitatively a minor class of the membrane glycerophospholipids, PS amounts to 2% - 10% of the total phospholipids in a typical mammalian cell (Vance & Steenbergen 2005). PS is predominantly located on the cytosolic leaflet of the plasma membrane along with PE and PI (Farooqui & Farooqui 2009). Subsequently, its appearance in the outer leaflet is an apoptotic signal for clearance of cell corpses by phagocytosis (Fadok *et al*. 2000; Züllig 2007). Moreover,

PS acts as an essential cofactor and activator of protein kinase C in the presence of Ca^{2+} and sn-1, 2-diacylglycerol (Bell 1991).

Phosphatidylinositol (PI) – exclusive to eukaryotes, this group of phospholipids makes up around 5% of membrane lipids (Vance 2002). These are acidic phospholipids with a phosphatidic acid backbone that were first recognized for their role as precursors of secondary messengers. This function is a direct result of PI's unique ability to be reversibly phosphorylated at three distinct positions (Wymann & Schneiter 2008). Products of this phosphorylation then serve as second messengers that initiate activation of protein kinases and release of Ca^{2+} as signaling cascades. Furthermore, soluble inositol polyphosphates (PIPs), mainly inositol 1, 4, 5-trisphosphate, have various functions ranging from gene transcription and RNA editing to modulation of cell growth, proliferation and protein phosphorylation (Paolo & Camili 2006). Phosphoinositides ability to generate constitutive signaling is employed as a detection code that is needed to define organelle identity. This characteristic stems from its ability to cooperate with one or more additional binding sites within the membrane forming a very stable membrane-protein interaction which in turn serves as a detection code in regulation of membrane-cytosol interactions and thus for the definition of organelle identity (Paolo & Camili 2006).

Lysophosphatidylcholine (LysoPC) – is a polar phospholipid generated from partial hydrolysis of PC by the enzyme phospholipase A2. Studies have shown that LysoPC functions as an immunomodulatory agent by inducing the expression of multiple genes essential for inflammatory action in variouse cell-types such as endothelial and T- cells (Matsumoto *et al.* 2007). As one of the essential lipid components of plasma, LysoPC assists in the transport of polyunsaturated fatty acids and choline to tissues. On the other hand, LysoPC can also have cytotoxic effects if its level is not regulated causing pathological conditions such as ischaemia and aretherosclerosis (Croset *et al.* 2000).

Table 1 Structure of major phospholipid classes (modified King 2011)

Phosphatidylcholine (PC)	Phosphatidylethanolamine (PE)
	
Phosphatidylserine (PS)	Phosphatidylinositol (PI)
	

1.3 Functions of lipids

Whereas proteins and genes are known to be very specific to the functions they perform, lipids on the other hand as membrane building blocks and fat depots, have been thought to play a fairly nonspecific role (Mouritsen 2005). This biased concept of lipids comes from the lack of an obvious link between molecular structure and function as compared to proteins whose function is readily governed by their structure. However, this view has changed considerably in recent years and now the central biochemical importance of lipids expands far beyond what was first presumed.

1.3.1 Lipids as Building Blocks

Lipids are most commonly recognized for their role in building membrane bilayers by self-assembly processes (Mouritsen 2005). Although biological membranes differ greatly in function and lipid composition, they all have the same basic construction principle consisting of a lipid bilayer into which proteins are inserted in a unique orientation (Tamm et al. 2002). These lipid bilayers of living cells are responsible for providing the fundamental architecture that delineates boundaries as well as for interactions of the cell with its environment. Furthermore, membranes are used to compartmentalize cells into different functional units, for protein synthesis and secretion and to support signal transduction and vesicular transport via fusion (Fagone & Jackowski 2009). The semi-permeable membranes tightly regulate the transport of various solutes across the bilayer via passive and active transport mechanisms (Cross 2001). Perhaps, the most important and recently discovered role of membrane lipids is their ability to modulate membrane protein functionality via covalent attachment to proteins (Martin 2001). Last but not least lipid bilayers provide communication platforms for cells to interact with the extracellular environment through signal transduction process mediated by transmembrane proteins (Salvaterra 2001). All this diverse functionality of membranes arises from different structural and chemical behavior of lipids.

1.3.2 Lipids as Energy Stores

The role of energy storage in lipids is most evident when considering adipose tissue which is the major long-term energy source in mammals. A typical adipocyte contains one or more cytoplasmic triacylglycerol-rich lipid bodies or droplets which are synthesized in specialized regions of the ER (Murphy & Vance 1999). Lipids are the preferred type of energy store because they enable a much larger reservoir of energy compared to carbohydrates or proteins. This in turn is a direct consequence of triglycerides ability to be stored in anhydrous thus less bulky form in adipose tissue. During oxidation of fatty acids ATP is generated yielding an energy source two-fold greater than other sources (Tronsted & Berge 2004).

1.3.3 Lipids as Signaling Molecules

After the Singer-Nicholson model (fluid-mosaic) of a uniform plasma membrane was proposed, it became evident that eukaryotic cell membranes were of heterogeneous composition and that certain lipids and proteins tend to form a more liquid ordered phases or micro-domains called *lipid rafts* (Pike 2009). These dynamic domains can sequester a subset of signaling proteins which preferentially partition into them and change their local concentration, composition and conformation according to the metabolic state of a cell (Rajendran & Simons 2005, Simons & Toomre 2000). Furthermore, lipid rafts on the cell surface, enriched with cholesterol and glycosphingolipids, have been implicated in such cellular processes as signal transduction, membrane trafficking, cytoskeletal organization, and pathogen entry (Hanzalbayer & Hancock 2007). The raft domains are ideal platforms for transmembrane signaling because many surface receptors tend to associate with them upon binding appropriate ligands. For instance, the mammalian inflammatory response mechanism sequesters several immune receptors to raft domains upon antigen presence. This binding by ligands leads to phosphorylation by Src-family kinases which in turn initiate a series of signaling cascades leading to immune cell activation (Cherukuri *et al.* 2001, Sedwick & Altman 2002). In addition, lipid rafts nowadays are implicated to have functional significance to different ion channels, which localize within these microdomains, such as K^+-, transient receptor potential- and Ca^{2+}-channels (Martens 2004). The mechanism employed by rafts could either be direct through protein–lipid interactions or indirect via post-translational modifications (Dart 2010). A common example here is PIP_2 modulation of ion channels through its ability to connect channels to various signaling mechanisms (Hilgemann et al. 2001). The ability of rafts to differentially associate signaling proteins with their ion-channel counterparts could serve as an efficient cell-surface compartmentalization technique (Martens 2004). Finally, it is worth noting that lipids role in cellular signaling extends beyond that of receptor activation, to that of a second messenger. For instance, opening of a calcium channel in the endoplasmic reticulum is followed by a signaling pathway which leads to receptor activation in the plasma membrane. This activates phospholipase C, which in turn

cleaves PIPs to produce diacylglycerol and inositol phosphate both of which can act as second messengers (Fisher & Jain 2009).

1.4 Lipidomics

The previous scientific revolution eras, i.e. genomics and proteomics, have resulted in major developments towards sequencing genes and identifying the multitudes of protein functions. Currently, a third and previously less explored science is coming into the metabolomics field, called *lipidomics*. This field owes its emergence and development to the advent of novel mass spectrometric techniques as well as sound theories on the structural and functional heterogeneousity of biological membranes. Thus with such effective tools, it is now possible to study lipids at the molecular level which in turn enables a more in-depth analysis of complex lipid mixtures in various biological matrices and metabolic pathways of lipids as well as their role in cell signaling (Fernandis & Wenk 2009). Moreover, modern lipidomics can sensitively identify changes in a cell's lipidome after cellular perturbations (Meer 2005). Consequently, lipidomics can now be applied for the development of biomarkers that are indicators of certain cellular functions.

1.5 Role of Lipids in Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) belong to multipontent type of adult stem cells which can differentiate to several cell lineages including osteocytes, chondrocytes, myocytes and adipocytes (Rubio *et al.* 2005). The plasticity of these cells makes them excellent candidates for tissue regeneration processes for normal turn-over, and to repair diseased or aging tissues (Mangi *et al.* 2003, Horwitz *et al.* 2002). Furthermore, MSCs can provide important cues for cell survival in damaged tissues, and also modify the response of immune cells (Blanc & Pittenger 2005). However, despite the extensive progress on their isolation and differentiation techniques, the full potential of MSCs is not yet realized due to the fact that there are still no reliable or consistent cell markers available for the identification of mesenchymal stem cells (Minguell *et al.* 2001).

Besides, gene expression and surface markers may differ between the original cell population and expanded MSC pool.

At present, lipidomics research aims to assist in the discovery of biomarkers in order to guarantee the proper identification and isolation of MSCs in addition to genomic and proteomic markers. There is evidence showing that the phospholipid composition of cells, especially that of stem cells, changes significantly during apoptosis (Fuchs *et al*. 2007, Xu 2000). Such cellular profiles can thus serve as a reliable measure of cell integrity as well as stem cell differentiation that can assist in the proper identification and separation of MSCs from other progenitor cell population (Fuchs *et al*. 2008).

1.6 Objectives

Eukaryotic cells' ability to interact with their external environment is mainly governed by the membrane localization and function of several signaling proteins which in turn are dependent upon their covalent modification by specific membrane lipids. This same phenomenon is also partly responsible for regulating proliferation, differentiation and migration of stem cells, which are the current focus of regenerative medicine. In order to accomplish this however, it is first necessary to understand the characteristics of stem cells, with regards to differentiation and proliferation potential. Therefore, there is a need for the efficient identification and isolation of a specific cell population, i.e. ascertain the stem cell identity before transplanting processes.

This project aims to provide potential characteristic BMSC biomarkers that can properly identify these pluripotent cell lines from the residual cells. The proposed hypothesis for this project was that compositional changes in stem cell lipidome profile from early to late passages could be a possible indicator of aging of the cells. Based on this, the main aim of the project was to identify healthy functional human mesenchymal stem cells. The specific goals for this thesis work were the following;

- Determine the phospholipid profile of the studied BMSC lines at class level

- ♦ Determine the species profile of the selected major phospholipid classes in each BMSC line and between the young and old donors as well.
- ♦ Perform a comparative analysis of the above data to be able to detect lipidome changes from early to late passages of the hBMSC
- ♦ Find functional implications for the observed lipidome changes from early to late passages of hBMSC.

Previous work on the lipidomics of stem cells has been insufficient to study the effects of sequential passaging on MSCs' phospholipid profile. Comparative analysis of stem cell lipidome profiles associated with a specific physiological condition, such as aging of the cells, can provide key markers for their functional stages. Furthermore, these types of biomarkers are essential for qualitative control of MSCs products, which in turn determines the effectiveness and patient safety during transplantations.

2 Materials and Methods

Human bone marrow mesenchymal stem cell lines (BMSC) were acquired from Red Cross Blood Service research and development department for this study. The BMSC samples were harvested, after informed consent, from ten individuals of which five were between the ages of 62 to 82 years (mean 74.6years) and the remainder five came from younger donors all under the age of 25 years (mean 23.2years). For the protection of anonymity the cell lines were coded 164, 172, 194, 268 & 271 for older donors and 81, 88, 89, 91 & 92 for younger donors. The BMSC cells were harvested up to 10 passages for cell lines 164, 172, 194, 91 & 92; cell lines 81 & 88 up to 12 passages; and the remaining cell lines 268, 89 and 271 were cultured up to 8, 9 and 14 passages respectively. The cell culturing was stopped when cells start dividing at a much slower rate and eventually senescence was reached. The cell pellet samples were marked with their codes and passage numbers. It should be noted that prior to cell culturing it was demonstrated at the Red Cross Blood Service that the BMSC were able to proliferate and subsequently differentiate (in-vitro) into different mesenchymal cell types, such as adipocytes, myoblasts, chondrocytes, tendon cells and osteoblasts.

2.1 Tissue Culture

BMCS samples were thawed at passage four and maintained in growth medium containing the following reagents; 86% α-MEM, 2% 1M HEPES buffer, 10% fetal bovine serum certified (FBS/msc), 1% Penicillin – Streptomycin 10000 U/ml and 1% L-Glutamine 200mM (100X), all purchased from Gibco Invitrogen, Paisely, U.K. The samples were plated into 75cm^2 tissue culture flasks and maintained in the above mentioned growth medium at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was removed and cultures were washed with Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS; Gibco), after waiting for two days that is needed to ascertain the cell-attachment. The cell culture continued for up to three weeks (late passages), meanwhile changing the medium twice a week until 70% - 80% confluence was reached. The cells were sub-cultured further at density of 1000 cells/ cm^2, from passage 4 until

senescence was attained. For lipid analysis, the human MSCs were washed with ice-cold phosphate-buffer saline (PBS), scraped on ice and transferred to silylated tubes. The adherent cells were detached using trypsin-EDTA solution (Gibco).

2.2 Total Lipid Folch Extraction

The cultured cells were stored at -70°C for a period of 4 to 8 months. The total lipids for each sample were extracted according to Folch *et al.* (1957). Initially the cell pellets were removed from the cold storage unit and allowed to warm up to room temperature. Next 400 µl millipore water was added to the cell pellet inside the kimax tube and vortex mixed. Consequently 3ml Methanol and 6ml Chloroform were enumerated onto the mixture and vortex mixed thoroughly after each addition. The solution was then incubated in the dark for 30 minutes, so as to let the lipid enter the solution, before centrifuging at 3000 rpm for 10 minutes that helped in precipitating the proteins to the bottom. The resultant clear supernatant was transferred into another tube and additional 1.8ml millipore water was put in and vortex mixed. Again this solution was centrifuged at 3000 rpm for 10 minutes resulting in the separation of the solution into upper and lower phases. The lower chloroform phase containing the lipids was transferred into another kimax tube and 4.5ml theoretical lower phase (Chloroform/Methanol/Water 86/14/1 by volume) was added to the remaining upper phase and vortex mix. This mixture was centrifuged for 10 minutes at 3000rpm and the resultant lower phase was extracted and mixed with the previous lower phase. Finally, the solution was evaporated to dryness under nitrogen flow, 1.5ml Chloroform/Methanol 1:2 were added immediately, and the sample solution was stored in a silylated vial in a freezer.

2.3 Lipid Standards

The MS instrument response for different head group classes varies greatly depending on many structural features of lipids, overall lipid concentration, solvent composition and instrument settings (Koivusalo *et al.* 2001). The fact that such a variety of factors affected the instrument response necessitated the addition of several internal standards

for each phospholipid class in order to obtain truly quantitative data. The dependency of the instrument response on variable chain length was corrected by including both short and long chain standards for each class of lipids analyzed. Moreover, the standards selected were mainly di-unsaturated molecular species mimicking the average degree of unsaturation of the natural lipid species as close as possible. This measure is to ascertain correct calibration of the instrument response. The internal standards (Table 1) were added to the solution prior to infusion.

Table 2 Lipid species included in the cocktail of internal standards

Lipid Standards	Monoisotopic molecular weight (m/z)	Concentration (pmol/µl)	Source
PC 14:1/14:1	674	50	Avanti Polar Lipids (Alabaster, AL, USA)
PC 20:1/20:1	842	50	
PC 22:1/22:1	898	50	
PE 14:0/14:0	636	30	
PE 22:1/22:1	856	30	Synthesized (Phospholipase D mediated transphosphatidylation)
PS 14:1/14:1	676	10	
PS 20:1/20:1	842	10	
PI 18:1/18:1	863	5	Avanti Polar Lipids (Alabaster, AL, USA)
PI 17:0/14:1	793	5	Lipid Maps (via Avanti Polar Lipids)
LysoPC 14:1	465	5	Avanti Polar Lipids (Alabaster, AL, USA)
LysoPC 22:1	577	5	

2.4 Triple quadrupole Mass Spectrometry (Tandem MS/MS)

The samples were stored at -70°C for a period of 2-3 months. The lipid molecular species profiles were analyzed by ESI tandem MS using a triple quadrupole instrument (*Micromass Quattro Micro, Manchester, UK*). The solution, containing the sample, along with the standard mix, NH_3 and Chloroform/Methanol solvent, was introduced into the equipment using a Harvard syringe pump at a rate of 7µl/min. The addition of NH_3 helps in the ionization of certain lipids and prevents formation of Na adducts (those molecules that are ionized by the addition of a sodium cation Na^+ rather than a proton H^+). The sample solution was infused into a bath gas at the tip of a thin capillary set to a very high voltage (4kV, either positive or negative). Consequently a charged spray is formed. The lipids at the surface of the evaporating droplets of the spray get ionized and travel down a pressure and potential gradient towards the vacuum orifice and finally enter the mass analyzer parts.

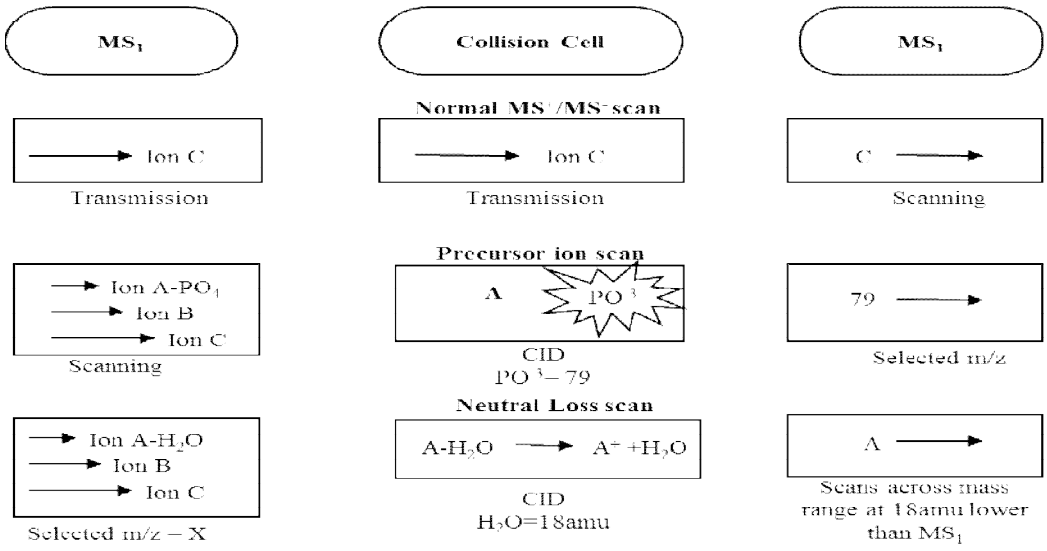


Figure 2 Scanning modes of analysis in a triple-quadrupole mass spectrometer. (Adapted from Graves & Haystead 2002)

This MS/MS instrument is equipped with three quadrupoles (Fig.1) of which the first (MS_1) and the last one (MS_3) act as mass analyzers while the second quadrupole (MS_2) operates as a collision cell. In the case of single-stage MS mode only MS_1 or MS_3 operate to produce positive or negative mass spectra of molecular ion species of polar lipids only. If collision induced dissociation results in ***A)*** *loss of the head group* as a *charged fragment* then the preferred scanning is the ***Precursor Ion mode*** in which the second mass analyzer (MS_3) is set to transmit only ions of the m/z value of the selected fragment ion and the first mass analyzer (MS_1) scans precursor masses. On the other hand if collision induces ***B)*** *loss of the head group* as *a neutral fragment* then ***Neutral Loss scanning mode*** is the preferred detection type. Here both mass analyzers i.e. MS_1 & MS_3 scan simultaneously in a synchronized fashion however MS_3 scans at a set offset from the first one. This offset is equivalent to the characteristic neutral loss for that specific class of compound. The samples were analyzed using internal standards and the aforementioned class specific detection modes, i.e. Precursor ion and neutral loss scans (Table 2). The selected scanning mode, detecting either a neutral fragment or fragment ion is characteristic to the lipid class of interest. Source temperature was maintained at 80°C and each run took approximately 3minutes.

Table 3 Summary of the phospholipid class-specific scan modes

Specificity	Polarity	Scan Mode	MS Range (m/z)	Collision Energy	Capillary Energy
PC & LysoPC	Positive	P 184	400 - 950	35	3.8kv
PE	Positive	NL141	600 - 900	19	3.8kv
PS	Negative	NL87	660 - 900	27	4.0kv
PI	Negative	P241	720 - 940	70	3.7kv

P= precursor ion scan, NL = neutral loss scan

2.5 Data Analysis

In quantitative mass spectrometric analysis of lipids the rate-limiting step, irrespective of the method used, is often data analysis (Koivusalo *et a*l. 2001). The paragraphs below include explanations for the steps taken and the MS software applied to remedy this limitation.

2.5.1 Acquisition of MS data

A software known as **MassLynx 4.0** (Waters Corp. Milford, MA) controlled the triple quadrupole MS as well as the data acquisition process. A new project file was created which contained the method files that configured the instrument settings for each type of detection and data acquisition used for the BMSC samples. The MS method settings were adjusted to six different modes i.e. MS+, MS- and the four class specific scan modes mentioned in **Table 1**, before the process of data acquisition was set to start. The data were represented by a 2-dimensional contour map, whereby the mass-to-charge (m/z) is plotted on the x-axis while the intensity is drawn on the y-axis.

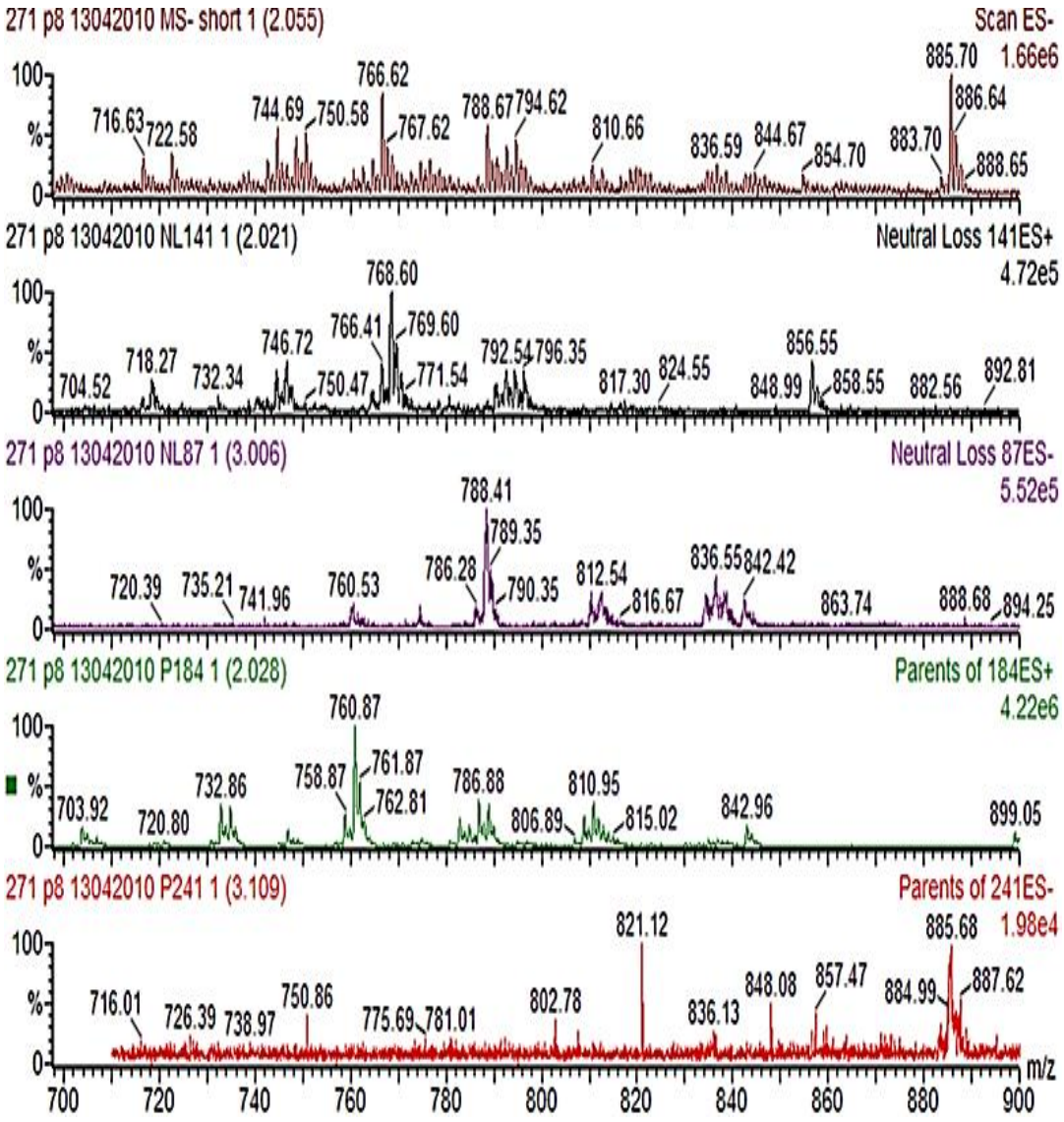


Figure 3 MS/MS spectra of cell line 271 at passage 8 taken from MassLynx 4.0 the spectra are overlaid and partial with mass range of 700m/z – 900m/z.

2.5.2 MS data analysis

The huge amount of data gathered by this analytical instrument was processed by a program called "**LI**pid **M**ass **S**pectrum **A**nalysis", **LIMSA** (Haimi *et al.* 2006). The

standard data processing steps that followed include peak detection, lipid identification, isotope correction of ^{13}C (one of the naturally occurring isotopes of ^{12}C with one atomic mass unit higher), detector response correction and quantification. Initially the raw data are exported to Microsoft Excel having LIMSA as an add-in program, which finds and integrates peaks in the mass spectrum pasted as list of masses and peak intensities. This program with its convenient user interface works as a Microsoft Excel add-in program and consists of dynamic library, written in ANSI C++, for identification, deconvolution, and quantification of lipid species from MS data. A list consisting of compounds with names, sum formulas and isotope abundances for relevant atoms obtained from the internal lipid database, as well as quantities of internal standards are prepared as part of the input for the LIMSA program. Peak finding and deconvolution of the overlapping peak patterns are done to obtain the corrected peak areas of the individual lipid species. The problem of isotopic overlap of lipids having mass values close to each other is corrected by using different algorithms such as linear fit model or Gaussian peak model. Finally, the found compounds and their concentrations are presented on a sheet in graphical and tabulated form.

2.5.3 Statistical analysis

Relevant information was extracted from the available multivariate data matrix using a type of applied mathematical modeling approach known as **P**rincipal **C**omponent **A**nalysis (PCA) available in a software package SIRIUS 6.0 and 7.0 (Pattern Recognition Systems, Bergen). This unsupervised method of mathematical modeling for exploratory analysis is useful to identify patterns or capture trends in high dimension data when there are correlated variables. PCA also helps in expressing the data in such a way that it highlights their similarities and differences by reducing the number of dimensions of the data without much loss of information. This reduction is brought about by creating new set of variables which are linear combinations of original variables finally resulting in specific principal components. The resultant principal components are independent and uncorrelated to each other with the first one set in the direction of the greatest variance in the data. Meanwhile, subsequent components are constructed orthogonal to the previous principal component in the direction of largest

remaining variance, with each new component accounting for progressively smaller and smaller amounts of variance. A principal component analysis proceeds in this fashion till the end, finally resulting in components that display varying degrees of correlation with the observed variables while at the same time remaining uncorrelated with one another. In addition to multivariate data analysis, an independent samples t-test was conducted to compare phospholipid species mol% values in initial passages and final passages of BMSC from young and aged donors.

3 RESULTS

BMSC samples obtained from the ten young and aged donors were subjected to ESI-MS and statistical analysis. Tandem MS produced ion spectra that are generated from two types of fragment peaks upon collision: headgroup fragment peak as in the case of PC, LysoPC and PI; or fragment peak detected from the neutral loss of the headgroup as in PE and PS. Exception was made to PEe which had no specific detection mode and thus these lipids were detected by using single stage scans in negative ion mode. The immense amounts of ion spectra or data acquired from ESI-MS were further statistically analyzed by the MassLynx and LIMSA programs to yield mol% profiles. Subsequently lipidome profiles for the BMSC samples' phospholipid class and their species were obtained.

3.1 BMSC Lipid Class Profile

The phospholipid class compositions for the BMSC samples from the young and aged donors were studied separately (Figs. 4, 5). The class totals were summations of mol% of individual molecular species from each phospholipid group identified in a cell line. Zwitterionic phospholipids PC (40-55%) and PE (34-48%) were the main phospholipid classes present in the BMSC samples. The remaining PI, PS and LysoPC classes had a relatively smaller portion (9-18%).

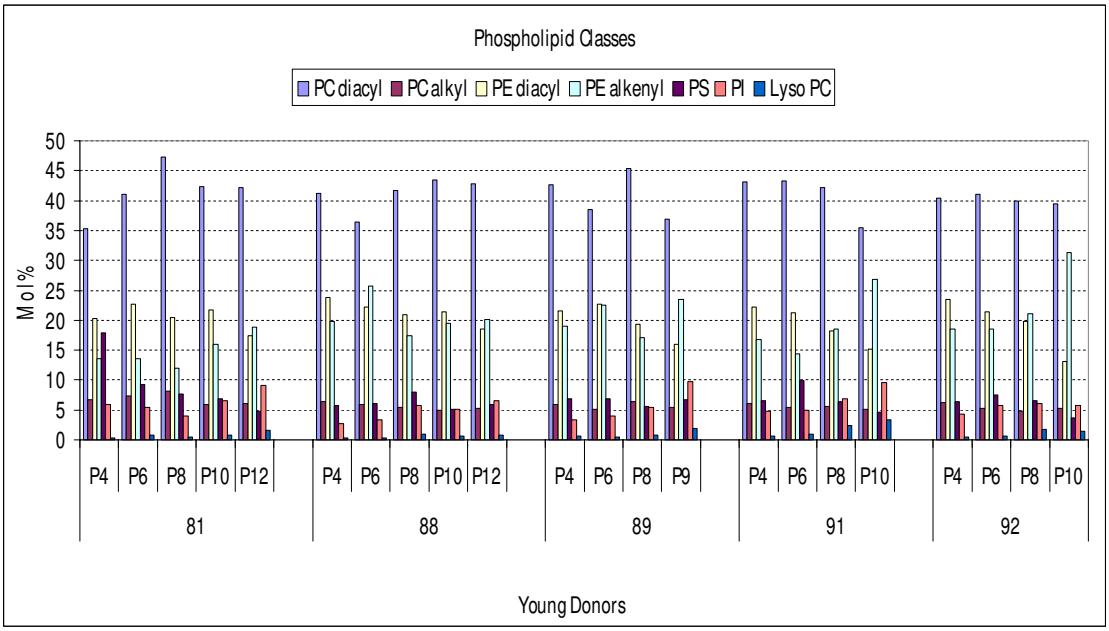


Figure 4 Mol% proportions of phospholipid classes detected in the BMSC samples from early to late passages of five young donors.

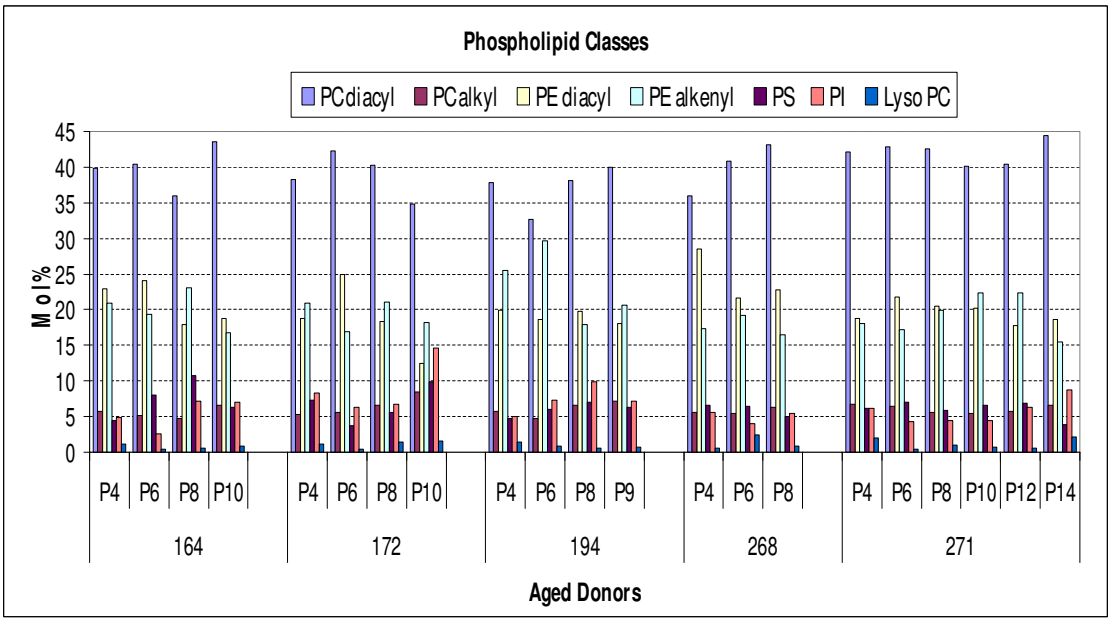


Figure 5 Mol% proportions of phospholipid classes detected in the BMSC samples from early to late passages of five aged cell lines.

Diacyl PC in the young BMSC had a higher mol% (>36) in most of its cell lines' initial passages when compared to similar passages of the older groups (< 36 mol %). Two of the young cell lines, 81 and 88 showed an increasing mol% towards their final passages (Fig. 6); in contrast, cell lines 89 and 91 had a lower value at their latest passages. Cell line 92 maintained its diacyl PC levels at a constant throughout its four passages. In the older BMSC (Fig. 7) most of the cell lines had an elevated mol% levels at the final passages with the exception of cell line 172. However, their earliest passages had a lower mol% (<36%) in comparison to the young BMSC counterparts. Ether-linked lipids such as alkyl-acyl PC make up one fifth of the overall phospholipids classes (Figs. 6, 7) and the total concentration had fluctuating levels between different cell lines. Despite this fluctuation, the PC total pool was relatively well conserved in both cell lines.

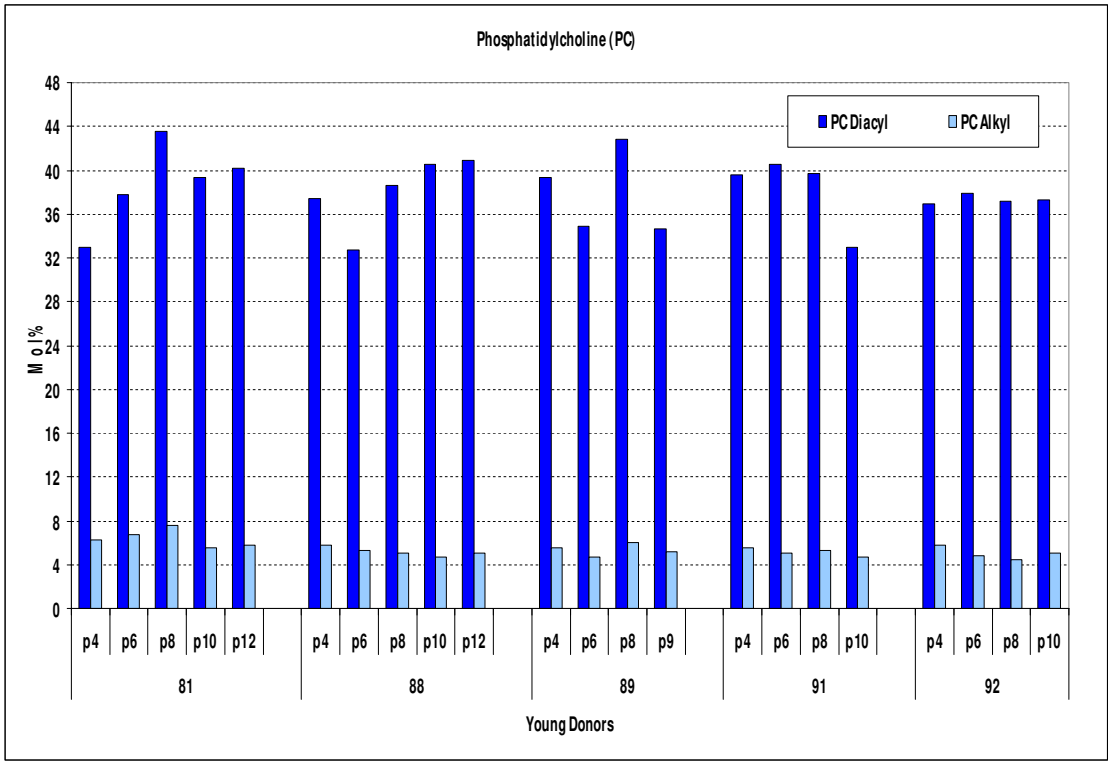


Figure 6 Mol% proportions of diacyl and alkyl-acyl PC detected in the BMSC samples from early to late passages of five young donors.

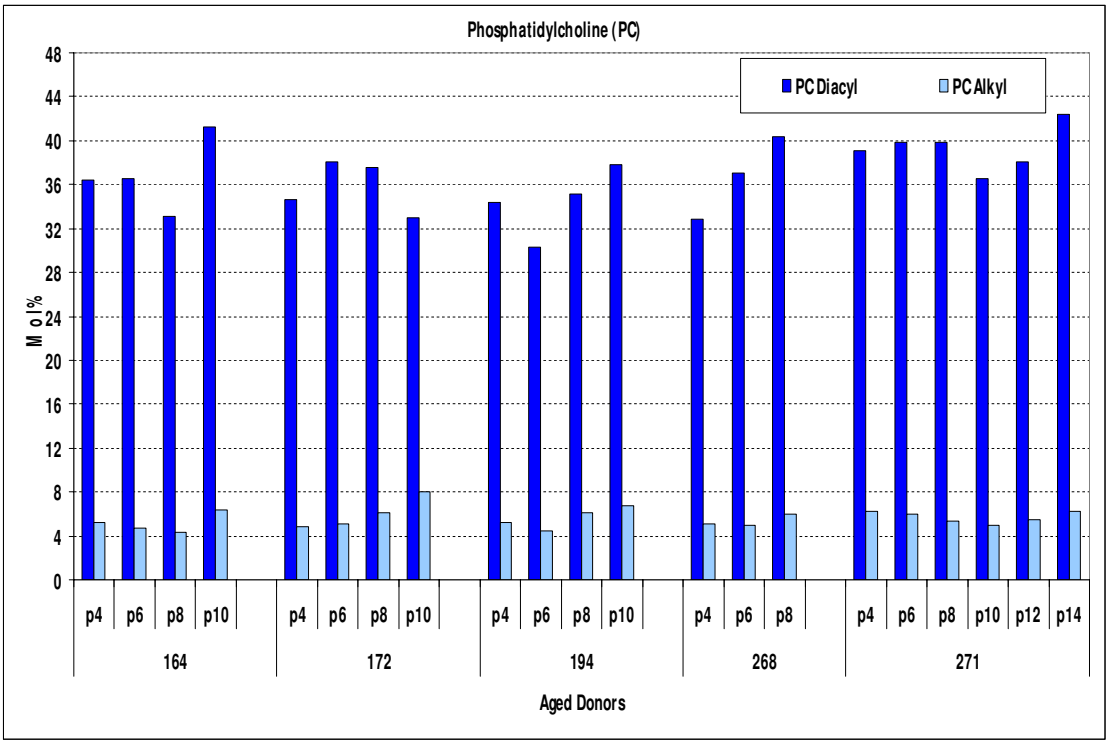


Figure 7 Mol% proportions of diacyl and alkyl-acyl PC detected in the BMSC samples from early to late passages of five aged donors.

LysoPC, present in trace amount, increased towards late passages of BMSC from the young cell lines (Fig.8) reaching upto 2% on average. However, cell line 91 showed a much higher level at similar passages. In contrast, the aged donors' profile of consecutive passages (Fig.9) showed wider individual variation. Cell lines 172, 268 and 271 all had an elevated late passage mol% while cell lines 164 and 194 had the reverse. Furthermore, initial levels at passage 4 were in general higher in the samples from aged donors than from young ones (Figs. 8, 9).

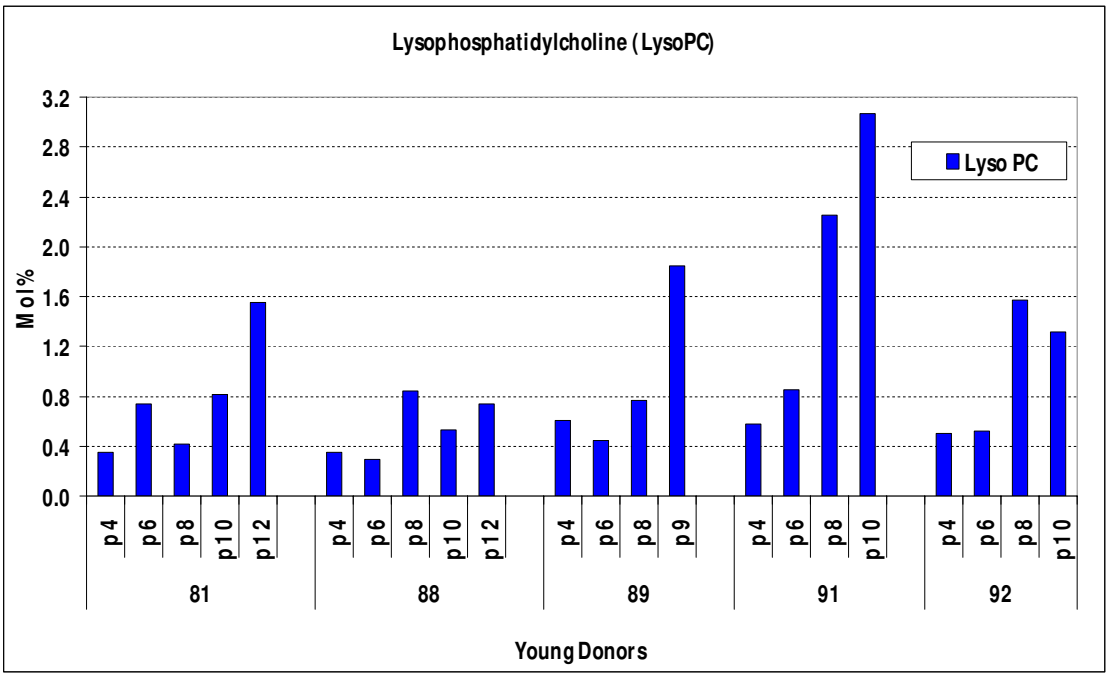


Figure 8 Mol% proportions of LysoPC detected in the BMSC samples from early to late passages of five young donors.

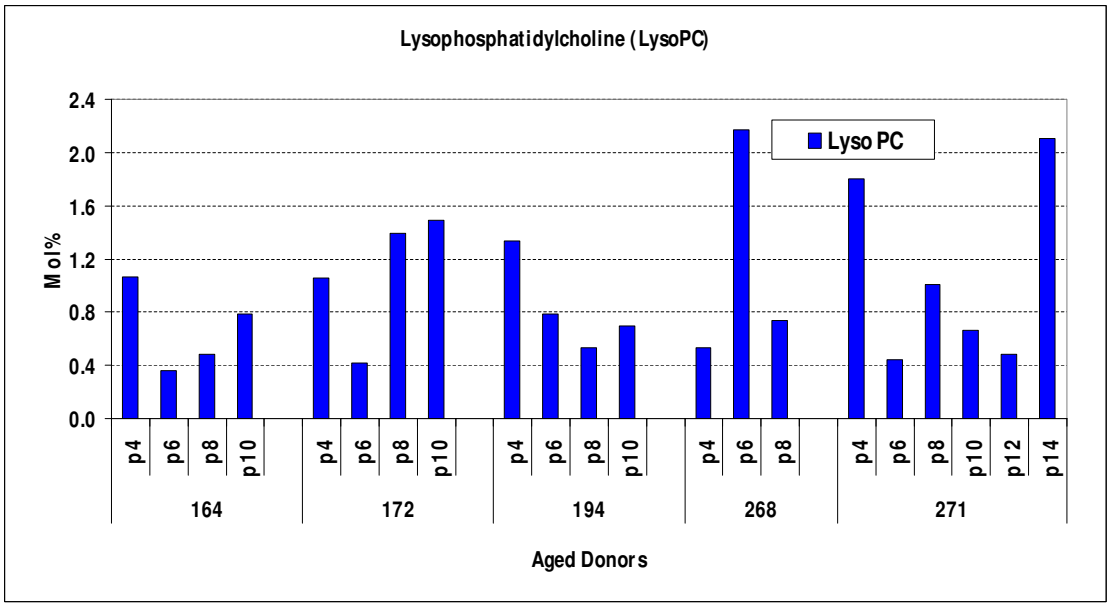


Figure 9 Mol% proportions of LysoPC detected in the BMSC samples from early to late passages of five aged donors.

Diacyl PE levels in BMSC of both the young (Fig. 10) and aged donors (Fig. 11) showed a rather decreasing trend from the early to late passages.

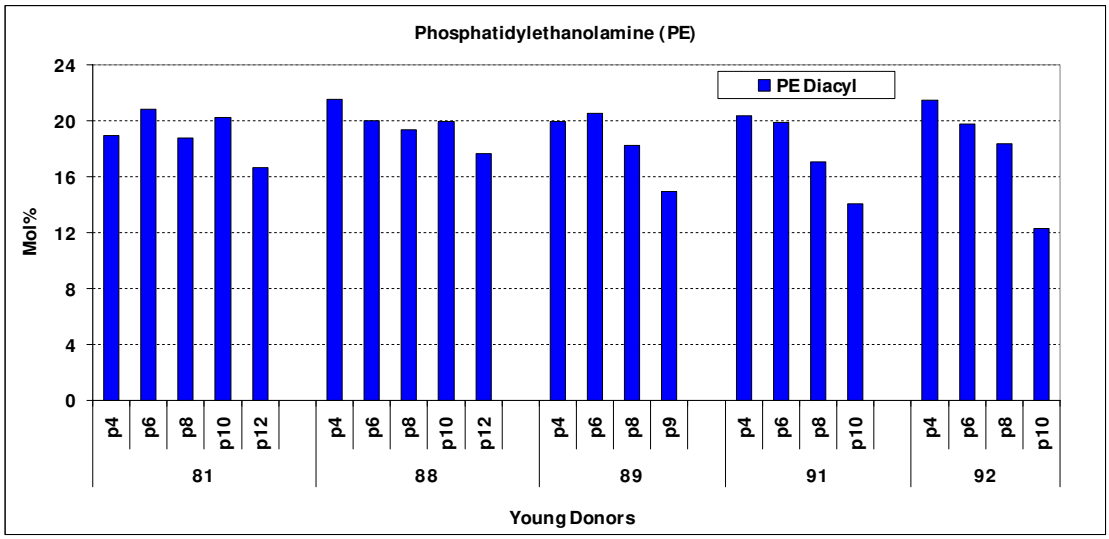


Figure 10 Mol% proportions of diacyl PE detected in the BMSC samples from early to late passages of five young donors.

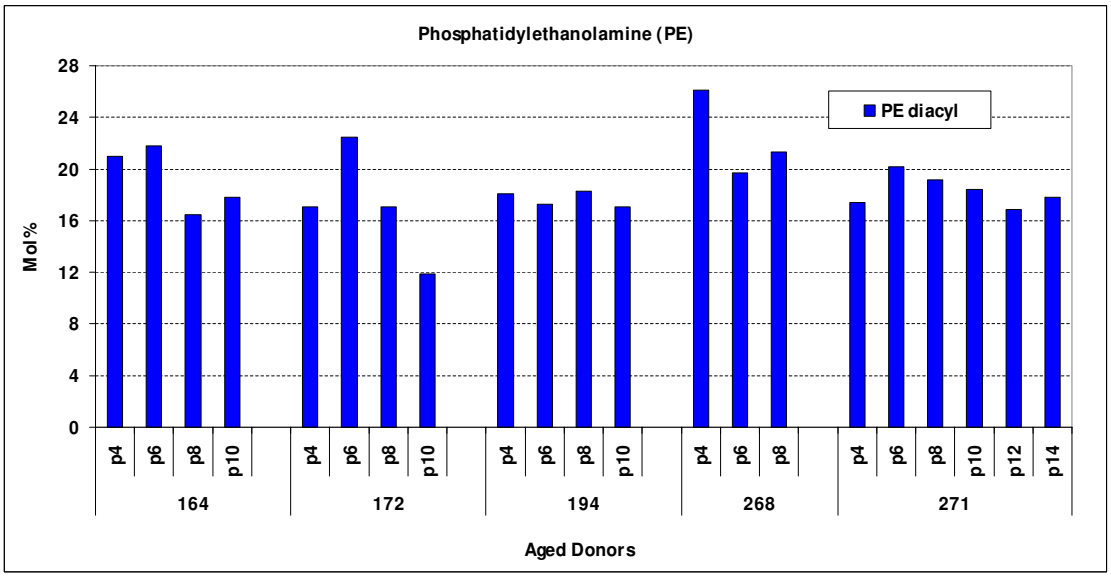


Figure 11 Mol% proportions of diacyl PE detected in the BMSC samples from early to late passages of five aged donors.

Alkenyl-acyl PE constitutes a considerable amount of the total phospholipids detected in the BMSC. The young cell lines' profile (Fig. 12) had an ascending molar percentage towards late passages with an almost two fold increase in the case of cell line 92. On the other hand the aged donors' (Fig.13) had a highly fluctuating profile even though the overall concentration was maintained below 24mol% in most cases. Nevertheless, the general trend seen was a decreasing one towards final stages.

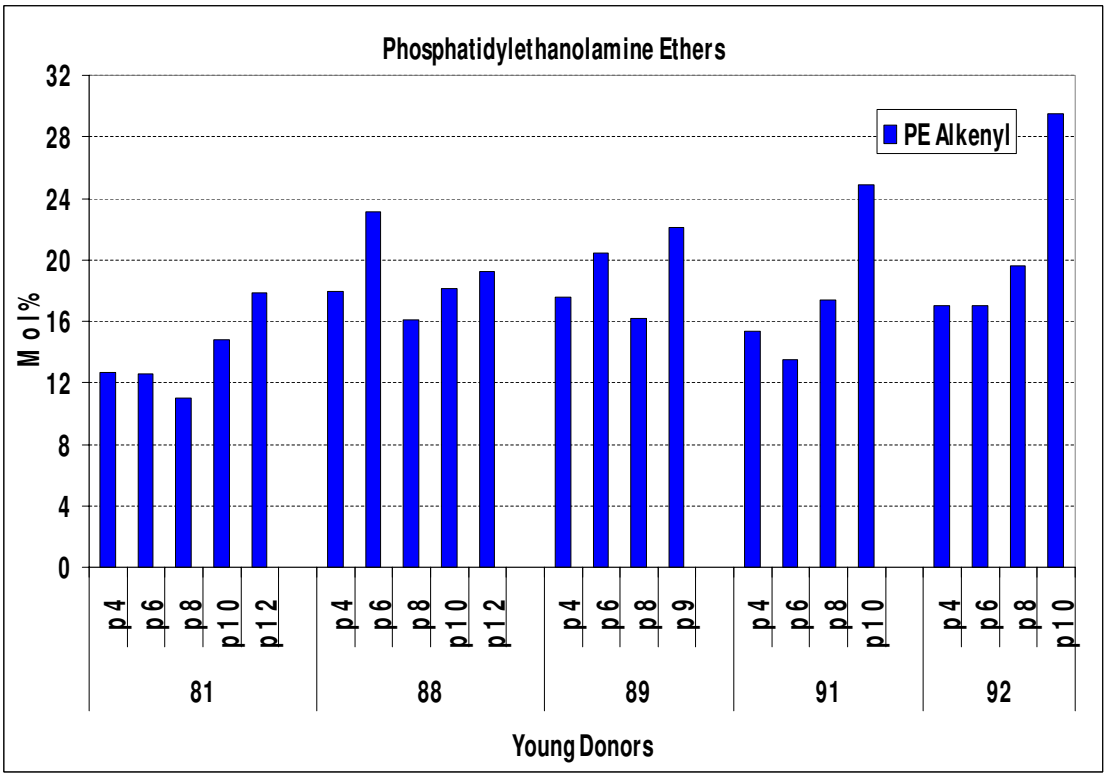


Figure 12 Mol% proportions of alkenyl-acyl PE detected in the BMSC samples from early to late passages of five young donors.

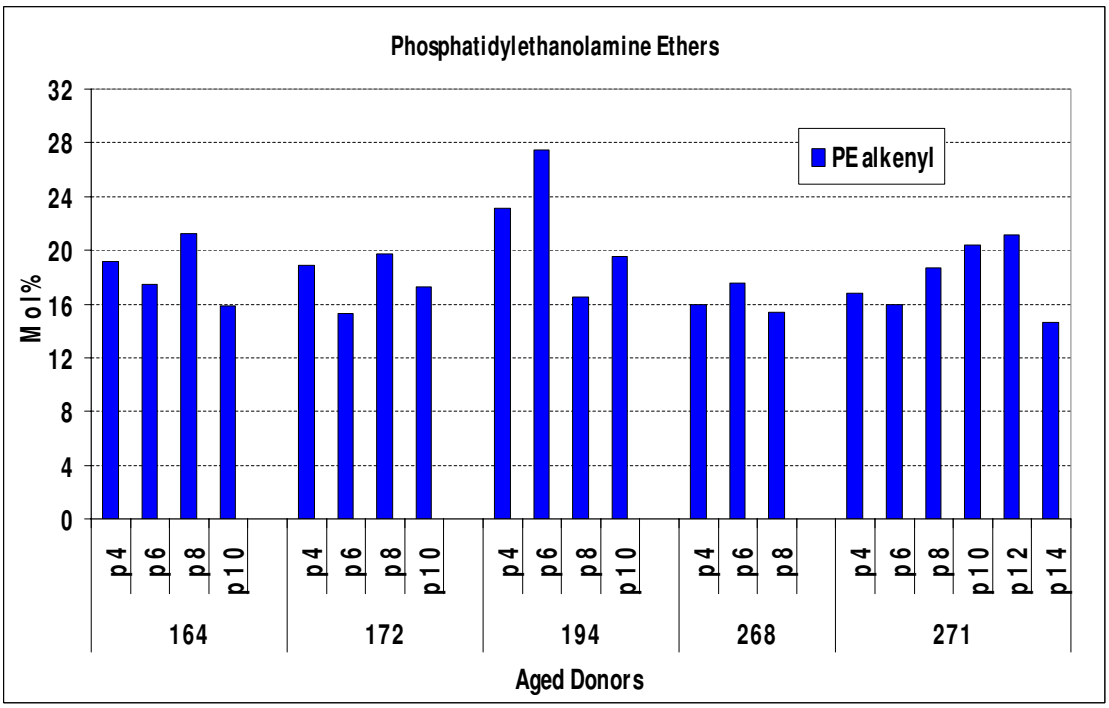


Figure 13 Mol% proportions of alkenyl-acyl PE detected in the BMSC samples from early to late passages of five aged donors.

The aminophospholipid, PS constituted less than 10% of the total phospholipid extracted from the BMSC samples. Young donors (Fig. 14) exhibited a decreasing mol% from initial to final passages with the exception of cell lines 88 & 89 which had a relatively stable profile. BMSC from cell line 81 passage 4 had an abnormal level of PS (>10%) which might be due to artifacts. The aged donors (Fig. 15) had varying mol%; the first three cell lines (164, 172, and 194) increased their mol% from passage 4 to the final passage 10. In contrast, the last two cell lines (268 and 271) had a decreasing mol% when comparing initial to final passages.

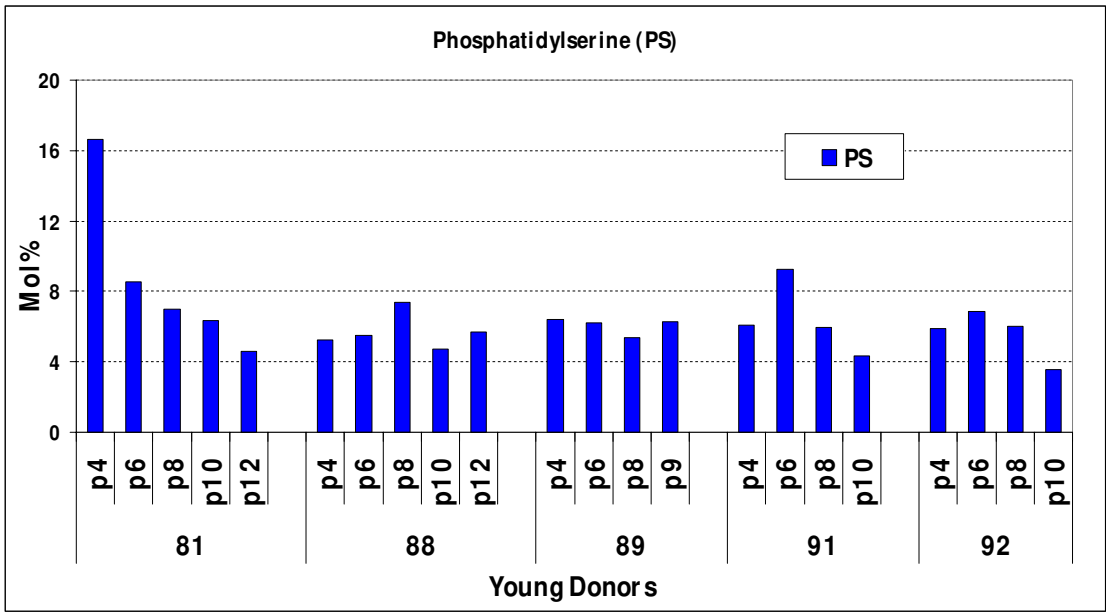


Figure 14 Mol% proportions of PS detected in the BMSC samples from early to late passages of five young donors.

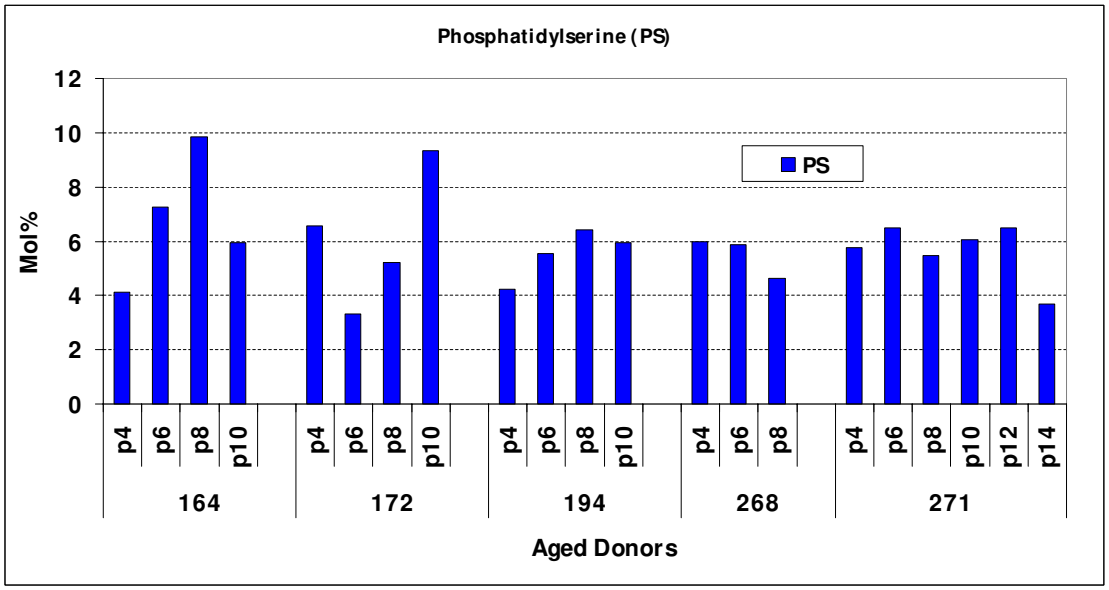


Figure 15 Mol% proportions of PS detected in the BMSC samples from early to late passages of five aged donors.

PI profiles for both young (Fig. 16) and aged (Fig. 17) cell lines illustrate an enhanced mol% towards late passages. However, this trend is not consistent throughout individual passages but highly fluctuated between initial and final passages for both the aged as well as young BMSC samples. Cell line 172 distinctively had an abnormal level of PS at its final passage exceeding 10%.

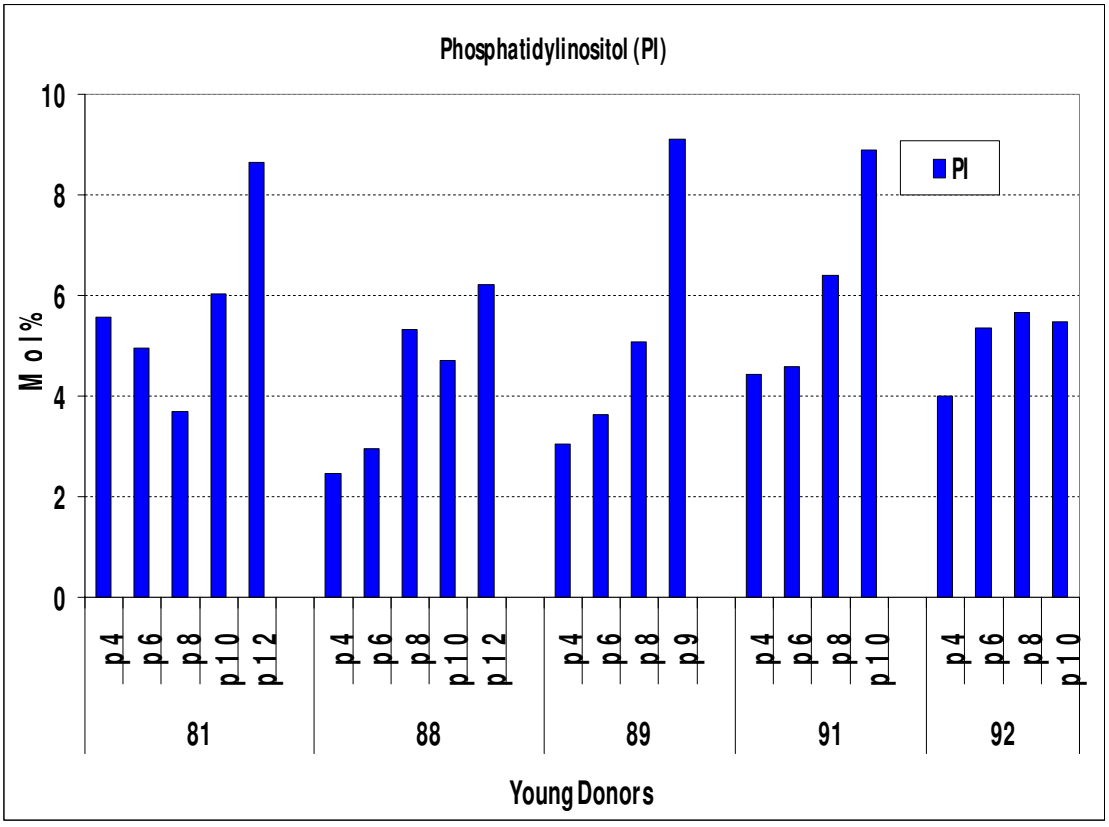


Figure 16 Mol% proportions of PI detected in the BMSC samples from early to late passages of five young donors.

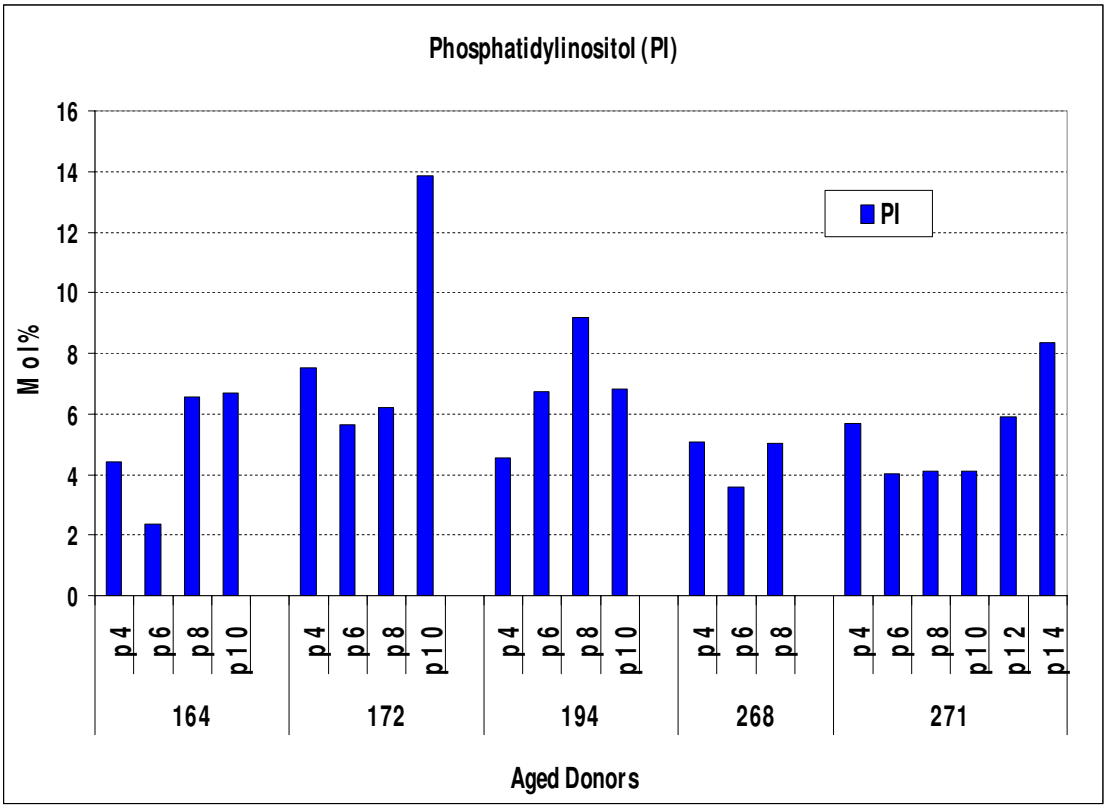


Figure 17 Mol% proportions of PI detected in the BMSC samples from early to late passages of five aged donors.

PI which made up less than 10mol% of the total phospholipid classes was contrasted against PS classes to detect a simpler pattern (Figs. 18, 19). The resulting profile ascertained the previous findings of a decreasing value for PS accompanied by an increment of PI levels. The ratio of PI: PS rose to more than 1% shortly before the last passage in most cases of the young BMSC cell lines (Fig. 18).

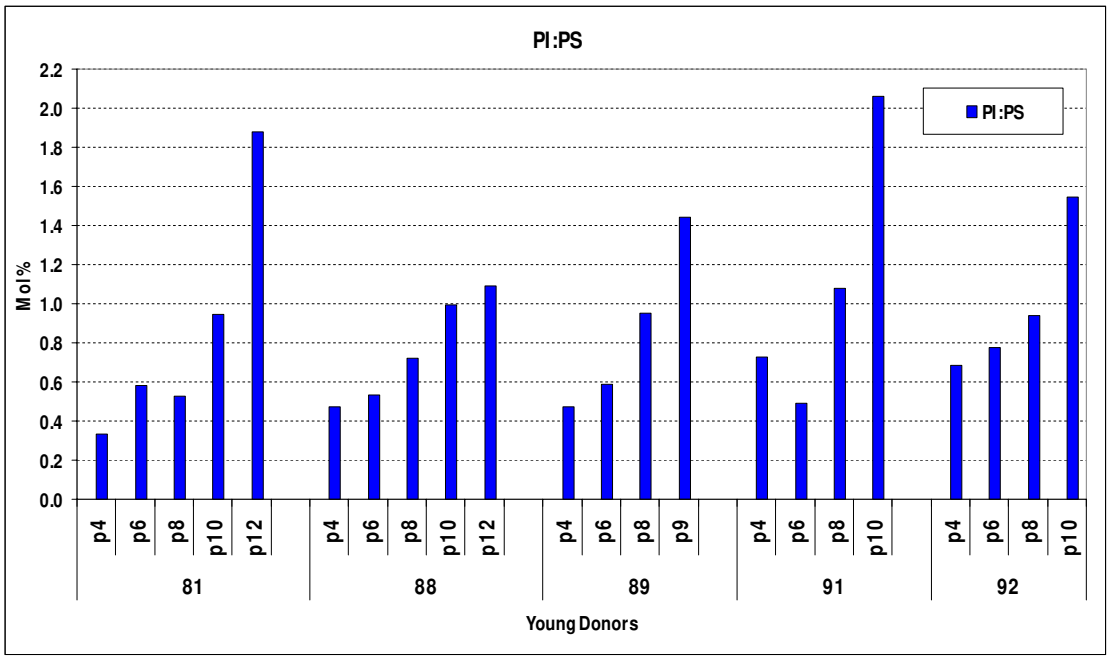


Figure 18 Ratio of PI totals to PS totals (PI: PS) on mol% basis of the BMSC from early to late passages in young donors.

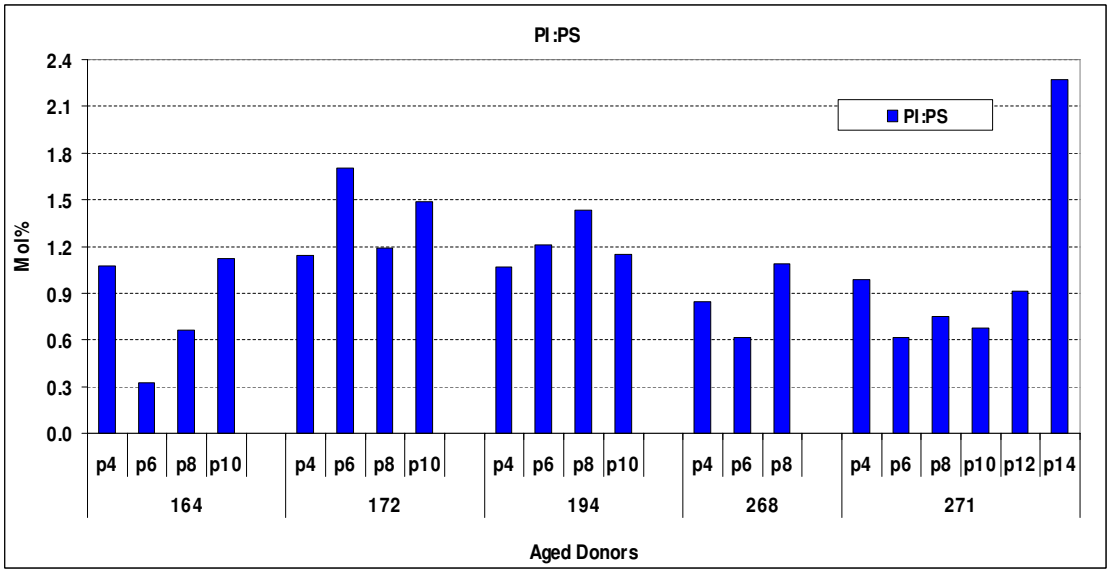


Figure 19 Ratio of PI totals to PS totals (PI: PS) on mol% basis of the BMSC from early to late passages in aged donors.

3.2 BMSC Molecular Species Profile in each phospholipid class

Concurrent to developing the lipidome profile at phospholipid class level, analysis of alterations in molecular species level were also carried out for individual BMSC donors and their respective passages. The large number of phospholipid species included in the analysis work is presented in the figures below. However, the mol% listed below represent the average of a specific species' total from initial passages and final passages likewise. T-test calculations have been performed to evaluate the difference between the means from the initial and late passages.

3.2.1 Phosphatidylcholine diacyl (PC) species

PC diacyl species of the studied BMSC samples from both young (Fig.20) and aged (Fig.21) donors had a strikingly similar profile in that a decrease in saturated and monounsaturated short acyl chained species such as 32:0, 32:01, 34:0 and 34:01 was observed towards latest passages. This trend was counter-balanced by an elevation of long acyl chain and polyunsaturated species 36:01, 36:04, 38:03 and 38:04.

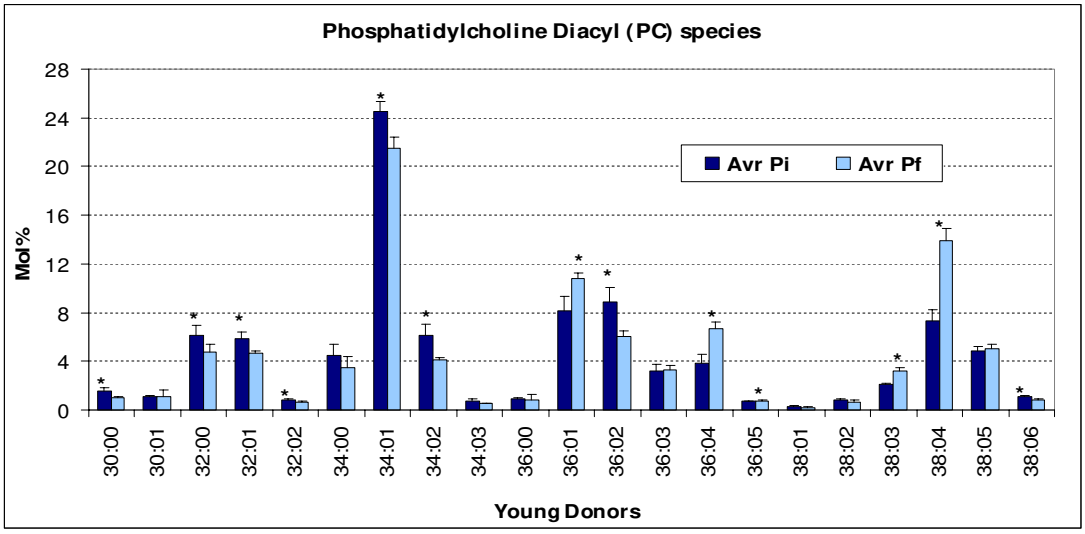


Figure 20 Main molecular PC diacyl species on mol% basis (means of five replicates ± SD) of means of initial (Pi) and final passages (Pf) of the BMSC from five young donors. * *statistical difference at p<0.05 level, t-test*

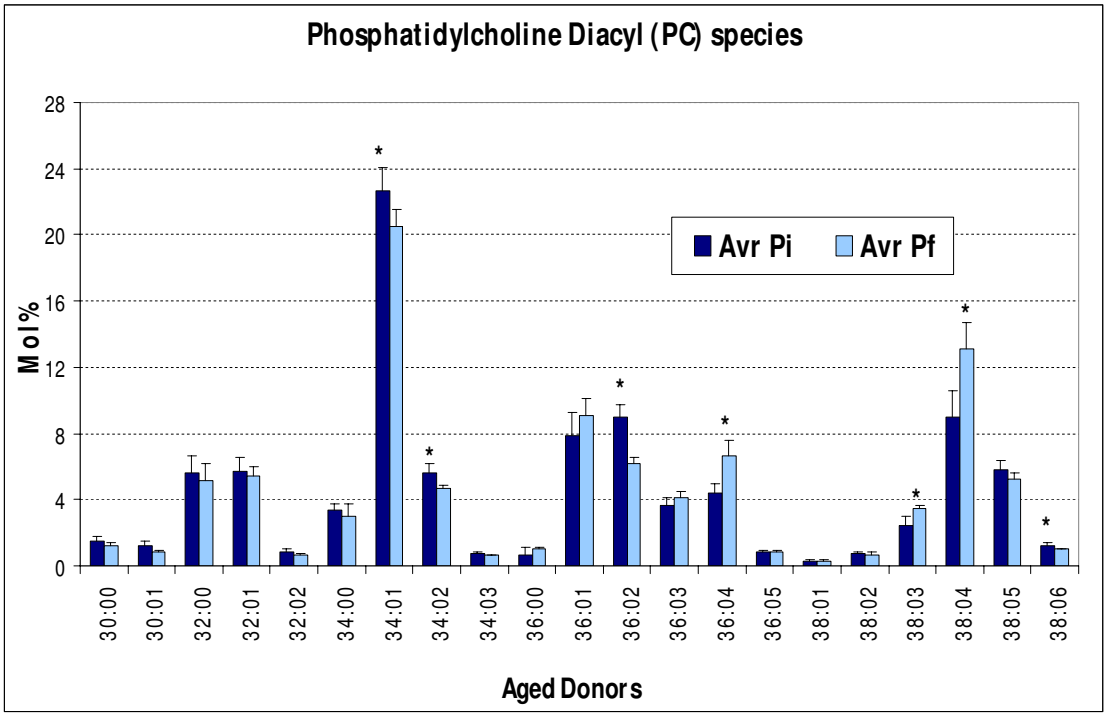


Figure 21 Main molecular PC diacyl species on mol% basis (means of five replicates ± SD) of means of initial (Pi) and final (Pf) passages of the BMSC from five aged donors.

3.2.2 Phosphatidylcholine ether lipid (PCe) species

PC alkyl mol% profiles in the young (Fig.22) and aged donors' (Fig.23) emulated each other as well as PC diacyl species profile. The quantitatively prevalent species 34:1 made up approximately 20% of the total alkyl-acyl PC species in the initial passages but later was reduced to nearly 16% in the final passage. This decrease appeared to be compensated by an increase in polyunsaturated long acyl-chain species specifically 38:4 and 40:2.

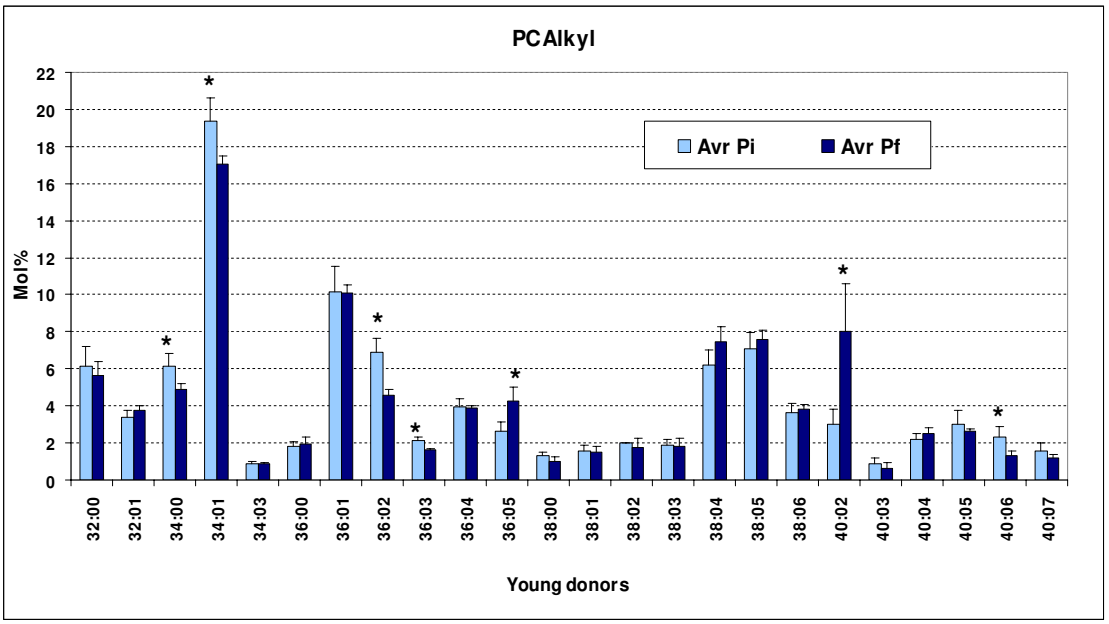


Figure 22 Main PC alkyl species on mol% basis (means of five replicates ± SD) of means of initial and final passages of the BMSC from five young donors.

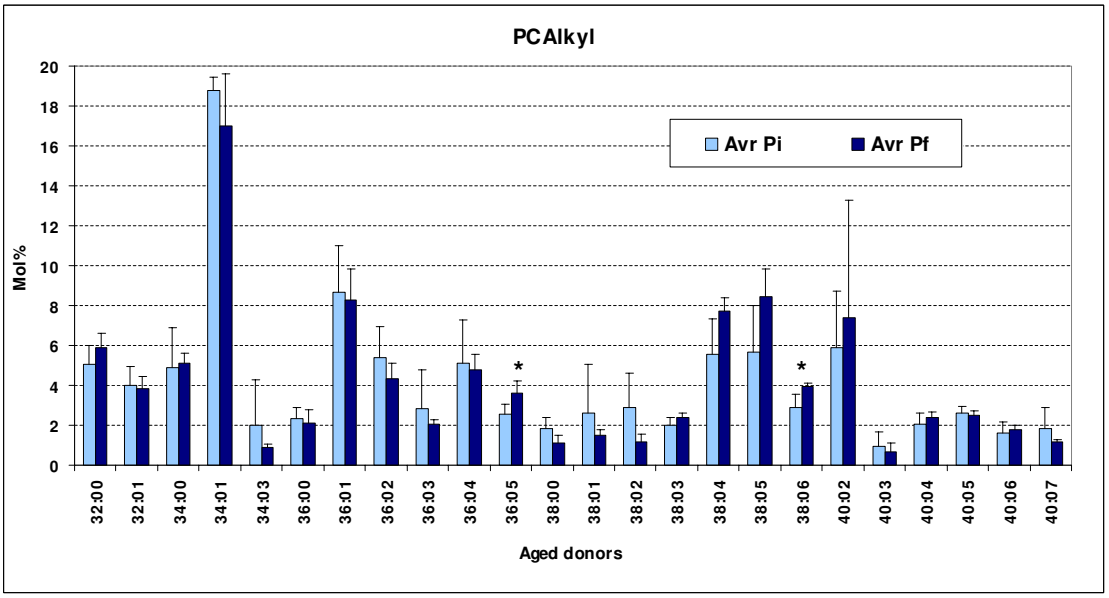


Figure 23 Main PC alkyl species on mol% basis (means of five replicates ± SD) of means of initial and final passages of the BMSC from five aged donors.

3.2.3 Phosphatidylethanolamine diacyl (PE) species

The profiles of PC and PE diacyl species exhibited comparable trends in their alterations from the early to late passages. For instance, short acyl chain molecular species had decreased mol% towards final passages in both young (Fig.24) and aged (Fig.25) donors' profile. On the contrary long acyl chain species had a rise in their mol% in the latest passages. Furthermore, elevated level of 38:04 in final passages was evident both in diacyl PC as well as PE profiles.

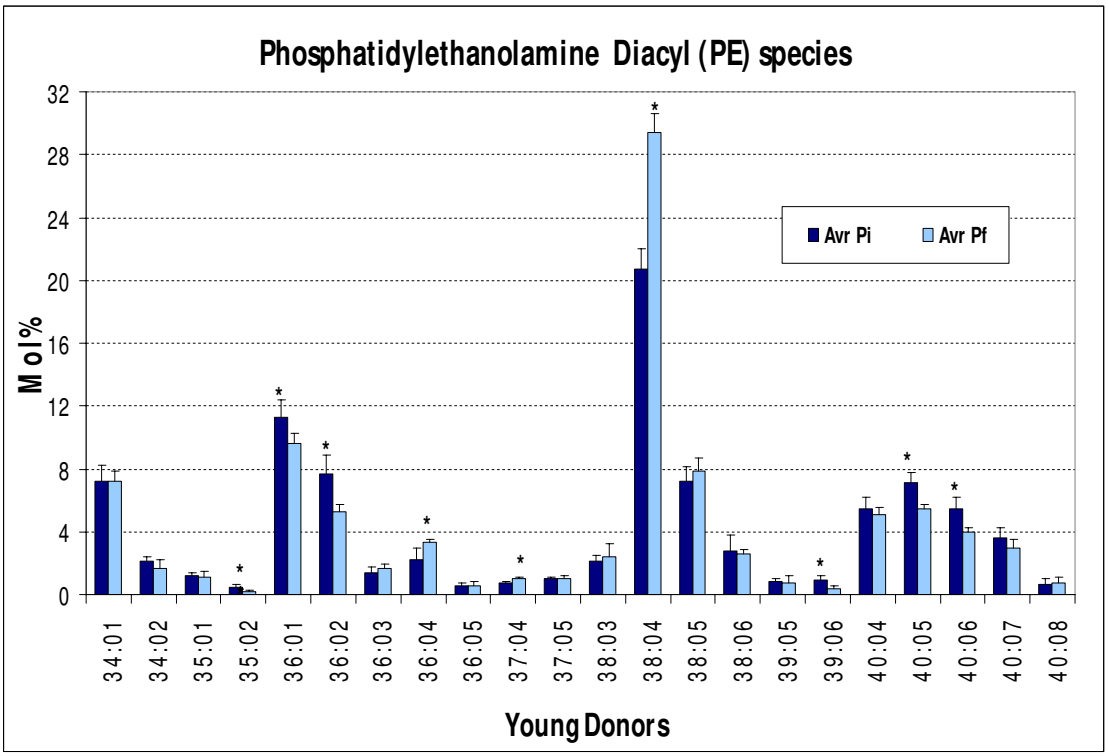


Figure 24 Main PE diacyl species on mol% basis (means of five replicates ± SD) of means of initial and final passages of the BMSC from five young donors.

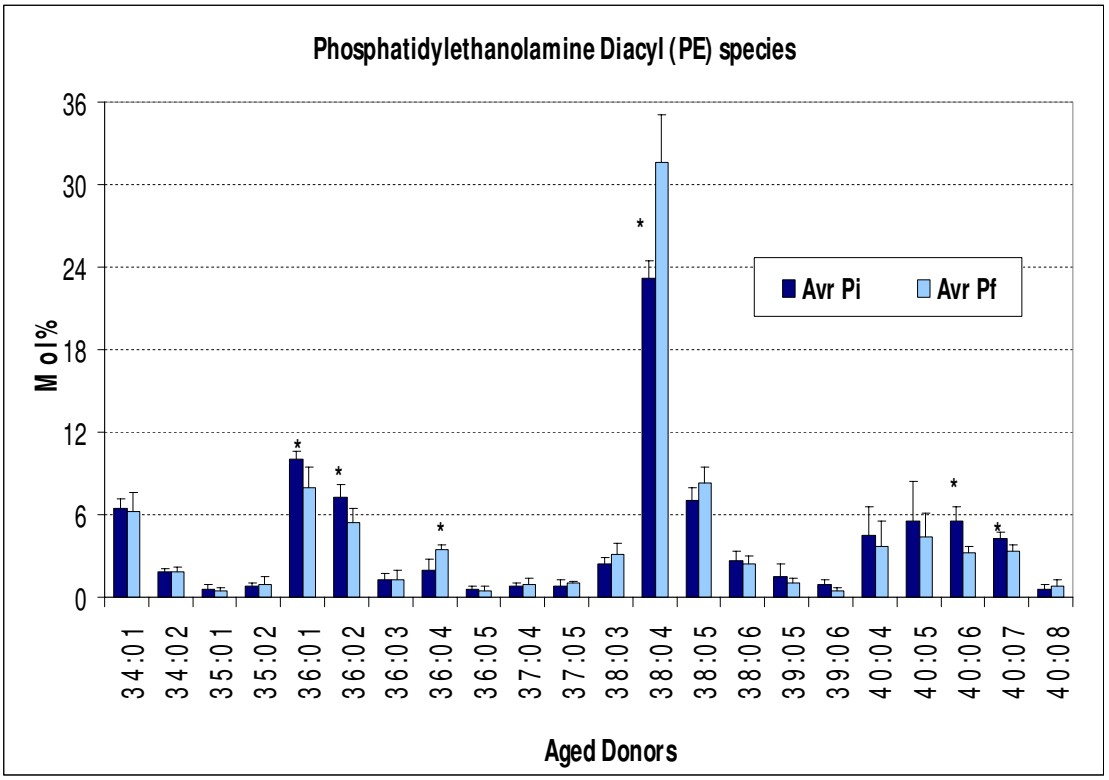


Figure 25 Main PE diacyl species on mol% basis (means of five replicates ± SD) of means of initial and final passages of the BMSC from five aged donors.

3.2.4 Phosphatidylethanolamine ether lipid (PEe) species

Only unsaturated PEe species were found in the BMSC samples from both groups of donors (Fig. 26, 27) with the main species being polyunsaturated: 36:4, 38:4 and 38:5. The mol% of these species decreased a significant amount in the end passages but this trend was altered in the heavier species with the exception of 40:4 and 40:5 which kept a similar decreasing progression.

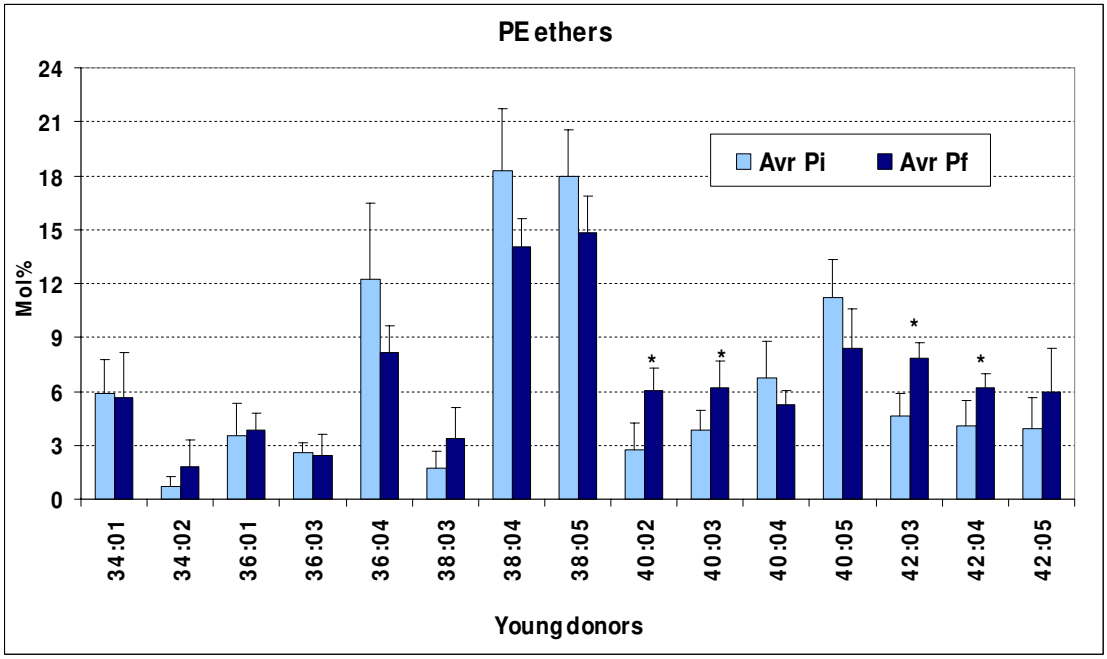


Figure 26 Main PE ether species on mol% basis (means of five replicates ± SD) of means of initial and final passages of the BMSC from five young donors.

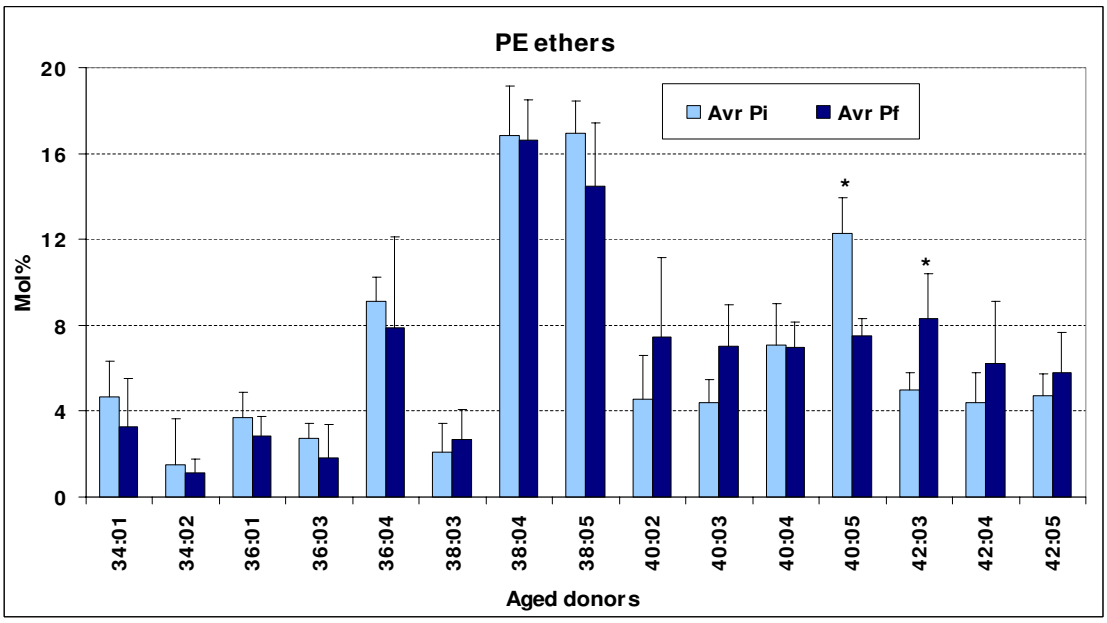


Figure 27 Main PE ethers species on mol% basis (means of five replicates ± SD) of means of initial and final passages of the BMSC from five aged donors.

3.2.5 Phosphatidylinositol (PI) species

The predominant PI species was 38:4 which accounted for about 50% of the total class detected in both the young and aged BMSC. The mol% of this species dwindled in the final passages. In contrast, short-acyl chained species such as 36:1, 36:2, and 36:4 saw a small rise in their mol% during the last passages (Figs. 28, 29).

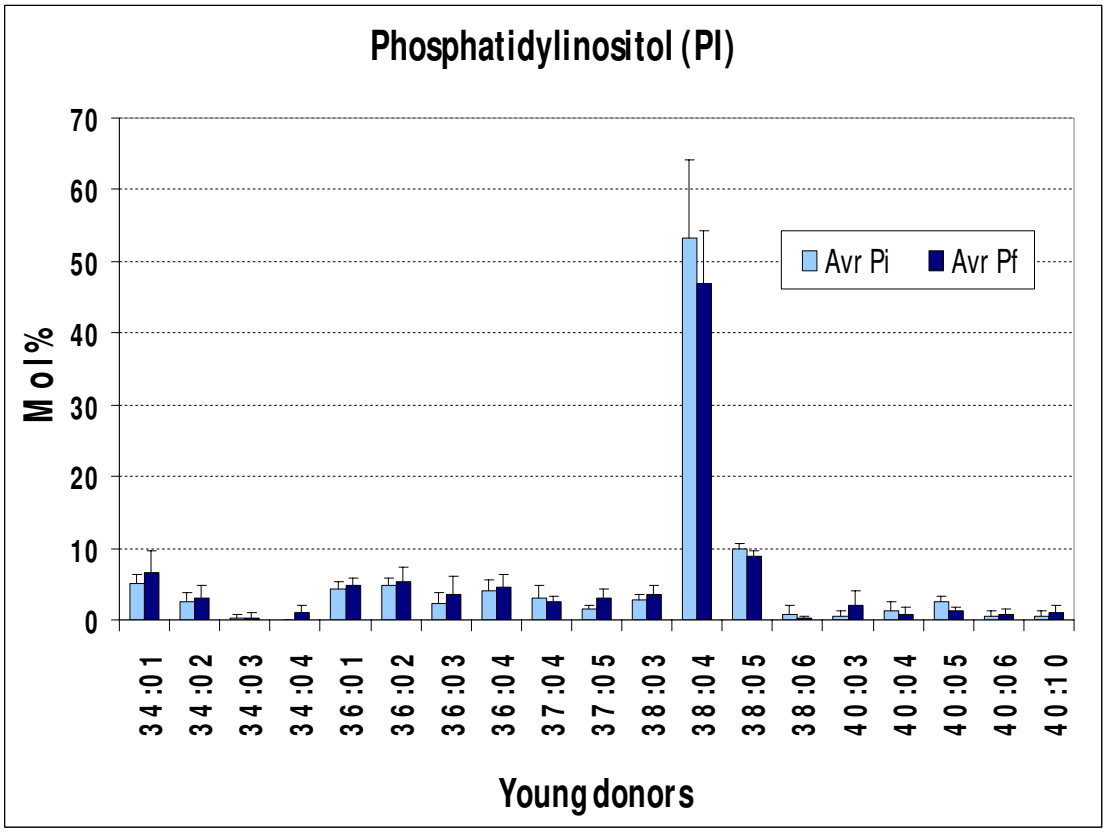


Figure 28 Main PI species on mol% basis (means of five replicates ± SD) of means of initial and final passages of the BMSC from five young donors.

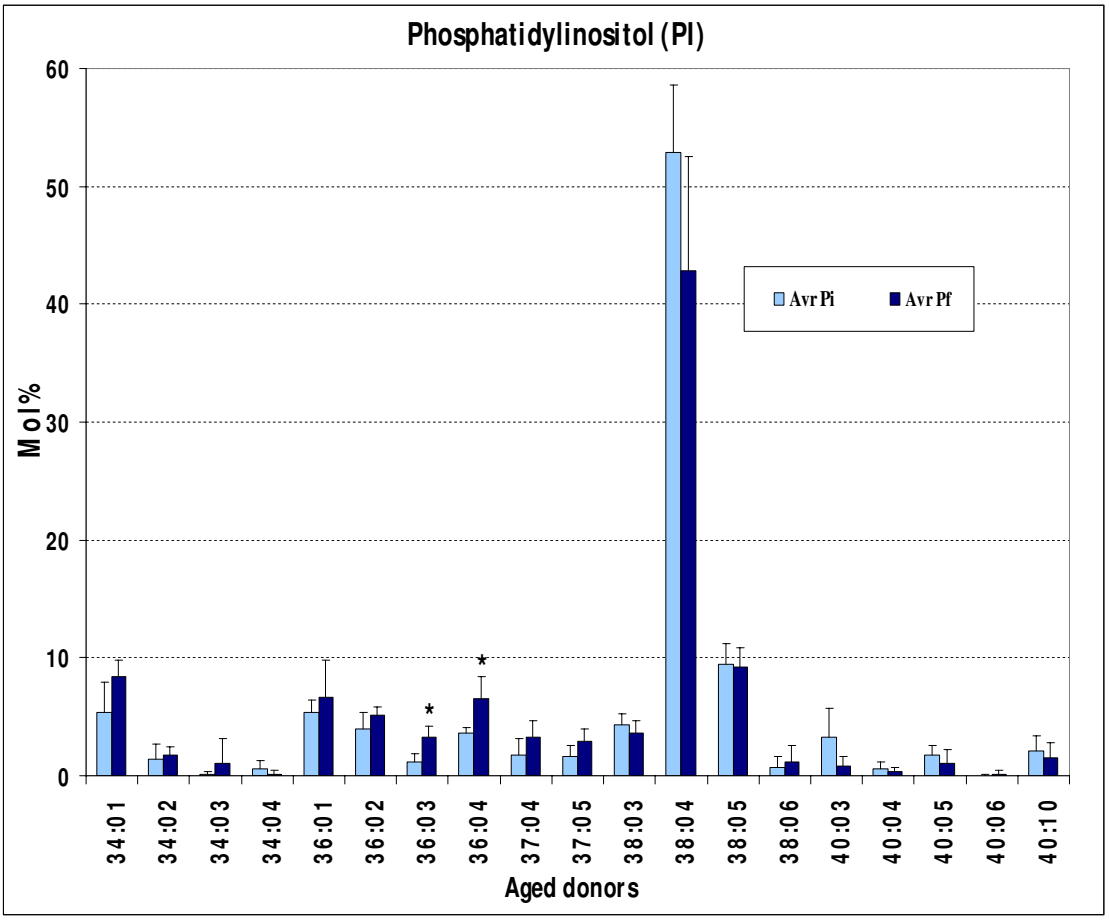


Figure 29 Main PI species on mol% basis (means of five replicates ± SD) of means of initial and final passages of the BMSC from five aged donors.

3.2.6 Phosphatidylserine (PS) species

About 40% of the total PS was comprised of 36:1 species in both aged (Fig.30) and young (Fig. 31) BMSC samples. Again here a common progress detected was a decrease in short chain species towards latest passages, accompanied by an elevation in the long chain species such as 38:3 and 38:4. Exceptions to this trend were the heavier acyl chain species such as 40:4, 40:5 and 40:6.

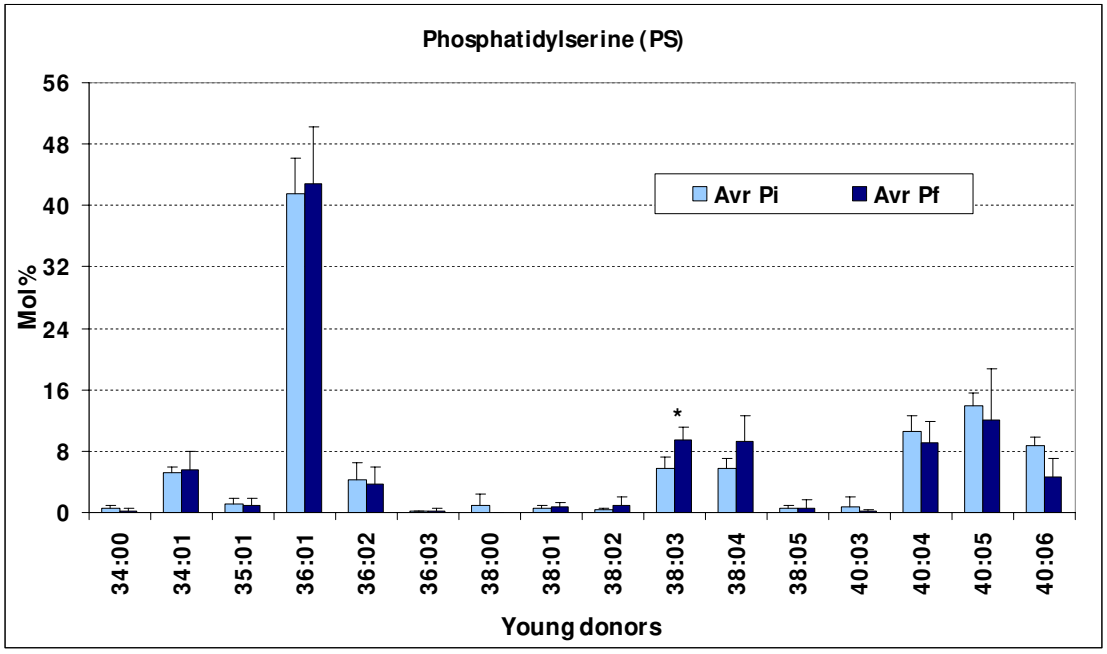


Figure 30 Main PS species on mol% basis (means of five replicates ± SD) of means of initial and final passages of the BMSC from five young donors.

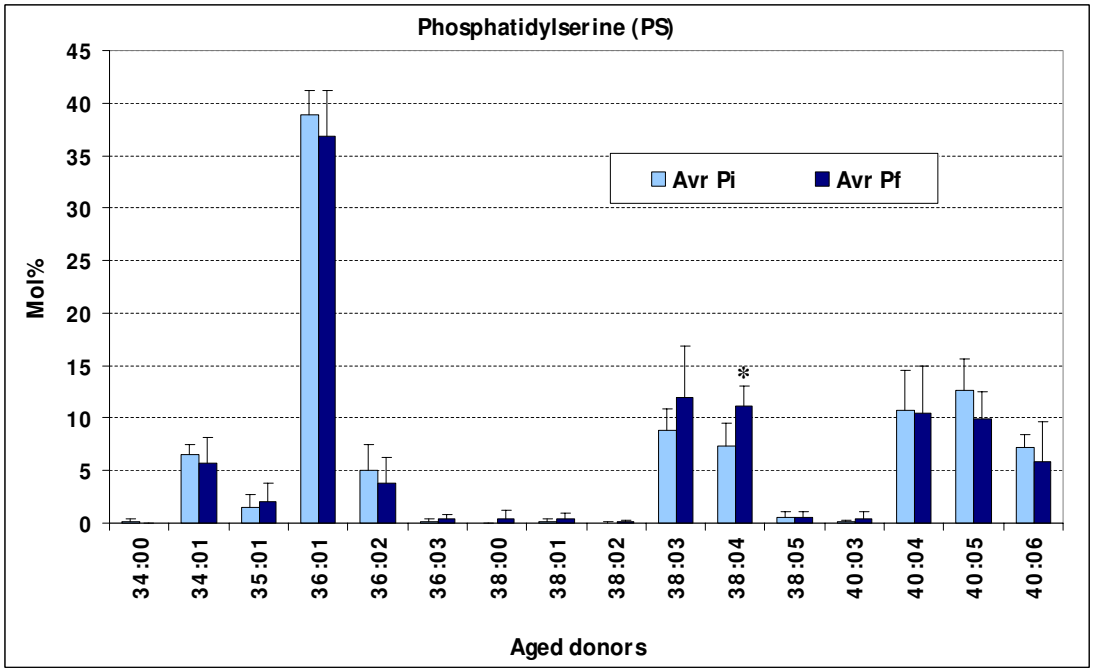


Figure 31 Main PS species on mol% basis (means of five replicates ± SD) of means of initial and final passages of the BMSC from five aged donors.

3.2.7 Lysophosphatidylcholine (LysoPC) species

The vast majority of LysoPC species from the BMSC samples were either saturated or monounsaturated ones. The main molecular species was 16:0 (28%) which decreased in the final passage. The long acyl chain species 18:0 and 20:4 on the other hand had a final higher mol% as compared to initial passages of both cell lines (Fig. 32, 33). However, aged donors' profile differed somewhat from the young ones because the mol% of 18:1 decreased slightly in the former one.

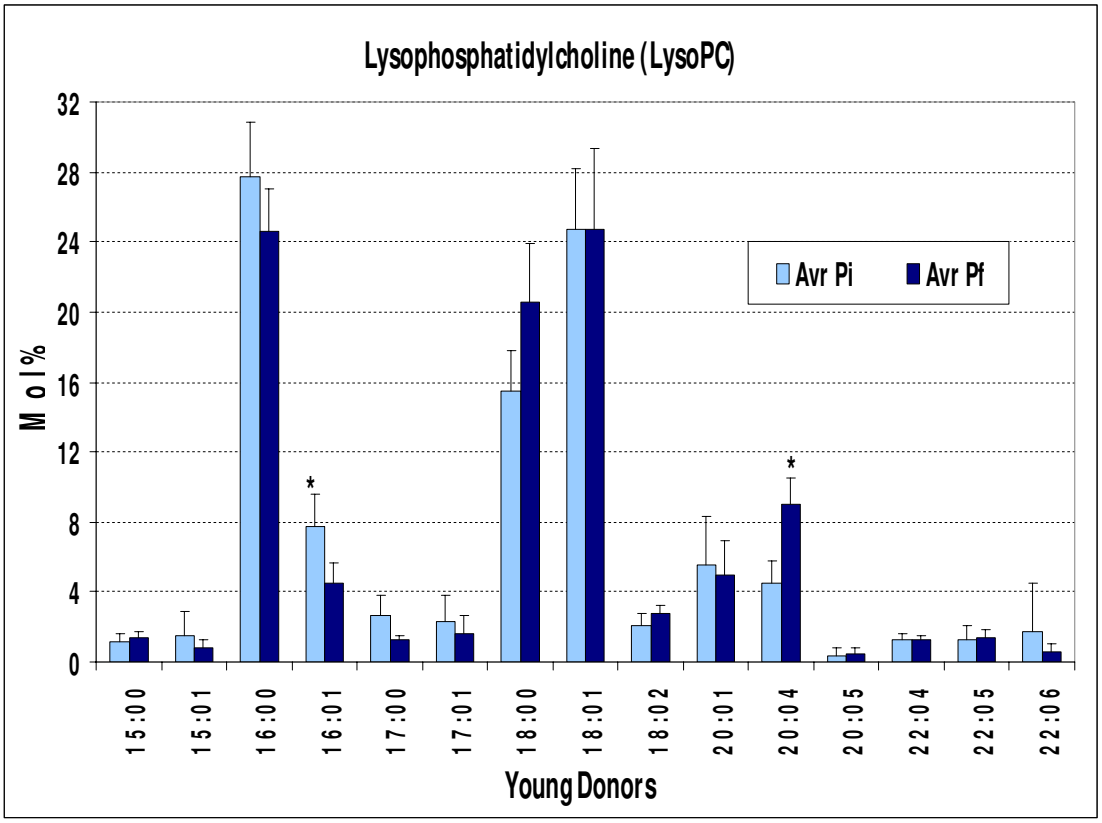


Figure 32 Main LysoPC species on mol% basis (means of five replicates ± SD) of means of initial and final passages of the BMSC from five young donors.

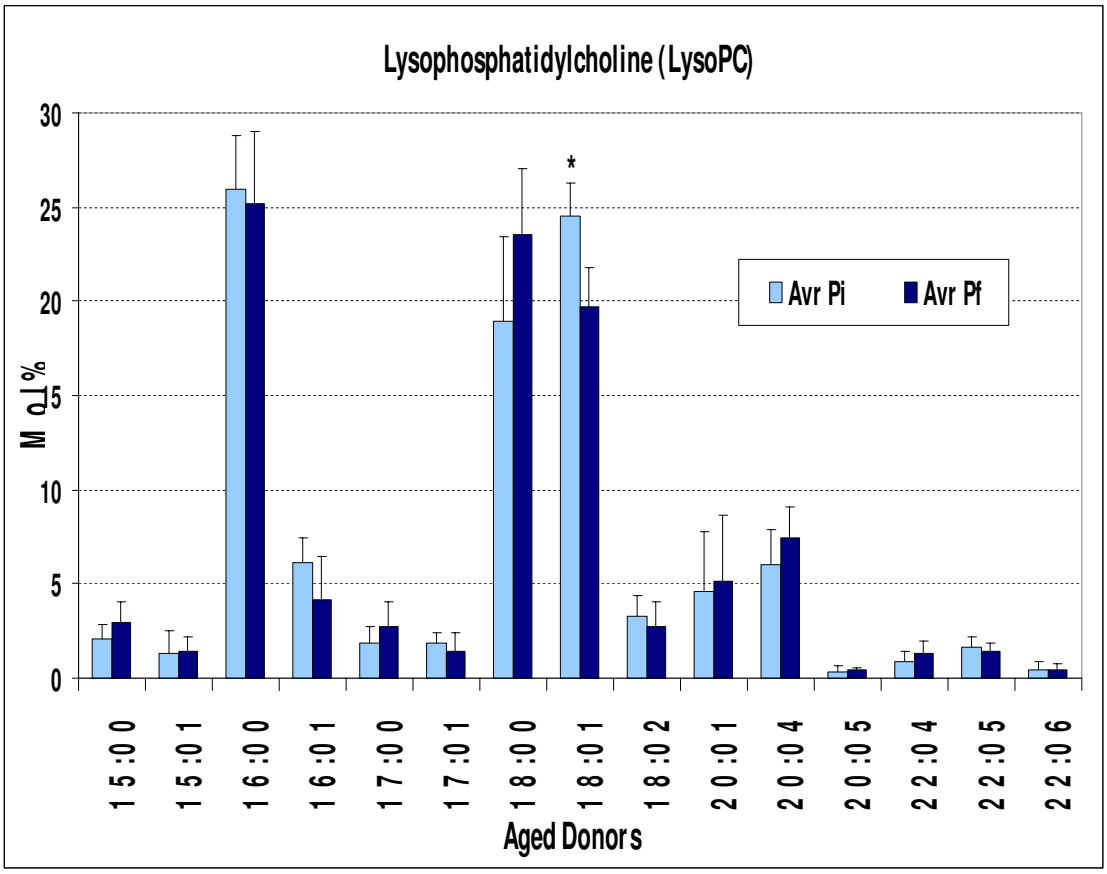


Figure 33 Main LysoPC species on mol% basis (means of five replicates ± SD) of means of initial and final passages of the BMSC from five aged donors.

3.3 PCA

Multivariate principal component analysis (PCA) was used to study the difference in phospholipid composition as a whole among the BMSC from the young as well as aged donors. The phospholipid classes were plotted together with BMSC from young and aged donors. The PCA biplot (Fig. 34) result demonstrated that the latest passages from both young and aged BMSC had a higher level of PI, LysoPC, and PE alkenyl whilst earlier passages were mainly rich in PC and PE diacyl classes. Furthermore the latest passages were poor in PC diacyl, PE diacyl and PS classes.

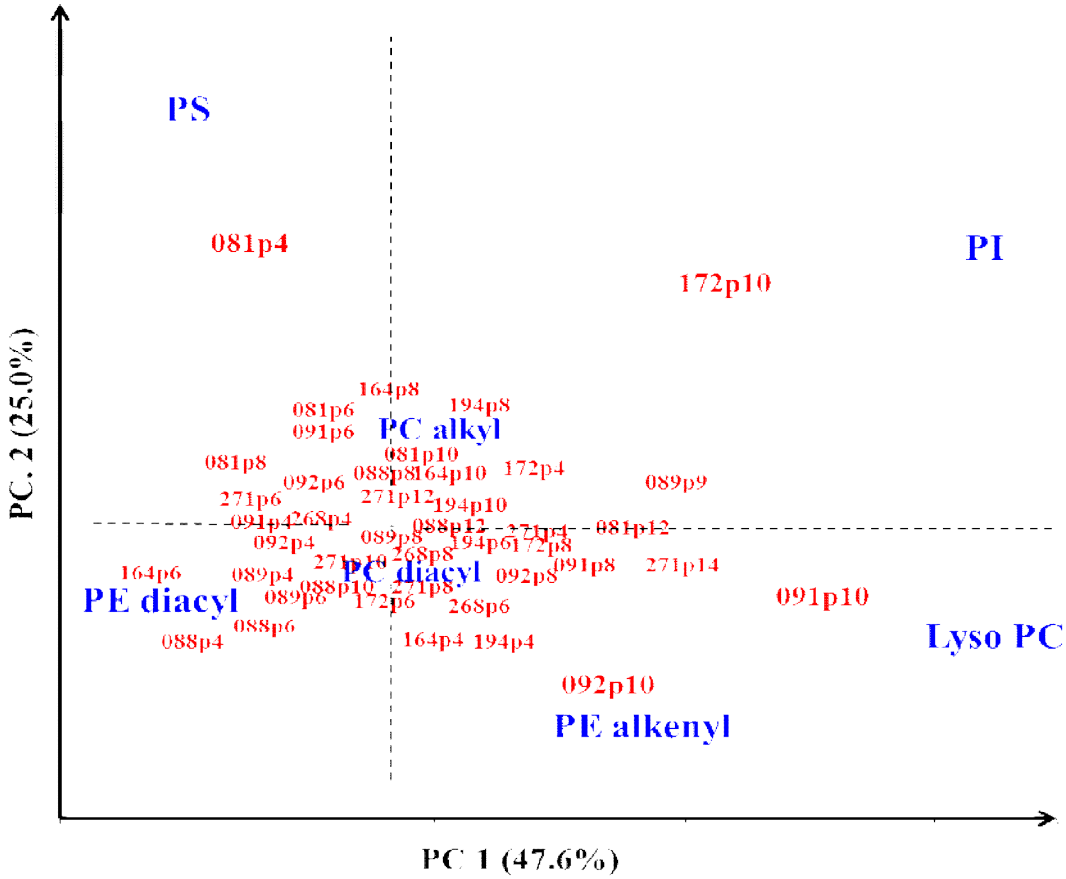


Figure 34 PCA biplot demonstrating similarities and differences among the BMSC samples from both young and aged donors in terms of their phospholipid class composition.

Fig. 34 ***Appendage: How to read the PCA biplot?***

The BMSC data studied had different dimensions to be taken into consideration. For this purpose a multivariate data analysis technique, PCA was used in order to find the various relationships that exist between the data. PCA highlights similarities and differences between data and displays these relationships in a plot as shown above with two axes PC1 and PC2. In PCA this high dimension data is compressed with minimum loss of information into only two dimensions, PC1 (47.6%) and PC2 (25%) which

represented the total variation amongst the ten BMSC lines in terms of their phospholipid classes.

PCA assigns PC1 to the direction of the largest variation detected among the samples and removes it so that it can then search for the next significant direction of variation and assign that to PC2. In this way, PC1 and PC2 serve as indicators of different and independent variables which are responsible for the changes in the composition detected in the samples. Thus the biplot is interpreted as follows. Samples with similar phospholipid class composition are clustered together. As the distance between two BMSC samples (marked red) increases the more they differed in terms of their phospholipid class profile. Similarly, the lipid classes (marked blue) are responsible for the variations observed in that particular direction. The correlation between the BMSC samples and the phospholipid classes are as follows. The further a BMSC sample is located from the origin and on the same side of the coordinate plane along with a specific lipid class, then this means it is richer in that specific lipid class and poorer in those phospholipids that appear on the other side of the plane. The angle formed when connecting two different BMSC samples via the origin denotes the degree of coincidence of different lipids in all studied BMSC cell lines. (e.g. 0° = total positive correlation, 90° = no correlation, 180° = total negative correlation between the mol% of those two lipids in the studied cell samples).

In the distribution of variation plot (Fig.35), principal component 1 (PC1) was the highest mostly in the latest passages of the BMSC from both the young and aged donors. On the other hand, the second principal component was equally significant in 172p10, 081p4 and 092p10. In BMSC from young donors, most of the variation was mainly due to PC1 while in BMSC from aged donors the response differed from cell line to cell line as well as through passages. Only cell lines 164p4, 172p10 and 271p4 behaved similarly to those BMSC from young donors.

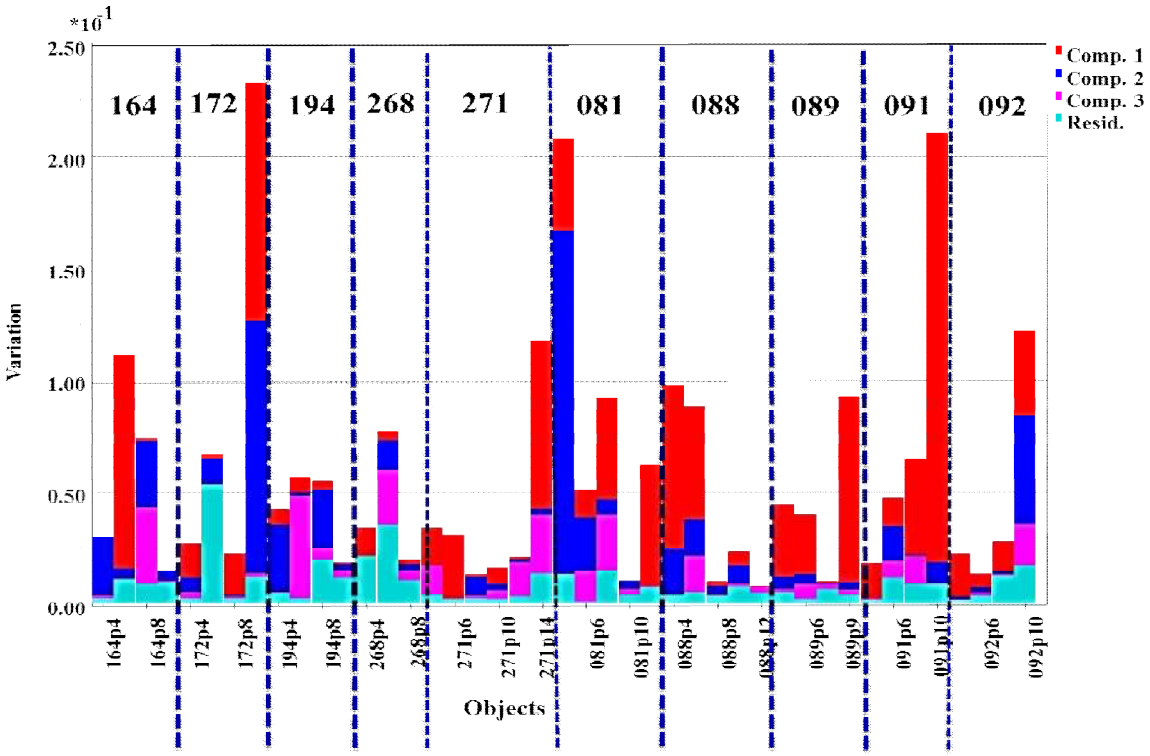


Figure 35 Distribution of variation in the phospholipid class composition for each BMSC sample, for the first three principal components and remaining residuals.

Due to the consistent increase in the totals of 38:4 (PC and PE) species in the latest passages from both the young and aged donors, combined PCA analysis of these two classes of phospholipids was performed. The results in Fig. 36 clearly indicated that latest passages of BMSC from both young and aged donors were rich in PC38:4 and PE38:4. Monounsaturated species from both PC and PE classes were found closer to earliest passages of young and aged BMSC. The angle formed between the lines connecting PC34:1 and PC38:4 via the origin, indicates a near full negative correlation.

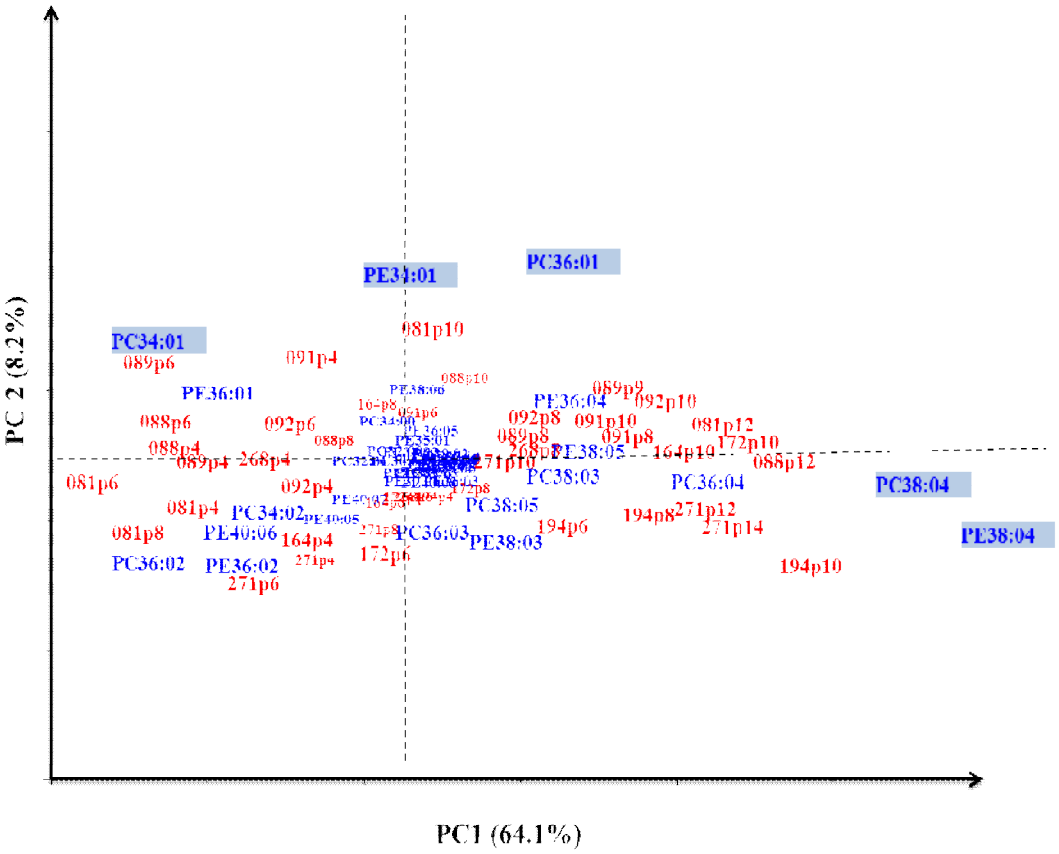


Figure 36 PCA biplot demonstrating similarities and differences among the BMSC samples in terms of their PC and PE species composition.

Again in the distribution of variation graph below (Fig. 37), PC 38:4 and PE38:4 are the main reason behind the largest variation noted as component 1. Other species such as PC34:1, PC36:4, PE34:1 and PE36:1 also contributed to the variations in BMSC in terms of their PC and PE species profile change.

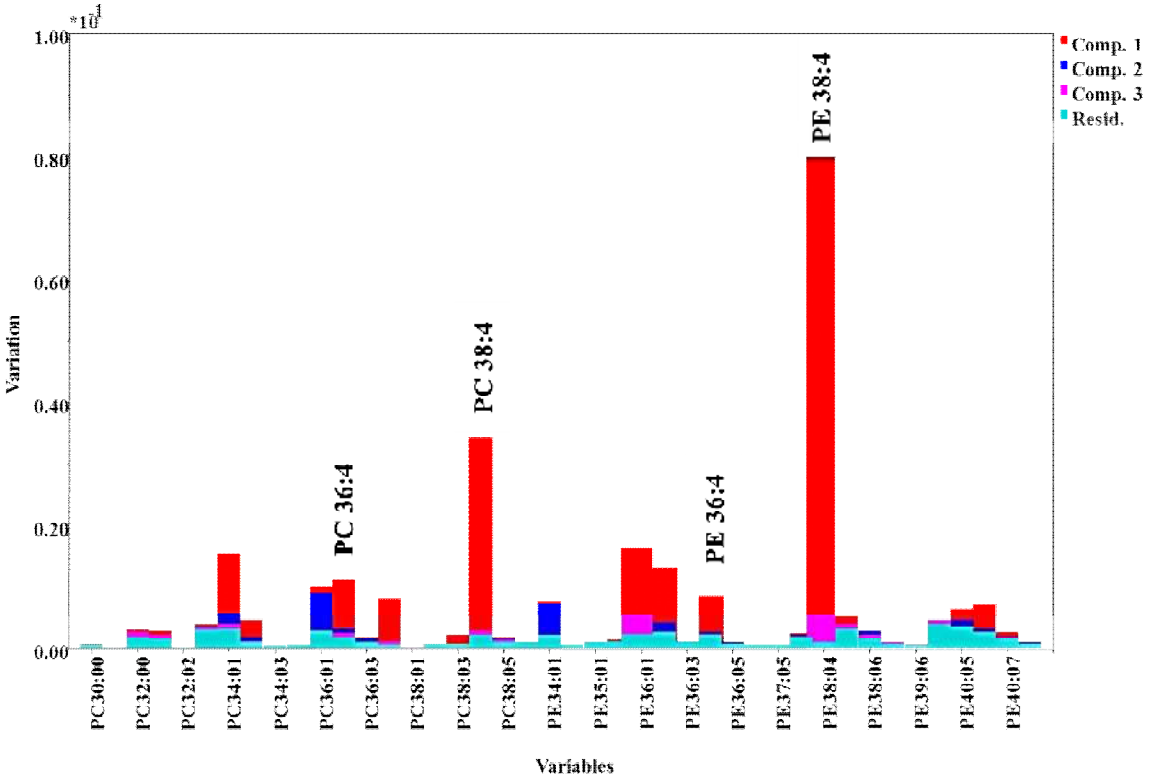


Figure 37 Distribution of variation in the phospholipid class composition for each BMSC sample, for the first three principal components and remaining residuals.

4 Discussion

MSCs' therapeutic potential emanates from their ability to regenerate damaged tissues, which in turn stems from their multipotency nature. However, the low abundance of these cells in the most commonly used sources of adult stem cells necessitates *in vitro* expansion. According to several studies, it is during these repeated culturing steps that progenitor cells lose their growth as well as differentiation potential (Brown and Dykin 1991, Fuchs *et al.* 2007, Tekkatte *et al.* 2011, Tuan *et al.* 2003). At the same time the phospholipid composition of cells was affected by the passage number along with different physiological conditions such as apoptosis induced by aging or inflammation. These challenges coupled with the need to ascertain the quality of transplanted MSCs' inspired the current study which was focused on investigating the effect of passaging and donor age on MSCs' phospholipid profile which can be a reflection of their functionality.

Phospholipid Classes

Regardless of donor age, the different BMSC from both young and aged donors were characterized by similar lipid class composition (Figs. 1 and 2). The phospholipid class proportions were in accordance with those commonly found in mammalian cell membranes, with PC and PE being the two predominant classes and others such as PS and PI present in smaller amounts (Leidl *et al.* 2008, Meer 2005). The results for each phospholipid class are discussed below.

PC. Since PC is the most abundant phospholipid in mammalian cells, and the main building block of the membrane outer leaflet, its cellular level needs to be tightly controlled to maintain the general architecture of cell membranes (Koumanov *et al.* 2004). PC is one of the neutral or zwitterionic phospholipids that have the ability to readily organize into bilayers due to its cylindrical shape as well as large polar head group. These characteristics make it an essential part of the membrane's outer leaflet the primary role of which is to act as a protective shield for cells against the extracellular environment. Despite certain individual variations in the PC totals of young and aged

donors, mol% levels from early to late passages from both sets of donors were maintained in general between 32 - 40%. The rate of PC synthesis via the Kennedy pathway has been shown to be regulated by biochemical processes, such as selective degradation, rather than by actual supervision of certain enzymes' transcription or translation (Sugimoto *et al.* 2008). For instance, excess amount of PC is often converted to DAG or other phospholipids by an enzyme that catalyses the final steps in the Kennedy pathway (Hermansson et al. 2011). In general, the biosynthesis, degradation and inter-organelle transport of phospholipids accompanied by the systematic coordination between these three processes play a major role in maintaining the lipid composition of cellular membranes (Somerharju et al. 2009).

The species profile in this class indicated that of the monounsaturated long-chain PC species, 34:1 was the predominant one present in both young and aged BMSC. It also revealed a decrease in unsaturated phospholipids such as 34:1 and 36:2 from early to late passages (Figs. 20, 21). On the contrary polyunsaturated species 36:4 (16:0/20:4n-6) and 38:4 (18:0/20:4n-6) increased towards late passages. This pattern was even more evident in the combined PCA biplot of PC and PE species (Fig.36) which showed that such PUFAs were enriched mainly in the latest BMSC passages from both the young as well as aged donors. Abundant relative levels of PUFAs supress the synthesis of monounsaturated fatty acids, oleate or palmitoleate especially, by inhibiting stearoyl-CoA desaturase (SCD), an enzyme that catalyses the addition of the first *cis* double bond into the Δ-9 position of the acyl chain (Bené et al. 2001, Sampath and Ntambi 2005). Moreover, Miller *et al.* (2010) recently presented a possible mechanism by which phospholipid species composition in a bacterium (*Pseudomonas aeruginosa*) is regulated. The mechanism involves transcriptional regulators which are able to detect changes in composition rather than concentration of specific fatty acids. Consequently the ratio of unsaturated vs. saturated fatty acids affects the abundance of individual phospholipid species using these fatty acids as structural components and indirectly plays a key role in the regulation of membrane lipid homeostasis. Furthermore, the shift in phospholipid composition in favour of more unsaturated species especially those containing 20:4n-6 may indicate the BMSC' decreased rate of cell proliferation and the

activation of certain lipid signalling pathways which are discussed in detail later on (Surette *et al.* 1999).

PCe. Unlike diacyl species the totals of PCe for this group of phospholipids did show age-related variations (Figs. 22, 23). BMSC's from young donors gradually decreased their alkyl-acyl PC totals towards final passages while the reverse was observed for those from aged donors. An exception to this trend was cell line 271 obtained from an aged donor which was able to maintain its alkyl-acyl PC mol% at constant level. According to Baburina and Jackowski (1999) increased alkyl-acyl PCs totals interfere with the regulated deacylation of excess phospholipids which in turn inhibits normal cell proliferation. Aged BMSC must therefore be exhibiting slower cell growth rates as opposed to the young ones which were able to reduce their alkyl-acyl PCs totals towards final passages.

LysoPCs. Higher LysoPC mol% was seen in late passages from both the young and aged BMSC (Fig. 8, 9). The earliest passages from old donors had double the level of LysoPC present in similar passages from young BMSC. Comparison of the LysoPC species composition of early to late passages from both young and aged BMSC indicated a decrease in palmitic acid (16:0) while in contrast stearic (18:0) and 20:4n-6 levels increased. Furthermore, the total 20:4n-6 content of total fatty acids released from different phospholipids was increased as well (*unpublished data, 2011*). As a bioactive pro-inflammatory lipid LysoPC can be a vital sign of cellular pathological activities (Matsumoto *et al.* 2007, Colles and Chisolm 2000). As a result higher concentration of LysoPC in later passages can be taken as a sign of oxidative stress or an indicator of cells diminished capability to fend off inflammation (Tselepis and John 2002, Watanabe *et al.* 2002, Okita *et al.* 1998). Furthermore, studies have shown that LysoPC accumulation causes an increase in the intracellular Ca^{2+} level with simultaneous stimulation of membrane associated protein kinase C (PKC) activity (Su *et al.* 1995). The influx of Ca^{2+} and associated PKC activity indirectly activate cytosolic phospholipase (PLA2) finally resulting in a rapid release of 20:4n-6 (Mayer and Marshall 1993, Wong *et al.* 1998). This is a possible bioactive mechanism by which

LysoPC contributes to the generation of pro-inflammatory activity in BMSC from older passages.

The species profile for LysoPC and PC emulate one another. This is to be expected since hydrolysis of PC by phospholipase A2 produces LysoPC. The predominant fatty acids present in both classes of phospholipids were palmitic and oleic acids. Besides, both PC and LysoPC mol% profiles had an elevated amount of 18:0 and 20:4n-6 species towards final passages in BMSC from both the young and old donors (Figs. 32, 33). As discussed above the gradual accumulation of 20:4n-6 towards older passages may indicate inflammatory activities and subsequent pathological responses.

PE. BMSC from both young and aged donors (Figs. 10, 11) had declining PE totals towards the latest passages. This pattern was consistent in both groups of donors with the exception of cell lines 194 and 271, which were able to maintain their PE mol% at a constant level throughout. Cellular turnover of PE is maintained by mechanisms currently unclear (Vance 2008). Nonetheless, studies conducted using mutant cell lines defective in PE biosynthesis, ascertained PE's essential role in regulating cytokinesis and normal cell growth (Dowhan and Bogdanov 2002). Thus the reduced PE mol% towards latest passages in both groups of donors may indicate poor cellular growth and division. There are more 20:4n-6 containing species in PE as compared to the other zwitterionic phospholipid, PC. In addition 38:4 (18:0/20:4n-6) was the main PE species that increased towards late passages in both the young and aged BMSC (Figs. 24, 25). Such an increase in 20:4n-6 containing PE species is possibly related to the decline in PS mol% which is used as substrate for the synthesis of polyunsaturated PE molecules via the PS decarboxylation pathway (Bleijerveld et al. 2007, Stone *et al.* 1998). PS is the major precursor for PE synthesis via its decarboxylation in mitochondria (Voelker 1990). Thus the decline in PS totals may be responsible for the decreased steady-state levels of PE. Overall, the progressive decline of PE totals towards older passages in both the young and aged BMSC, may compromise its role in neutralizing the net negative charge effect of certain transmembrane proteins (Dowhan 2009).

PEe. Latest passages in BMSC from young donors had higher PEe (Fig. 12) while the reverse was true for BMSC from aged donors (Fig. 13). However, the overall PE total pool for both the BMSC from young and aged donors was primarily composed of Pee towards older passages. This was in contrast to early passages which contained a higher proportion of PE diacyl species instead. Similarly, of the total PE pool present in inflammatory and tumor cells, approximately 70% was comprised of ether lipids (Vance 2008). A potential function for PEe is to act as sink for polyunsaturated fatty acids (Nagan and Zoeller 2001). Indeed the species profiles from the BMSC from young and aged donors (Figs. 26, 27) support this view by having 36:4, 38:4 and 40:5 as predominant species. Enhanced susceptibility of PEe to oxidative attack indirectly helps to protect other phospholipids from similar damage (Zoeller *et al.* 1999, Brites *et al.* 2004, Wallner and Schmitz 2011). This role of PEe as scavengers of reactive oxygen species could explain the need to increase their molecular levels as seen in the latest passages of BMSC from aged donors. Thus, theoretically the inability of BMSC from aged donors to up regulate PEe synthesis may suggest higher oxidative stress.

PI. An increase in the cellular levels of PI from early to late passages was evident in both BMSC from young as well as aged donors (Figs. 16, 17). Since 20:4n-6 is the primary precursor in the *de novo* synthesis of PI (Nuwayhid *et al.* 2006), enhanced levels of PI could be linked with increased levels of LysoPC liberating 20:4n-6 as mentioned above. Characteristic to animal cells studied previously the BMSC from both young and aged donors (Figs. 28, 29) had a high content of 18:0 and 20:4n-6 species. Consequently, PI is taken to be the primary source of arachidonic acid in animal cells. This freed 20:4n-6 in turn is required for the synthesis of eicosanoids and prostaglandins by the action of phospholipase A_2 (Zhou and Nilsson 2001). Furthermore, the ability of PI to be phosphorylated at various sites renders it an important precursor for several second-messenger molecules such as inositol 1, 4, 5-trisphosphate and DAG. Thus PI is needed to initiate different lipid signaling pathways that affect proliferation and differentiation of cells and contribute to various inflammatory functions (Wymann and Schnieter 2008). PI is mainly located in the inner leaflet of the plasma membrane

alongside PS. Thus the increase in PI totals as well as in PI: PS ratio asserts the enhanced PI-related signaling towards the late passages.

PS. Anionic phospholipids PS and PE are the main amino phospholipids present in the internal leaflet of plasma membrane. These two phospholipids also have interrelated biosynthetic pathways; PS is made by base-exchange reactions in which polar head groups of either PC or PE are replaced by serine, or vice versa, while an alternative synthesis route for PE involves the decarboxylation of PS (Kanfer 1980, Bleijerveld et al. 2007). The PS totals in cell lines 81, 91, 92 from the young donors and 268 and 27 from the aged donors (Figs. 15, 16) had lowered mol% in the late passages. On the contrary, cell lines 164, 172 and 194 from aged donors had increased PS mol% towards the latest passages.

Not much is known about the regulation mechanism involved in neither PS biosynthesis nor its decarboxylation into PE in mammalian cells. Several studies speculate that there is a feed-back mechanism that keeps PS turn over and level constant (Kuge et al. 1998, Vance and Steenbergen 2005). In other words the activities of enzymes that catalyze PS synthesis are regulated by the end-product, PS. Thus several reasons can account for the rise and fall in PS totals observed in the BMSC. PS is synthesized mainly from PC and to a lesser extent from PE molecules by base-exchange reaction which requires Ca^{+2} (Kanfer 1980, Kuge *et al*. 1998, Stone and Vance 1999). Since PC totals were shown to be maintained at a constant level, shortage of PC precursors needed for head group exchange reactions can be ruled out as a reason for the observed fall in PS totals. However, PS synthesis is also known to be regulated by the docosahexaenoate (22:6n-3) levels which decreased towards late passages (unpublished data, 2011) and thus shortage of this preferred fatty acid constituent might hinder PS biosynthesis (Gracia *et al*. 1998). A possible alternative explanation for the reduced PS synthesis may be depletion in Ca^{+2} from ER stores (Pelassy *et al*. 1992). On the other hand, higher concentration of PS, as seen in some of the aged donors, may signal over expression of the catalytic enzymes involved in the base-exchange reaction as well as inhibition of PS decarboxylation to

limit the conversion of PS to PE. Furthermore, studies suggest that this type of increase in PS synthesis is characteristic of apoptotic cells (Aussel et al. 1998).

In summary, the phospholipid compositions of the BMSC from both young and aged donors were close to earlier records for MSCs (Fuchs *et al.* 2008, Meer 2005). However, certain exceptions did exist in the case of PE totals which had a slightly higher mol% at an average of 36% for BMSC from both young and old donors. Phospholipid Although the BMSC from the different donors were cultured in similar conditions individual variation were expected due to the donors' age, hereditary reasons, physiological condition, diet and other such factors. Consequently, certain differences were evident between BMSC from the different donors especially in the totals of LysoPC and PS.

The variation in the phospholipid class and molecular species profiles among the donors was smaller than that observed between consecutive passages i.e. between early to late passages. In general, the early passages maintained the normal cellular phospholipid profiles but this regulation was gradually lost towards the late passages. As the univariate t-test of single species and the multivariate analyses for example the compiled distribution of variation graph for both PC and PE species showed, earlier passages of BMSC from both young and aged donors were rich in saturated and monounsaturated molecular species (*e.g.* 34:1, 36:1). Whereas, latest passages were poor in these monounsaturated species, they were highly enriched in polyunsaturated species, especially 20:4n-6 -containing ones (36:4, 38:4). The biological significance of these findings mainly relates to the lipids function as signalling molecules. Upon activation of certain lipid signalling pathways, the cellular responses may go in either direction of proliferation, differentiation or programmed cell death depending on the type of signal received. Several cellular signalling pathways originate in membranes when 20:4n-6 is deliberated from phospholipids especially for eicosanoid signalling, of special interest to this study.

The main precursors for eicosanoid synthesis are PUFAs such as 20:4n-6, which is liberated from the *sn-2* position of phospholipids by the action of phospholipase A_2 (Buczynski *et al.* 2009). Upon release 20:4n-6 is subsequently converted to prostaglandins, leukotrienes and lipoxins collectively termed as eicosanoids (Cheng *et al.* 2006). These bioactive lipids have different physiological and pathological roles including vascular homeostasis, inflammation, cell proliferation and differentiation (Harizi *et al.* 2008). Moreover, eicosanoid biosynthesis increases during inflammation whereby they act as chemoattractants and platelet aggregators.

As the main finding supporting lipid-mediated functional changes in the late passages of the BMSC, the lipid molecular species profiles and the PCA demonstrated that 20:4n-6 - containing phospholipids markedly increased towards the late passages. Of particular interest were the 38:4 species in both PC and PE profiles which increased their proportions from early to later passages. These species of lipids can be used as biomarkers that can be monitored in association with MSCs' passaging or cell aging. In addition, another efficient biomarker could be the PI: PS ratio which showed a consistent rise throughout sequential passaging. In this perspective lipid's role as biomarkers assists in providing tested, viable and functional cell lines needed to ensure efficient therapeutic MSC applications that guarantee patient safety.

5 References

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