Development of novel isolation methodologies for microvesicles and exosomes, as potential biomarkers for health (Therapeutic Lifestyle Changes), and use of microvesicle-delivered β-Gly in erythroleukaemia

Presented by

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Thesis submitted in partial fulfilment of the requirement

for the degree of

DOCTOR OF PHILOSOPHY (Ph.D.)

October 2016

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DEDICATIONS

I dedicate this thesis to anyone who is suffering adversity and wishes to create a life of transformation, joy, fulfilment, and happiness, not just for themselves, but so they can share the experience with family and loved ones. Be the change. Aim higher. Inspire.

It is a great honour to dedicate his thesis to Trevor Little, Aunty Rosanne, Aunty Saras, all people I deeply valued, whom I have lost throughout the duration of this thesis.

I would also like to dedicate this to Dr. Bill Revel, a fellow motorcyclist, and inspiring lecturer. The loss of Bill, and surviving my own experience of a horrific motorcycle accident, that has left me with life changing injuries, has really made me question how much time any of us have left. Realising none of us know when our number is up, I asked myself what would happen if I died today? What is my legacy? - This became jet fuel for finding my purpose and combined with passion, really unlocked my productivity.

Thank you to Dr. Susannah Jenner at iCope, Dr Knight for the ongoing support, managing my condition has been extremely challenging. Thank you for being here to make a positive impact.

I would like to dedicate this to Isiah Hankel, Founder of the Cheeky Scientist Association, I am well on my way to becoming a prolific public speaker and I aim to travel the world over and over again. I would like to inspire the elevation of consciousness. Thank you for publishing a bestselling book, Black Hole Focus, this was truly a life changing book. Thank you for inspiring me to understand and harness the power of purpose.

Thank you to my many mentors, renowned economist; Paul Zane Pilzer. Earl Nightingale, Founder of the Personal development industry; Marcus Aurelius for the Meditations, Steven Covey, Tony Robbins, Jim Rohn, Zig Ziglar, Brian Tracy, Bob

Proctor, Joe Vitale, Napoleon Hill, Malcom Gladwell, Robert Kiyosaki, Deepak Chopra, Daniel Priestley, Grant Cardone, Vishen Lakshiani and Tai Lopez, all whom have online digital vaults that have provided invaluable counsel, and primed me for productising my passion.

Thank you to the non-conformists, Dr Rupert Sheldrake, Dr Joe Dispenza, Dr. F. Batmanghelidj, for shifting the scientific paradigm in the face of scrutiny. Thank you, Dr Bruce Lipton, Prof. Rudolph Tanzi for expanding my understanding of epigenetics and expanding my scientific horizon, particularly in relation to the science behind the mind-body- spirit connection, and the role this plays in immunotherapy, wellness and disease prevention.

Thank you to The School of Life, who has accelerated my journey of self-discovery- I recommend you try the game- “Know Yourself” with friends and family- this is the key to understanding human behaviour and increasing awareness. For more self-knowledge – Go Here:

http://www.thebookoflife.org/know-yourself/

Finally, I would like to dedicate this thesis to my family, friends and colleagues who have contributed endlessly towards my health and radical wellbeing.

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ACKNOWLEDGEMENTS

Firstly, thank you God, I am still here with us today.

I would like to express my heartfelt appreciation to my immediate and extended family, especially my mother, Luxmi Rose Grant, my father Ian Duncan Grant, and beloved sister, Melissa Anne Grant, beloved brother, Duncan Grant, and my Grandmother, all of whom have always inspired and encouraged me to be the change I wish to see, and actively challenged my views, a rivalry much appreciated, that spurred my growth from an infant through adulthood. Thank you for providing great intellectual stimulation.

I would express great thanks to Makeda Kumrai, Raphael King and Loben Henry, loving friends. Thank you for all the support and encouragement, insisting my physical body and mind where always nourished and rested. Without all of the above support, this thesis would not have been possible. I thank myself for following through to completion, even when the tough times got tougher. I am thankful for my relatively good health, even throughout times of severe adversity. I now know I am capable of so much even throughout the harshest of circumstances.

I would like to thank my team for their support, Dr. Ahmad Haidery, Dr. Samireh Jorfi, Dr Sharad Kholia, & Dr. Dan Stratton. I would also like to extend my thanks to my colleagues Dr. Chris Bax, Prof. Chris Palmer, Memory Pinchbeck, Sheelagh Heugh, in addition to Dr Una Fairbrother. Thank you, Dr B Angst, Dr. B Awamaria, Ruth Eyob, Louis Stott and Eric Coleman for their provision, assistance and intellect. Thank you, Cornell Coggins and Administrative staff, including Doreen Henry and Mel Goldsmith for your provision of resources and administrative skills.

I would like to thank London Metropolitan University for the award of the Vice Chancellors Scholarship, which enabled my further tuition and funded my learning, I am most great-full.

I would like to give special thanks to Prof. Jameel Inal for his continuous, advice, support, and guidance, both professional and scientific. Thank you very much for always making time to listen and provide exceptional support even throughout very challenging times, this is what true friendships and teams are made of, thank you for being exemplary. Thank you for keeping me focused and keeping me on track to completion. Your service and support are unrivalled. You have always inspired me to aim higher, and thus I hope I have become a continuous source of your own inspiration with my work. Thank you for the privilege of being your student, it is great to see as I have grown, so have you.

I wish you a life of eudaimonia.

I wish the following quote serves you well.

"Know Yourself"

In Ancient Greece, the philosopher Socrates famously declared that the unexamined life was not worth living.

Asked to sum up what all philosophical commandments could be reduced to, he replied: 'Know yourself.'

Knowing yourself has extraordinary prestige in our culture. It has been framed as quite literally the meaning of life.

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ABSTRACT

Both MVs and exosomes are isolated by similar methodologies such as differential centrifugation of cell culture supernatant (S/N), filtration, floating on chemical gradients or using antibody labelled magnetic beads. Hence, the isolation techniques, commonly in use, provide low levels of purification and therefore require improvements and an in-depth analysis of observed results. This research has established a protocol for isolation of these vesicles and a means to identify them using known markers and their respective properties. The isolation techniques used and developed in this research include differential centrifugation, sucrose gradient centrifugation, and filtration. MVs and exosomes were later identified and distinguished according to their relative ability to bind FITC and PE conjugated annexin V, anti-LAMP1, anti-CD63 and anti-CD11b antibodies using flow cytometry. Protein gels were run with MVs and exosome samples isolated by different techniques to identify some distinct proteins in MVs/exosomes that may be uniquely present on one or either and hence that could be used as a marker to distinguish MVs from exosomes.

To prevent exosomal contamination of MV preparations, in a further improvement of the newly developed reverse filtration technique, gentle disruption of exosome aggregates by gentle water sonication was used and measurements of plasma MV levels made from a population of healthy donors. This found plasma MV levels to range between 0.51 and 2.82 x 10^5 MVs/ml. The MVs were characterised as phosphatidylserine-positive and ≥0.2 μm in diameter. Most of the variables looked at in this study including

freezing at -20ºC, gender and subject age did not alter MV absolute counts significantly. For the first time the effect of fasting on MV levels has been studied. Fasting individuals had a wider spread and appeared to have higher MV levels ($2.8x10^5$-$5.8x10^5$ MVs/ml). This could be defined as the normal baseline fasting reference range, which is almost 3-fold higher compared to the non-fasting group reference range (0.9-$1.5x10^5$) MVs/ml. These results suggest that when analysing total MVs, further investigations should use fasting subjects, as for quantification of other fasting analytes.

Prospective markers of (monocyte-derived) MVs, IL-1α, RANTES, G-CSF, CCL-1 and IL-17E are proposed for further investigation. MVs were shown to inhibit phagocytosis of apoptotic bodies more effectively than exosomes. With a view to understanding likely pathways being stimulated during MV release, and to also shortlist possible pharmacological reagents capable of being used to therapeutically inhibit Y27632, calpeptin and methyl-β cyclodextrin were shown to inhibit MV release. MV release from K562 cells was inhibited by Y27632 and calpeptin but less effectively by chlorpromazine or methyl-β-cyclodextrin, MV release from THP-1 acute monocytic leukaemia cells was greater than from peripheral blood monocytes which were in turn shown to express more phosphotidylserine than on exosomes.

With a view to using MVs as drug delivery vehicles, THP-1 MVs were found to fuse/hemifuse to K562 erythroleukaemia cells, but not at 4°C or if the MV surface was blocked with annexin V. β-Gly (10 μM) (as well as MVs carrying β-Gly [β-Gly MVs]) was found to inhibit proliferation at least 3-fold compared to untreated control. β-Gly and β-Gly MVs increased the doubling time but

neither induced apoptosis of K562 cells (unlike MVs from apoptotic cells). Finally, β-Gly increased the percentage of cells in G0/G1 whilst decreasing the cells in G2/M and appears to induce erythroid differentiation as seen by the increase in the percentage of benzidine-stained cells.

Key words: MVs, exosomes, reverse filter, sucrose gradient ultracentrifugation, Lifestyle, Cancer Prevention, Epigenetics.

Abbreviations

AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride
AML	Acute myelocytic leukaemia
An V	Annexin V
BSA	Bovine serum albumin
C5a	Complement 5a Protein
Calp	Calpeptin
$[Ca^{2+}]_i$	Intracellular calcium
DAF	Decay accelerating factor
DNA	Deoxyribonucleic acid
DTT	Dithio threitol
ECM	Extracellular matrix
EGTA	Ethylene glycol-bis (2-aminoethylether) N,N,N',N'-tetraacetic acid
ELISA	Enzyme immunosorbent assay
ER	Endoplasmic reticulum
Exos	Exosomes
FACS	Fluorescent activated cell sorter
FGF-1	Fibroblast growth factor -1
FSC	Forward scatter
g	G-force
H_2O	Water
Hsp	Heat-shock protein
HUVECs	Human umbilical vein endothelial cells
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17E	Interleukin 17 E
ILV	Intraluminal vesicles
KH_2PO_4	Potassium dihydrogen phosphate
MIF	Macrophage inhibitory factor

MNA	Methyl nadic anhydride
MPs	Microparticles
mRNA	messenger Ribonucleic acid
MVs	Microvesicles
MVBs	Multivesicular bodies
Na_2HPO_4	Disodium hydrogen phosphate
NI	Non-Induced
PB	Peripheral blood
PBB	Permeabilization buffer
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline – Tween
PC	Phosphatidylcholine
PEAM	Platelet expressed adhesion molecules
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphatidyl inositol-3-kinases
PL	Phospholipid
PM	Plasma membrane
PMN	Polymorphonuclear neutrophils
PMSF	Phenylmethanesulfonyl Fluoride
PMP	Platelet microparticles
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
RANTES	Regulated on activation normal T expressed and secreted
RBCs	Red Blood Cells
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
SSC	Side scatter
SM	Sphingomyelin
TEMED	N, N, N-tetramethylethylenediamine
TF	Tissue factor

TFG-β1	Transforming growth factor β 1
TfR	Transferrin receptor
TPA	Tissue plasminogen activator
v/v	Volume per volume
w/v	Weight per volume

Original Publications

Grant, R.C., Inal, J.M. (2012) Isolation of microvesicles and exosomes by microfiltration and estimation of normal reference range in blood plasma. Microvesiculation and disease; 13-14 September 2012. *Biochem Soc. Trans.* 41, Abstract P008

1st International meeting of the International Society for Extracellular Vesicles, Gothenburg:

Grant, R., Ansa-Addo, A., Stratton, D., Antwi-Baffour, S., Jorfi, S., Kholia, S., Krige, L., Lange, S., Inal, J.M. (2012) Isolation of microvesicles and exosomes by filtration and estimation of normal reference range in blood plasma. *Journal of Extracellular Vesicles* Vol 1, Abstract 18

Stratton, D., Antwi-Baffour, S., Williams, G., **Grant, R.**, Lange, S., Inal, J. M. (2012) Characterisation of microvesicles released from cells constitutively and upon stimulation. *Journal of Extracellular Vesicles* Vol. 1, pg 2. 99th American Association of Immunologists meeting, Boston:

Inal, J.M. Stratton, D., Antwi-Baffour, S., **Grant, R.** (2012) Two subtypes of Plasma Membrane-derived Vesicles can be isolated from tissue culture. *J. Immunol.* 188 Abstract 1336625.

Grant R., Ansa-Addo E, Stratton D, Antwi-Baffour S, Jorfi S, Kholia S, Krige L, Lange S, INAL J. (2011) A filtration-based protocol to isolate human plasma membrane-derived vesicles and exosomes from blood plasma. *J Immunol Methods.* 371, 143-51.

1. Introduction

1.1 First reports of extracellular vesicles

Exosomes are released from cells and are of endosomal origin but besides these vesicles, cells release membrane-derived vesicles that are released by direct budding form the cell surface. Over seventy years ago in the 1940s, the first clue that there might be pro-coagulant subcellular entities in plasma was proposed, when it was found that platelet-poor plasma (PPP) took longer to clot after it had been centrifuged at high *g* forces (reviewed in Inal et al., 2012). Twenty years later in 1967, it was shown for the first time showed that activated platelets shed membrane fragments, which he termed “Platelet Dust” (Wolf et al., 1967). Wolf and colleagues went on to show that the platelet-derived membrane fragments were associated with the phospholipid-related procoagulant activity, otherwise now known as platelet factor 3 (PF3).

1.2 Ectosomes, microparticles, plasma membrane derived microvesicles, otherwise known as ‘Microvesicles’ (MVs)

Ectosomes, Microparticles (MPs), plasma membrane-derived microvesicles (PMVs), are one in the same entity and for the purposes of this thesis will be referred to as microvesicles (MVs). Microvesicles are membrane vesicles that are phospholipid rich and that carry various membrane receptors including other intravesicular proteins from their parental cells. MVs are released by every eukaryotic cell type directly from the plasma membrane upon activation, apoptosis, necrosis or through stress. MVs are formed by pinching off from the cell membrane and range in size from 0.1 µm to 1 µm (Coombes et al., 1999), and platelet MVs, some of the first recognised MVs, were shown to have a role in thrombosis and inflammation (Wolf et al., 1967). Prior to links with inflammation and immune and infectious disease as well as cancer biogenesis and spread, MVs were considered inert material.

1.2.1 Mechanisms of microvesicle biogenesis

The precise mechanism of MV biogenesis has not yet been fully elucidated and it is likely that there are several overlapping pathways, leading to different MV sub-types. MV release now appears to be a highly controlled process. Cells can be activated by different stimuli and MV release does not appear to be a random process such as when the plasma membrane of a dying cell undergoing necrotic degradation. Four cell membrane phospholipids, namely phosphatidylcholine (PC) and sphingomyelin (SM) on the external leaflet of the lipid bilayer and phosphatidylserine (PS) and phosphatidylethanolamine (PE) on the inner leaflet maintain cell membrane integrity through five enzymes, gelsolin (which is only found in platelets), aminophospholipid translocase, floppase, scramblase and calpain (Zwaal et al., 1997). In the steady state, phospholipids are moved towards the outer leaflet whilst aminophospholipids are moved towards the inner leaflet of the lipid bilayer. However, either during cell activation, apoptosis or necrosis, the cytosolic concentration of Ca^{2+} rises, resulting in a change of the steady state affecting the ability of the cell to maintain this membrane asymmetry and in the stable exposition of PS on the external surface of the plasma membrane.

Cytosolic calcium concentration is indeed a central and fundamental condition to initiate MV formation. Gelsolin, in platelets, removes the capping proteins at the end of actin filaments (maintaining the cytoskeletal structure) resulting in increased Ca^{2+} levels in the cytosol (and therefore MV release) (Piccin, et al., 2007). Increased levels of cytosolic calcium inhibit the activation of aminophospholipid translocase that carries out ATP dependent transport of PS, against an electrochemical gradient to the inner layer of cell membrane from outside (Seigneuret et al., 1984). Floppase or phospholipid translocase may work along with aminophospholipid translocase and facilitates transport of phospholipids from the inner to the outer leaflet (Connor et l., 1992). Increased cytosolic Ca^{2+} also results in scramblase is activated but flippase inhibited thus allowing a random movement of phospholipids between both leaflets, leading to a loss within minutes of the

lipid asymmetry (Williamson et al., 1995). Lipid scramblase, activated at higher cystolic Ca^{2+} concentrations, allows the movement of phospholipid cross the cell membrane and thus PS is expressed stably on the outer surface (Andrea et al., 2007; Zwaal et al., 1993). Calpain, again activated by increased cystolic Ca^{2+}, cleaves cytoskeletal filaments, and as interactions between aminophospholipids on the inner leaflet of the lipid bilayer with skeletal proteins are disrupted. The resulting effects on cell shape and membrane mechanical stability result in microvesicle shedding and eventually in the activation of apoptosis through procaspase 3 and BclxL (Andrea et al., 2007); Rho associated kinase 1 (ROCK-1) is essential for apoptotic membrane blebbing and membrane blebbing is associated with MV release in apoptotic cells. Depending upon the mechanism of MV formation their size and constituents vary (Jimenez et al., 2003).

Release of platelet MVs (often referred to as platelet microparticles, PMPs) can occur during cell activation by adrenaline, adenosine diphosphate, thrombin, collagen, Ca^{2+} ionophore A23187, complement membrane attack complex (MAC) and shear stress (S. P. Ardoin et al., 2007; Simak et al., 2006; Horstman et al., 1999). Other stimuli of microvesiculation include microbial peptides such as formyl-methionyl-leucylphenylalanine (fMLP) (Gasser et al., 2003; Hess et al., 1999), and phorbol esters. MVs also take surface proteins and other contents of the originating cell (Martinez et al., 2005) while they are shed. As well as biological stimuli, shear stresses imposed on cells are important mechanical factors able to induce microvesicle release from cells. Furthermore, it should be noted that some of these MV-inducing stimuli are likely to be additive or possibly synergistic.

1.2.2 Genetic elements in MV biogenesis

The ATP-binding cassette transporter A1 (*ABCA1* gene) modulates the transbilayer distribution of phosphatidylserine (PS) at the outer leaflet of the plasma membrane and also controls the initial steps of reverse cholesterol transport, that is the release of cellular phospholipids to lipid-free apolipoproteins (Combes et al., 2005). Curiously, the ABCA1 transporter is

a major element controlling cerebral malaria susceptibility. In this study authors have shown ABCA1-/- mice to have significantly reduced numbers and pro-coagulant activity of MVs compared to wild type ABCA1 +/+ mice. Interestingly it was demonstrated that ABCA1-/- knockout mice in fact develop complete resistance to cerebral malaria. It was also shown that red blood cells (RBCs) from ABCA1-/- mice have a reduced capacity to externalise PS and hence to release MVs. Moreover, flippase activity of ABCA1 is largely responsible for the lipid transbilayer remodelling and translocation of PS to the external leaflet of the lipid bilayer in the plasma membrane (Daleke DL., 2003). MVs contain glycosylphosphatidylinositol-anchored CD14 molecules and the GM_1 (Phu T et al., 2006), both being markers of plasma membrane rafts which appear to be enriched on MVs (Satta, N et al., 1994; Parton, R. G et al., 1994); it is likely that vesiculation to release MVs involves plasma membrane raft domains.

1.2.3 Composition of MVs

The composition of MVs largely depends on the state and constituents of original cell and type of inducing agent(s) used along with the mechanism of MV release. However, the rules of incorporation of different proteins in MVs are not yet understood. PMPs express Gplb (CD42b), platelet endothelium adhesion molecule (PECAM-1, CD31) (Andrea et al., 2007; Losy et al., 1999), integrin aIIb ß3 (GpIIb-IIIa)(Schwarz et al., 2004), P-selectin (CD62P) (George et al., 1986), CD63 (Tschoepe et al., 1990), CD41a and CD 61 (von Ahsen et al., 2001). CD42 found in platelets is not expressed in PMP revealing that a differential sorting mechanism is involved in MV formation. MVs from endothelial cells express CD31, CD34, CD51, CD54, CD62E (E-selectin) (Mutin et al., 1997) CD62P (P-selectin), CD105 (endoglin) and CD146 (S endo 1). The protein composition very much relies on whether MVs are released upon cell activation or apoptosis and the comparison of both showed that constitutive endothelial markers (CD31 > CD105) were more in MVs from apoptotic cells, while inducible endothelial markers (CD62E > CD54 > CD106) (Jimenez et al., 2003; Hussein et al., 2003) were

increased in MVs from activated cells. Furthermore, MVs from apoptotic cells have more annexin V and CD31 (Horstman et al., 2004).

1.2.4 Functions of MVs

After several years of microvesicle research it is only now becoming clear that MV release impacts on a broad range of biological processes, including intercellular communication, signal transduction, coagulation and transfer of functional receptor proteins, such as the chemokine receptor, CCR5, a co-receptor for HIV from mononuclear cells to cells not expressing it, and that this transfer renders the recipient cells able to be infected (Mack et al., 2000). These activities are possible because of the phospholipid rich surfaces of MVs and the particular surface molecules they express. One of the early biological functions of MV release that was delineated came in the field of complement biology. Here it was shown that microvesiculation protects against complement attack by removing the C5b-9 membrane attack complex (MAC) from the cell surface as shown for polymorphonuclear leucocytes (PMNs), oligodendrocytes, and even erythrocytes (Scolding et al., 1989; Iida et al., 1991; Campbell et al., 1985). Stein and Luzio coined the term ‘ectocytosis’ for the release of right-side-out oriented vesicles (ectosomes) from the surface of PMNs attacked by the sublethal concentration of complement (Stein and Luzio, 1991). However, the function of ectocytosis is not only the removal of C5b-9 complex, but also the specific sorting of membrane proteins and also lipids into the shed MVs. Hess et al. have provided evidence that when PMNs are stimulated *in vitro* with formyl-methionyl-leucylphenylalanine (fMLP) or exposed to calcium ionophores, they release small vesicles in minutes which have many of the properties expected for MVs due to complement attack (Hess et al., 1999). These MVs expressed a specific set of membrane proteins, including the complement receptor 1 (CR1), and in addition, proteases such as myeloperoxidase (MPO) and elastase, suggesting that they might function as extracellular antibacterial organelles as shown later (Timar et al., 2013). There are numerous other biological functions that have been described. For example, IL-1 is rapidly secreted by THP-1 monocytes, because it is present in the

MVs that the cells shed (MacKenzie, et al., 2001). Furthermore, platelet and monocyte-derived MVs induce the coagulation cascade and inflammatory processes by activating endothelial cells (Nomura et al., 2001; Satta, et al., 1994; Satta, et al., 1997). In other work MVs from leucocytes, mainly PMN or neutrophils were shown to activate endothelial cells (Mack et al., 2000) and chondrocytes which were found to continuously release MVs loaded wtih annexin I, which induces calcium precipitation and bone formation (Anderson, 2003).

Being smaller in size, MVs can readily be transported through the blood stream reaching the cells and tissues, which the cell of origin could not have communicated with. MVs can transfer surface molecules like receptors, to other cells and exchange membrane and cytoplasmic proteins (Ardoin et al., 2007; Fritzsching et al., 2002; Barry et al., 1998). PS and tissue factor exposed on MVs play a role in the coagulation cascade. Platelets and PMP both contribute to haemostasis (Freyssinet et al., 2003). MVs from endothelial cells contain von Willebrand factor multimers, which promote and stabilize platelet aggregation (Jimenez et al., 2005). MVs from patients having bleeding and thrombosis-like disorders have procoagulant activity (Nieuwland et al., 2000; Jimenez et al., 2001). MVs are shown to have inflammatory properties. PMPs can induce binding of monocytes to endothelial cells and promote survival of haematopoietic cells (Baj-Kryworzeka et al., 2002). P-selectin can possibly promote leukocytes aggregation and are found on MVs (Forlow et al., 2000). MVs can also secrete IL-1β (Mackenzie et al., 2001) having proinflammatory roles.

Apart from the above-mentioned biological conditions MVs are also associated with many diseases and immune conditions like atherosclerosis, diabetes, metabolic syndrome, malignancy, pregnancy, multiple sclerosis, rheumatoid arthritis, vasculitis, systemic lupus erythematosus and antiphospholipid antibody syndrome.

Some researchers consider MVs as ‘vectors’ of waste removal which are in turn removed by macrophages, but even if that is the case the evidence is

mounting that they most likely play other important roles in inflammation, even curtailing inflammation as shown for anti-inflammatory cytokine release from macrophages that had phagocytosed neutrophil-derived MVs (Gasser, 2004); by contrast neutrophil MVs express enzymes able to attack microorganisms which they may well do at sites of acute infection (Gasser et al., 2003), thus likely protecting the organism from excessive inflammation at the site of infection. Exosomes for example are able to elicit specific immune responses (Andre et al., 2004; Skokos et al., 2003; Thery et al., 2002). Whilst the MVs released from chondrocytes support ossification (Anderson, 2003), endothelial cell, monocyte and platelet MVs enhance thrombosis (Sabatier et al., 2002; Satta et al., 1994).

In yet other studies it was shown, at least *in vitro*, that platelet MVs are capable of increasing the adhesion of monocytes to endothelial cells. MV-stimulated HUVECs cells increased the expression of ICAM-1, but not that of VCAM-1, P-selectin or E-selectin. Receptor complexes on monocytes, CD11a/CD18 and CD11b/CD18 were upregulated and MVs induced chemotaxis of monocytes (Marcos et al, 2000). Interestingly, the induction of monocyte endothelial adhesion through platelet MVs was mimicked by arachidonic acid isolated from MVs. In fact, platelet MVs modulate platelet, monocyte, and vascular endothelial cell function by the direct effect of MV arachidonic acid and by its metabolism to bioactive prostanoids. However, platelet activation required prior metabolism of MV arachidonic acid to thromboxane A2 (TXA2) (Barry et al., 1998).

1.2.5 Present status of MV isolation

Differential centrifugation is the most commonly used method for isolation of MVs but it has its limitations. This technique involves centrifugation cycles to remove cells and debris, followed by high speed ultracentrifugation to pellet MVs. Table 1.1 summarises how differential centrifugation has been adapted by different authors. Clearly there is no standard centrifugal force and time criteria set for isolation of MVs as there are various factors to be

considered during centrifugation such as the density of the medium MVs are in, and the distance they need to travel to form a pellet. As the centrifuges and rotors used by authors differ, the centrifugal force and time have to be optimized by each.

Microparticles Preparation		Pre-stoge	Washing Buffers	AnV	Authors
PFP plasma	Microparticles pellett				
1550 g × 20 min 20 °C	17500 g × 20 min 20 °C	−70 °C	apopbuffer	yes	*Nieuwland et al Blood 2000*
1550 g × 20 min 20 °C	18.000 g × 30 min 20 °C	−80 °C	PBS-citrate	yes	*Biro J et al Thr. Hemost 2001*
1550 g × 20 min 20 °C	17.570 g × 30 min 20 °C	none	PBS CaCl mouse serum	yes	*Joop et al. J Thr. Hemost 2001*
1550 g × 10 min 20 °C 1300 g × 10 min 20 °C	100.000 g × 60 min × 2times 20 °C	−80 °C	HEPES	yes	*S. Shet et al Blood 2003*
2700 g × 2 times	19800 g × 10 min 20 °C	−80 °C	HBSS	yes	*J. Simak et al BJH 2004*
1550 g × 20 min 20 °C	17570 g × 30 min 20 °C	−80 °C	PBS	yes	*Diamant M Cirulation 2002*
1550 g × 15 min	1300 g	na	na	yes	*Combes V J Clin invest 1999*

Table 1.1 Variations in differential centrifugation protocols for isolating microvesicles. Adapted from Piccin, A., et al., 2007

Gasser et al., in 2003, used two consecutive runs, 20 min each of 4000 *g* at 4°C and concentrated cell culture supernatant 50-fold using Centriprep centrifugal filter devices (10,000 MW cut off, Millipore Corporation, Bedford, MA, USA) to isolate ectosomes (actually MVs). Interestingly electron microscopy of this ectosome sample shows vesicles sized smaller than 100nm, which does not fit into the defined size for MVs/ectosomes but does for exosomes and hence poses a question on the isolation protocol used here. Such mistakes may often occur when identifying MVs/ectosomes and exosomes. One of the aims of this research is to accurately identify and distinguish between MVs and exosomes.

1.2.6 Microvesicles defend against complement-mediated cell lysis

Colleagues at the Cellular and Molecular Immunology Research Centre found that intracellular *Trypanosoma cruzi* induces the release of host cell derived MVs, which protect the parasite from host complement (Cestari et

al., 2012). The mechanism of resistance against complement-mediated lysis includes removal of membrane attack complex (MAC). MAC is a C5b-9 complement protein complex, a membrane-damaging complex that can form pores on the membrane leading to lysis of the cell. Exposure to sublytic levels of MAC can trigger Ca^{2+} influx, activation of phospholipases, generation of diacylglycerol (DAG) and ceramide, and activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathways (Kraus et al., 2001; Morgan et al., 1985; Niculescu et al., 1993; Shirazi et al., 1989). CD46, CD55 and CD59, complement regulators, restrict binding of complement to the cell surface (Kojima et al., 1983; Meri et al., 1990; Nicholson-Weller et al., 1994) thus providing resistance to complement lysis. HSP (Fishelson et al., 2001; Gasser et al., 2005) and ERK (Kraus et al., 2001) are other proteins that provide protection against complement lysis. PKC activation depends on ERK and treatment with ERK inhibitor has been shown to reduce the number of proteins released upon sublytic MAC exposure as observed in K562 cells (Pilzer and Fishelson, unpublished). Hence, ERK seems to be involved in MAC removal. Mortalin (found in mitochondria, ER, cytoplasm and cytoplasmic vesicles) an HSP70 family protein has recently been shown to be involved in MAC vesiculation (Wadhwa et al., 2002; Ran Q et al., 2000). Cells release mortalin, soon after exposure to sublytic MAC (observed by Pilzer and Fishelson in K562 cells); mortalin in binding to C8 and C9 might then enable MAC to be assembled to vesicles (Pilzer et al., 2005).

Cells release MAC upon exposure to sublytic C5b-9 complex by membrane vesiculation either by ectocytosis (releasing MVs) or endocytosis followed by exocytosis, releasing exosomes. Both these vesicles contain some similar proteins that are essential for protection against complement lysis. Erythrocytes secrete vesicles on complement attack that are enriched in proteins anchored to the plasma membrane via GPI anchors such as acetylcholinesterase and decay accelerating factor (CD55, DAF) (Butikofer et al., 1989; Whitlow M et al., 1993). In erythrocytes, GPI anchored proteins seem to be required for ectocytosis. In epithelial cells and Ehrlich tumour

cells (Carney et al., 1985; Kerjaschki et al., 1989; Morgan et al., 1987) C5b-9 complexes may be subjected to proteolytic degradation upon endocytosis (Morgan et al., 1986). C5b-9 complexes on the epithelial cell surface are endocytosed in MVBs and then released as exosomes upon exocytosis.

Both exosomes and MVs are induced in a Ca^{2+} dependent manner and studies suggesting MAC increases intracellular Ca^{2+} levels have been reported (Campbell et al., 1981; Campbell et al., 1979). Regulated endocytosis as described earlier is Ca^{2+} dependent. Increased level of Ca^{2+} upon sublytic MAC exposure may internalize membrane bound complement components in the form of cytoplasmic vesicles. Such MAC contained vesicles are released upon exocytosis thus eliminating MAC. Increased Ca^{2+} influx causes imbalances in enzyme systems maintaining the integrity of cell membranes, resulting in further exposure of PS on the outer surface, in turn leading to blebbing and pinching off of plasma membrane to form MVs. MVs thus formed upon sublytic exposure to MAC may incorporate cell surface bound complement components thus eliminating MAC.

1.3 Exosomes

Exosomes are small membrane vesicles that are secreted by all antigen presenting cells through a process termed exocytosis. Like MVs, exosomes are enveloped by a lipid bilayer but have a diameter of 30 nm to 100nm, this being one of their defining criteria. Owing to their smaller size they can only be observed using electron microscopy. Microscopic observations have revealed them as having flattened spheres, having a ‘saucer-like’ structure, although this was subsequently shown to be an artefact from the procedures required to prepare samples for transmission electron microscopy (Thery et al., 2002). Exosomes arise as internal vesicles of Multi Vesicular Bodies (MVBs) and are released upon exocytosis. Exosomes were described, initially, as microvesicles containing 5’-nucleotidase activity that were released from neoplastic cell lines (Trams, et al., 1981) and also thought to be involved in the process of removing cell surface molecules, first observed in reticulocytes as a mode of removal of transferring receptor (Keller et al.,

2006). However, more recent studies have suggested them to have important roles in other biological functions, such as immunological response(s) and cellular communication.

1.3.1 Biogenesis of Exosomes

Eukaryotic cells continually communicate with the extracellular environment and other cells by the uptake and secretion of macromolecules whether they be proteins, receptor, or nutrients. Endocytosis and exocytosis are the mechanisms for uptake and secretion, respectively, of such macromolecules (Stoorvogel et al., 2002).

Earlier studies involving reticulocytes showed them to secrete small vesicles, which were endocytic in origin (Thery et al., 2002). These vesicles contained transferrin receptors (Pan et al., 1985; Harding et al., 1983) and were formed by the reverse budding at the limiting membrane of the late endosome. Fusion of the limiting membrane of MVBs with the plasma membrane (exocytosis) results in the release of the internal vesicles of MVBs (termed intraluminal vesicles and later on release ‘exosomes’). Upon release, these exosomes can be purified by a series of low speed centrifugation steps followed by high speed ultra-centrifugation cycles of cell culture supernatant to pellet the exosomes. The actual fusion of MVBs with the plasma membrane was first observed in RBCs (Pan et al., 1983; Harding et al., 1984) and later for a variety of cell types.

1.3.2 Endosomal Origin

The molecular composition of the purified exosomes is found to be similar to the internal vesicles of MVBs (Kleijmeer et al., 2001; Raposo et al., 1996; Escola et al., 1998; Thery et al., 1999). Exosomes contained few membrane markers compared to the abundant cystolic markers (Stoorvogel et al., 2002) indicating them to be different from that of other vesicles shed directly from the plasma membrane, in other words MVs. FACS analysis of exosomes from different cell types revealed them to be depleted in cell surface receptors like Fc receptors in dendric cell (DC) (Thery et al., 2001); CD28,

CD401 and CD45 in T-cell derived exosomes (Blanchard et al., 2002) and transferrin receptor in B-cell derived exosomes (Raposo et al., 1996; Clayton et al., 2001). Cystolic proteins involved in the endocytic pathway like RAB5/RAB7 (Gruenberg et al., 1995), annexin II (Gruenberg et al., 1995) and TSG 101 (Babst et al., 2000) were however found on the exosomes. Immuno-electron microscopy revealed that the antibodies against the cystolic proteins that are found in exosomes, such as HSP70 and annexin do not label unlysed exosomes but that the antibodies against the extracellular domain of the transmembrane protein, such as MHC class II molecules, CD9 and αMβ2 integrin, do (Thery et al., 1999). These vesicles do not expose phosphatidylserine (PS) on their outer surface. However PS has been observed on the outer surface of exosomes secreted by DCs (Thery et al., 1999) and platelets (Heijnen et al., 1999) but to a lesser extent (only 18%). These observations have strengthened the hypothesis supporting the endocytic origin of exosomes.

1.3.3 Endosomal Pathway: MVB Biogenesis

The endosomal system consists of endocytic vesicles, early endosomes, late endosomes, MVBs and lysosomes (Stoorvogel et al., 2002). The activated transmembrane receptor-ligand complex at the plasma membrane is endocytosed, in a clathrin or non-clathrin dependent manner, forming an endocytic vesicle. These activated transmembrane receptor-ligand complexes were thought to undergo full degradation upon uptake in endosomes followed by the fusion of MVB with lysosome; this concept has however now been modified in view of more recent findings.

MVBs are thought to develop from late endosomes (though many have considered both to be the same structure) as a result of the accumulation of exosomes by intraluminal budding or reverse budding at the limiting membrane of the late endosome (Johnstone et al., 2006). A model for MVB formation was suggested 30 years ago (Hirsch et al., 1968; van Deurs et al., 1993). The endocytic vesicle carries biomolecule cargo to the MVB limiting membrane. The next step involves sorting of selected cargo to recruit

proteins and lipids in MVB followed by dissociation of cargo and later the inward budding in the MVB (Stoorvogel et al., 2002) giving rise to exosomes or intraluminal vesicles inside the MVB.

1.3.4 Exosome biogenesis: Cargo sorting

Proteins and lipids are important for the biogenesis of MVBs and hence exosomes. Ubiquitation of selected proteins is one of the most common, though not the only protein sorting mechanism in MVBs (Hicke et al., 2001). For example, in *Saccharomyces cerevisiae* ligation of a single ubiquitin moiety to the substrate protein serves as a sorting signal for incorporation of that protein in MVBs as an internal vesicle. However, ligation of multiple ubiquitin units to the substrate protein serves as a signal for degradation of the protein (Keller et al., 2006). Here, G-protein coupled receptor (GPCR) Ste2 is ubiquitinated and mutation of lysine residues in Ste2 blocks downregulation (Keller et al., 2006). Similarly, c-Cbl (ubiquitin ligase for EGFR) is required for sorting EGFR in MVBs (Levkowitz et al., 1998). Fifteen yeast VPS E class mutant genes are identified and reported to be important in MVB biogenesis (Lemmon et al., 2000). Hetero-oligomeric protein complex ESCRT-1 in yeast, orthologous to mammalian TSG101, is thought to interact with VPS23 to recruit ubiquinated proteins. The selected protein cargo near the limiting membrane dissociates and releases ubiquitin, ESCRT-1 and clathrin coat (proteins involved in the MVB pathway) before assembling into the internal vesicle in MVBs. Tertaspannins are recruited in MVBs without ubiquitylation and get incorporated to form rafts (lipid rafts)/microdomains in the vesicles (Stoorvogel et al., 2002; Hemleret et al., 2001). Tetraspannins can interact with many other proteins and so become incorporated in the MVBs without ubiquitination. Similarly, clustered proteins can be recruited into MVBs. For example, lack of transferrin receptor-HSC70 interaction results in aggregation and clustering of proteins, which get recruited into MVBs and hence in exosomes and which are thus released upon exocytosis in association with exosomes (Geminard et al., 2001).

1.3.5 Exosome biogenesis: Post endocytosis

The vesicles inside the MVB may be processed in different ways that are driven by their composition and the cell's requirement. Accordingly, these internal vesicles have three fates as illustrated below using a few examples and summarised in Fig. 1.1.

i) Activated transmembrane receptor-ligand complexes are internalised by endocytosis and taken up in MVBs. Such receptor-ligand complexes might undergo full degradation upon fusion of MVBs with lysosomes.

ii) The internal vesicles containing MHC-II loaded with antigen peptide fuse with the plasma membrane and thus the antigen loaded MHC-II is exposed at the cell surface where it can be recognised by $CD4^+$ T cells.

iii) Upon the fusion of the limiting membrane of MVBs with the plasma membrane, internal vesicles of MVBs are released into the extracellular space. These vesicles are called exosomes. The process of exocytosis may be constitutive (non-Ca^{2+} triggered), as in EVB transformed B-cells, DCs and epithelial cells (Raposo et al., 1996; Thery et al., 1999; van Niel et al., 2001) or regulated (Ca^{2+}-triggered) as in mast cells and T-cells (Blanchard et al., 2002; Raposo et al., 1997). The exact mechanism of these fusions is not known. However, a proposed protein mechanism is described, which depends on one of the membranes containing v-SNARE [vesicle-SNAP{soluble NSF(N-ethylmaleimide-sensitive factor)-attachment protein}-attachment protein receptor] while the other should contain t-SNARE (target-SNARE) (Thery et al., 2002).

These three possible fates of the vesicles inside MVBs are outlined in Figure 1.1 below.

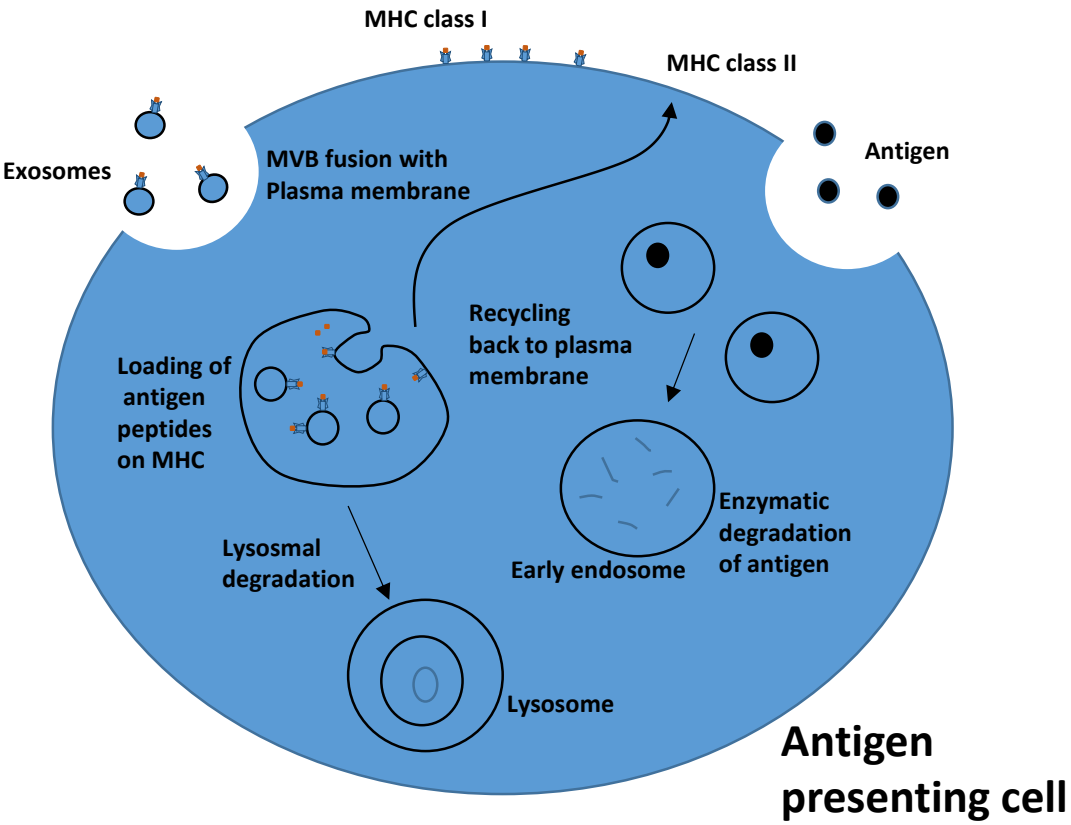


Fig. 1.1 Representation of the process of endocytosis and possible fates of internal vesicles formed inside MVBs in an antigen presenting cell.

1.3.6 Molecular composition and structure of Exosomes

Exosomes contain common as well as cell-specific proteins. Therry et al observed that 80% of exosomal proteins are conserved between mouse (Thery et al., 1999 and 2001) and human DC-derived exosomes (Thery et al., 2002). Table 1.2 lists some protein families commonly observed in exosomes from different cell types. MHC class I and II (Blanchard et al., 2002; Wolferset al., 2001); heat shock protein 70 (HSP70) HSC70, HSC73 and HSP90 (Thery et al., 2001; 2002); and tetraspannins CD9, CD63, CD 81 and CD82 are a few common proteins found in exosomes. Ubiquinated proteins help to load antigen peptide onto MHC molecules. HSPs carry misfolded proteins to their degradation site. Mortalin, an HSP70 family

protein, is being studied for its possible role in cells' defence against complement lysis (Pilzer et al., 2005). Indeed, Pilzer D. et al., in 2005, suggested that mortalin is involved in MAC vesiculation on sublytic complement exposure. Further, Graeme I. Lancaster and Mark A. Febbraio in 2005 suggested exosome dependent trafficking of HSP70.

Marker	Type	Exosome source	Effect	Reference
Amphiregulin	Protein	Breast, colorectal cancer	Invasion	Higginbotham et al. (2011)
CD44v6	Protein	Rat pancreatic adenocarcinoma	Pre-metastatic niche formation	Jung et al. (2009)
Del-1	Protein	Mesothelioma	Angiogenesis*	Hegmans et al. (2004), Ho et al. (2004)
EGFR	Protein	Pancreatic cancer, brain cancer	Proliferation*, signal transduction*	Adamczyk et al. (2011), Graner et al. (2009)
Hsp90α	Protein	Invasive carcinomas	Migration, invasion	McCready et al. (2010)
LMP1	Protein	Nasopharyngeal carcinoma	Signal transduction	Meckes et al. (2010)
LRG1	Protein	Non-small cell lung	Signal transduction*, cell adhesion*	Li et al. (2011)
MUC1	Protein	Breast cancer	Growth*, inhibition of Apoptosis*, invasion*	Staubach et al. (2009)
TSPAN8	Protein	Rat pancreatic adenocarcinoma	Endothelial cell activation, angiogenesis	Nazarenko et al. (2010), Gesierich et al. (2006)
Let-7	miRNA	Lung cancer, metastatic gastric cancer	Downregulate Ras*	Takamizawa et al. (2004), Ohshima et al. (2010)
miR-21	miRNA	Ovarian cancer, glioblastoma, breast, pancreatic cancer	Downregulate PDCD4*	Taylor and Gercel-Taylor (2008), Skog et al. (2008)
CDK8	mRNA	Colorectal cancer	Cell division*	Hong et al. (2009)
EGFRvIII	mRNA	Glioblastoma	Proliferation*, signal transduction*	Skog et al. (2008), Graner et al. (2009)
RAD21	mRNA	Colorectal cancer	Mitosis*, DNA repair*	Hong et al. (2009)

*Effect of marker is implied based on other published research.

Table 1.2 Summary of markers, proteins, miRNA, and mRNA found in exosomes from cancer cells as described by Henderson and Azorsa, 2012.

Exosomes also express CD55 and CD59 which have been shown to protect against complement mediated lysis (Clayton et al., 2003). The Tetraspannins, which are commonly found on exosomes, have four transmembrane domains and 2 extracellular domains that can interact with many other proteins like MHCs and integrins (as well as with themselves) thus allowing them to form large multimeric protein networks.

Exosomes contain cell-specific proteins that link them with cellular function; exosomes from antigen presenting cells (APCs) for example are particularly rich in MHC-II. DC-derived exosomes also contain CD89, aMb2, b2 (Thery et al., 2002) and EGF factor VIII (Thery et al., 1999); T cell derived exosomes

contain T cell receptor and B2 integrin. Additionally, B cell, DC and tumour derived exosomes contain a lysosomal associated membrane protein 1 (LAMP-1) and HLA-DM (Thery et al., 1999; Hammond et al., 1998). Fig. 1.2 schematically represents the structure of exosomes and other extracellular vesicles (microvesicles and apoptotic vesicles) including the location and probable

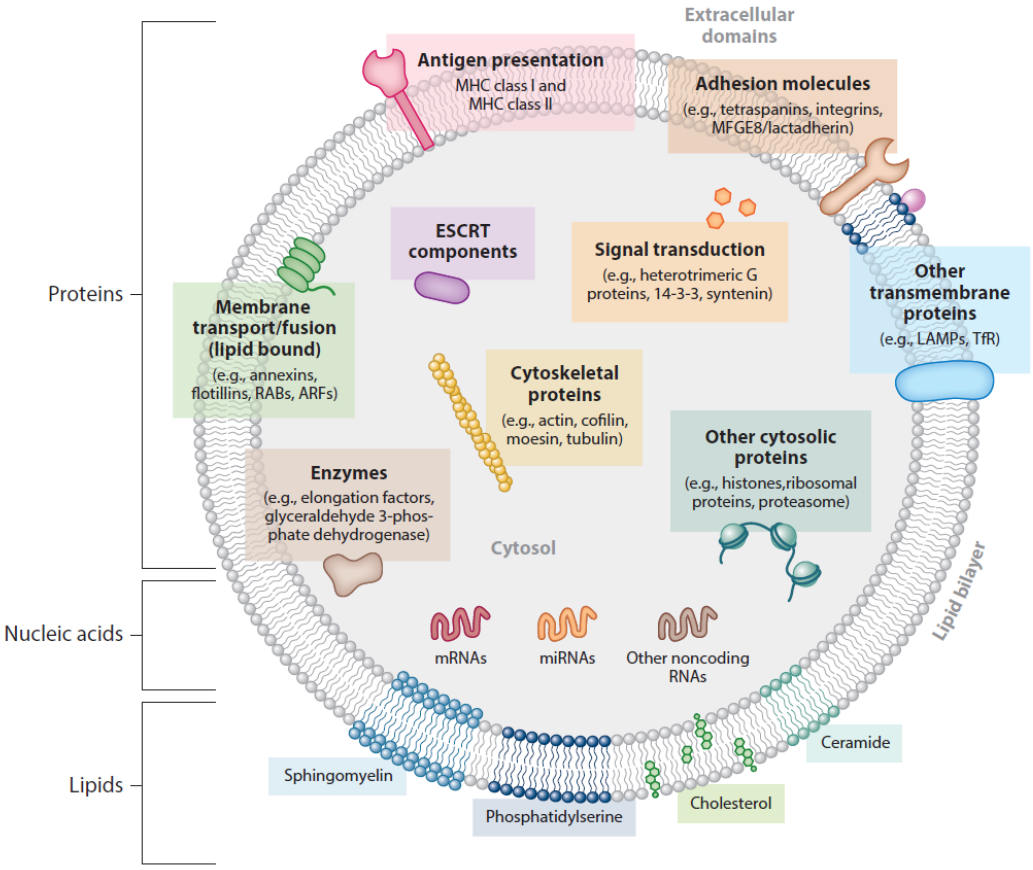


Fig. 1.2 Summary of the composition of Extracellular Vesicles (including Exosomes and Microvesicles (EMVs) and apoptotic vesicles). The molecules represented include proteins, lipids and nucleic acids. Abbreviations: ARF, ADP ribosylation factor; ESCRT, endosomal sorting complex required for transport; LAMP, lysosome-associated membrane protein; MHC, major histocompatibility complex; MFGE8, milk fat globule-epidermal growth factor-factor VIII; RAB, Ras-related proteins in the brain; TfR, tranferrin receptor. Colombo et al., 2014.

functions of proteins found in them (Colombo et al., 2014). Additionally, CD63 (LAMP3) is particularly enriched in exosomes (Heijnen, H. F. et al., 1999).

Despite much proteomic data, miRNA content and long noncoding RNA content, little is known about the lipid composition of exosomes. Lysobisphosphatidic acid (LBPA) (Kobayashi et al., 1999) and N-lissamine rhodamine B sulfonylphosphatidylethanolamine (Vidal et al., 1997) are found in exosomes. Exosomes from B cells are rich in cholesterol and sphingomyelin (Stoorvogel et al., 2002) both involved in raft formation (Stoorvogel and Schwartzman, unpublished results). It has been described that tetraspannins are involved in forming raft-like microdomains. Glycosylphosphatidylinositol (GPI)-anchored proteins are also incorporated into these rafts (Ikonen et al., 2001).

1.3.7 Functions of exosomes

Exosomes were initially thought to be involved as part of the machinery for removal of surface receptors, in other words as an alternative to lysosomal degradation and this role for exosomes was first observed for the removal of transferrin receptor from the mature RBCs (Pan et al., 1983). Raposo et al., showed that exosomes derived from EBV transformed B cell can stimulate $CD4^+$ T cells *in vitro*, to a lesser extent though, compared to B cells themselves (Raposo et al., 1996). Exosomes produced by 0.5×10^6 and 1×10^6 mouse DCs with tumor peptide produced anti-tumor responses mediated by T cells (Zitvogel et al., 1998). Exosomes from DCs carrying antigen loaded MHC are indeed found to transfer such MHC-antigen complexes among other DCs thus allowing the recipient DCs then to stimulate a $CD4^+$ T cell response (Andre et al., 2004; Hsu D.H. et al., 2003). Exosomal proteins such as milk fat globule elongation factor 8 (MFG-E8), CD11a, CD54, CD9 and CD81 have been shown to mediate the transfer of exosomes among DCs (Morelli et al., 2004). Mast cell exosomes when incubated with splenocytes for example induced blast formation, proliferation and production of IL-2 and INF-γ (Skokos et al., 2001). Also mast cell derived

exosomes induced maturation of DCs by regulation of MHC II, CD40, CD80, and CD86 (Skokos et al., 2003). Zhang et al., in 2006, made an observation supporting the role of exosomes in inflammation showing that synovial fibroblast-derived exosomes, derived from rheumatoid arthritis patients contained TNF-α, which was sensitive to the L929 cell line. Tumour antigens like MART1 positive exosomes can be found in cancer patients, and these have been shown to provide protection against tumour establishment (Andre et al., 2002). The mechanism involved in this protection is the transfer of tumour antigen in DCs, which can then activate cytotoxic T cells (Andre et al., 2002). Rabesandratana et al., in 1998, showed that glycosyl-phosphatidylinositol (GPI)-anchored proteins acetylcholinesterase (AchE), decay accelerating factor (DAF-CD55), membrane inhibitor of reactive lysis (MIRL, CD59), and LFA-3 were released in exosomes during *in vitro* maturation of reticulocytes (Kinoshita et al., 1986; Rollins et al., 1991). Furthermore, DAF and MIRL present on exosomal membranes may play a role in regulating membrane attack complex (MAC) formation.

1.3.8 Present status of exosome isolation and purification

The most commonly used isolation technique for exosomes is differential centrifugation followed by purification and identification by sucrose gradient centrifugation, which is described in detail in this thesis under Materials and Methods. However, the isolation protocol of exosomes has recently received attention and protocols have been designed to produce clinical grade exosomes with the removal of 90-95% of contamination. Dhellin Olivier et al., US patent 6,899,863 B1 accepted in 2005, defines a protocol to isolate and purify exosomes for clinical use as claimed by the inventors. The technique involves anion exchange chromatography under pressure (ion exchange HPLC) using supports such as mixtures of agarose, dextran, etc. in the form of beads having a diameter between 0.1 µm and 1 µm. The authors also describe the use of gel permeable chromatography in addition to anion exchange chromatography for the isolation of exosomes. The

authors go on to suggest that although differential centrifugation provides sufficiently pure samples for research purposes, it is not suitable for clinical use. Essentially, when it comes to the question of production at an industrial scale the differential centrifugation technique has its limitations.

Cheruvanky et al., in 2007, described the isolation of urinary exosomes using a nanomembrane ultrafiltration concentrator using commercially available nanomembrane concentrators (Vivaspin). Urine samples cleaned of cells and debris by 17, 000 *g* centrifugation for 15 min was added to Vivaspin and then centrifuged at 3,000 *g* for 10 mins. Urinary exosomal proteins were subsequently recovered from the concentrator by adding, unheated or preheated at 95ºC, 2X solublizing buffer [2X Laemmli buffer with 400 mM dithiotheritol (DTT)]. This technique can be used to effectively isolate exosomes from very small sample volumes down to ~0.5 ml.

1.3.9 β-glycyrrhetinic acid

β-Gycyrrhetinic (β-Gly) (β-Gly) acid is derived by hydrolysing glycyrrhizic acid (Sato, T. et al., 2009) from the root of the liquorice plant (Glycyrrhiza glabra L.) (Wu, F et al, 2013). It's steroid-like structure, a triterpenoid, shown below, has a structural similarity to corticosteroids, (Liu, D et al, 2007) and it is because of these structural similarities to steroids, that they are able to inhibit metabolic enzymes for adrenocorticosteroids (Ha, Y.M., et al., 1991). GA is the aglycon of glycyrrhizin, a component of Glycyrrhiza, the liquorice plant. When glycyrrhizin is given orally, it is metabolized to GA by intestinal flora whereupon it is absorbed into the circulation (Takeda, et al., 1996).

This natural steroid is thus able to reduce inflammation and has therapeutic value for example for patients with eczema and psoriasis (Kang, et al., 2014). β-Gly was also shown to enhance the action of hydrocortisone (Teelucksingh, S et al, 1990) and to bring about the apoptosis of monocytes (Schmidt et al, 1999). Furthermore, in terms of regulating immunity and inflammation β-Gly was also shown to inhibit 15-hydroxyprostaglandin dehydrogenase and delta-13-prostaglandin, thereby enhancing the action of

prostaglandins (Reddy et al,1992). β-Gly is a potent agent which has been derivatised to drugs such as carbenoxolone, used to treat ulcers, liver disease and been found to be anti-oxidant and immuno-regulatory (Chintharlapalli, S et al, 2007). Extracts from the liquorice root have been used in traditional Japanese and Chinese medicine for some time where they are used to treat ulcers, asthma, hepatitis and eosinophilic (Kroes, B et al, 1997).

1.3.10 Microvesicles as drug delivery vehicles

The obvious advantage of using a MV as a drug delivery vehicle is that it gives the drug it carries an added degree of protection from for example immune effector mechanisms. Compared to liposomes for example, MVs are much more resistant to complement attack as they carry all the complement inhibitory receptors that the parent cell carries (Inal et al., 2012). The half-life of MVs *in vivo* ranges from hours to days (Inal, personal communication) so clearly the choice of the cell from which to derive MVs for this purpose is an important consideration. One of the most important examples of extracellular vesicle (in this case exosomal) drug delivery which also addresses the targeting aspect to using any vesicle for drug delivery but also is interesting for having used an immune cell derived exosome is the seminal work of Matthew Wood in Oxford (Alvarez-Elviti et al., 2011). In this study exosomes derived from mouse dendritic cells were used. The study showed that these DC exosomes (sometimes called dexosomes) could be targeted to the mouse brain by engineering the expression of the neuron specific RVG peptide fused to Lamp2b. They went on to show that siRNA targeting BACE1, could knock down expression of this protein, which is an important therapeutic target in Alzheimer's disease by 62%.

1.3.11 Microvesicle interaction with recipient cells

The manner in which MVs released from one cell interacts with a particular target cell is still very much the subject of ongoing research in the field of cell biology (Mulcahy et al., 2014). It is broadly believed that there are several means of MV-cell interaction, none of which is likely to be exclusive with any

one cell and that in fact for any given MV cell interaction that this may occur by more than one of these means. Looking at endocytosis, this could be mediated as receptor-mediated uptake for example if a protein ligand expressed on the surface of a MV is able to interact with its cognate receptor on a recipient cell thus triggering receptor-mediated endocytosis. A non-receptor-mediated form of endocytosis could also be triggered if for example macropinocytosis is triggered such as with Epidermal Growth Factor or HB-EGF (heparin-binding Epidermal-like Growth Factor). This means of uptake involved cytoskeletal rearrangement and may result in the MV following a degradative pathway or being recycled to the cell surface (Faille, D., et al., 2012). If a MV interacts with a recipient cell by fusion, it is still not known if this is true fusion or hemi-fusion. Ongoing research in this field is using techniques such as lipid mixing with a lipid dye called octadecylrhodamine or various PKH dyes but also the more elegant FRET (Fluorescence Resonance Energy Transfer) by microscopy. It is also being speculated that any kind of MV-cell fusion may be preceded by some protein-protein interaction.

1.3.12 The Cancer Epidemic

There are almost 200 different types of cancer in humans, which can commence in a wide range of body tissues and then metastasise into other body tissues. There is also a range of identified causes of cancer which include viral infections (e.g. human papilloma virus, bacterial infections (e.g. *Helicobacter pylori*) or parasitic infections (e.g. *Schistosoma mansoni*) old age, mutations, diet, weight, environmental factors including environmental pollutants and life style practises. According to Cancer Research UK (cruk.org) and the Office of National Statistics (ona.gov.uk) cancer is the leading cuase of death in the UK, in 2015 accounting for 28% of deaths which was ahead of cardiovascular diesease at 26% e.g. heart disease and stroke The 530,000 deaths recorded in 2015 represent an increase of just over 5.5% compared to that in 2014. These increases are not wholly unexpected with an ageing population, but it is also important to acknowledge that patient survival has doubled in the last 40 years.

The European Prospective Investigation into Cancer and Nutrition (EPIC) is a study investigating the relationships between diet, lifestyle and the incidence of chronic disease including cancer. This is one of the largest cohort studies in the world, with over 0.5 mi participants from 10 European countries over the course of 15 years. The EPIC study is headed by Prof. Riboli (Imperial College London) and Dr. Romeiu and Brennan (International Agency for Research on Cancer, Lyon). The study accumulated over 6 million person-years. As shown in the table below, over 47,000 participants were diagnosed with cancer (13,000 breast; 3,500 lung; 5,300 colorectal and 5,100 prostate). There were 37,000 deaths and 8,500 participants developed at least two deadly diseases, including cancer, cardiovascular diseases and diabetes. As a result, epidemiological studies of co-morbidities were thus also possible. Just this year EPIC has resulted in a plethora of studies on cancer, nutrition and with some recommended life style changes (Assi et al, 2016; Bakker et al, 2016; Emaus et al, 2016; Merritt et al, 2016) including reducing prothrombocytic microenvironments (Rak, J et al, 2006).

Table 1. Number of incident cancers and deaths in EPIC in 2010

Sex	Country	*N*	Person-Years	No. of incident cancers	No. of incident deaths
Women	France	74 524	1 103 492	7313	4038
	Italy	32 577	394 213	2594	978
	Spain	25 808	352 213	1466	775
	United Kingdom	60 970	775 025	5141	5001
	Netherlands	29 751	379 272	2607	1843
	Greece	16 614	159 377	577	833
	Germany	30 255	341 989	2101	991
	Sweden	30 329	422 044	3400	2507
	Denmark	29 875	352 610	3658	2190
	Norway	37 200	406 473	2357	975
	Total	**367 903**	**4 686 708**	**31 214**	**20 131**
Men	France	0	0	0	0
	Italy	15 168	188 503	1268	730
	Spain	15 630	209 831	1421	1197
	United Kingdom	26 917	335 112	3160	4586
	Netherlands	10 260	130 580	563	543
	Greece	11 947	106 722	560	1313

Germany	22 833	253 868	2342	1845
Sweden	23 494	320 353	3406	3273
Denmark	27 178	311 900	3591	3359
Norway	0	0	0	0
Total	**153 427**	**1 856 869**	**16 311**	**16 846**

By 2015, the number of cancers that may develop in the cohort is projected to increase to more than 96 000 and will include approximately 25 000 cases of breast cancer, 12 000 of colorectal cancer, 8000 of lung cancer, 10 000 of prostate cancer, 2000 of gastro-intestinal cancer, 4400 of bladder cancer, 2500 of pancreatic cancer, 3700 of endometrial cancer, 1000 of liver cancer, and 1300 of thyroid cancer (Table 2). A large number of incident cancer cases with prospectively collected lifestyle data and blood specimens will allow EPIC to address state-of-the-art hypotheses about the etiology and prevention of several forms of common and rarer cancers.

Table 2. Sex-specific expected frequency of major cancer sites available by 2016

Sex	Breast	Colorectal	Lung	Prostate	Stomach	All cancers
Men	0	4907	4152	10 771	1198	32 334
Women	24 899	7669	3852	0	1010	64 242
Total	**24 899**	**12 576**	**8004**	**10 771**	**2208**	**96 576**

The new cancer strategies adopted in England (CRUK.org) include early diagnosis, immediate access to the appropriate treatment and importantly disease prevention which includes lifestyle changes such as stopping smoking, improved diet and exercise. As reported by CRUK in 2011 (cruk.org/preventable) these measures include stopping smoking, drinking

less alcohol, moderate exposure to sun, consumption of less processed food and red meat, eating a high-fibre diet, reducing salt intake, doing exercise, reducing exposure to asbestos, certain infections such as HPV, minimising exposure to radiation, e.g. x-rays, breastfeeding, and reducing time on HRT.

There are many environmental pollutants associated with disease in general but also cancer As shown in a small study of umbilical cord blood carried out in the US in 2004 (http://www.ewg.org/research/body-burden-pollution-newborns#.WcJUsdWPKJA) up to 287 chemicals and pollutants were detected including polyaromatic hydrocarbons, pollutants derived from burning petrol, which accumulate in the food chain and that are linked to cancer. Perfluorinated chemicals, polychlorinated biphenyls, organochlorine pesticides, polychlorinated dibenzodioxins, all also linked to cancer were also found.

Over 40% of cancer cases in the UK are linked to fourteen life-style factors (Parkin et al., 2010). The higher proportion of males (45%) versus females (40%) is attributed to differences in smoking habits. Over half a million cancers are deemed preventable in the UK over a 5 year period (www.cancerresearchuk.com 2014) by changing lifestyle. Examples include fasting, hydration and detoxification strategies which represent a more holistic approach to health akin to one of the oldest such medical systems, that of Ayurvedic medicine using herbal products (Pole, 2012) and other plant extracts (http://www.cancerresearchuk.org/about-cancer/cancer-in-general/treatment/

complementary-alternative-therapies/individual-therapies/ayurvedic-medicine).

MVs released by tumor cells are detectable in patients with cancer and their number in the circulation correlates with poor prognosis. Due to their pleiotropic effects, MVs may play a role in the prothrombotic state associated with cancer as well as in cancer development and progression. Recent findings show that Microvesicles can be involved in the epigenetic reprogramming of neighbouring cells (Camussi et al., 2011; Quesenberry, 2008, 2010, 2012). With the current advances in MV detection and

characterisation and general use in as diagnostic tools, it may be that in the near future that cancer progression and regression may be monitored by assessing MV types and levels.

1.4 Aims of thesis

MVs and exosomes are shed by most eukaryotic cells constitutively (without stimulation) and upon exposure to inducing agents (induced). The Isolation techniques in place do not give absolutely pure preparations of either membrane vesicles and hence results obtained using these isolation techniques are likely to give inaccurate associated functions with vesicle type. Both MVs and exosomes have similar proteomes and biological functions and furthermore a correct identification becomes necessary when dealing with either of them.

Using previously established properties, such as size, enrichment of specific marker proteins etc., the aim was to use sucrose gradient density centrifugation (currently used to purify exosomes) to also isolate MVs and to test novel filtration methods for the isolation of plasma MVs. A further aim was to use this methodology to make measurements of plasma MV levels in donors and to begin to look at various factors that may affect these levels such as gender, age, and for the first-time fasting status.

The possibility of using MVs as drug delivery vehicles was also to be investigated, at first from the point of seeing how they interact with potential target cells, in particular to ascertain whether membrane fusion occurs, which would be important for delivery of intravesicular content into the target cell cytosol. The effect of β-Glycyrrhetinic acid, derived from liquorice was also investigated on erythroleukaemia cells and its capacity to limit cellular proliferation estimated upon delivery within MVs.

2. Materials and Methods

2.1 Solutions and reagents

RPMI1640, FBS, Antibiotics, PBS, Sucrose, HEPES buffer, PMA, Anti CR1 antibody, FITC, octadecyl rhodamine (R18), DAPI, Fluorescein isothiocyanate (FITC) conjugated anti-human CD107a (LAMP-1) antibody, anti-goat IgG antibody, anti CD11b antibody, Phycoerythrin (PE) and FITC conjugated anti AnnexinV antibody and PE conjugated anti CD63 (LAMP-3) antibody. All fluorescence conjugated antibodies were from SIGMA.

2.2 Cell culture

THP-1 (Human acute monocytic leukaemia cell line) cell cultures were maintained in complete medium - RPMI 1640 with 10% fetal calf serum (FBS) and 1% antibiotic. Minimum of $2x10^6$ cells per ml was maintained in sets of three 20ml flasks at 37°c. Cells were split twice a week into the fresh medium and the supernatant was stored at 4°C till further requirement.

2.3 Isolation of MVs and exosomes

Majority of the work in this research was on isolation protocol for MVs and no stage inducing agents were used. Different isolation protocols were used involving 2 common steps

1) Centrifugation of cell culture for 5 mins at 160*g* to pellet cells and collect supernatant (SN).

2) Cell free SN is centrifuged again twice at 4,000*g* for 30 mins to remove the cell debris (clean SN).

NOTE: SN was stored at 4°C, if not required to process immediately at any of these two steps. The temperature was set at 14°C for all centrifugation cycles unless otherwise mentioned.

2.3.1 Differential centrifugation

Clean SN was subjected to ultracentrifugation for 1 hr and 30 mins at 200,000*g*/40,000rpm (in sorvall ultracrimp 35ml centrifuge tubes – 40000rpm max.) or for 2 hrs at 150,000*g*/30,000rpm (in sorvall ultracrimp

11.5ml centrifuge tubes – 30,000rpm max) to pellet MVs. SN was collected and ultracentrifuged for 16 hrs at 200,000g or 18 hrs at 150,000g to pellet exosomes. To wash MVs and exosomes, pellets were resuspended in 1ml PBS (in 1.5ml eppendorf tubes) and centrifuged at 25,000g for 60 mins to regain a pellet, which was again resuspended in 200 µl PBS and stored at -80°c till further requirement. At the later stage in the research, clean SN was first sonicated for 4 mins, to any break MVs or exosomes that might have aggregated to form clusters, and then subjected to high speed ultracentrifugation step. In one of the experiment CD63 preincubated MV sample was split into 2 equal aliquots and one of them was filtered with 0.22 µm filter. Both were then subjected to FACS analysis.

Few experiments required to have both exosomes and MVs in a single preparation. A MVs and exosomes (MV + ex) mixed sample was obtained by 16hrs centrifugation at 200,000g or 18 hrs centrifugation at 150,000g of clean SN. The pellet so obtained was washed in 1ml PBS and again resuspended in 200 µl PBS as described in earlier steps. [All high-speed ultracentrifugation was performed in Sorvall SE 90 Discovery centrifuge (unless otherwise mentioned) using sorvall T-865 fix-angled rotor – thanks to IBCHN department London Metropolitan University].

2.3.2 Sucrose gradient

40% to 10% continuous decreasing sucrose gradient was made in 11.5ml sorvall ultracrimp tubes using gradient mixture. The gradient was allowed to settle undisturbed for 90 mins. MV + ex sample (in 200 µl PBS) was kept in an ultrasonic bath for 4 mins and then layered on top of the gradient and centrifuged for 90 mins at 150,000 *g* in the sorvall fix-angled rotor (T-865) or for 60 mins at 200,000 *g* in Beckman's swinging bucket rotor. Ideally, eleven 1ml fractions were collected from the top of the tube (it was not possible to collect all equal 11 fractions every time due to difficulties in drawing a sample with a syringe) and the pellet was resuspended in 200 µl PBS. The density of each fraction was measured by weighing and 20 µl of the sample from

each fraction and pellet was subjected to FACS analysis to check the number of vesicles.

Two approaches were used to eliminate sucrose for the fractions i) centrifugation for 90 mins at 25,000 *g* or ii) overnight dialysis followed by centrifugation at 25,000 *g* for 60 mins. Alternatively, fractions were pooled in 2 groups i.e. Pool 1 (P1- fractions 1-6 from top) and Pool 2 (P2- fractions 7 and later from top) and dialyzed as described later and then centrifuged for 60 mins at 25,000 *g* to pellet P1 and P2. Pellet obtained during sucrose gradient centrifugation step would be referred as sucrose gradient pellet (SGP) from here on to avoid any confusion.

Dialysis

Adequate length of dialysis membrane was cut and clipped form one end and the sample was introduced from other end and clipped. The membrane was kept in a container having PBS in 100 times the volume of the sample to be dialyzed. The container was kept overnight on a magnetic stirrer. Sample from inside the membrane was collected into eppendorf and centrifuged to pellet vesicles, which was then resuspended in 200 µl PBS that was either processed as per the requirement or stored at -80^{o}C.

2.3.3 Filter recovery

This was a novel protocol designed with the basic idea to recover the bigger vesicles that do not pass through the filter. Clean SN was kept in an ultrasonic bath for 4 mins and vortexed for 30 secs. A filter membrane with uniform pore size of 0.22 um was placed in the filter apparatus and screwed up tightly. 13-15ml of clean SN was passed through the filter before membrane blocked. The filtrate was collected in 50 ml tubes for exosomes isolation. The filter was then unscrewed and the filter membrane was reversed and placed back in the filter apparatus and tightly screwed. Two approaches were used from here on

2.3.3.1 MVs without high speed centrifugation

1ml of PBS was passed through the filter, after reversing the membrane as described above, and the filtrate was collected in a 1ml eppendorf tube. Filter membrane was taken out from the apparatus and was cut into 6-7 pieces so they can fit in eppendorf tube that contains the filtrate. This eppendorf tube was then kept in ultrasonic water bath for 4 mins and then vortexed for 1 min. Filter pieces were removed from the tube and the sample was stored at -80°C or processed as required.

2.3.3.2 MVs concentrated by high speed centrifugation

4 ml of PBS was passed through the filter after reversing the membrane and the filtrate was collected in 20 ml tube. The membrane was placed back to its original position in the filter apparatus. And again, clean SN was passed through the filter till the membrane was blocked (roughly 9-10ml). Filter membrane was again reversed and 4 ml PBS was passed through that was collected in the same 20 ml tube containing filtrate. Filter membrane was taken out, cut into 4-5 pieces and was kept in the tube with the 8ml filtrate. This tube was kept in an ultrasonic bath for 4 mins and vortexed for 1 min. Eight 1ml aliquots were taken in eppendorf tubes that were centrifuged at 25, 000 *g* for 30 mins to form a pellet. SN was discarded and all pellets were taken together resuspended in 200 µl PBS.

2.4 FACS analysis

All FACS analysis was performed on Guava EasyCyte Plus system and express plus assay was used for all sets of experiments in 96 wells plate. FACS was mainly used to determine the relative fluorescence as a result of FITC or PE conjugate antibody that binds the sample and to determine the number of vesicles. All experiments involving FITC and PE conjugate antibodies were performed in dark followed by incubation in dark at 4°C on a shaker unless otherwise stated.

2.4.1 FACS for differential centrifugation and filter recovered samples:

For antibody binding assay (on FACS) of differential centrifugation samples a standard protocol followed for all antibodies. In general, 20 µl of exosomes and/or 20 µl of MVs sample were taken from 200 µl aliquots. 5 µl of FITC conjugate anti LAMP1 antibody, 5 µl of PE conjugate anti CD63 antibody, FITC conjugate anti CD11b antibody was added to 20 µl of sample in individual microfuge tubes and the volume was topped up to 100 µl with PBS. This preparation was incubated overnight. Samples were washed by centrifugation at 25,000 *g* for 60 mins to remove excess unbound antibody and to pellet antibody labelled vesicles that were resuspended in 200 µl PBS and subjected to FACS analysis to detect green and yellow fluorescence intensities. In a similar manner 20 µl of filter recovered MVs and 20 µl of differential centrifugation MVs samples were incubated overnight with 5ul FITC conjugated anti annexin V antibody and the volume was topped up to 100 µl with HEPES buffer. The preparation was washed and subjected to FACS analysis as described earlier. Unlabelled exosomes or unlabelled MVs (in a few experiments isotype control FITC conjugated anti IgG antibody was used) were used for FACS settings and also as controls. Settings were made to observe maximum possible population on forward scatter (FSC) Vs. side scatter (SSC) on plot 1, on green and yellow fluorescence on a log scale on plot 2. Population shift, depending upon fluorescence that is produced due to binding of FITC or PE conjugated antibody to the sample was compared to the unlabelled control on marker2 region in plot 3 (marker3 region was not used and was set at 0%) for either green or yellow fluorescence.

2.4.2 FACS for sucrose gradient sample

20 µl of the sample from sucrose gradient fractions and the pellet was subjected to FACS analysis for quantification of vesicles in each sample. In

a few experiments, exo + MV pellet in 200 µl buffer (PBS for antibody [LAMP1, CD63, CD11b] and HEPES for annexinV) was incubated overnight with 50 µl FITC conjugated anti LAMP1 antibody and PE conjugate anti-annexinV antibody. The preparation was subjected to sucrose gradient ultracentrifugation at 150,000 *g* for 90 mins and the fractions were collected as described earlier. 200 µl of the sample from each fraction was loaded in 96 wells plate and observed on FACS for fluorescence intensity due to FITC and PE conjugated antibody binding to vesicles. Unlabelled exosomes/MVs form differential centrifugation was used for settings and as controls.

2.4.3 Expression of CD63 as marker for vesicles in pool 1 and of CD11b as marker for vesicles in pool 2

P1, P2 and SGP that were in 200 µl PBS suspension were divided in 4 equal aliquots (set1, set2, set3 and set4) into 12 eppendorf tubes and volume in each was topped up to 100 µl adding PBS. On day one, 20 µl of antibody was added to set1 (P1, P2 and SGP) and incubated overnight in the dark on a shaker at 4°C. On the second day, 5 µl of FITC was added to set 1, 5 µl FITC to set2 and 20 µl of FITC conjugate anti IgG antibody to set3. All tubes were then incubated overnight. Excess unbound was removed after washing with PBS by centrifugation at 25,000 *g* for 60 mins. SN was discarded and the pellet was resuspended in 200 µl PBS. Set4 was unlabelled samples. All 12 samples were again spilt into 2 equal sets giving a total of 24 samples. One of these sets was filtered using 0.22 µm Millipore filter and hence named filtered set viz. FP1, FP2 and FSGP. A clear understanding of pooling and naming is given in results. Set4 samples were used in FACS analysis for settings. All 24 samples were loaded on 96 wells plate and the volume was topped up to 200 µl using PBS and subjected to FACS analysis.

2.5 Protein estimation assay (BCA)

BCA assay was performed only when the equal concentration of protein samples was required in the experiments. Mainly it was used before running SDS-PAGE or agarose gel and doing ELISA to measure TGF-β in MVs and exosomes.

Nine bovine serum albumin (BSA) standards with concentration ranging from 0 µg/ml to 2,000 µg/ml were made by serial dilutions in distilled water. Working reagents were made as stated in thermo scientific BCA kit protocol. 200 µl working reagent was added to all required wells in 96 wells plate. 20 µl of required exosome or MV samples were lysed using 5 µl 1X triton lysis buffer. 25 µl of nine BSA standard and samples were loaded into the wells with 200 µl working reagent. The plate was agitated gently for 30 secs and incubated at 37°C for 30 mins. After incubation the samples were allowed to cool at room temperature for 5 mins and absorbance was measured at 562 nm using FLUOstar Omega microplate reader. The protein concentration of samples was estimated by comparing absorbance to that of standards.

2.6 SDS-PAGE

2.6.1 Sample preparation for SDS-PAGE

All required samples - MVs from differential centrifugation and filter recovery; exosomes; filtered and unfiltered pools and pellet samples from sucrose gradient centrifugation were lysed with 1X triton lysis buffer and centrifuged at 5,000 rpm for 5 mins. SN that contains proteins was collected and protein concentration was estimated using BCA kit and was equalized in all the samples. 5ul bromophenol blue and 1 µl DTT were added to every sample and heated at 95°C in water bath for 5 mins.

The 10 % gel was placed in the electrophoresis apparatus with tris buffer. The comb was removed from the top of the gel and 20 µl samples were loaded into the wells. Molecular weight markers were also loaded. 20 mA current was passed through the gel for 2 hours or until the dye front had reached the bottom of the gel.

The gel was then stained as described below. Firstly, Coomassie stain was prepared by dissolving 0.4 g of Coomassie blue R350 in 200 ml of 40% (v/v) methanol in water with mixing. The solution was then filtered to remove any insoluble material and 200 ml of 20% (v/v) acetic acid in water added. The final concentration was 0.1% (w/v) Coomassie blue R250, 20% (v/v) methanol, and 10% (v/v) acetic acid. To stain the gel, it was gently agitated in Coomassie stain for 1 h. To prepare the destain solution 500 ml of HPLC-grade methanol was added to 300 ml of water. 100 mL of acetic acid was then added and, after mixing, the total volume adjusted to 1 L with water. The final concentrations were 50% (v/v) methanol in water with 10% (v/v) acetic acid. To destain, the gel was covered with several volumes of destain solution and mixed overnight at room temperature. A piece of tissue in the destain solution meant aspiration of the destaining solution was not needed until the next day. Destaining was continued until the protein bands became distinct with little background staining.

If the bands were not clearly visible due to suspected lower concentrations of protein in the sample, silver staining for maximum sensitivity was performed according to the protocol described below. Silver stained gels were then photographed and compared to Coomassie blue stained gel. Bands in different lanes were analysed to detect presence/absence of proteins or level of protein expression in different samples.

2.6.2 Silver Staining Procedure

Silver staining is over 100 times more sensitive than Coomassie Brilliant Blue R25o staining of proteins and is particularly useful for detection of low abundance proteins, allowing detection of down to 0.05 - 0.6 ng of protein per band. Silver Staining uses silver nitrate to bind proteins at a weakly acidic pH and these silver ions are subsequently reduced to metallic silver by formaldehyde at an alkaline pH.

Preparation of working solutions for silver staining

1. Preparation of fixative solutions:

Prepare the following two gel fixing solutions as outlined. The prepared solutions can be stored in tightly closed bottles at room temperature. Add the reagents to the clean glass bottles.

Gel fixing solution 1:
Ethanol 50 ml 50% (v/v)
Glacial acetic acid 10 ml 10% (v/v)
Deionized water 40 ml

Gel fixing solution 2:
Ethanol 60 ml 30% (v/v)
Deionized water 140 ml

2. Preparation of staining solutions:

Prepare the following four gel staining solutions using reagents provided in the kit. For best results the solutions should be prepared the same day prior to staining. Add the formaldehyde only immediately prior to staining. The provided volumes are sufficient for staining one mini-gel (8 x 10 cm, 0.75-1.5 mm thick). Mark clean glass bottles and add the indicated volumes of reagents.

Sensitizing solution:
Sensitizers concentrate 0.4 ml
Deionized water to 100 ml

Staining solution:
Staining reagent 4 ml
Deionized water to 100 ml
Formaldehyde 54 µl*
* Add immediately prior to use.

Developing solution:
Sensitizer concentrate 10 µl

Developing reagent 10 ml
Deionized water to 100 ml to 100 ml to 100 ml 92 ml
Formaldehyde 27 µl*
* Add immediately prior to use.

Stop solution:
Stop reagent 8 ml
Deionized water 92 ml

Before using the Developing reagent was checked for precipitation and if so precipitated salts were redissolved by warming the solution at 37°C, before cooling back to room temperature.

To carry out silver staining, firstly the gel was placed in a staining tray and rinsed briefly with deionized water. After adding 100 ml of gel fixing solution #1, the gel was microwaved for 30 s at maximum power, taking care not to boil the solution. The tray was then gently mixed for 10 min. If necessary the staining could be stopped at this stage or if necessary continued overnight. After discarding the gel fixing solution #1, gel fixation was carried out twice. For this, 100 ml of gel fixing solution #2 was added and the solution once more microwaved for 30 s at maximum power. After gently mixing for 10 min, washing was carried out twice. For this 100 ml of deionized water was added with gently mixing for 20 s and then discarded. For sensitizing and washing 100 ml of sensitizing solution was added with gentle mixing for 1 min and then discarded. Washing (100 ml deionized water and gentle mixing for 20s) was carried out twice. For staining, 100 ml of staining solution was added for 20 min with mixing, the solution discarded and washing as before being performed twice. To develop the gel 100 ml of developing solution was added with gentle mixing for 4 min until the protein bands appeared distinctly. The developing solution was discarded and the reaction terminated by adding 100 ml of Stop solution with gentle mixing for 5 min.

The gel could then be stored in deionized water having discarded the stop solution.

2.7 Detection of nucleic acids in isolated MVs and exosomes by agarose gel electrophoresis

A 0.8% agarose gel was prepared in 1X TAE (Tris Acetate EDTA) and wells were created using a comb. 5 µl exosomes, differential centrifuged MVs and SGP was lysed using 1X of 15 µl of triton lysis buffer. Samples were centrifuged for 5 mins at 5,000 rpm (using a bench top microfuge, Eppendorf) and SN was collected. 5 µl bromophenol blue premixed with ethidium bromide (EtBr) was added to all the samples. 5 µl molecular weight marker was loaded in the first well followed by 20 µl samples. 60 mA current was applied until the dye front had travelled about 75% of the total length of the tray. The gel was subsequently taken to the UV light box and photographs taken.

2.8 ELISA for TGF-β1 of isolated microvesicles and exosomes

Quantikine Human TGF-β1 kit was purchased from R&D systems. Protocol given in the kit was followed. In brief 50 µl of Assay diluents RD1-21 was added to each well followed by addition of 50 µl standard/control/activated sample. The plate was covered with tape and incubated for 2 hrs at room temperature. All wells were washed 3 times with 400 µl wash buffer. 100 µl of TGF-β1 conjugate was added to each well and incubated for 2hrs at room temperature followed by a wash as described in the previous step. 100 µl of substrate solution was added and incubated in dark for 30 mins at room temperature. Finally, a 100 µl stop solution was added to each well and readings were taken immediately on an omega microplate reader at 450 nm.

The plates are pre-coated with a monoclonal antibody specific for TGF-β1. Any TGF-β1 present in sample/control/standard will be immobilized on the plates (first incubation) while unbound would be removed upon wash. Later a polyclonal antibody specific to TGF-β1 is added to sandwich TGF-β1 that was immobilized. Unbound antibody is again removed by washing followed

by addition of substrate that will develop the colour proportional to the amount of TGF-β1 present in the sample.

2.9 Cytokine array analysis of isolated microvesicles and exosomes

Human cytokine array panel A array kit from R&D Systems that can detect multiple cytokines in a single sample was used and protocol given in the array kit was followed. In brief, selected capture antibodies are spotted in duplicate on nitro-cellulose membranes. Equal aliquots (as measured by BCA kit) of lysed exo + MVs, filter recovered MVs and exosomes were mixed with biotinylated detection antibodies and incubated with Human cytokine array panel A array of different membranes. Washing was performed followed by addition of Streptavidin-Horseradish Peroxidase and chemiluminescent detection reagents. The light produced at each spot in proportion to the amount of cytokine bound was imaged and developed for 1-10 mins by exposure to X-ray. Images were taken and the coordinates were compared to the reference coordinates given in kit.

2.10 Histopaque-1077 procedure for monocyte isolation

In a 15-ml conical centrifuge tube, 3.0 ml HISTOPAQUE-1077 (Sigma Aldrich) was added and this was brought to room temperature. 3.0 ml whole blood was carefully layered onto the HISTOPAQUE-1077 which was then centrifuged at 400 *g* for 30 min at RT. (Centrifugation at lower temperatures, such as 4°C, may result in cell clumping and poor recovery). After centrifugation, the upper layer was carefully aspirated with a Pasteur pipette to within 0.5 cm of the opaque interface containing mononuclear cells, the upper layer is discarded. The opaque interface was then carefully transferred, with a Pasteur pipette, into a clean conical centrifuge tube. 10 ml Isotonic Phosphate Buffered Saline Solution was then added, with mixing with gentle aspiration. The tube was then centrifuged (250 *g*; 10 min) and the supernatant aspirated and discarded. The cell pellet was resuspended in 5.0 ml Isotonic Phosphate Buffered Saline Solution and mixed by gentle aspiration with a Pasteur pipette followed by centrifugation at 250 *g* for 10

min. Washing was repeated, the pellet is resuspended in 5 ml isotonic PBS. As heavy contamination with RBC and platelets was found with this procedure, even when a buffy coat was used instead of whole blood, Ficoll-Hypaque procedure (below) was used.

2.11 Ficoll-Hypaque procedure for monocyte isolation (adapted form de Almeida MC, et al., 2000)

Firstly, EDTA anticoagulated fresh blood was centrifuged at 400 x *g* for 35 min.

In a separate 15-ml conical centrifuge tube, 3.0 ml Ficoll-Hypaque gradient (density= 1.070, Sigma Aldrich) was added and the mixture brought to room temperature. The buffy coat layer was carefully layered on the Ficoll-Hypaque and this was centrifuged (400 *g*; 35 min). The supernatant was carefully transferred with a Pasteur pipette, onto a slightly hyperosmolar Percoll gradient (density = 1.06 g/mL) in another clean conical centrifuge tube.

Isosmotic Percoll was prepared by mixing one volume NaCl 1.5 M with nine volumes of Percoll (Pharmacia, density =1.130 g/mL) and hyperosmolar Percoll by mixing 1:1 (v/v) isosmotic Percoll with PBS/Citrate (NaH_2PO_4 1.49 mM; Na_2HPO_4 9.15 mM; NaCl 139.97 mM; $C_6H_5Na_3O_7.2H_2O$ 13 mM; pH 7.2). After centrifugation (400 *g*; 35 min) the supernatant was carefully aspirated without disturbing pellet into another clean centrifuge tube to which was added 5 mL of Phosphate Buffered Saline into with gentle mixing and centrifugation (250 *g*; 10 min). The cell pellet was resuspended in 5.0 ml Isotonic Phosphate Buffered Saline Solution and mixed by gentle aspiration with a pasteur pipette. This washing was repeated and the cell pellet finally resuspended in isotonic PBS.

3. Development of Sucrose gradient- and reverse filtration-based methodology to isolate microvesicles and exosomes

3.1 Introduction

Since the ‘formalisation’ of the extracellular vesicle field by the founding of the International Society for Extracellular Vesicles, in 2012 and the holding of its first meeting in April of that year in Gothenburg, the community has been grappling with the lack of standardisation of extracellular vesicle isolation and analysis. Four years on, despite position papers published in the prime extracellular vesicle journal (Witwer et al., 2013) and working groups having been set up to address the issue, it is still clear from a look at the literature in this field that besides there being little standard practise in operation, many research groups are continuing to address the issues of isolation and analysis with a view to improving existing techniques. This is a fast-changing field in which groups are finding inconsistencies in previously established procedures and then attempting to rectify the problems.

The aim of this first section of the thesis was to explore alternative methodologies capable of isolating microvesicles (MVs) and exosomes with high yield and a high degree of purity. The most used method is that of differential centrifugation. It was decided to explore the possibility of using filtration and sucrose gradients which are currently used to show that exosomes have been isolated.

Although it may be important to separate MVs and exosomes for example when they need to be used as therapeutic vehicles, in terms of studying a population of vesicles released from a cell with a view to understanding a biological process, it may actually be preferential to work with extracellular vesicles (EVs) (that is MVs and exosomes) this being more representative of what is released from cells. If MVs or exosomes are investigated in terms of their relative contribution to say disease pathology, however, then it should be born in mind that running vesicles through some kind of density gradient, such as a sucrose gradient, whilst likely yielding a vesicle population that is highly pure, certainly from the point of view of surface proteins that they may have acquired, this may actually not represent a very ‘physiological’ vesicle.

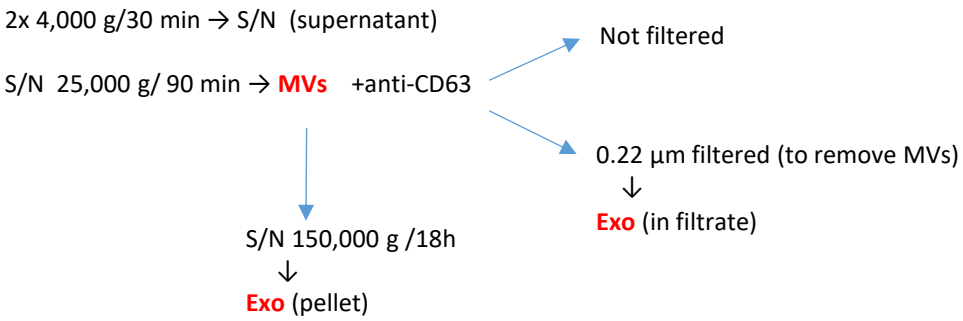
3.2 Materials and Methods

The detailed procedures for isolation methods adopted in this chapter are described fully in Chapter 2, the Materials and Methods chapter of this thesis. The exosomes and microvesicles (MVs) used in this study were isolated from THP-1 monocytes. In Figures 3.1 and 3.2 summary flow charts of these procedures are summarised. Fig. 3.1 outlines the standard procedure of differential centrifugation which is widely used to isolated MVs, after removal of cellular debris by a centrifugation at 25,000 *g* for 1h. Also, in Fig. 3.1 the use of a sucrose gradient has been outlined. This is used as a way of isolating exosomes but has so far not really been considered as a possible means of isolating microvesicles, probably because it was thought that they are less likely to have a specific buoyant density, as for exosomes, (1.12-1.18 g/ml) (Heijnen et al., 1999; Inal et al., 2012) because of their large variation in described diameter (ranging from 100 nm up to 1 μm). In this case two pools of fractions were analysed for MVs and exosomes. Finally, in Fig. 3.2 a novel method in which a mixture of exosomes and MVs was filtered is described. Essentially this procedure involved passing the EMVs (exosomes and MVs), having removed cells and cell debris by low speed centrifugations at 4,000 *g* through a Millipore filter of pore size 0.22 μm. Most of the MVs are likely to deposit on the membrane, the smaller exosomes (50 – 100 nm) being found in the filtrate. Upon reversal of the membrane, the deposited MVs could be washed. The possibility of carrying out a light sonication of the EMV mixture was also investigated with a view to breaking down any exosome clumps in order to improve purity and yield of exosomes and microvesicles respectively.

3.2.1 Flow cytometry analysis of isolated exosomes and microvesicles

To prepare settings and controls, unless otherwise stated, EMVs, whether exosomes or MVs as isolated from a range of methodologies were diluted four-fold in PBS. The settings for flow cytometry were adjusted to observe the maximum number of possible events in the forward versus side scatter plots (plots #1 and #2), and such as to obtain a bell-shaped histogram in plot #3 (GRN-HLog or YLW-HLOg).

1. Differential Centrifugation



2. Sucrose Gradient

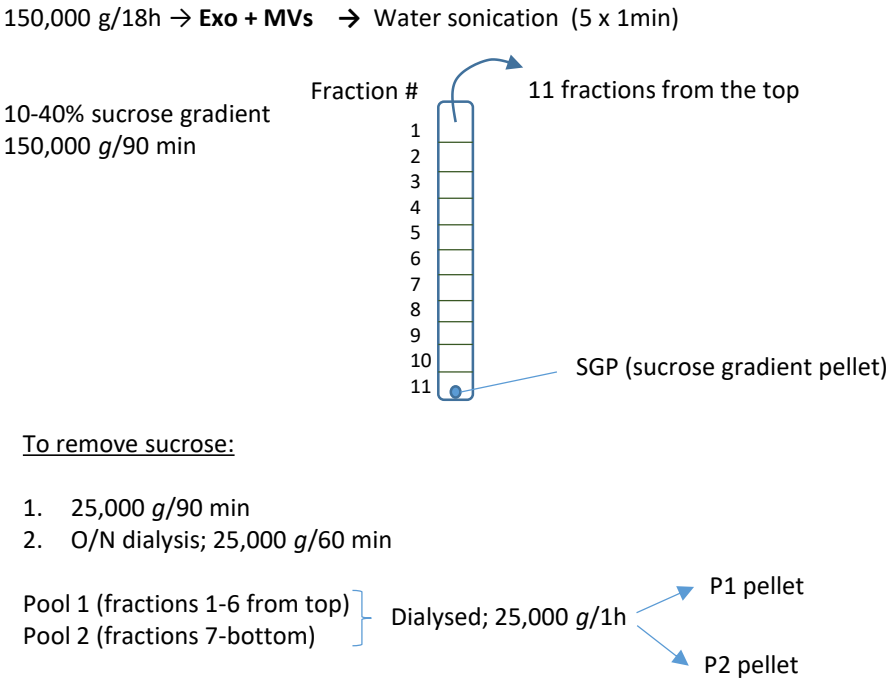


Fig. 3.1 Differential centrifugation and sucrose gradient isolation of MVs and exosomes. Use of differential centrifugation using 25,000 *g* to isolate MVs and using the resultant supernatant to isolate exosomes at 150,000 *g* for 18 h (1). The use of a sucrose gradient (10-40% sucrose; 150,000 *g* for 90 min) and the pooling of fractions into two pools, to isolate exosomes and microvesicles (2)

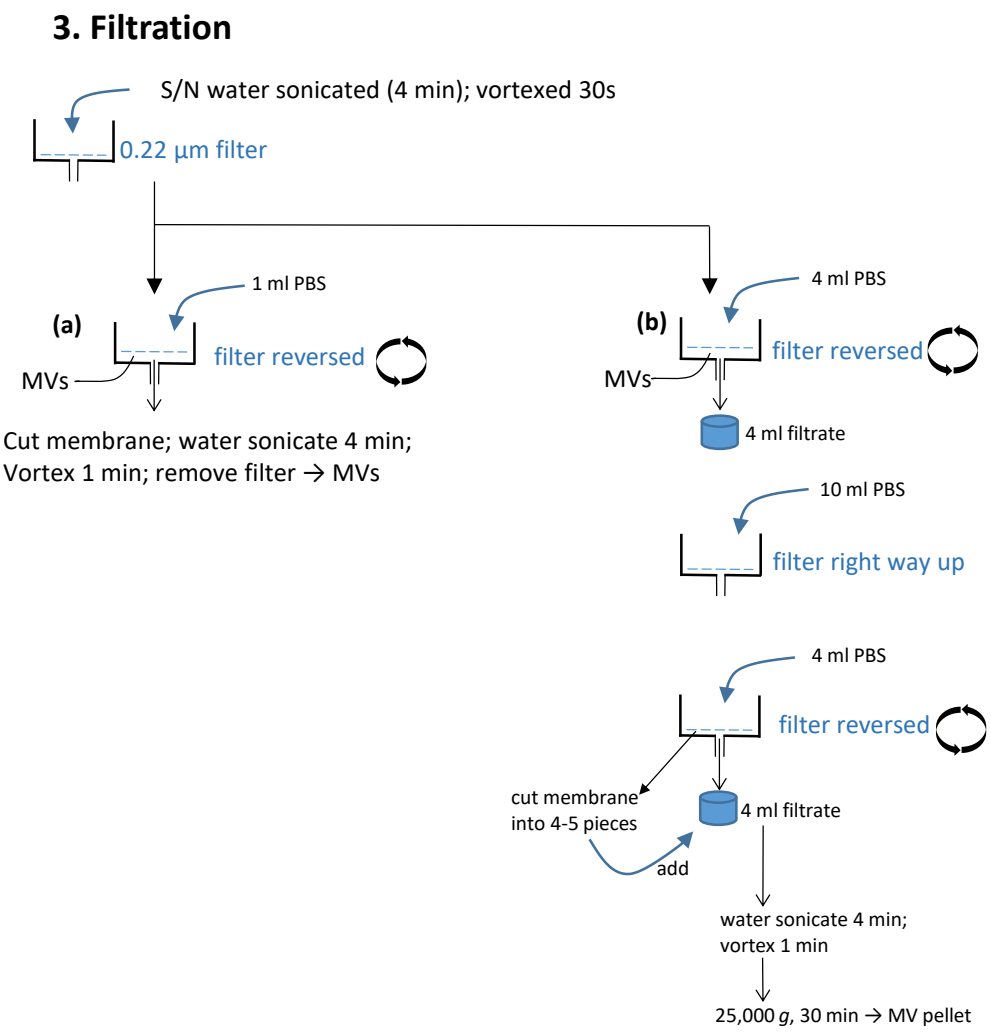


Fig. 3.2 Filtration and reverse filtration to isolate exosomes and MVs. "S/N" as prepared in Fig. 3.1 under 'differential centrifugation' (by removal of cells and cell debris) but containing a mixture of exosomes and MVs was then filtered through a 0.22 µm pore size filter. This was treated either as shown in (a) or (b) in which the filter was reverse-filtered, washed and sonicated to remove MVs.

3.3 Results

3.3.1 Differential centrifugation of microvesicles (MVs) and exosomes

Differential centrifugation was carried out according to the method outlined in Fig. 3.1. Two exosome markers, Lysosome Associated Membrane Protein, LAMP-1 and the tetraspannin, CD63 were used and the best current marker of MVs, phosphatidylserine (observed by staining with AnnexinV-FITC).

To observe the maximum shift in the fluorescence intensity in samples labelled with FITC or PE conjugated antibody, gating was performed. This was carried out in one of two ways. Either the marker #2 region in plot #3 was set from the point of intersection of histograms to the end, whereupon events in this region were considered as positive events, or else a rectangular gate was placed in plot #2 with GRN-HLog versus YLW-HLog scales on the same coordinates and scales (as for plot #3) and events occurring in this gate being taken as positive data. Once these gatings had been set, the same settings were applied for controls as well as labelled samples.

In the assay for Lysosome Associated Membrane protein 1, LAMP-1, unlabelled exosomes as well as those stained with FITC-conjugated anti IgG antibody (isotype control) were used as controls. Exosomes pre-incubated with FITC conjugated anti LAMP1 antibody (exo + LAMP-1 antibody) resulted in 41% positive events compared to 7% for MVs (Fig. 3.3).

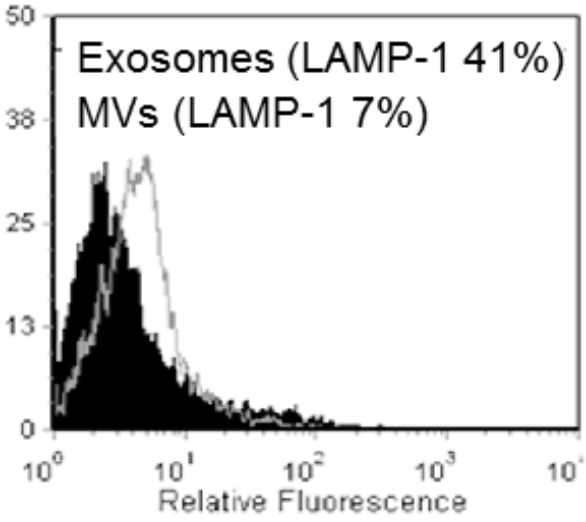


Fig 3.3 Lysosome associated membrane protein-1 (LAMP1) is more highly expressed on exosomes than microvesicles. Binding of Exosomes and MVs on GRH-HLog scale using IgG FITC isotype control.

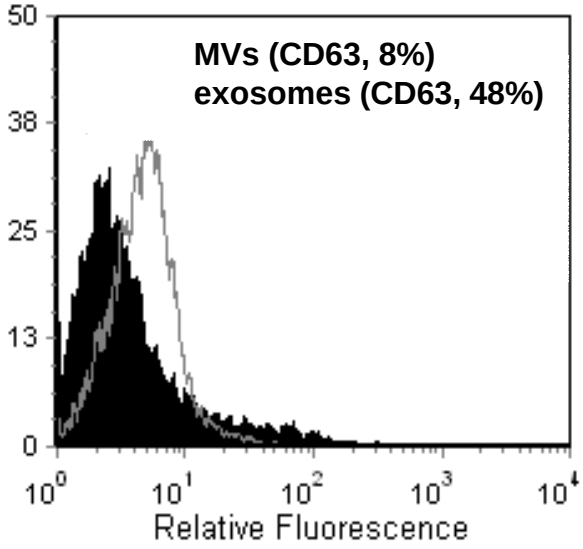


Fig. 3.4. LAMP-3 (CD63) is more highly expressed on exosomes than microvesicles. MVs and exosomes, pre-incubated with an anti CD63-PE antibody, on YLW-HLog scale.

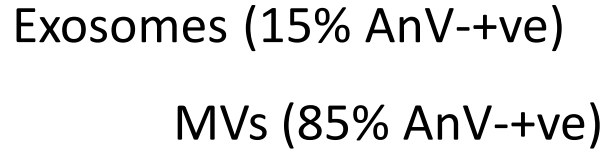


Fig. 3.5. Microvesicles have a greater exposition of phosphatidylserine (PS) on their surface than exosomes. MVs and exosomes, pre-incubated with annexin V-FITC, on YLW-HLog scale.

Exosomes were also stained with PE conjugated anti CD63 antibody and this resulted in 48 % positive events on the YLW-HLog scale while MVs alone gave 8%, suggesting that CD63, is indeed more highly expressed on exosomes than MVs. By way of negative control, unlabelled exosomes and MVs were used and these were set at 2% positive events, the histogram showing a comparison of the relative fluorescence intensities upon binding to an anti-CD63 antibody, Fig. 3.4. As a marker for MVs, the binding of Annexin V-FITC to indicate phosphatidylserine exposition, was used. Whilst MVs (25,000 *g* pellet) gave 85% positive events with AnV-FITC, there were only 15% with exosomes (Fig. 3.5).

In an experiment to begin to understand the merits of filtration in terms of MV and exosome isolation, a sample of MVs that had been pre-incubated with anti-CD63 antibody was divided into two equal groups, one of which was filtered through a Millipore filter of pore size 0.22 µm. Both groups were then observed by flow for fluorescence (yellow). It was observed that there was a significant decrease in the number of vesicles in the filtered group, reduced from 3.1×10^6 to 2.1×10^4 as enumerated by flow cytometry. It was notable that there was a significant, concomitant increase in fluorescence intensity in the filtered sample, increasing from 4% in the unfiltered sample to 54% in the filtered sample (Fig. 3.6), suggesting that the filtered sample was enriched for exosomes.

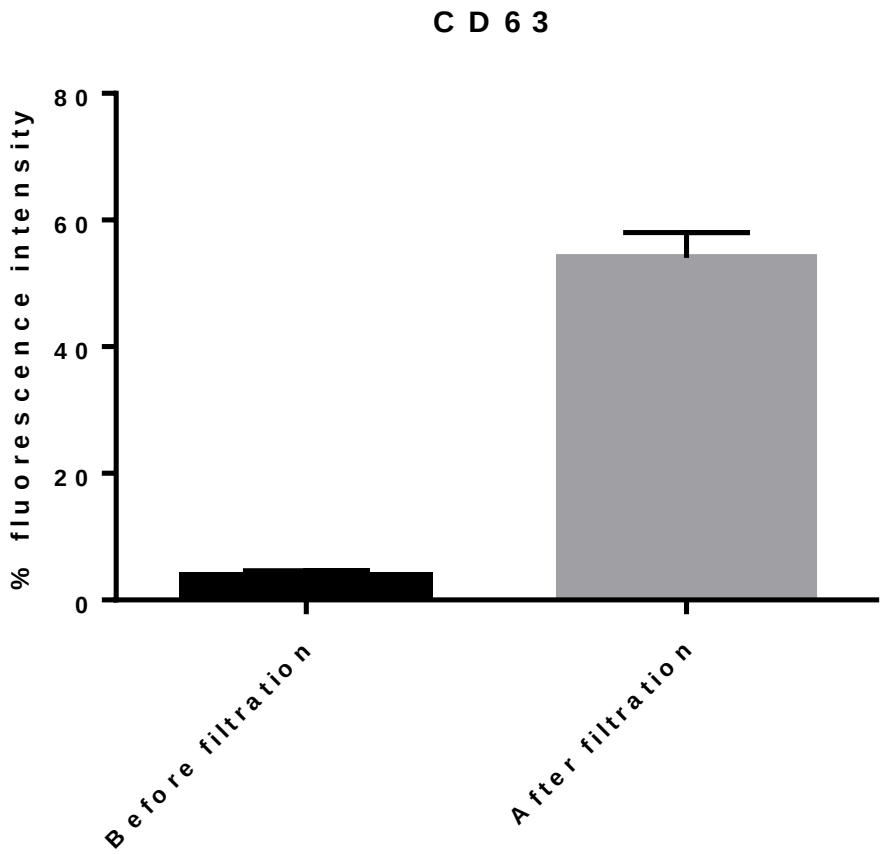


Fig. 3.6. Enrichment of exosomes after filtration through a 0.22 µm pore size filter. An EMV (exosome and MVs) sample (devoid of cells and cell debris by centrifugation (2x 4,000 *g*, 30 min) was pre-incubated with PE conjugated anti CD63 antibody, and analysed by flow cytometry for % fluorescence before and after filtration. The Y-axis represents percentage fluorescence intensity for each sample.

Although Fig. 3.5 shows increased PS exposition on MVs compared to exosomes and colleagues at CMIRC have continually shown this to be the case for the last 6 years (Ansa-Addo, E. *et al*., (2010) Anti-Baffour, S. *et a*l., (2010); Hind, E*. et al*., 2010; Cestari *et al*., (2012); Inal *et al*., (2012) ; Inal *et a*l., (2013); Stratton *et al*., (2013); Stratton *et al.,* (2015a); Stratton *et al*., (2015b); Kholia et al., (2015)) the lack of any other described markers of MVs has prompted a search by CMIRC for alternative markers. Proteomic analysis (Inal, unpublished personal communication) indicated that there may a significant difference in expression level of some of the integrin proteins between exosomes and microvesicles. Flow cytometry of MV and exosome expression of CD11b (αM integrin), was therefore perfomed, using the settings described above and using PE conjugated CD11b antibody. This indeed showed that MVs express more CD11b (51 % positivity) compared to 13 % of positive events for exosomes. Unlabelled MVs and exosomes were used for settings and as control and were set at 3% positive events. Fig 3.7, below, shows the histogram to compare the relative fluorescence intensities of exosomes and MVs sample when stained with PE-conjugated anti CD11b antibody.

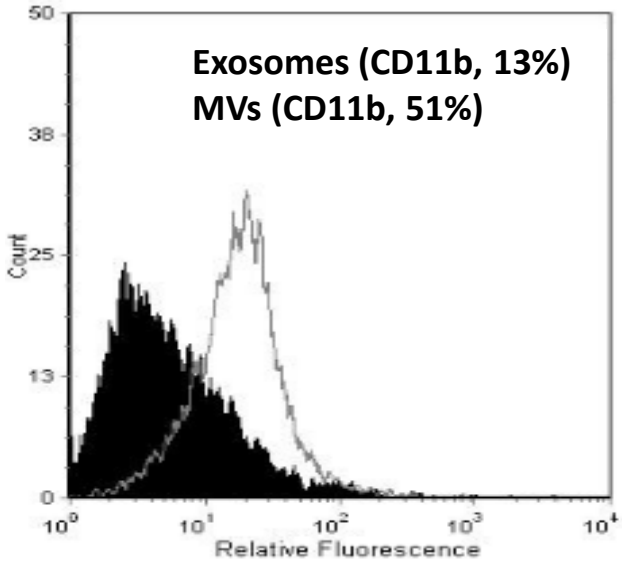


Fig. 3.7. Microvesicles express higher levels of αM integrin (CD11b) than exosomes. Both vesicle types were pre-incubated with anti-CD11b-PE antibody on YLW-HLog scale.

3.3.2 Phosphatidyl serine exposition on MVs isolated by filtration versus MVs isolated by differential centrifugation.

Microvesicles were isolated by filtration as outlined in Fig. 3.2 and by differential centrifugation as outlined in Fig. 3.1 (1). Annexin V (AnV) binding was then used to assess relative phosphatidylserine (PS) exposition and this confirmed that the vesicles recovered from the filter were indeed MVs. FSC vs SSC dot plots of both of these samples appeared similar to those that had been described at various times in the literature (Inal et al., 2010). For these experiments unlabelled MVs obtained after differential centrifugation was used for settings and control, and gated as described earlier. In detail, marker #2 on plot #3 was set from 100 to 10,000 on the GRN-HLog scale, giving 27 % positive events, for the control, in the marker #2 region. As a result, the population of MVs incubated with FITC conjugated annexin V shifted higher on the GRN-HLog scale. Under the same settings, the MVs obtained by differential centrifugation when incubated with FITC conjugated annexin V gave reading of 42% positivity and vesicles isolated by filtration gave readings of 49 % positivity. Annexin V binding on vesicles isolated by filtration was thus 7% higher than on MVs isolated by differential centrifugation and thus non-significantly different (Fig. 3.8).

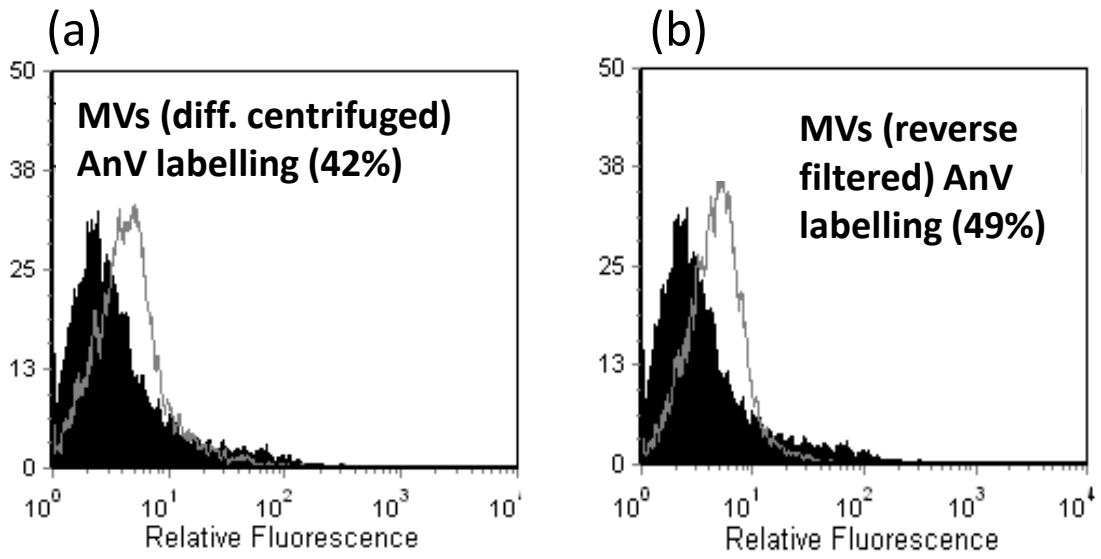


Fig 3.8. Annexin V-positively labelled vesicles (PS-positive) isolated by differential centrifugation and those isolated by reverse filtration are not significantly different in their levels of PS exposition. AnnexinV binding on GRN-HLog scale, for MV isolated by differential centrifugation and b) filter recovered MVs, on GRN-HLog scale

3.3.3 Sucrose gradient isolation of EMVs (exosomes and microvesicles) and analysis using Annexin V and anti-LAMP 1

The samples of exosomes and microvesicles (so-called S/N, i.e. EMVs free of cells and cellular debris) collected after low speed (4,000 *g* / 2X 30 min) centrifugation were isolated as described earlier in the materials and methods section. This time unlabelled exosomes were used as a control and to create the settings. The degree of Annexin V binding averaged at 8%, in the first 6 fractions from the top of the gradient and this increased significantly to 20% in the 9th fraction, 38% in 10th fraction, and back to 12% in 11th fraction (Fig. 3.9A) and 30% in the sucrose gradient pellet (SGP) (not shown).

Interestingly, the percentage of LAMP1 expression was higher in the top fractions, specifically 18% in 1st, 34% in 2nd and 32% in 3rd, then falling to an average of 10% for the remaining fractions (Fig. 3.9B).

It should be noted that unlabelled SGP when applied to the flow cytometry for observing side scatter versus granularity (FSc v SSc), that the vesicles produced a typical tapered dot plot as might be expected for MVs (Fig. 3.9C).

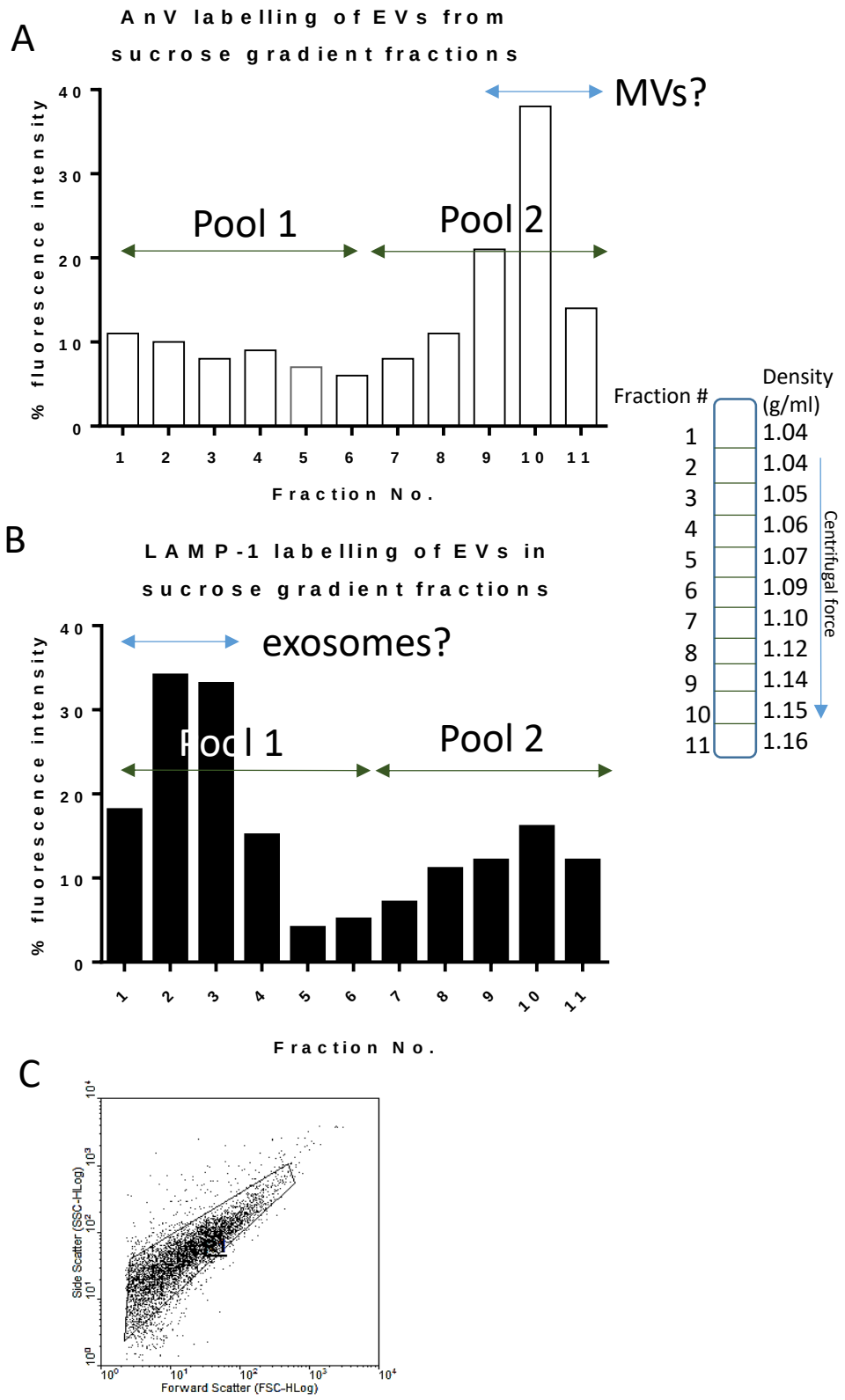


Fig. 3.9. a) AnnexinV and b) LAMP1 binding in sucrose gradient fractions. c) FSC Vs. SSC dot plot for SGP.

3.3.4 Use of Sucrose gradient and analysis of pools of fractions obtained for expression of αM integrin (CD11b) and CD63 (Lysosome Associated Membrane Protein, LAMP-3)

The sample of EMVs was isolated as described before and the experimental procedures for sucrose gradient were performed as described in the Materials and Methods section. Samples were read on the GRN -HLog scale and FITC conjugated anti IgG antibody was taken as the isotype control. The primary antibodies were not conjugated and so samples were incubated with FITC to be able to observe fluorescence on the flow cytometer (GuavaEasyCyte). In addition, samples that had been labelled with FITC were read and used as a control to enable removal of any background fluorescence produced by the fluorescein (FITC) itself.

In comparing samples labelled with FITC compared to those labelled with FITC conjugated with anti IgG antibody it was found that there was actually a very high background (i.e. high non-specific binding). It was therefore decided to use FITC as a standard control to look for a shift in fluorescence intensities in the sample population. The Marker #2 region on plot #3 was set at ~15% positive events for FITC labelled samples, on GRN-HLog scale. The same settings were then applied to IgG and samples labelled with a primary antibody (CD63 and CD11b). FITC-conjugated anti IgG antibody labelled samples are in fact excluded from the graph in Fig. 3.10 due to the extremely low percentage of binding. All unfiltered samples (incubated with FITC conjugated anti-CD63/CD11b antibody) showed significant CD63 and CD11b binding that increased from 58% (P1) and 50% (P2) to 82% for SGP. Interestingly, the percentage of fluorescence intensity decreased from 27% in FP1 and 7% in FP2 to 4% in FSGP.

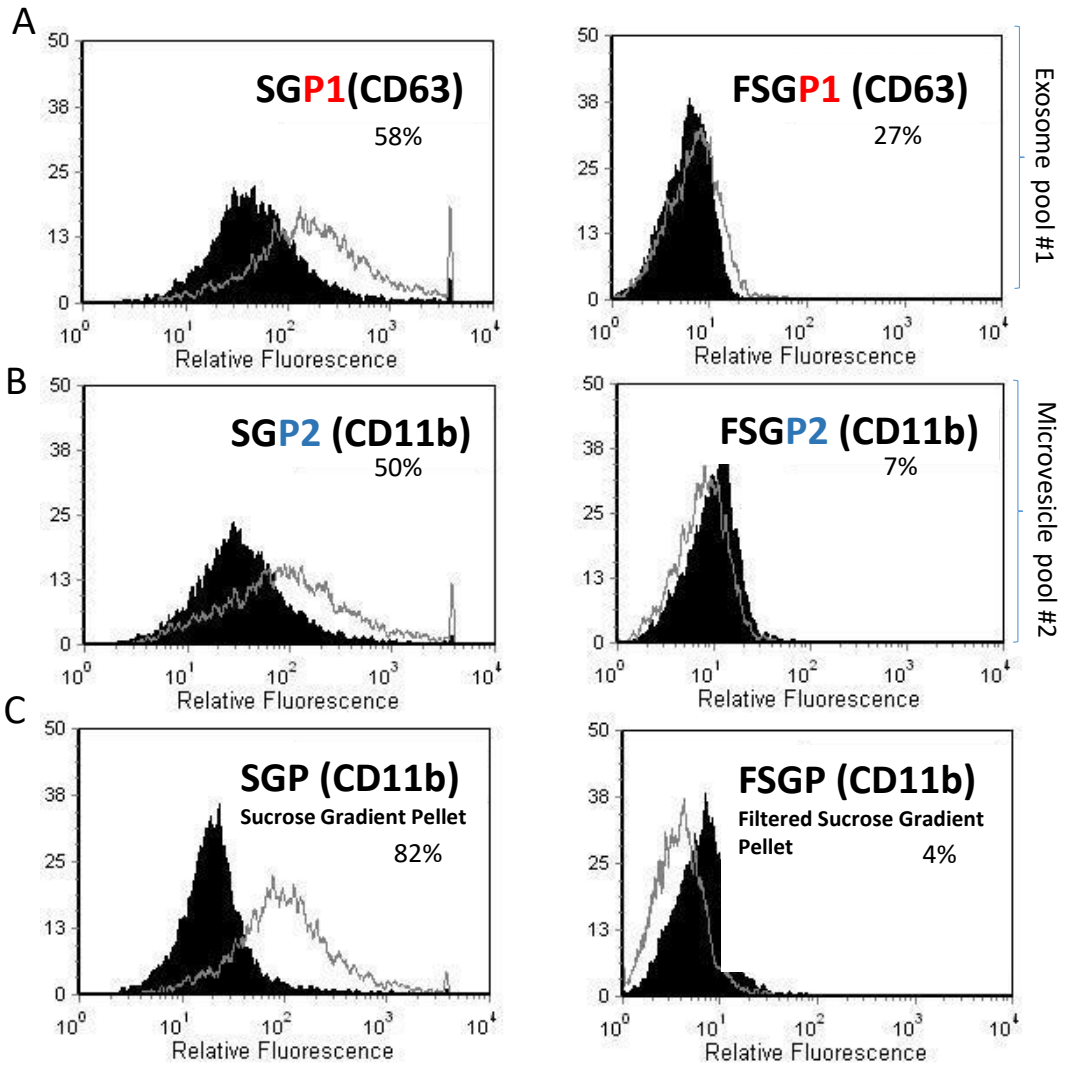


Fig. 3.10. Detection of CD63-expressing exosomes in pool #1 recovered from a sucrose density centrifugation as well as CD11b-expressing MVs in pool #2 and MVs in the pellet. (A) Pool #1 vesicles recovered expressing CD63 and (B) pool #2 vesicles expressing CD11b. (C) sucrose gradient pellet and filtered sucrose gradient pellet vesicles expressing CD11b. Isotype control is excluded from the graphs as binding was very low.

3.4 Flow cytometric analysis of microvesicles and exosomes purified by sucrose gradient and reverse filtration suggests that both MVs and exosomes can be isolated by sucrose density gradient centrifugation.

The use of flow cytometry analysis of EMVs aims to be able to detect the expression of a particular marker ideally able to distinguish microvesicles (MVs) and exosomes. In previous work Thery et al., in 1999 suggested that exosomes derived from dendritic cells are LAMP-1 positive. Also, THP-1 acute monocytic leukaemia cells express LAMP-1 and this protein can be released in association with exosomes. This was shown in the lab at CMIRC and the work presented here confirms binding of LAMP-1 antibody on exosomes by flow cytometry (Fig. 3.3). Although it has largely been suggested that MVs are typically not expressing cytosolic and lysosomal marker proteins, it was nevertheless found that low percentages of MVs do actually express LAMP-1. Exosomes are particularly enriched for CD63 (LAMP3) (Heijnen et al., 1999) compared to MVs as shown by Western blotting and corroborated by the results presented here for MVs and exosomes isolated by differential centrifugation and analysed by flow cytometry (Fig. 3.4). It was also found that the expression of CD11b is higher in MVs compared to exosomes (Fig. 3.7) and that MVs have a higher level of AnV binding indicating PS exposition on MVs compared to exosomes (Fig. 3.5) as was also found by Heijnen et al., 1999).

It is well accepted that exosomes have a natural buoyant density in sucrose density gradients in the range 1.12 and 1.18 g/ml (Heijnen et al., 1999; Inal et al., 2012) but very curiously nothing has ever been reported as to whether MVs might reside within a sucrose gradient. Flow cytometry of fractions obtained from sucrose density gradients gave some tantalisingly interesting results in which fractions were found to have fewer numbers of vesicles than SGP, the sucrose gradient pellet. Furthermore, the forward versus side scatter plots (FSc v SSc) of all the fractions were similar (data are not shown) and yet significantly different from SGP (Fig. 3.9c) In looking carefully at the FSc v SSc dot plot for SGP there can be discerned the first clue that the SGP could actually indeed be MVs. Annexin V is the most common marker

associated with MVs, having a high affinity for PS, the exposition of which is widely regarded as a characteristic and distinguishing feature of MVs, but not exosomes. Fractions towards the bottom of the tube (where the bigger particles would be retained, and therefore amongst the vesicle population, presumably MVs) were found to have higher annexin V binding compared to the top fractions (where smaller particles are likely to be retained, presumably exosomes). Alongside the annexin V binding results it was possible to carry out LAMP1 binding assays. Here it was observed that fractions towards the top of the sucrose gradient had a higher binding of LAMP-1 compared to the bottom fractions. It is known from previous studies that LAMP-1 is a lysosomal protein associated with exosomes. Taking all the data together, including FSc Vs SSc of SGP, and annexin V and LAMP-1 binding as obtained from the isolation of MVs and exosomes using the sucrose gradient, it is clear that SGP and the two fractions above it contain the majority of the MV population (SGP being the least contaminated of the three). Furthermore, the fractions above likely contain a mixed population of smaller MVs and exosomes and first 5 fractions likely contain mainly exosomes. By using fluorescent antibody staining it has thus been possible to detect exosomes as well as microvesicles by flow cytometry. Essentially the use of anti-LAMP-1 FITC conjugated antibody showed vesicles presumed to be exosomes in the higher fractions obtained from the sucrose gradient whilst in the lower fractions vesicles were detected that stained positively with annexin V-FITC (and hence presumably microvesicles). Subsequent to this work other members of the Cellular and Molecular Immunology Research Centre in unpublished work indeed confirmed the nature of the vesicles obtained from the sucrose gradient by electron microscopy. Of interest to the field of Extracellular Vesicles isolation and analysis, the sucrose density gradient can be used according to the data presented to separate and purify both MVs and exosomes.

The vesicle sample obtained through filtering resulted in a similar FSc v SSc dot plot and similar histogram, due to Annexin V-FITC binding as was obtained for the differentially centrifuged sample (Fig. 3.8). Of note and an

important new development for EMV isolation methodologies, when using filtration, the supernatant was gently sonicated for 4 minutes in a water sonicator before passing through the filter (0.22 µm pore size) in order to break up most of the exosomes that may have aggregated into clumps. As a result, any small MVs, exosomes and other debris smaller than 0.22 µm will pass through the filter into the filtrate whilst any vesicles and debris greater than 0.22 µm will be retained by the filter. A further washing step of the vesicles recovered on the filter would then be recommended to obtain a cleaner sample. Also, of note the integrity of exosomes after sonication was maintained, no breaking up of exosomes (or MVs) being detectable by transmission electron microscopy (not shown).

The expression of CD11b has not hitherto been described in exosomes. In the work presented in this chapter, using pools of fractions obtained from sucrose density gradients, the unfiltered samples, SGP1, SGP2 and SGP were found to be positive for CD11b/CD63, which was not the case for the filtered samples. There was also a significant loss of CD11b/CD63 expression in the filtered samples, FSGP1, FSGP2 and FSGP, although there were other differences, such as that the loss in CD63 expression being from 58% (SGP1) to 27% (FSGP1), compared to a much greater reduction from SGP (82%) to FSGP (4%) upon filtration through the 0.22 µm filter. It is important to note, however, that at this point samples were not sonicated prior to filtration and hence that there was a possibility that exosome clusters were retained in the filter; however, FP1 still retained CD63 binding up to 27%. The reason for a decreasing CD11b expressing sample in the lower fractions (pool 2), upon filtering might be that the larger vesicles settling in these lower fractions, were being removed upon filtration. Any CD11b positivity is completely lost in FSGP2 and FSGP; this is evident from the CD11b fluorescence values being beneath or at best equal to those for isotype control. From the results obtained for SGP1 and FSGP1 it can be said that exosomes are CD63 positive but that because of the data obtained using the SGP (sucrose gradient pellet) that CD11b is highly expressed by MVs (SGP results).

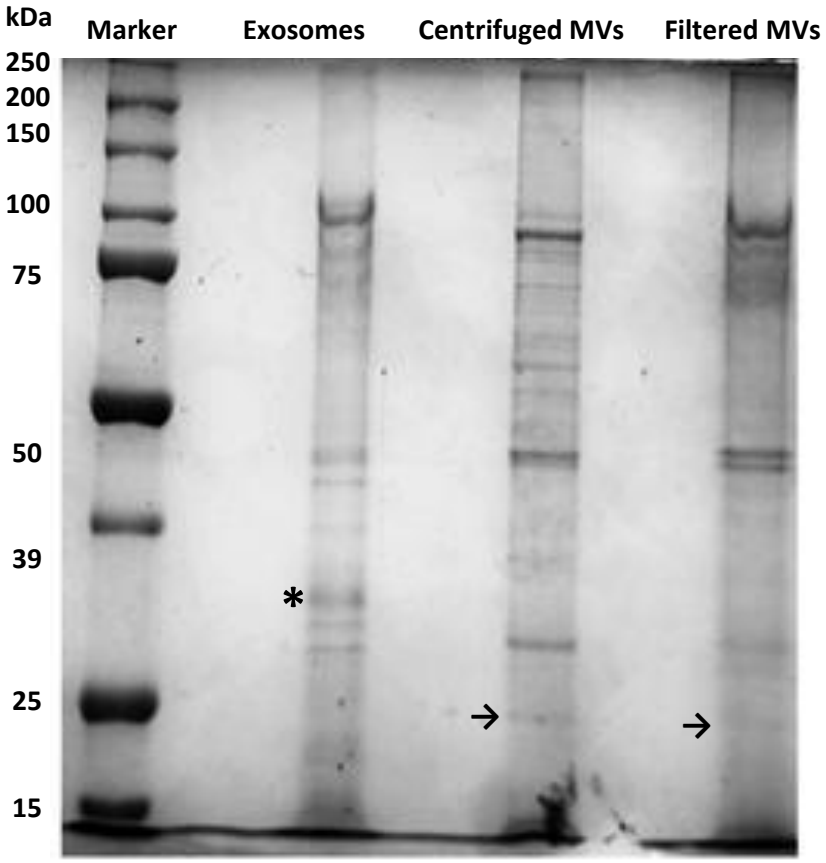


Fig. 3.11. Coomassie Brilliant Blue staining of 10% reducing gel for exosomes, differential centrifuged MVs and MVs isolated by filtration. 3µg protein (as determined by a BCA protein assay) was loaded for each sample. Asterisk denotes protein uniquely expressed in exosomes and arrows denote protein uniquely expressed in MVs. Samples were separated electrophoretically at 100V for 3h and then stained in Coomassie Brilliant Blue R250.

3.5 Comparative analysis of protein profile of isolated microvesicles and exosomes

MVs isolated by differential centrifugation were compared with those isolated by filtration by SDS-PAGE and Coomassie Brilliant Blue staining. 10% resolving gels were used to also separate exosomes. As can be seen from Figure 3.11, distinct proteins bands can be seen in all three samples. The largest number of bands, lie within the 40 to 80 kDa range for the sample of MVs separated by differential centrifugation. There were some differences also noted such as an approximately 31 KDa protein (asterisked) expressed in exosomes not being expressed in other samples and a 25 KDa protein (arrows) expressed in differentially centrifuged MV and MVs isolated by filtration, not being expressed in exosomes (Fig. 3.11).

In further experiments, pools obtained from the sucrose gradients of vesicles that had not been filtered or that had been filtered were run on 10% resolving gels. However, the exceedingly low protein concentrations (below ng level) meant that bands were not visible with Coomassie Blue staining (Fig. 3.12a) and therefore silver staining was employed (Fig. 3.12b). The latter revealed many protein bands, notably some proteins that were expressed in SGP but not in other samples, such as a protein band of ~60 kDa (asterisked). This protein appeared to be also lost in the FSGP sample. By contrast, a 100 kDa band (marked with the symbol °) was strongly expressed in SGP and FSGP but is weakly expressed in all other samples.

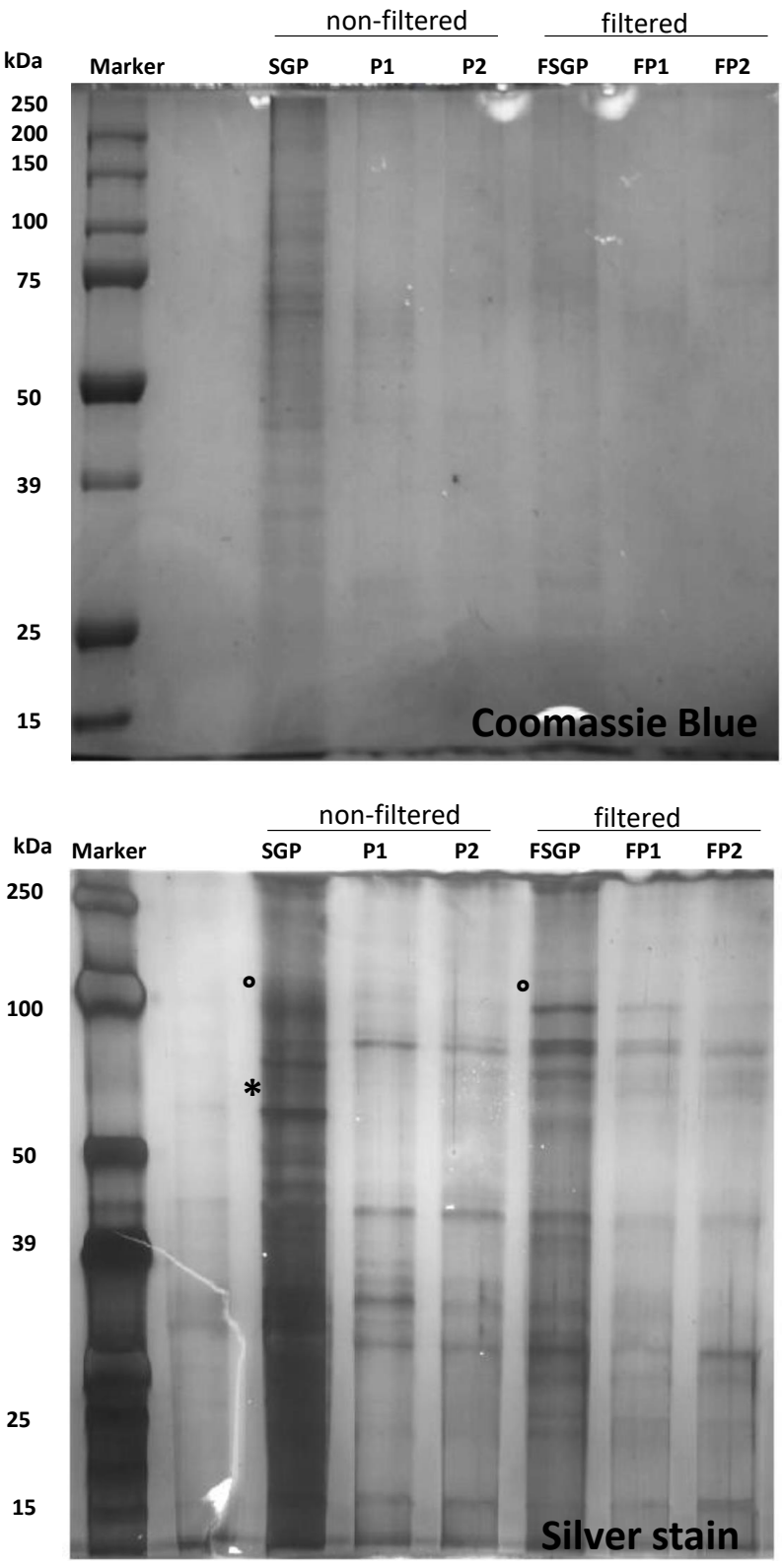
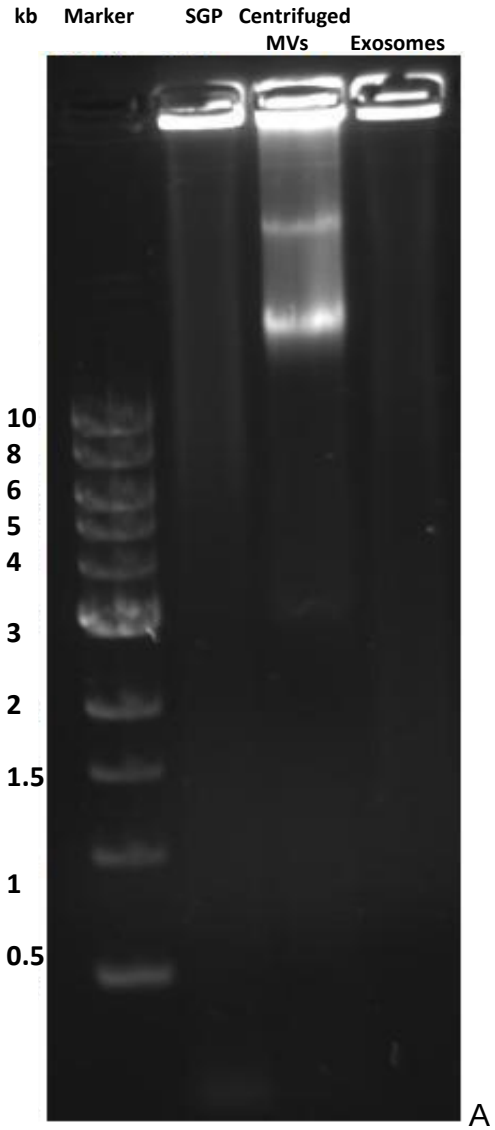


Fig. 3.12. Sucrose gradient samples of MVs and exosomes analysed by SDS-PAGE and Coomassie blue and silver staining. Coomassie blue (a) and silver staining (b) of 10% reducing gel with sucrose gradient samples (3

μg per lane). Samples were separated electrophoretically at 100V for 3h and then stained in Coomassie Brilliant Blue R250.

3.6 Agarose gel electrophoresis of isolated microvesicles and exosomes

The final means of analysis of isolated MVs and exosomes employed was to look at nucleic acids in the samples obtained. Therefore SGP, differentially centrifuged MVs and exosomes were analysed by agarose gel electrophoresis and ethidium bromide staining and all three indicated the presence of nucleic acids after exposure to UV. Where there was little or no electrophoresis of nucleic acid out of the well (in a 0.8% gel) it was assumed that such bands represented genomic DNA (gDNA). The sample of differentially centrifuged MVs showed two more bands, larger than 10 kb, which were absent from the exosome or SGP samples, suggesting the presence of contaminating DNA fragments being co-purified in the pellet after high speed ultracentrifugation to isolate MVs (Fig. 3.13A). Of interest, when MVs were lysed and only the intravesicular contents separated by agarose electrophoresis (Fig. 3.13B) there were no such similar bands of presumed gDNA visible (D. Stratton, personal communication). Firstly, of note, the MVs collected in the sucrose gradient pellet (SGP) are clearly devoid of contaminating gDNA present in the differentially centrifuged pellet. This suggests that centrifugation through the sucrose medium has removed gDNA from the MVs. As the MVs collected in 3.31A were collected by centrifugation at 25, 000 *g* for 60 min, which had been the centrifugation routinely used at the start of this project, the constitutively released MVs in Fig. 3.13B were centrifuged at 11,000 *g*. Once more this highlights the problem of co-isolation of associated macromolecules (such as gDNA) or possibly of various soluble proteins by higher speed centrifugation.



A

B

Fig. 3.13 Nucleic acids in samples of Exosomes and Microvesicles (sucrose gradient pellet and differentially centrifuged microvesicles and exosomes). (A) Unlysed samples of MVs isolated by sucrose gradient (SGP) and differential centrifugation together with unlysed exosomes were run on a 0.8 % agarose gel, stained with ethidium bromide (1 µg/ml for 1 minute) and viewed on a UV transilluminator. (B) Constitutively released

MVs (cMVs) and exosomes were gently lysed with RIPA buffer and similarly electrophoresed on a 0.8% agarose gel (from D. Stratton, PhD thesis, 2013).

3.7 Comparison of residual surface cytokine, TGF-β1, by ELISA, on microvesicles isolated by differential centrifugation and filtration

Another important analysis of EMVs that needed to be made was that of the cytokine TGF-β1. This is of particular interest as it is a cytokine that is carried on the surface of MVs (Ansa-Addo et al., 2010), and that can be removed from parent cells upon vesiculation. (Ansa-Addo, et al., 2010). It is also known to be present as an inactive protein within a latent complex that is released upon activation by low pH or by the action of plasmin or even interaction with integrin proteins (Khalil, 1999; Klingberg et al., 2014).

A TGF-β1 ELISA was therefore used to estimate TGF-β1 in filter purified MVs versus those isolated by differential centrifugation. On the basis of the nature of the method it might have been expected that the shear forces involved in differential centrifugation as opposed to filtration might result in a comparatively greater loss of surface TGF-β1 from the centrifuged MV sample. To make the comparison possible, equal protein concentrations of both samples were estimated using a BCA protein assay kit. As shown in Figure 3.14, the concentration of TGF-β1 in samples of MVs recovered by filtration was just over 100 pg/ml while unexpectedly being two-and-a half-fold higher at 250 pg/ml for the sample of MVs obtained by differential centrifugation (Fig. 3.14). Perhaps it is in fact the MVs recovered by filtration that does not have any cellular-derived TGF-β1 associated because of the nature of the purification method, and the level of TGF-β1 detected represents that which is truly on the MVs. Indeed, the higher levels of TGF-β1 on the ultracentrifuged samples might simply be due to cellular levels of TGF-β1 being co-purified.

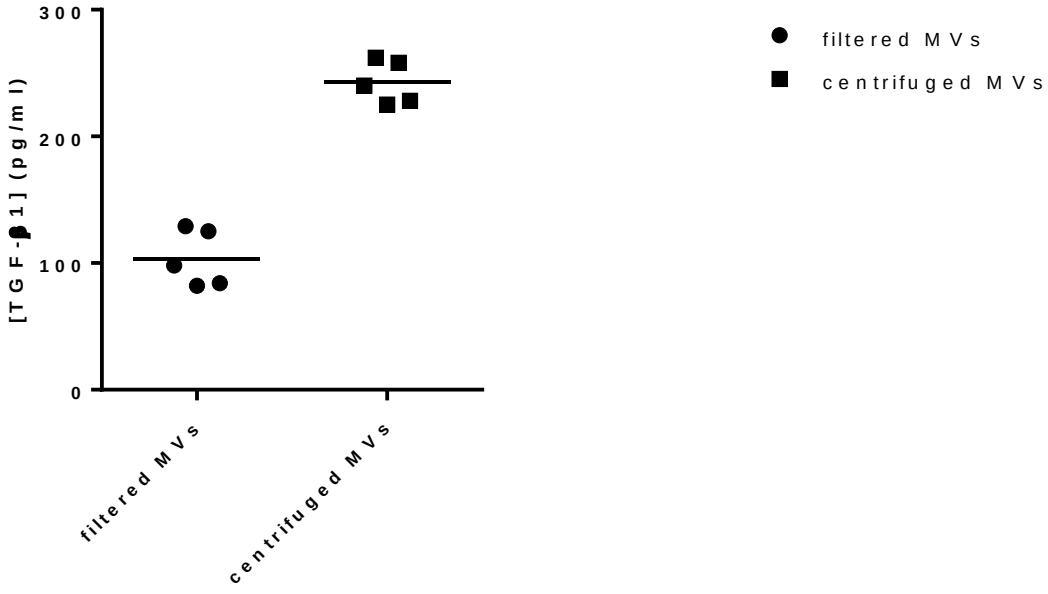


Fig. 3.14. Higher levels of the surface cytokine TGF-β1 are recovered by differential centrifugation than by filtration. TGF-β1 level as tested by Quantikine human TGF-β1 ELISA kit (R&D Systems) for MVs recovered by filtration and those recovered by differential centrifugation.

In effect the differentially centrifuged MVs appear to give a strongly false positive reading for surface TGF-β1 which is likely due to biding or association of cellular TGF-β1 that is getting incorporated into the pellet of vesicles after high speed centrifugation. Current studies at CMIRC are looking at the expression of TGF-β1 in MVs, their specific location and importantly their means of activation and release as an active 25 kDa homodimer able to interact with the cognate receptor on recipient cells.

3.8 Cytokine array of MVs and exosomes obtained by filtration and differential centrifugation

The MVs with exosomes sample was used as a reference to determine the presence or absence of particular cytokines in MVs purified by filtration in comparison with the exosome sample. In summary, all the cytokines found in the MVs + exosomes sample were present in the MVs only sample, but a few particular ones were absent in the exosome samples (Figure 3.15 and Table 3.1A). Sixteen cytokines were found in the samples and these are shown in bold in Table 3.1B. Table 3.1A gives a comparison of ten of these cytokines, which were present in the MVs + exosome sample and in the MVs sample; interestingly half of these were not or were only weakly expressed on exosomes. As a result of this study it is possible to propose candidate proteins that would warrant further investigation as possible markers of MVs (as opposed to exosomes). The paucity of MV markers and plethora of exosomal marker proteins means the results of such cytokine arrays provide important clues to possible future markers. As a point of interest, it may seem strange that C5a would be detected, as this very small anaphylatoxic peptide is released as a result of the C5 convertase enzyme complex cleaving complement C5 and this would only occur upon complement activation which actually was the way the MVs were prepared, the releasing cells having been stimulated with sublytic complement in the form of the normal human serum used to stimulate them. This has been shown to indeed be due to complement lysis as MVs are not produced if the NHS is depleted of

complement C9 or when it is heat inactivated. Exosome release does not need the stimulation of cells and no complement C5 was detected with exosomes, suggesting that its presence (on MVs) is less likely to be due to a co-isolation by centrifugation. Although C5a would not form part of the membrane attack complex (C5b-9), one of the first functions of MV release from cells was proposed by complementologists, many years ago, namely to remove deposited, potentially lytic MAC complexes from the plasma membrane of cells (Morgan et al., 1985, 1986, 1987).

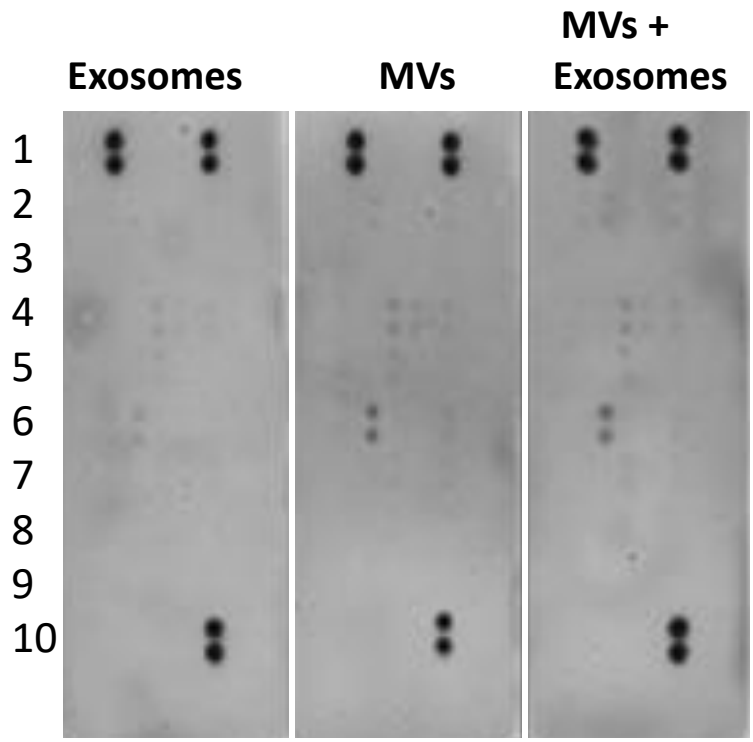


Fig. 3.15. Cytokine array showing the expression of 36 growth factors cytokines and acute phase proteins in samples of MV and exosomes. Samples were lysed with Triton X-100 and placed on the cytokine microarrays. Membranes were processed as described by the manufacturers, essentially by exposing the membranes with lysed MVs and exosomes to the detection antibodies, chemiluminescent signals being developed by exposure to the substrate luminol. Microarrays were then and developed by 10 mins exposure to X-ray film. Controls are the two duplicate dots of high intensity in row 1 and the one duplicate dot in row 10.

MVs+Exosomes	MVs	Exosomes
C5a	+	-
IL-1α	+	-
IL-32α	+	+
RANTES	+	-
IL-12 p70	+	+
G-CSF	+	-
IL-1ra	+	+
IL-13	+	+
IL-6	+	+
MIF	+	+
CCL-1	+	-
IL-17E	+	-

(A)

NO	A	B	C	D	E
1	**Control**	-	-	-	**Control**
2	**C5a**	**IL-1 α**	IL-10	**IL-32 α**	**RANTES**
3	CD154	IL-1 β	**IL-12 p70**	P-10	SDF-1
4	**G-CSF**	**IL-1 ra**	**IL-13**	**I-TAC**	**TNF-α**
5	GM-CSF	IL-2	**IL-6**	MCP-1	**sTREM-1**
6	GRO-α	IL-4	IL-17	**MIF**	-
7	I-309	IL-5	**IL-17E**	**MIP-1α**	-
8	sICAM-1	IL-6	IL-23	MIP-1β	-
9	IFNγ	IL-8	IL-27	**Serpin E1**	-
10		-	-	-	**Control**

(B)

Table 3.1. (A) A comparison of the presence or absence of the cytokines detected on MVs and exosomes. **(B)** Cytokines found present at the respective coordinates on the cytokine microarray are shown in bold. The

remaining cytokines that were potentially detectable but not found in either exosomes or MVs are in non-bold.

3.9 Purified exosomes are less able to block phagocytosis of apoptotic bodies than are microvesicles

Macrophages were obtained from 48 h after adding 1 μM PMA to THP-1 monocytes as shown by increased CD11b expression (Fig. 3.16A-C). These differentiated cells also had enhanced expression of CD14 (not shown). The resulting macrophages had the capacity to carry out phagocytosis as shown in Fig. 3.16D-F in which apoptotic bodies (stained in red with octadecylrhodamine [R18]), the nucleus being stained blue with DAPI (Fig. 3.16E) that had been opsonised with complement (heat-inactivated Normal Human Serum, NHS). MVs labelled with FITC seemed to have been phagocytosed by macrophages along with the R18 labelled apoptotic bodies (ABs). (Yellow = Green + Red, White = Red + Green + Blue) (Fig. 3.16F). As shown in Figure 3.17, where phagocytosis was assessed quantitatively by flow cytometry, it was seen that if macrophages were given an excess of MVs, that this competed with the uptake of apoptotic bodies, and that although exosomes could also diminish phagocytosis, it was not as effective as the reduction achieved with MVs.

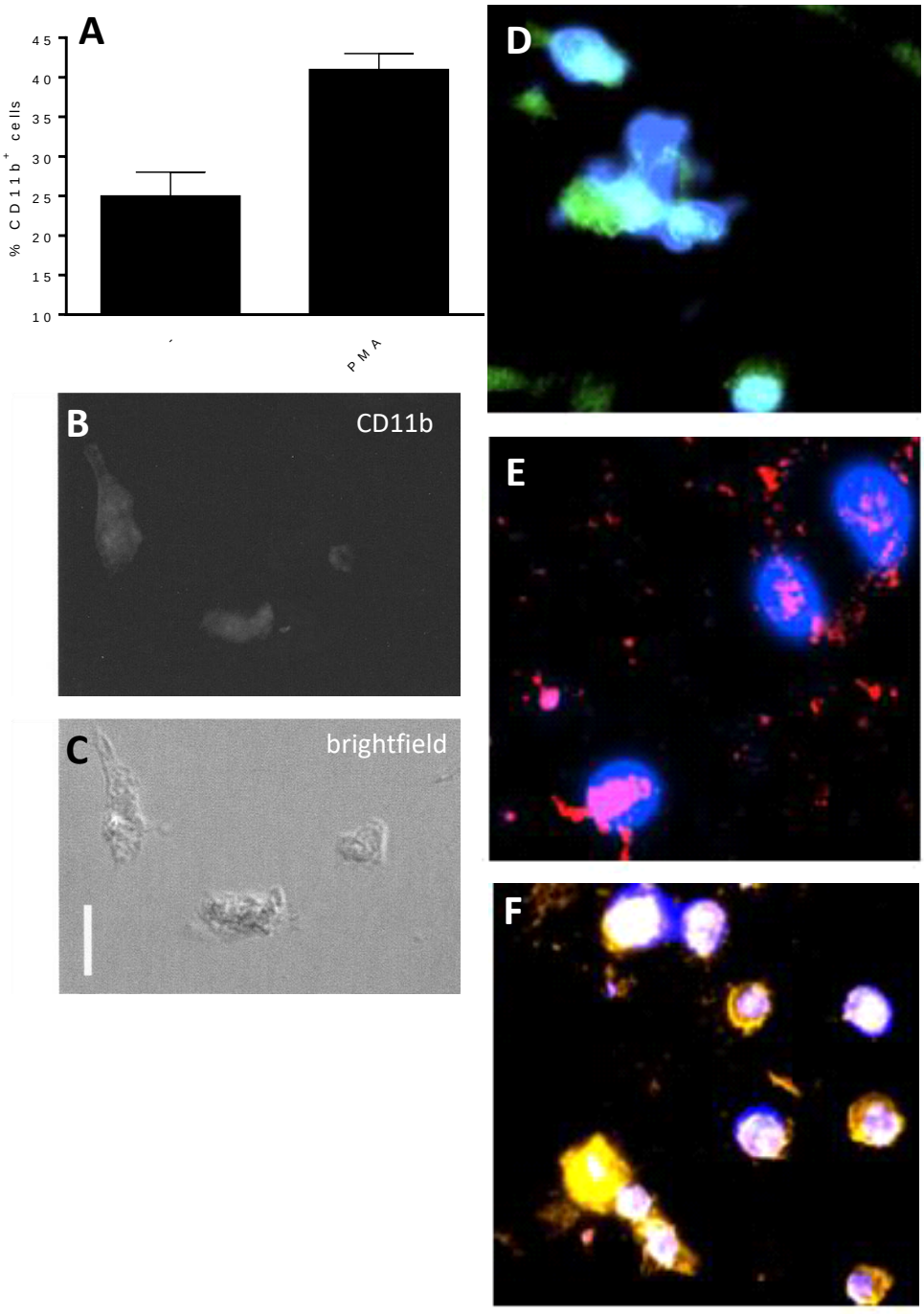


Fig. 3.16. PMA-differentiated macrophage phagocytosis of apoptotic bodies is reduced more effectively by microvesicles than exosomes. Fluorescence microscopy was obtained as follows: In (A) macrophages are labelled with anti-CD14-FITC antibody and stained with DAPI. In (B) macrophages were incubated for 1 h with R18-labelled apoptotic bodies (from Jurkat cells) and then stained with DAPI. In (C) macrophages were incubated for 1 h with R18-labelled Jurkat apoptotic bodies (FITC labelled) and then stained with DAPI. In panels (D-F) FITC is green, DAPI blue and R18 red. Yellow fluorescence is produced when red and green fluorescence combines while white fluorescence indicating all three fluorescence colours merging. All images were taken at 40X magnification.

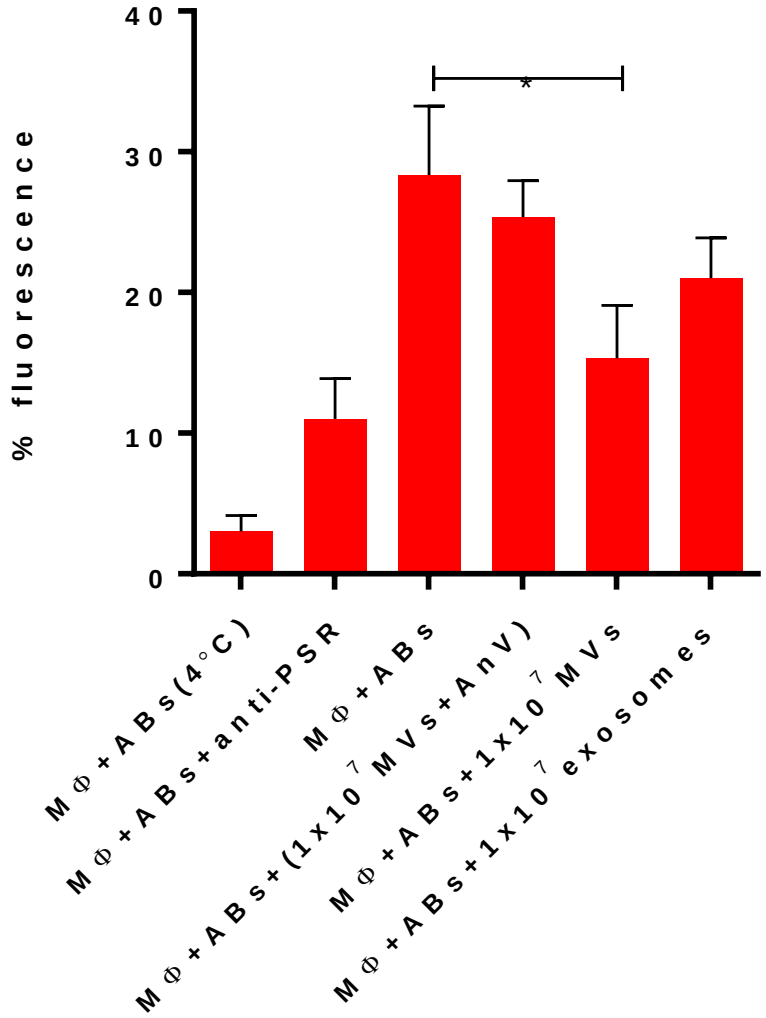


Fig. 3.17. Fluorescence intensity of uptake into macrophages by phagocytosis of R18-labelled apoptotic bodies (ABs) in the presence of unlabelled MVs or exosomes. Uptake was negligible at 4°C and was reduced significantly by blocking anti-phosphatidylserine receptors (anti-PSR) on the macrophages. The reduction of phagocytosis of ABs by providing competing MVs was however reversed when competing MVs had their exposed PS blocked with AnV. The error bars represent standard error of the mean, S.E.M. from 3 experiments in triplicate.

3.10 Discussion

Protocols to isolate and analyse exosomes and microvesicles are continually being re-evaluated. This has especially been the case and indeed one of the objectives of the International Society for Extracellular Vesicles since it was founded in 2012. The first meeting of ISEV was held in April of that year in Gothenburg and it was very apparent that researchers in the field of exosomes and microvesicles were very often using quite different procedures, as was communicated to me by Professor Inal, who chaired a session on 'Isolation and analysis of microvesicles.' as highlighted in our own meeting, 'Microvesiculation and Disease,' hosted at London Metropolitan University 5 months later (Inal et al., 2013). The current standard procedures are recommended in an article in the *Journal of Extracellular Vesicles* (Witwer, *et al*., 2013) and include differential centrifugation for the isolation of microvesicles with the additional sucrose gradient for exosomes.

The work presented herein began by comparing the exposition of PS on MVs isolated by differential centrifugation with that of MVs isolated by a novel filtration protocol developed at the start of this thesis. This turned out not to be that different (49% PS exposition, as AnV+ vesicles for those isolated by filtration versus 42% for MVs isolated by differential centrifugation) and this work also confirmed the use of PS as a marker of MVs (85% AnV+) versus exosomes (at only 15% AnV+). As there are a plethora of markers used to identify exosomes, it was also important to confirm MV markers and to this aim it was shown that exosomes are positive for LAMP-1 (41%) and LAMP-3/CD63 (48%) but that MVs were comparatively negative for both markers. Presence of αM integrin (CD11b) was confirmed as a useful marker for MVs with 51% positivity versus only 13% for exosomes. That filtration or 'reverse filtration' the term used to more accurately describe the method developed resulted in an enrichment of exosomes, was shown by the increase in CD63 expression from 5% before filtration to 55% after filtration. An interesting finding which has not been reported hitherto was the flotation of MVs in a sucrose gradient and the apparent concentration within a comparatively wider range of fractions ranging in density from 1.12 to 1.16 g/ml, with the

exosomes concentrating within a tighter range of 1.04 to 1.05 g/ml. This may reflect the greater heterogeneity of size of MVs as reported earlier (Stratton et al., 2015) in the nanosight profile of average microvesicular size, but of more relevance still due to the variation of protein content, and therefore the density of MVs (Stratton et al., 2015). As a final comparison of MVs isolated by differential centrifugation versus those isolated by filtration, the level of a cytokine that is found on the surface of the MV, TGF-β1 (Ansa-Addo et al., 2010) was compared. As this cytokine is released as a 25 kDa homo-dimer tethered to the extracellular matrix in a latent complex (LAP) it was deemed a sensitive marker for the harshness of the two isolation procedures. Although the level of TGF-β1 was greater on the MVs isolated by differential centrifugation compared to those isolated by filtration, the difference was marginal and non-significant. The comparison of vesicles should instead have been performed on exosomes where to centrifuge these much smaller (100 nm in diameter) vesicles, much higher centrifugal forces (100,000 *g*) are required as opposed to 11,000 to 15,000 to be able to pellet the larger MVs (100 to 1000 nm in diameter). When MVs isolated by differential centrifugation were run on an agarose gel and stained with ethidium bromide it was apparent that there was contaminating genomic DNA, which would favour a further step of sucrose density gradient centrifugation in order to 'clean up' the samples of contaminating macromolecules such as DNA. This would be of paramount importance if MVs are to be used as therapeutic or drug delivery vehicles.

In comparing MVs and exosomes, isolated by both procedures it is still very important to make any comparative studies of physiological parameters of these vesicle types as such comparisons are very few; for example, whereas there are now numerous studies highlighting proteomic analysis of MVs or exosomes there are barely any such comparative studies. The cytokine array analysis of MVs and exosomes isolated revealed potential markers at least for monocyte MVs that warrant further investigation, including RANTES, G-CSF, CCL-1 and IL-17E.

Finally, exosomes were compared with MVs for their capacity to block the phagocytosis of apoptotic bodies (ABs). It was assumed that the exosomes having lower levels of exposed phosphatidylserine, that they would be less able to inhibit the process compared to MVs. This was following on from previous studies that had shown MVs to inhibit the ability of macrophages to phagocytosis apoptotic cells (Antwi-Baffour, 2010). The hypothesis was confirmed and it was indeed found that exosomes were less able than MVs to inhibit the phagocytosis of ABs. This sort of information should be borne in mind especially when considering vesicle-mediated delivery of drugs with respect to the type of target cell, and for example whether it expressed PS receptors. Using cMVs (constitutively released MVs) and sMVs (formed after stimulating cells) which both express different levels of phosphatidylserine (PS) it would also be interesting to compare their relative ability to inhibit the macrophage induced phagocytosis of ABs.

4. Development of improved reverse filtration methodology for clinical isolation of MVs, and its use in making physiological measurements of plasma MV levels in the population

4.1 Introduction

In the previous chapter a sucrose density gradient which is used in the procedure to isolate exosomes was used to see if MVs could also be isolated. One potential advantage of running MVs through a sucrose gradient is that the viscosity of the sucrose can itself provide a degree of 'washing' of the vesicles and to remove any macromolecules that can potentially be co-purified during high speed centrifugation. One important physical factor that must be considered by any procedure that involves centrifugation is that of the potential for shear forces to physically break apart the MVs. To this end, another procedure, with the potential to separate out vesicle populations according to size, that could be considered, is that of filtration. Using a filter with a pore size of 0.1 μm, which based on current knowledge is the closest to a size at which MVs and exosomes can be separated, it would be hoped that MVs would be retained on the filter and exosomes pass through into the filtrate. These new methodologies, adapted for a clinical laboratory, could then be used to enumerate plasma MV levels within a population and to try to relate their levels to various parameters such as age, gender, fasting status, etc.

The standard and most often used protocol for the isolation of MVs involves differential centrifugation. Such protocols typically result in much contamination of MV pellets with exosomes mainly because the lower size limit of vesicles that can be pelleted at a particular centrifugation force covers a range of vesicle sizes. Furthermore, there is the problem that many of the smaller vesicles, typically exosomes are able to form clumps and therefore be centrifuged at the same force as some of their larger vesicle counterparts, MVs.

Once a novel method for isolation of plasma MVs is developed, the aim will be to make measurements of plasma MV levels in the population and to make preliminary assessments of any influences of gender, age, fasting and smoking status on these MV levels.

4.2 Materials

0.2 µm Polysciences Polybead Microspheres

Annexin V- PE

Annexin V- Binding Buffer 10x Concentrate

Biocleanse Biocidal cleaner

BD FacsCalibur Flow Cytometer Analyser

BD Plastipak 2.5 ml Syringe

BD TruCOUNT Tubes

Calcium Ionophore A23187

DiMethyl SulfOxide (DMSO)

Dulbacco's Phosphate Buffered Saline (DPBS)

Hanks' Balanced Salt Solutions (HBSS)

Horiba ABX Diagnostics P120 Haematology Analyser

Millipore 0.1 µm Hydrophillic Durapore membranes

Swinnex 13mm filter holders

Wooden Applicator sticks

4.3 Methods

4.3.1 Analysis of MVs by flow cytometry and enumeration of MVs using Trucount beads

MV measurement was carried on filtered citrate plasma. Annexin V-PE was used to detect phosphatidylserine (PS) on the surface of MVs, AnV-PE positive vesicles being quantified in the FL-2 channel. The absolute count of MVs was calculated on the analysis template using the Trucount tube lot number information and expressed in number of MVs/ml.

4.3.2 Specimen Requirements

Samples were collected in 1.8 mL vacutainer tubes containing 100 µL of 1M Sodium Citrate 1mL blood (0.109M, final concentration). Venous blood **(**1.8 mL) blood was drawn slowly and gently into a syringe, and immediately transferred to the tube. The use of a vacutainer was avoided to ensure that the cells would not become activated, resulting in increased microvesiculation and giving falsely elevated results. Ethical approval for this work was obtained from Hammersmith Medicines Research and from the ethics committee of the Dept. of Health and Human Sciences at London Metropolitan University.

Samples were processed within 1 hr of drawing the blood and analysed within a time frame of 3h. Samples that had been centrifuged and from which MVs isolated were on occasion frozen at -20°C, until further analysis. Samples were however never repeatedly freeze/ thawed.

4.3.3 Isolation of MVs and exosomes by filtration using a filter of pore size 0.1 µm

4.3.3.1 Preparation of samples

Within 1 hour of being collected, samples were labelled with a lab reference number (LRN) and centrifuged at 160 *g* for 5 min followed by two centrifugations for 30 min at 4,000 *g*. All the aliquots and transfer tubes for the same sample were labelled with the same LRN. This enabled correct tracking, storage and retrieval of samples when required.

The plasma samples (0.5 ml) were collected in a labelled microfuge tube for reproducibility and to enable a comparison of sample stability. Samples were then stored immediately at -20ºC.

The remaining 0.5 ml of plasma was then filtered using a Swinnex 13 mm filter holder with a 0.1 µm sized Millipore Hydrophillic Durapore filter placed inside. The O-ring was positioned on top of the filter. Each sample was passed through a filter holder using a disposable 2.5 ml BD Plastipak syringe inserted at the female inlet. The filtrate containing exosomes was used for

further analysis if needed. The MVs (of average diameter 0.1-2 µm) were thus deposited on the filter membrane.

The filter membrane was then carefully removed using a new set of disposable wood applicator sticks for each sample, discarding sticks after use. The filter was placed in 3 ml PBS in a 15 mL conical tube (this volume having been kept consistent across all samples). Sonication of the conical tubes for 15 min at RT, was followed by 5 min of very gentle vortexing to remove all MVs from the filter membrane. During vortexing the tube containing the membrane was placed at an angle to enable the vibrating liquid to help remove MVs from the membrane. The filter membrane was the removed & discarded from the conical tube using new applicator sticks for each sample. All used filter holders were soaked in Biocleanse Biocidal cleaner for 15 mins followed by sonication for a further 15 mins to decontaminate and rinsing five times with distilled water. After air drying over an absorbent paper towel in a cupboard to prevent dust collecting they were ready to be re-used.

4.3.3.2 Preparation of controls

Within 1 h of being taken, samples were stimulated with 1 mM Calcium Ionophore A23187 (mixed Ca^{2+}, Mg^{2+} salt). The resulting early apoptosis and cell activation resulting from the stimulation of plasma cells with Calcium Ionophore A23187, was deemed cause an increase of intracellular calcium resulting in a subsequent increase in the general number of MVs.

To prepare the Calcium Ionophore A23187 reagent, 10µl 50mM Calcium Ionophore concentrate was added to 250µl HBSS and 240 µl DMSO. This was used within one hour of preparation and kept protected from light. To stimulate the plasma cells, 36 µl of the prepared Calcium Ionophore A23187 reagent was added to each 1.8 ml citrate tube to give a final concentration of 20 µM. The citrate tubes were incubated in a 37ºC incubator for 1 hour and filtered as for samples using 1 ml plasma.

4.3.4 Staining Method

4.3.4.1 Staining samples with AnV-PE

The PBS supernatant was discarded carefully such as to ensure that the pellet of MVs was not disrupted. The pellet was then resuspended in 100 µl AnV Binding Buffer (AnVBB) mixing thoroughly with a Gilson pipette. In this way the original 0.5 ml of plasma sample was concentrated 5-fold. The re-suspended MV pellet (100 µl) was then transferred from the conical tube to a Trucount tube where it was stained with 5 µl Annexin V-PE (annexin V conjugated to phycoerythrin, PE). The reaction mixture was incubated for 15 min at RT in the dark and 395 µL of 1x AnVBB (prepared *in situ* from 10x Binding Buffer concentrate) added to each tube. The results were analysed by flow cytometry within 1 hour.

4.3.4.2 Preparation of controls for flow cytometry

Once the PBS supernatant had been carefully discarded, the MV pellet was re-suspended in 200 µl PBS (5x concentrate). Aliquots (100 µl each) were frozen in a BD Falcon tube. The aliquots were then ready for use as positive and negative controls. These controls (as described in the table below) were stained on the day of use and run within 1 hour.

Table 4.1. Annexin V PE staining method for controls:

Reaction		Negative Control	Positive Control / Patient Samples
MVs re-suspended in PBS		100µl	100µl
AnV- PE stain		-----	5µl
AnV Binding Buffer	Pre-incubation	100µl	100µl
	Post-incubation	300 µl	295 µl
Total reaction volume		500µl	500µl

4.3.5 Calibration/Quality Control

A 3-colour calibration was performed daily, using BD CaliBRITE 3 beads (SLE017). The calibration reports of the 'lyse-wash' and the 'lyse-no wash' assays were printed out, signed and dated and filed in the FACSCalibur Calibration data file.

Polystyrene beads (0.2 µm) were run as a size control before each batch of samples (displayed in the histogram).

4.3.6 Sample Acquisition and analysis using the BD FACSCalibur flow cytometer (running samples manually)

Having opened the appropriate acquisition template in *Facsdrive1/ Acquisition Templates* folder, the *Acquire* menu was selected, followed by '*Connect to cytometer*,' whereupon the *Browser* window would open automatically. It could also be opened from the windows menu by selecting *Show Browser.* The appropriate instrument settings were then opened by selecting *Instrument Settings* and *Open* from the Cytometer menu and

selecting FACStation G4/1.25/WT, BD Files, Instrument Setting Files, and the relevant settings, MV settings. Once the settings had been chosen it was necessary to click the *Set* icon, then *Done*.

It was also necessary to choose the correct location for saving the data to be acquired in the *Browser* window, by clicking on *Change* next to *Directory.* The appropriate folder e.g. Facsdrive1/HMR code/Date/Time point/Patient file was then selected. Sometimes it was necessary to create the above files.

Throughout Sample ID was the laboratory reference number (LRN); Patient ID, was any or all of the following: patient initials and D.O.B & Randomisation Number.

To check the speed of acquisition, *Counters* from the *Acquire* menu was selected, to open the counters window. The following shortcuts were used: Apple 1,2, 3 and 4 to open *Detectors/Amps*; *Threshold; Compensation* and *Status* windows. These could also be accessed from the *Cytometer* menu.

Fluidics was pressurised by flicking the black knob between the sheath and waste reservoirs from ‘vent’ to ‘pressurise’ whilst ensuring the cytometer to be set on LOW for MV detection, and RUN. The sample was first gently mixed and then placed in the sample injection port (SIP). With the *Set-up* ticked *Acquire* was selected. The operator then had to acquire until sufficient events had accumulated to be able to judge the gates. Adjustments were made if necessary and then *pause* and *abort* clicked.

With the *Set-up un*ticked, *Acquire* was selected. The cytometer then acquired the pre-set number of events and the data was automatically saved (10,000 in this investigation). This was continued until all tubes had been analysed. The details of the next subject were then entered in the Browser, the location to which they were to be saved selected and then the samples run.

Once the run was complete, the SIP (SLE016) was cleaned. To optimise the uptake of FACSClean and FACSRinse, the cytometer had to be set on HI. When the clean was finished, 1 mL distilled water was aspirated, and RUN selected (on HI) for another 5 min.

4.3.6.1 Analysis of flow cytometric data

The data was analysed after the acquisition was complete, to ensure that the correct populations for obtaining the mean count of MVs had been selected and gated. For this the appropriate analysis template was opened from the *Facsdrive1/ Analysis Templates* folder. The light scatter and FL-1 was set at logarithmic gains, and 10,000 total events were collected during each sample analysis. These events were processed by an Apple computer in conjunction with the Cellquest Pro Software (BD Biosciences UK). After identifying MVs, their absolute counts were calculated automatically on the analysis spreadsheet after inputting the following formula provided by BD Biosciences UK:

$$\frac{\#\ of\ events\ in\ region\ containing\ MVs}{\#\ of\ events\ in\ absolute\ count\ bead\ region} \times \frac{\#\ of\ beads\ per\ test\ *}{Test\ volume\ (mL)}$$

= MV ABSOLUTE TOTAL COUNT

** this value is found on the TruCount Absolute Count Tube foil pouch label, and will vary from lot to lot.*

Each data file was printed and analysed. To print, *Apple* and *P* were pressed simultaneously.

4.3.7 Statistical analyses

The precision of the methodology was determined by calculating percentage coefficient variance (% CV). Boxplots were used to indicate MV absolute count spread amongst the human population, and the various variables (age, gender, fasting status, etc.) looked at. Regression Analysis for the Age variable, and t-Tests were performed to test the significance of the differences in the produced number of MVs using SPSS Version 17 .0. Statistical significance was considered when *p*-values were less than 0.05.

4.4 Results

4.4.1 Development of a new method for MV isolation based on filtration

In terms of developing novel methods for MV isolation it is necessary to remember the other vesicle type that cells release, namely the exosome. Figure 4.1 presents a reminder of the two vesicle types being considered here. The exosomes have an endocytic origin, being formed by the intraluminar budding of a multivesicular body and being released on the fusion of the MVB with the plasma membrane; their size is typically 50 – 100 nm and they expose much lower levels of phosphatidylserine (PS) than MVs. The larger MVs (100 – 1000 nm) are released directly from the plasma membrane, this process being initiated by an increase of intracellular calcium.

One goal of the filtration protocol, as opposed to any differential centrifugation protocol, and as summarised in Fig. 4.2 was to yield a population of MVs with as little contamination with exosomes as possible. The protocol designed was to be used to isolate MVs and exosomes from venous blood. For this purpose, 1.8 ml was collected in a vacutube containing 109 mM Na citrate. After centrifugation (2,200 *g*; 15 min) 0.5 ml of plasma was then subjected to a light sonication (five one-minute pulses) in a sonicating water bath. The reason for adopting this approach was that it has been previously reported that exosomes may form clumps (Heijnen et al., 1999; Zakharova et al., 2007) and therefore would be likely to co-purify with MVs. The resulting EMVs comprising MVs and exosomes were filtered

through a Millipore filter of pore size 0.1 µm. It was then possible to recover exosomes from the filtrate, exosomes averaging at 50 – 100 nm in diameter, whilst MVs were recovered from the filter itself by 5 x 1 min sonication's in a sonicating water bath followed by gentle vortexing for 1 min. Finally, the MVs were collected by centrifugation at 25,000 *g* for 30 min. As demonstrated by Stratton, D (personal communication) ~~and shown in the appendices (Fig. A1),~~ MVs could also be isolated with much reduced centrifugation speeds (e.g. 10,000 *g*, 1 h).

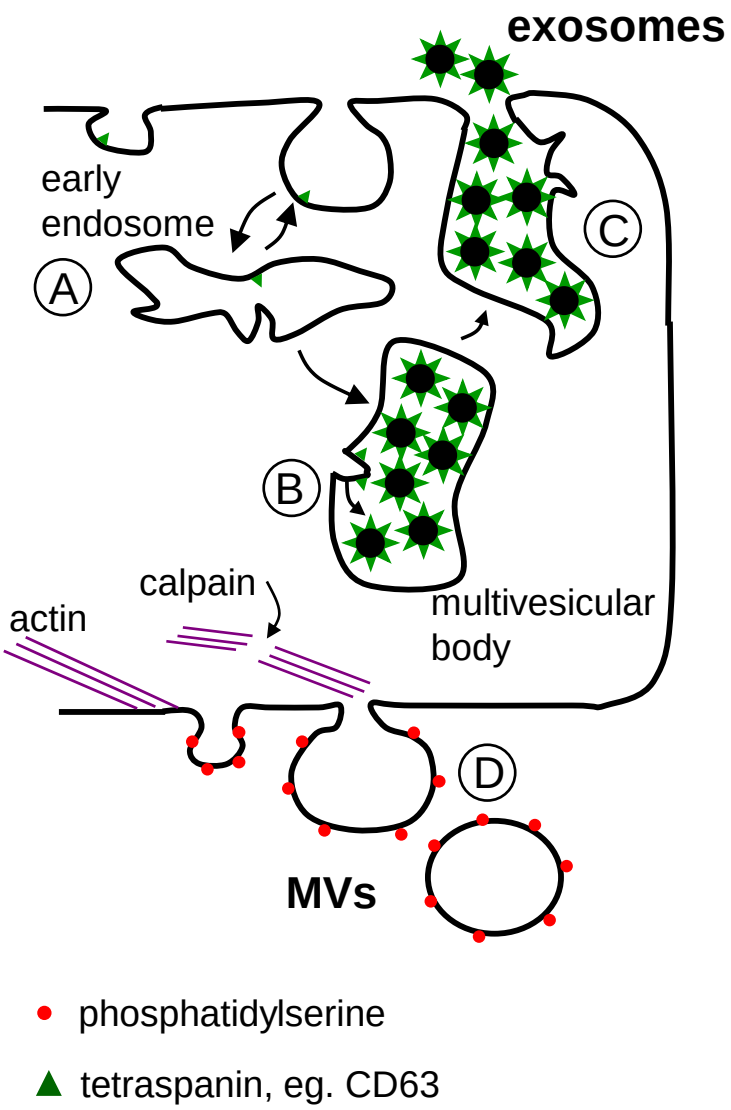


Fig. 4.1 Microvesicle (MV or MV) and exosome biogenesis from a typical eukaryotic cell. As a result of endocytosis, early endosomes and formed which may either recycle to the surface or form MultiVesicular Bodies (MVBs) by intraluminal budding to form IntraLuminal Vesicles (ILVs) (A). The ILVs are released as exosomes as the MVB fuses with the plasma membrane (B). Microvesicles (MVs), sometimes called Plasma Membrane-derived Vesicles (MVs) or even ecosomes are released (D) directly by budding form the plasma membrane upon activation resulting in increases of cytosolic calcium concentrations, or in early apoptosis, resulting in loss of membrane asymmetry, cleavage of the actin cytoskeleton by calpain. The MVs lie in the range 100 – 1000 nm in diameter whilst exosomes range from 50 – 100 nm. Exosomes carry tetraspanin proteins such as CD63 and expose less phosphatidylserine (PS) than MVs.

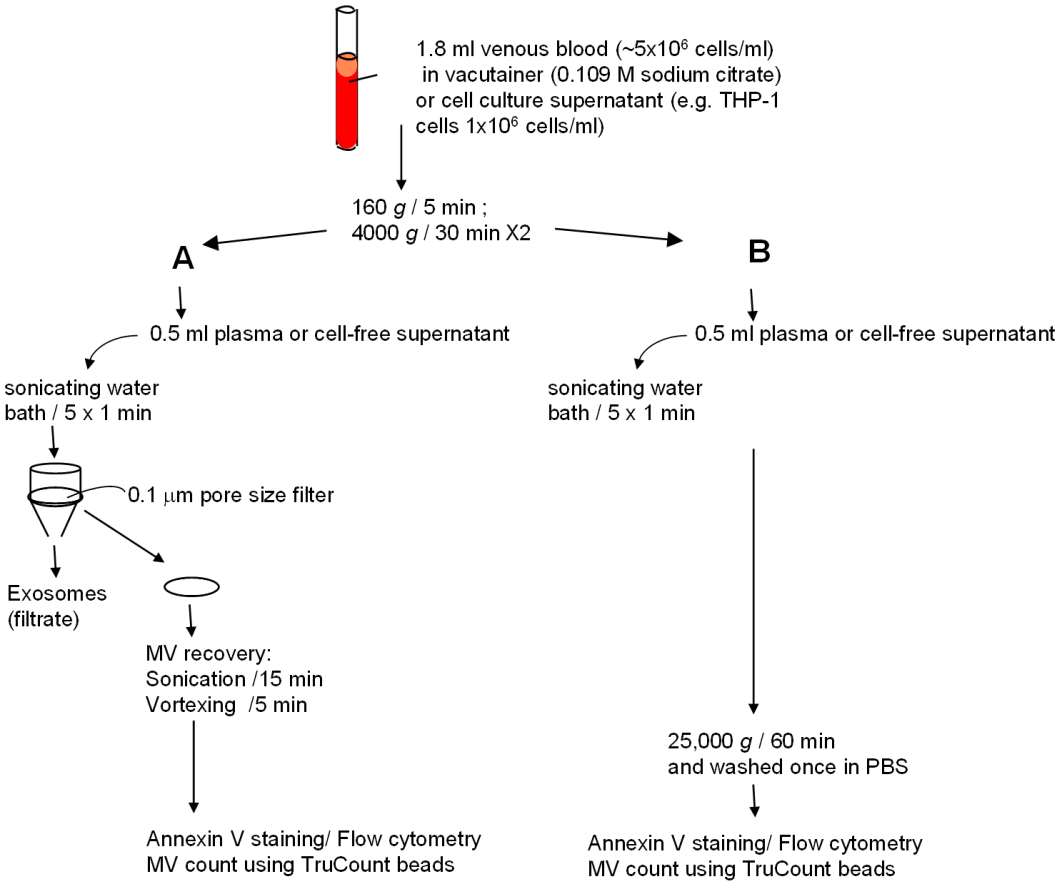


Fig. 4.2 Summary of newly developed filtration-based protocols (A) and of existing, modified differential centrifugation-based methods for MV/exosome isolation. (A) After collection of venous blood in a vacutube, and low speed centrifugation, plasma (0.5 ml) is gently sonicated (5 x 1 min pulses) in a sonicating water bath and then passed through a 0.1 µm pores size millipore filter. MVs are recovered from the filter by sonicating as before, gentle vortexing and finally centrifugation (25,000 *g*/60 min) MVs so obtained were analysed by annexin V binding using a Facs Calibur™flow cytometer and counted using TruCount beads. In (B) a differential centrifugation protocol is outlined that can be used to separate MVs and exosomes. Firstly, samples are centrifuged (160 *g* for 5 min and then twice at 4,000 *g* for 30 min). This removes all cell debris and the supernatant is then gently sonicated in a sonicating water bath (5 pulses for 1 min each) to disrupt exosomes clumps. MVs are collected by centrifugation (25,000g for 1h). They are then washed and identified by AnV labelling as PS-positive using a flow cytometer.

As an added confirmation that the two vesicles subtypes had been isolated, the vesicles obtained by filtration (MVs) and the filtrate (exosomes) were analysed by electron microscopy (Figure 4.3) to ascertain a size range. They were additionally analysed for particular marker proteins by ELISA or flow cytometry. The MVs isolated ranged typically from 200 nm to 1200 nm (Fig. 4.3A) and the exosomes were much less heterogenous in size, most of them consistently measuring 100 nm in diameter (Fig. 4.3B). These readings were obtained for n=500 vesicles. Size is a particularly useful parameter to distinguish the smaller exosomes from MVs. Looking at specific markers it was found that TGF-β was expressed at 5-fold greater levels on MVs than exosomes (Fig. 4.3C) and that PS was expressed at least at 3-fold greater levels also on MVs (Fig. 4D). CD63 which is regarded as a marker for exosomes was expressed at 2-fold greater levels than found on MVs (Fig. 4.3E). The filtration methodology, therefore, seemed to result in a very good separation of MVs and exosomes.

4.4.2 Calibration of flow cytometer using calibration beads and use of Trucount beads

Every day that the flow cytometer (FACSCalibur) was used, and before any new batch of samples was analysed, 20 µM of calcium ionophore was used to stimulate the quality control plasma. Great care was taken not to ‘spike’ all samples in the study to ensure that there would be no problems with inter-individual variations in response to the stimulus. By maximizing the general number of plasma MVs in the QC plasma, this ensured there to be enough MVs to gate the flow cytometer and to be able to generate quantifiable results.

Furthermore, calibration beads (0.2 µm) were run every day before overlaying the donor samples. Both were gated for AnV (G2) and also with no gate. The Trucount bead population was also included. To validate that the AnV-positive MVs samples being observed at different wavelengths (FL1

and FL2) were in fact the same population ($p < 0.001$), Kolmogorov Smirnov statistics was employed and histograms overlaid. As shown in Figure 4.4A, where a representative display of unstimulated blood is shown revealing AnV positive MVs, of diameter $\geq$ 200 nm, the MVs are found in the box with a hashed line. Using the formula described in the material and methods section of this chapter, (considering the Trucount bead count to be 50595.00 [Lot no. 2731]), Fig. 4.4B, the calculation of the absolute MV count revealed a number of 168473.86 /ml.

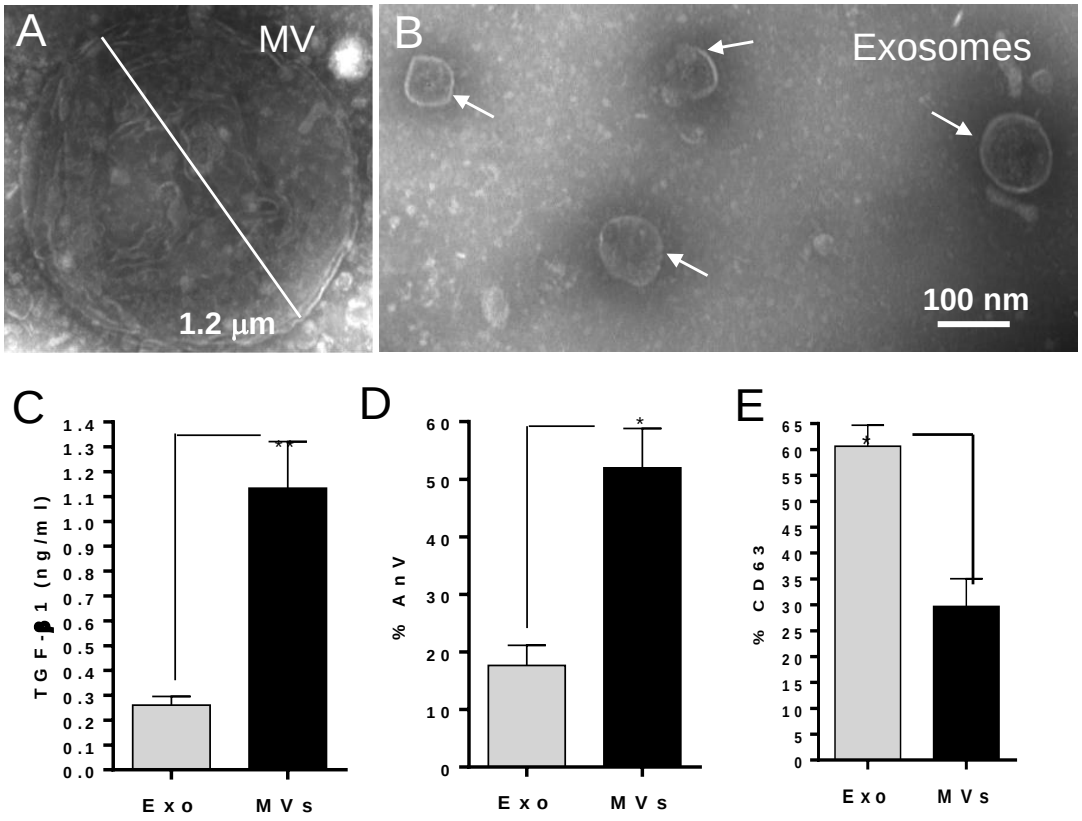
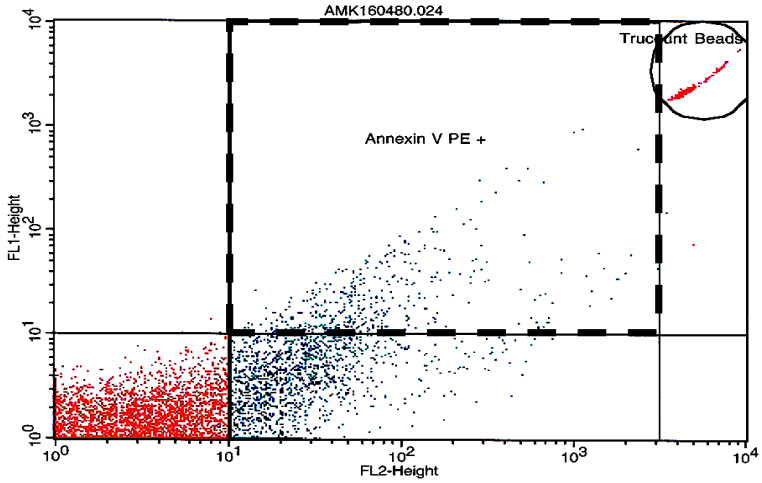


Fig. 4.3 Electron microscopy of MVs and exosomes isolated by filtration to show average diameter in nm and detection of specific markers as a means of delineating the main two different vesicle types (ranging up to 1200 nm). Ms (A) and exosomes (B) were collected from the filter (A) and from the filtrate and prepared by negative staining for electron microscopy. MVs ranged from 100 nm to 1200 nm in diameter, whilst exosomes ranged from 50 to 100 nm. By ELISA (C), TGF-β1 was more highly expressed on MVs (as determined after lysis with 1% (v/v) Triton X-100, and there was a greater exposition of PS, as determined by AnV-FITC binding using the Guava EasyCyte flow cytometer (D). However, as expected there was more of the tetraspanin, CD63, expressed on exosomes than MVs (E).

A



Region	Events	% Gated
>0.2um Calibration	8634	100.00
Annexin V PE +	1913	22.16
Trucount Beads	1149	13.31

B

$$\frac{\text{No. of events in region containing MVs}}{\text{No. of events in absolute count bead region}} \times \frac{\text{No. of beads per test}}{\text{Test volume (mL)}} * = \textbf{MV count}$$

Absolute MV count (before activation) = 1913/1149 x 50595/0.5
= 168474 MVs/ml

Fig. 4.4 Flow cytometric analysis of MVs isolated by filtration using a Facs Calibur™ flow cytometer. MVs isolated from unstimulated peripheral blood using the newly developed filtration method were analysed using a Facs Calibur™ flow cytometer. Trucount beads were used and those MVs with a diameter of greater than 200 nm that had also stained positive for Annexin V-PE, are indicated within the dashed box on the dot plot (A). These were counted and an absolute MV count determined (B) using the formula, those MVs of diameter greater than 0.2 μm and staining positive for annexin V-PE binding, shown in the dashed box on the scatter plot (A) were enumerated and an absolute MV count calculated (B) using the formula indicated in B and in Materials and Methods.

4.4.3 Use of the newly developed MV and exosome isolation protocol to assess plasma EMV levels within the population.

Having developed the filtration method for isolation of MVs and exosomes, this protocol was used to ascertain plasma MV levels firstly to simply establish a normal baseline reference range within a sample of the healthy human population. For this a total of 57 subjects was used, both male and female, and of age ranging from 19 to 71 years.

4.4.3.1 The effect of freezing and thawing on MVs levels measured

Unlike much of the recent literature suggesting MV levels measured to be increased upon freeze/thaw (Piccin et al., 2007) in this study when MVs levels were compared between freshly prepared samples and from those that had been frozen (-20°C), no significant difference was noted (p=0.226), Fig. 4.5A. Of note, however the samples that had been frozen at -20°C in the current study were only thawed once, about 30 minutes prior to analysis. These findings suggest that frozen MV samples, frozen and thawed once will give as reliable results in terms of MV numbers as freshly obtained MV samples. Frozen samples can therefore be used with confidence in any routine laboratory analysis. Therefore, in terms of reproducibility MV counts can be validated even if not freshly prepared.

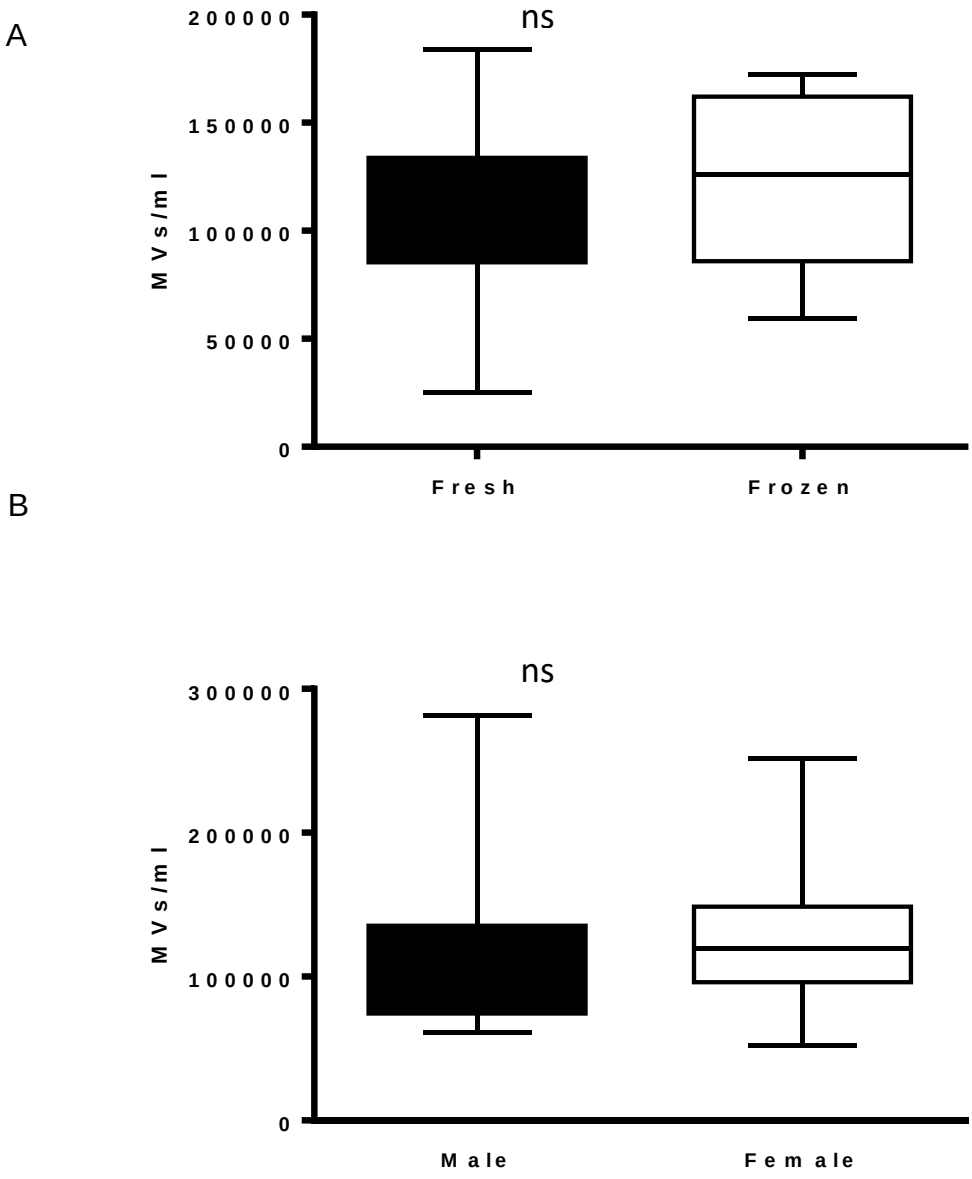


Fig. 4.5 Freezing has no effect on plasma MV levels and there is no different in plasma MV levels between males and females. Plasma MVs were counted as described in materials and methods using the Facs Calibur flow cytometer. Comparing the same plasma MV samples, freeze/thawing had no effect on the measurable MV levels (A) (n=38) and there was a negligible difference between males and females (B) (n=26 male; n=31 female). The results are presented as mean ± Standard Deviation.

4.4.3.2 Gender has no effect on plasma MV levels

To begin to compare mean plasma MV levels between males and females, 26 males and 31 females were compared (Fig. 4.5B). This data enabled a normal baseline reference range to be defined, as shown in the box plot in Fig. 4.5B as 0.55 $x10^5$ – 1.8 x 10^5 MVs/ml. For males the reference range is slightly smaller, 0.65 $x10^5$ – 1.35 x 10^5 MVs/ml, the lowest quartile starting at a slightly higher level with males. It was also noted that females had a higher median (1.25 $x10^5$ MVs/ml) compared to males (0.95 x 10^5 MVs/ml). As a result, it was possible to state that on the basis of gender there was no significant difference in plasma MV levels (p = 0.160).

4.4.3.3 Age has no effect on plasma MV levels measured

We had expected that the older subjects in the sample measured would have higher levels of MVs due to an increased general amount of cell death, apoptosis and associated MV production, but this was not observed (Fig. 4.6). The slightly elevated level of plasma MVs in younger subjects observed may be attributable to the higher cell turnover or metabolism, in younger subjects. By contrast elderly individuals may have reduced cell turnover or waste elimination. In either age group elevated plasma MV levels may then be indicative of disease.

4.4.3.4 Subjects in a fasting state appear to have higher base plasma MV levels than those in a non-fasting state

In this study, the possibility of obtaining subjects that could be compared in terms of plasma MV levels for fasting (which for the purposed of the study was defined as having had nothing to eat or drink, except water, for 12 h) and non-fasting was limited. In fact, only 4 subjects from the whole cohort were analysed both fasting and non-fasting and this was in great part due to the inconvenience logistically to the volunteer donors as well as to have to incorporate various other variables in the study. Within a large clinical trial, however, a much more statistically viable number of donors matching the fasting/non-fasting criteria would be met. Even though the population size was small (n = 4), using an independent sample *t*-test (not having assumed equal variances), a statistical significance was found, Fig. 4.7.

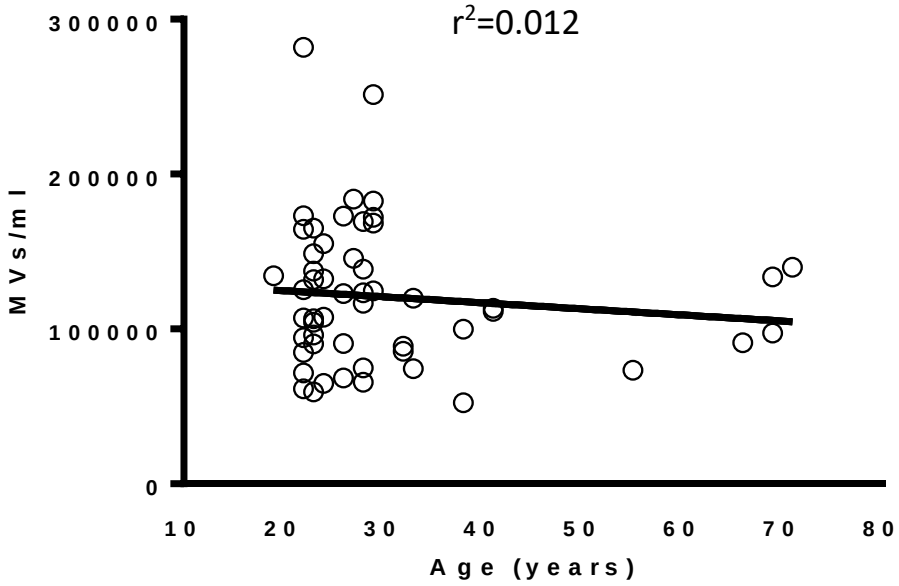


Fig. 4.6. Effect of age on plasma MV levels. MVs isolated from plasma were enumerated as described. Comparing the same plasma MV samples, although most samples were obtained from donors in the 20–30 age range, there was no effect of age on MV levels (n=57).

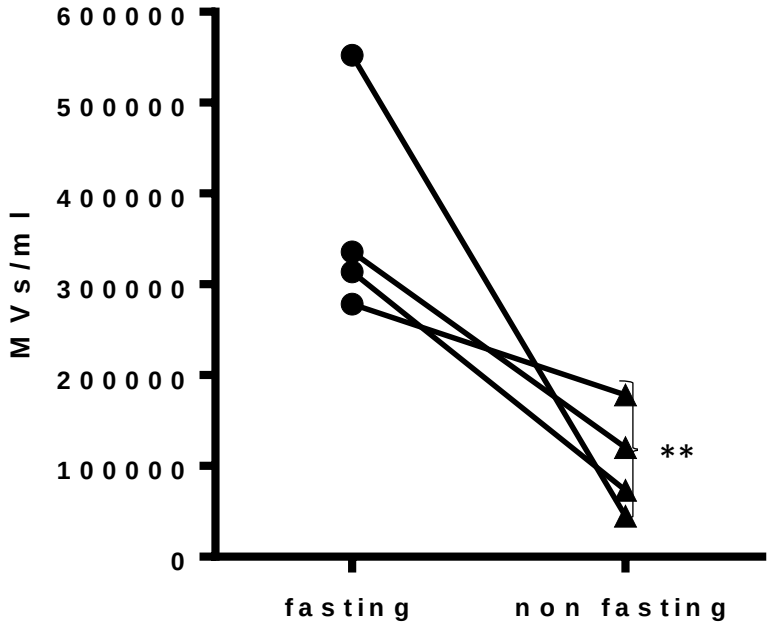


Fig. 4.7. Effect of fasting on plasma MV levels. MVs isolated from plasma were enumerated as described. Comparing the same plasma MV samples, although only four samples could be compared between the same donors after fasting or non-fasting, MVs levels were higher after fasting).

4.4.3.5 Smokers have reduced levels of plasma MVs

As shown in Figure 4.8 plasma MV levels were significantly reduced in smokers compared to non-smokers. Although cigarette smoking is likely to contribute more than one component capable of inhibiting MV release, it was considered that nicotine, as calpeptin, which is capable of inhibiting apoptosis (Dasgupta et al., 2006) could therefore ~~and therefore~~ be at least one of the components of cigarette smoking likely to inhibit microvesiculation, as microvesiculation is believed to be one of the effects of early apoptosis (Inal et al., 2012).

In vitro experiments were therefore performed in which increasing concentrations of nicotine were employed to see if this would result in decreasing levels of early apoptosis in THP-1 leukemic monocytes, as measured by annexin V binding, as well as decreasing levels of MV release; similar results were expected with calpeptin. and indeed, found to be the case (Fig. 4.9). As for calpeptin, nicotine was found to dose-dependently decrease the number of cells in early apoptosis (AnV^+, $7AAD^-$) but not those in late apoptosis (AnV^+, $7AAD^+$) and to also cause a decrease of MV release, as the cells were treated with increasing concentrations of nicotine, up to ~50 µM.

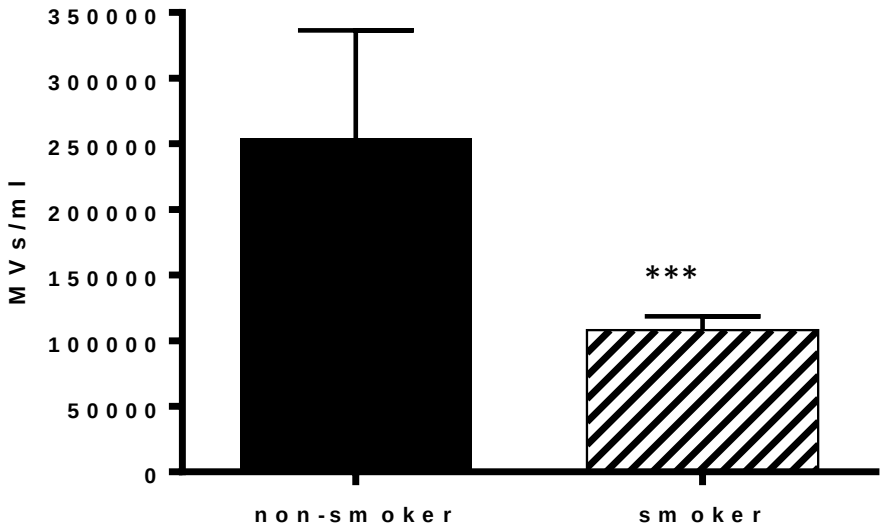


Fig. 4.8. Smokers have significantly reduced levels of plasma MVs compared to non-smokers. MVs isolated from plasma were counted using the FacsCalibur flow cytometer as described. Comparing the same plasma MV samples, smokers had reduced MV levels.

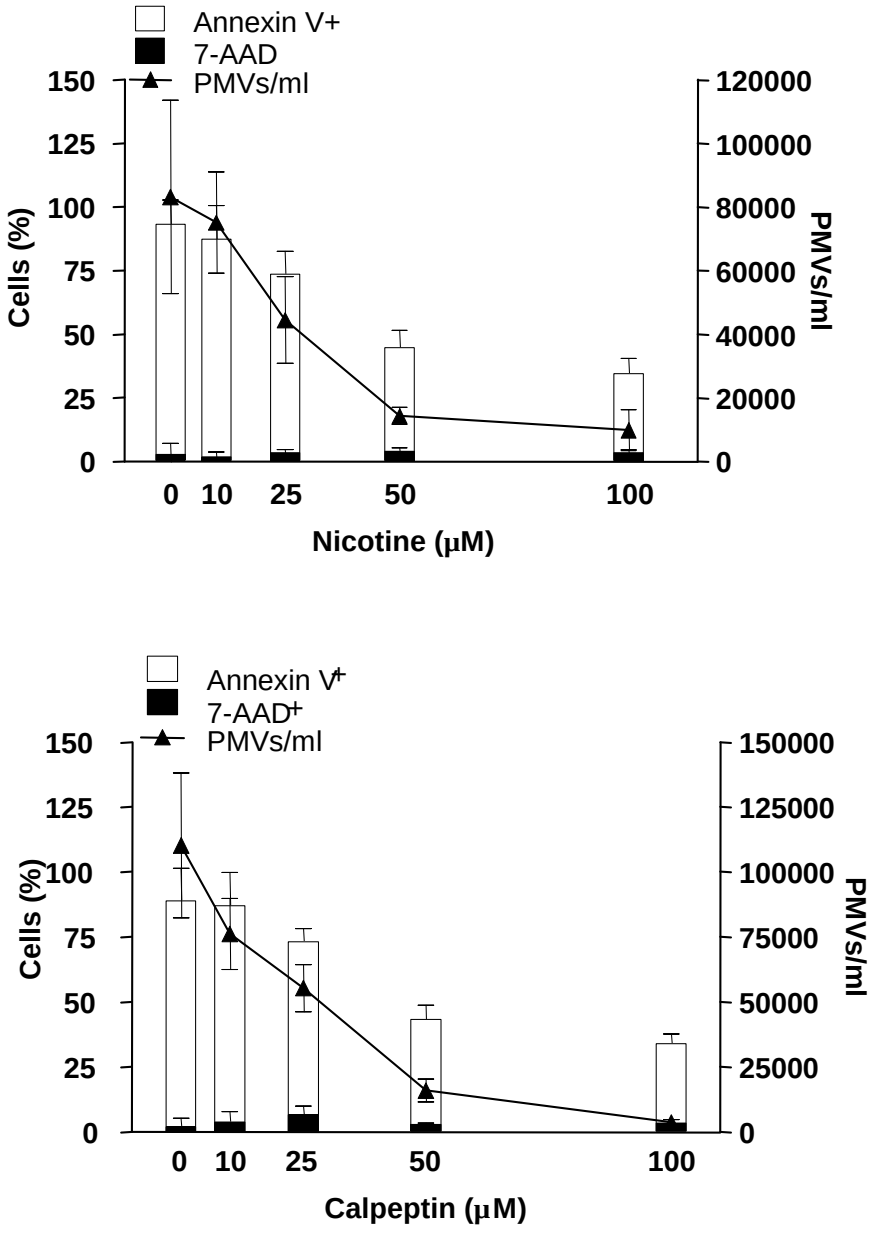


Fig. 4.9. Nicotine and calpeptin both decrease levels of cells in early apoptosis and also decrease microvesiculation levels, measured as number of MVs/ml. MVs isolated from plasma were counted as described. Increasing doses of nicotine (A) or calpeptin (B), reaching saturation at 50 μM, dose-dependently decreased annexin V binding, as a marker of early apoptosis, as well as the number of MVs released. These latter experiments with calpeptin and nicotine were repeated twice in triplicate.

4.5 Discussion

4.5.1 Sample preparation for MV isolation

Pre-analytical variables are of paramount importance with regard the measurement of MVs as has been underlined by researchers in the field (Ahmad et al 2005). In the same vein, the documentation of methodology is also essential before analysis is begun. In preliminary work in this study, plasma MV data was obtained from homogenizing Platelet-Free Plasma (PFP), using the ABX PentrP60 Haematology Analyser, which eliminated erythrocytes, platelets and leukocytes, and other such sources of cell debris, that could interfere with measurements. The result was a comparatively pure plasma sample that could be used in flow cytometry analysis, but that needed further purification by filtration through a 0.1 µm pore size membrane, to remove smaller debris, thus retaining all > 0.1 µm material on the membrane filter. To be sure that the sample was not lost, this was subsequently subjected to gentle sonication in a sonicating water bath and also to gentle vortexing in Dulbecco's PBS (DPBS) prior to staining with Annexin V-PE.

4.5.2 Preparation and analysis of controls for flow cytometric enumeration of microvesicles

MV release was raised by the use of calcium ionophore which stresses cells and increases levels of early apoptosis. In this way MV Control Results were able to provide an optimal way of determining whether the staining procedures were working properly. On every day that samples were run, by omitting the stain in the negative control on every day that samples were run and comparing this to the number of AnV positive events in the negative control (7609) to the positive control (stained) (5161), it was clear that only MVs positively stained for were being counted (in the gate set) indicating the stain was working prior to batch analysis every day.

4.5.3 Flow Cytometry Analysis Templates

The use of flow cytometry templates allows large amounts of data to be overlaid and thus collated for each donor. Calibration bead (0.2 µm) (purple) were run every day before overlaying donor samples. Both calibrations beads and donor samples were gated for AnV (G2) (green) and with no gate (pink). The Trucount bead population is also visible. Flow cytometry analysis templates allow a lot of data to be overlaid and collated per patient result. 0.2 µm calibration beads (purple) were run every day before samples could be overlaid with the patient sample, both gated for AnV(G2) (green) and with no gate (pink). The Trucount bead population is also visible. The KS stats & Overlaid histograms also validate that the AnV^+ MV samples being looked at on the different wavelengths (FL1 & FL2) are the same population (as p = <0.001).

4.5.4 MV Absolute Total Counts

This study has resulted in the development of a novel filtration-based method for the isolation of plasma MVs that are PS-positive (by staining with Annexin-V-PE). By taking measurements from healthy donors (n=57) it was found that plasm MV levels fell in the range 0.51-2.82 x 10^5 MV/ml. If the number of cells in venous blood is considered as ~5 x 10^6 cells/ml, the level of release can be considered as about 1 MV per 32 blood cells. This is comparable but as might be expected lower than the release figures from cells in culture where it was found to be about 1 MV per 8 monocytic leukaemia cells, which as I found and report in chapter 5 release twice as many MVs as their normal peripheral blood monocyte counterparts. As discussed below, the filtration method was then used to see the effect of various parameters and it was found that neither freeze thawing, gender or subject age significantly affected the absolute MV count. Fasting gave a wider spread and higher levels however (0.9-1.5 x 10^5 MVs/ml), in the order

of 3-fold higher; this could be considered to be an estimates normal baseline fasting reference range.

4.5.5 Smoking Status affects plasma MV levels

The number of subjects that could be obtained for comparing the effect of smoking versus non-smoking on plasma MV levels was limited. This was mainly due to the inability to recruit more smokers, hence a randomised sample of four non-fasting, non-smoking subjects was compared against four non-fasting smokers. It was found that the non-smoker group had a wider spread of MVs (especially the 2nd and 3rd quartiles), with a slightly increased median in comparison to the smoking group (>10,000). However, due to the small population size no inference could be made on statistical significance ($p = 0.326$ (>0.05 Paired t-Test). Experiments were also carried out on THP-1 monocytic leukaemia cells and in the same way that calpeptin, a known inhibitor of apoptosis, resulted in decreasing levels of early apoptosis (AnV^+ cells) and decreasing levels of MVs released, nicotine (and it should be noted that THP-1 cells are indeed positive for nicotine receptor [Morgan et al., 2001]) had the same effect.

4.5.6 Freezing does not affect plasma MV levels

MVs in the plasma samples that were not frozen were compared to those that had been frozen and then thawed (-20ºC group) (n=36). As shown in the Boxplots very similar total counts of MVs, with a Median of 119744.8 and 134937.4, for the Freshly Prepared and Frozen Groups, respectively was found, there being no significant differences.

4.5.7 Fasting increases plasma MV levels

The Boxplot figures shows that the Fasting group had a wider spread (especially the 3rd and 4th quartiles), ($2.8 \times 10^5 – 5.8 \times 10^5$) MVs/mL This

can be defined as the normal baseline Fasting reference range, in comparison to the Non- Fasting group reference range (0.98 x 10^5 – 1.5 x 10^5) MVs/mL.

The median was also greatly increased (3.8 x 10^5) MVs/mL, which is almost 3-fold in comparison to the non-fasting group (1.1 x 10^5) MVs/mL. Although the population size of this group is small (n=4) there is a statistical significance as an independent sample t-test (*p* value = 0.005 (equal variances not assumed) <0.05. However, this was not observed as a paired sample t-Test (*p* value= 0.117 >0.05 as the sample size was too small.

The transmission of the stain fluorescence to the flow cytometer may also be a factor.

There are various reasons why the absolute MV concentrations appear to be raised in subjects that had fasted. This may be due to other compounds found in the plasma in the non-fasting state. For example, there may have been lipaemic samples (a factor that often interferes with many routine laboratory tests). Whether samples were lipaemic was actually not noted, but importantly is something that should be considered in future studies. Furthermore, any food or other related contaminants could easily increase turbidity, thereby inadvertently masking or reducing MVs or even interfering with AnV labelling of the MVs, because of increases of the viscosity of plasma. A recent study enumerating plasma-derived microvesicles and exosomes also showed an increase postprandially in the MV gate delineated using Megamix™ beads (160 nm-500 nm in diameter) (Sodar et al., 2016). This showed an increase in the number of MV-sized vesicles1.5h, 3h and 6h after a meal. It should be noted that the fasting samples measured in this thesis were overnight but they also showed elevated levels of plasma MVs compared to non-fasting. The work by Sodar et al., showed interestingly that some of these increased MV levels up to 4 h postprandially compared to up to 30 min postprandially could be accounted for by cyclomicrons which are lipoprotein particles made up mainly of triglycerides but also phospholipids, cholesterol, and proteins and are responsible for transporting dietary lipids.

4.5.8 Age does not affect plasma MV levels

Scattergraph of Age vs Non-Fasting MVs is fairly randomly scattered, suggesting no correlation. ANOVA Statistics for The Correlation Coefficient was very low (p = 0.755>0.05 (at 95% Confidence interval) and hence is unlikely to be correlated. Age is hence not significant enough to be an explanatory variable.

If any trend there is a slightly negative correlation, such that as Age increases MV levels decrease. Regression Analysis Model Summary shows the R2 value of 0.005 x 100= 0.5% of MV total count variation is explained in this model using age as the independent variable. It is expected that the older subjects of this population would have higher levels of MVs due to an increased general amount of cell death, hence apoptosis and MV production. A healthy life style may result in less disease whereas by contrast elderly individuals may have reduced cell turnover waste elimination etc, and these same elevated levels may cause concern.

4.6 Conclusion

The data presented in this chapter summarises the development of a novel filtration method for the isolation of microvesicles (and potentially of exosomes). An important advantage over differential centrifugation is that larger volumes can be processed and that therefore this method would serve well in the clinic and in a research, lab setting where cell culture supernatants could be easily processed. The flow cytometer chosen for this study and for which instrument settings and methodology were validated was the BD Facs Calibur™. This instrument and the methodology developed would allow for use of this technology as a high throughput test. It has not been possible as yet to compare the Facs Calibur with the other main flow cytometer type that relies on a different technology whereby fluid is sucked up (e.g. Guava EasyCyte 8HT and 12HT) which would be less likely to have a clinical setting, being more likely to be used in research laboratories. It would be important to be able to compare samples on these two flow cytometers. Furthermore, since the MV measurements were made on circulating plasma MVs, it would be interesting to also make measurements on MVs isolated from lymph. The differentiation of different plasma MVs from their cell of origin by using specific markers is also an ongoing improvement being investigated at the Cellular and Molecular Immunology Research Centre.

When plasma MV levels were measured in a small cohort of normal donors (n=57) it was found that were levels were compared to age of donors, Regression Analysis gave an R2 value of 0.005, in other words that only 0.5% of variation in total MV count could be explained in this model using age as the independent variable. Freezing plasma MV samples seemed to have no effect on the number of MVs that can be measured. This of course has no bearing on the functional capacity of frozen versus non-frozen MV samples, but is important to know where enumeration of MVs is needed. Gender also had no bearing on measurable plasma MV levels.

The finding within this study that plasma MV levels appear increased in the fasting state suggest that fasting status should be considered when measuring plasma MV levels and that this should be a new standard adopted

in MV enumeration, in the same way that other fasting analytes, such as glucose, are measured in routine laboratories.

4.6.1 Fasting and Intermittent Fasting

The lysosome is an organelle that contains enzymes that digest proteins, lipids and carbohydrates. Lysosomes are sometimes found with whole organelles or other cellular components within them and the way these components are delivered to them is via autophagosomes, organelles that themselves form around damaged proteins and damaged organelles. As a result of autophagy (Mizushima et al, 1998), cells can quickly obtain energy and acquire building blocks for cellular renewal. Autophagy is thus essential as a response to cellular starvation and stress. In terms of removal of damaged and therefore potentially harmful proteins but also other macromolecules such as RNAs, in an attempt to maintain a healthy cell, it is now being shown that, in addition to autophagy, exosomes may be involved in the removal of these damaging molecules (Baixauli et al, 2014). This work describes how the role of exosomal removal of intracellular stress, by removal of damaged and harmful proteins in exosomes, may co-ordinate with the autophagy/lysosomal pathway to preserve the homeostasis of cellular proteins.

A fast is a period (whether intermittent or extended) without eating or drinking and may be performed for a range of reasons, including medical, spiritual or for weight loss. Certain fasts may allow the taking of fluids such as water, tea or coffee but some fasts have no fluids. Fasting is a world-wide practice and is found in most religions, including Hinduism, Christianity and Islam. For the prevention and as part of treating of certain chronic diseases, clinical trials would still need to be carried out to see the health benefits. From current observations it is already possible to see that fasting in medically supervised cases (up to 500 kcal intake per day) for periods of 1 to 3 weeks, besides giving a rest to the digestive system is useful in the treatment of chronic pain, metabolic syndrome, hypertension and in rheumatic disease.

5. Promonocytic cell-derived MVs and exosomes: analysis of fusion with target cells and potential use as therapeutic vehicles in Chronic Myeloid Leukaemia

5.1 Introduction

5.1.1 Chronic Myeloid Leukaemia (CML)

Every year about six hundred people are diagnosed with this particular form of leukaemia. CML, a myeloproliferative condition which is also known as Chronic Granulocytic Leukaemia results from a change at the genetic level in a pluripotent stem cell. This change is in fact a translocation of the *ABL* gene from chromosome 9 to 22 containing the *BCR* gene (Gupta, R., 2003; Hehlmann et al., 2007). Figure 5.1 represents the described translocation and shows how the chimeric gene *BCR-ABL* is formed on the Philadelphia chromosome (Ph), essentially by fusion of the chromosome 9-derived *ABL* oncogene with the region in chromosome 22, t (9,22) q (34, q11) (Mauro and Druker, 2001). As the BCR-ABL tyrosine kinase becomes overexpressed this leads to the chronic phase of the disease, in just under 70% of patients, where up to 30% of blast cells are located to the bone marrow or blood. In this phase blood counts (which may be part diagnostic, over 1 x 10^{10}/litre) are very high, with an abnormally high level of myeloid cells at various differentiation stages, including neutrophils and may lead to gout. With the uncontrolled proliferation and effective evasion of the immune system, the disease moves to the blast-crisis phase (Howard, M., 2008) where over one third of the blasts are in the blood/bone marrow and which is accompanied by other features such as thrombocytosis, and splenomegaly. CML is more common in older people (Bain, B., 2004). As a result of immune effector mechanisms, CML often goes undiagnosed for up to five years before a diagnosis is made and treatment commenced.

The standard treatment for CML today includes chemotherapy using spryceli, imatinib and tasigna (Pray, L., 2008) or radiotherapy and for high-risk patients, stem cell transplants may be used (Howard, M., 2008)

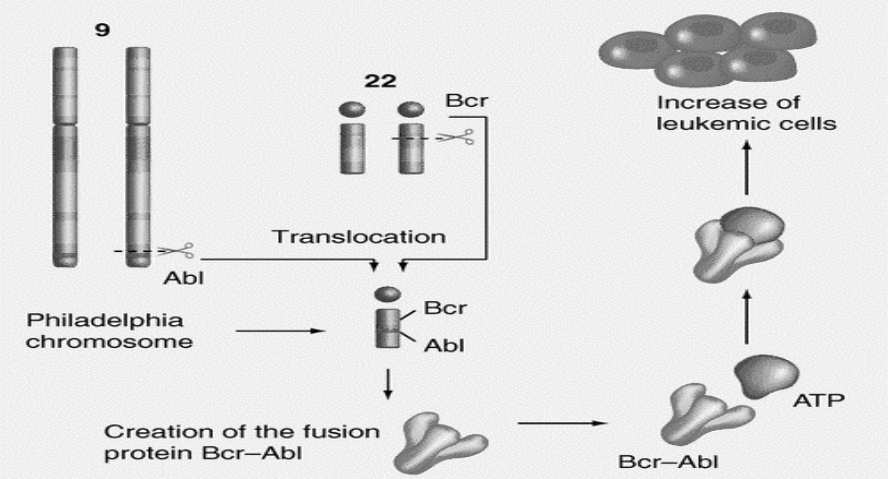


Fig. 5.1 Translocation of the Philadelphia chromosome through *BCR-ABL* fusion produces an increase in leukemic cells (Tanaka, R et al., 2008)

An understanding of the pathology of the disease at the molecular level has brought about a number of therapies, including ones based on interferon, or tyrosine kinase inhibitors of BCR-ABL which limit the proliferation of myeloid cell through the induction of apoptosis. Another important therapy is that of allogeneic stem cell transplantation. Unfortunately, the blast phase of the disease is untreatable at the moment (Goldman, 2009) which is why it is imperative that novel drugs are developed capable of very specifically limiting the proliferation of blast cells.

5.1.2 Erythroleukaemia cell line, K562

The K562 erythroleukaemia cell line was obtained from a chronic myeloid leukaemia patient (CML patient) in blast stage crisis (Lozzio and Lozzio, 1975). Morphologically these cells are essentially undifferentiated blast cells, positive for the Philadelphia chromosome and carry mutated *p53* and which are strongly positive for acid phosphate (Koeffler, H. and Golde, D., 1980); K562 cells have a doubling time of 12 h. The cytoplasm is basophilic and they have granulocyte-specific enzymes in the cytoplasm (Gewirtz et al., 1982) although they do not stain with Sudan black unlike the staining pattern seen for granulocytes. K562 cells do not have lymphocytic markers and as

they are derived from precursor cells or erythrocytes, unlike their more mature counterparts, lack the Ia antigen (Koeffler, H and Golde, D, 1980).

Furthermore, K562 cells lack Igs, and receptors for EBV and Herpes-like virus as well as MHC I receptors, meaning they are unable to inhibit NK cell activities. K562 cells do, however, strongly express Fc receptors but are not phagocytic.

K562 cells can be induced to express erythroid cell markers such as sialoglycoprotein or glycophorin (Andersson,1979). Furthermore, these cells can be differentiated along the megakaryocyte lineage or to progenitors of granulocytes, monocytes/macrophages or erythrocytes (Gewir, A. et al., 1982).

5.1.3 β-Glycyrrhetinic acid

β-Gly the structure of which is shown in Fig. 5.2 has been shown to inhibit 11-β-hydroxysteroid dehydrogenase (Edwards and Stewart, 1991), an enzyme found in the kidneys, inhibition of which results in the accumulation of hydroxycortisone.

In other interesting work β-Gly was found to have anti-inflammatory properties by inhibiting the complement-mediated inflammation. β-Gly has a direct anti-inflammatory effect by selectively inhibiting a complement cascade. β-Gly was specifically shown to inhibit the classical pathway of complement activation but not the alternative and it was found that this inhibition happened, dose-dependently, at the level of complement C2, allegedly by binding to C2 and in so inhibiting its subsequent cleavage by the serine protease complement C1 (Kroes, B. et al., 1997).

Working with a different myeloid cell line, HL-60, it was shown that β-Glycyrrhetinic inhibits proliferation of these leukemic cells (Liu, D. et al., 2007). On the breast cancer cell line, MCF-7 β-Glycyrrhetinic in combination with glycyrrhizin, the combination therapy was found to be toxic and to induce apoptosis as shown by TUNEL staining (Sharma, J. and Ward, P.,

2012). On this basis I wanted to see the efficacy of β-Glycyrrhetinic on K562 cells.

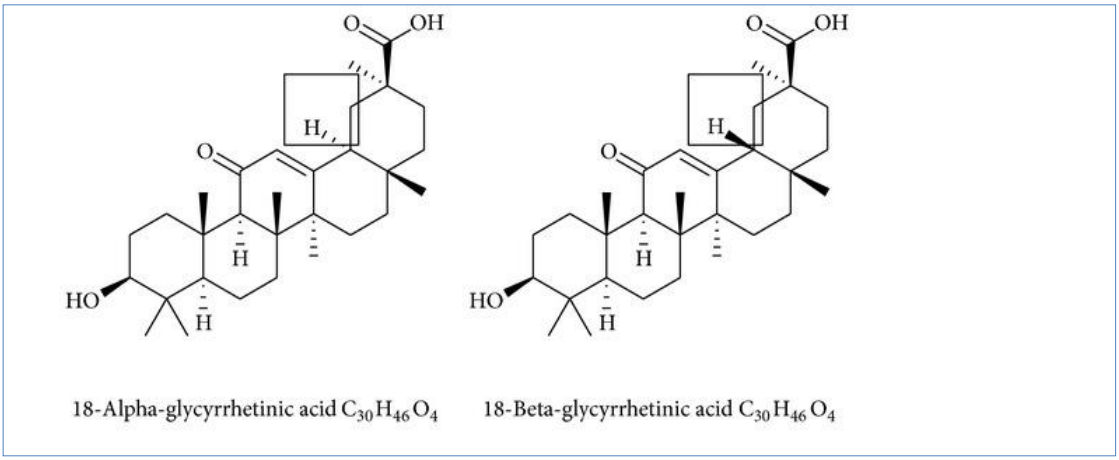


Fig. 5.2 **18α-Glycyrrhetinic acid and 18β-Gly molecular structures. 18α is a much rarer optical isomer than the 18β- form (Kang et al., 2014).**

In the last ten years β-Gly together with the derivative drugs developed has been investigated for its anti-cancer properties. Most significantly a synthetic analogue of β-Gly called CDODA-me has been found to inhibit the proliferation of prostate, colon and pancreatic tumour cells without affecting surrounding healthy cells (Chintharlapalli et al, 2007). As most tumour cells are able to resist pathways leading to apoptosis, the vast majority of cancer drugs are designed to interfere with this pathway β-Glycyrrhetinic and its derivatives were found to render the human leukemic cell line, HL60 apoptotic by acting on the role mitochondria play in apoptosis (Liu et al,2007**)** but the action on different tumor cells may involve other mechanisms that limit proliferation. β-Gly has also been shown to inhibit Gap junctions and hemi-channels in a range of cancers.

5.1.4 Aims

In this chapter I have begun to address the pathways of biogenesis of MVs by looking at potential pharmacological inhibitors of MV release. It is known that MVs contribute to tumorigenesis and tumour growth and that they are involved in promoting angiogenesis. Furthermore, MVs released from cancer cells may act as decoys and interfere with antibody-based immunotherapies. As was shown by the Inal group, inhibition of MV release from prostate cancer cells rendered the cells sensitive to chemotherapeutic drugs, both *in vitro and in vivo*, such that 100-fold lower concentrations of docetaxel could be used with the same efficacy (Jorfi *et al*., 2015). The reason for this was that as shown by Inal and others, tumour cells expel cancer drugs through the release of MVs. Therefore, an understanding of the release of MVs from cells has a very important potential application in the development of cancer therapeutics.

In this chapter I have also endeavoured to test the potential of a drug derived from liquorice, β-Gly, firstly on its own against K562, an erythroleukaemia cell line. In addition, I aimed to investigate firstly whether monocyte-derived MVs could bind K562 cells, by using a lipid mixing or octadecylrhodamine dequenching assay, and if so, whether monocyte-derived MVs harbouring β-Gly could limit proliferation and/or induce apoptosis of K562 cells, in other words to see if they could be used as drug delivery vehicles. As another strategy, using one of the drugs shown to limit MV release, it was to be used in conjunction with an anti-cancer drug, such as β-Gly, to see, following on from previous work (Jorfi et al., 2015), whether this would enhance the efficacy of the cancer drug by inhibiting its efflux from the cancer cells.

5.2. Materials and Methods

5.2.1 Cell culture

Cryogenic vials containing K562 cells were removed from liquid nitrogen and placed into a water bath at 37°C for about 5 minutes, to let the cells thaw. Each vial was then disinfected with 95% ethanol (IMS 95) and then transferred aseptically into a centrifuge tube with 10 ml of RPMI 1640 medium, centrifuged at 160 *g* for 5 min. The pellet was gently resuspended in complete growth medium (CGM) and placed at 37°C in a humidified incubator (5% CO_2). CGM is used for maintaining the cells and comprises 500 ml RPMI 1640, 50 ml 10 % Foetal bovine serum (FBS), and 5 ml of 100 x Penicillin / Streptomycin to prevent bacterial growth. Cells were then maintained and regularly cultured to carry out different experiments.

To minimise the chance of contamination, all cell culture procedures were carried out under aseptic conditions. Biosafety cabinets represent the simplest and cheapest way to provide aseptic conditions and were used in this experiment.

Gloves were worn at all time and IMS was used to wipe hands and the outside surfaces of containers, flasks, pipette tip box, pipettes, and centrifuge tubes before being placed inside the cabinet. Sterile 10 ml graduated wide tips (individually wrapped), with an appropriate pipette pump, were used to pour media and cells directly into or from the flask, using tips only once to avoid cross contamination.

Cells were sub-cultured every 2-3 days to prevent confluency of the cells in the culture flask (high confluency) and ensure optimal density for continued growth and proliferation. Following seeding and lag phase, cells will proceed to the log phase (exponential growth) until the till their number exceeds the capacity of the medium to sustain further growth, and cell proliferation decreases or stops altogether.

After transferring the cell suspension (in the culture flask) directly into a sterile 50 ml centrifuge tube under aseptic conditions, centrifugation was

carried out at 160 *g* for 5 minutes. The supernatant containing dead cells debris was removed with the use of a 10 ml sterile tip taking care not to disturb the pellet containing the live cells. 5 ml of RPMI 1640 (used here to wash the cells) was then added, followed by centrifugation at 160 *g* for 5 minutes. The pellet was resuspended in 5 ml of RPMI 1640 and centrifugation repeated once more resuspending in an equal volume to then make additional dilutions. A 1:20 dilution was then carried out (1 ml of cells in RPMI 1640 mixed with 19 ml of CGM) in a 75 cm^2 culture flask, aiming to produce a seeding density of approximately $1x10^6$ cells/ml. As already mentioned a higher density could affect the capacity of the medium to sustain further growth. However, a density that is too low could prevent the cells to produce communication signals and growth factors, hence affecting their growth and proliferation. The flask was subsequently incubated at 37°C, 5% CO_2.

Cells were viewed under an inverted microscope at 24-hour intervals to check on their health status and investigate any morphological changes taking place during growth and differentiation.

5.2.2 Cell count and study of normal growth pattern of K562 cell line

Cells were maintained and sub-cultured as necessary and on the first day of the experiment cells were washed with RPMI 1640 (see washing procedure above), and the pellet resuspended in 5 ml of RPMI 1640. 10 µl from this was transferred into an Eppendorf tube, together with 10 µl of 0.5% trypan blue, mixed thoroughly via pipetting the entire volume up and down 6-10 times, and the count then performed soon after by using a haemocytometer or the flow cytometer (see below).

The haemocytometer had been previously prepared by cleaning the chamber and the cover slip with bio guard, and then cover slip dried and fixed in position. 10 µl of the homogenous suspension was applied to the

edge of the cover slip and allowed to be sucked up by the capillary action taking care not to overfill the chamber. The chamber was placed in the inverted microscope under a x10 objective, focusing on the grid lines, particularly on one set (of four) of sixteen corner squares, counting the cells in every square including those located on the boundary lines on the right or bottom. An estimate of the number of cells per ml was calculated and subsequent dilution with Complete Growth Medium (CGM) was performed under aseptic conditions to achieve a seeding density of $5x10^4$ cells/well.

400 µl was then pipetted into each of three wells (three replicates) in the centre of a 24-well microtitre plate. A fresh pipette tip was used each time and the plate only uncovered immediately before inserting the tip into the well to avoid contamination.

Also counts performed with the haemocytometer give the number of cells per ml. However, the cells had to be seeded at a density of $5x10^4$ cells/well where the final volume in each well is 400 µl. The plate was subsequently incubated at 37°C, 5% CO_2. The count of viable cells was then evaluated over a period of four days.

On each day a 10 µl aliquot from each well was transferred by using a 2-20 µl pipette (mixing of cells in the well was performed with the tip of the pipette prior to the transferring process), under aseptic conditions, in three separate clean eppendorf tubes (using tips only once). The tubes were then labelled (replicate 1, 2, and 3) and 10 µl of trypan blue was added prior to counting. As above for every replicate individual counts for each of the four sets of sixteen corner squares in the chamber were performed and averaged. After completion of a successful count the 96-well microtitre plate was then replaced in the incubator.

5.2.3 Cell count and study of growth pattern of K562 cell line in the presence of β-Gly

The K562 erythroleukaemia cell line was seeded into a 24-well plate along with different reagents (histamine, imidazole, and 18β-Gly) at a density of $2x10^4$ cells per well in triplicate. To observe the dose response of the chemicals on the cells, the reagents were added at a range of concentrations (1 μM, 5 μM, and 10 μM,). A set of control samples (where no reagent was added) prepared in triplicate was also set up to compare the normal growth pattern of the cells with the growth pattern of the cells seeded along with the reagents, allowing observation and analysis of any variation taking place.

Counts were performed using a haemocytometer as above but over a 3-day period (see results/discussion).

5.2.4 Trypan blue (cell viability) assay

Trypan blue at 0.5 % w/v was used to calculate the percentage viability of K562 cells, cells that were alive being distinguished from those that were dead by observing whether they had absorbed the blue stain (dead) or not (alive). It was important to not overstain (leaving the cells in trypan blue for more than 3 minutes because after this the dye becomes cytotoxic and can affect the results. For all experiments, cell viability at the start of an experiment was >95%. Having counted the numbers of blue and white cells, the percentage viability was calculated using the following formula.

% of viability = (No. of viable cells / total No.of cells) x 100

5.2.5 Cell viability using a Guava EasyCyte flow cytometer

The ViaCount Assay from Guava Millipore represents a quicker and more accurate assay for viability that the haemocytometer method described above. This method was carried out exactly as described by the manufacturers. Essentially 175 μl of well-mixed suspension of K562 cells was taken from the 24-well plate into an Eppendorf tube where 25 μl of Guava ViaCount Reagent was added before analysis on the Guava EasyCyte.

5.2.6 Microvesicle Isolation and Quantification

The K562 cells were seeded in 12 well plate $6x10^4$ cell per well and the total volume of 1ml per well. Three different concentrations of β-GA were used, 40µmol, 50 µmol, 60 µmol and volume used were 4 µl, 5 µl,6 µl respectively. Control samples and test samples made in triplicate and the experiment was followed twice. The culture plate was incubated for 24 h at 37 ° C/ 5% CO_2.

After 24 hours, cell morphology was observed using the inverted microscope. Cell culture in each well was suspended properly using a sterilized pipette tip and transferred to 15ml centrifuge tubes labelled appropriately. The 4 centrifuge tubes (control sample, test samples 1 µM, 10 µM and 50 µM β-Gly) were first centrifuged at 4,000 *g* for 5 min to remove the cells. The supernatant was then centrifuged at 4,000 *g* for 1 h to remove cell debris. The supernatant was then transferred to labelled Eppendorf tubes and centrifuged at 15,000 g for 99 minutes (Micro centrifuge 5417R). Finally, the supernatant was carefully removed without disturbing the microvesicle-containing pallet and the MVs were suspended in 100 µl of 0.22 µm pore size filtered PBS.

A 10µl of microvesicles suspension was diluted in 190µl of PBS and placed into 96 well plate in triplicate. A calibration sample was prepared by suspending 5µl of the control sample with 195µl filtered PBS. Then the microvesicles were quantified on Guava EasyCyte flow cytometer using Easy Check software. Bar graphs for test samples were generated against control sample.

5.2.7 Lipid mixing or octadecylrhodamine dequenching fusion assay to assess membrane (microvesicle/recipient cell) fusion

A fluorescence increase was used to monitor the fusion of THP-1 cell derived MVs to recipient (K562) cells. Essentially such fusion activity would be due to the fusion activity involving octadecyl rhodamine B chloride (R18) with which the MVs were labelled and the unlabelled cells. The protocol was a modified version of one used in this lab before (Ansa-Addo et al., 2010). MVs (30 µg protein/ml) were labelled in 1 mM R18 (30 min/room temperature) in

10 mM MES buffer (10 mM MES (Sigma); 5 mM KCl; 145 mM NaCl). Unincorporated R18 was removed by passing the labelled MVs through an Exosome Spin Column, with a molecular cut off of 3000. To estimate the fluorescence of 15 µg R18 labelled MVs, the fluorescence after the unincorporated probe was removed was compared to that before removal by using a spectrofluorometer (560 nm excitation/ 590 nm emission). After equilibrating for 20 min, 1 x 10^6 K562 cells were added to the R18-labelled THP-1 MVs and the experiment continued for a further half an hour before adding 0.3 % (v/v) Triton X-100 as well as 50 mM octylglucoside, a non-ionic surfactant to obtain maximum probe dilution. The percentage Fluorescence Dequenching, FD, was determined as a percentage using the formula [Ansa-Addo et al., 2010]

%FD=((F-Fi)/Fmax-Fi)x100, as before (Ansa-Addo et al., 2010) where Fi is initial fluorescence and Fmax the fluorescence after lysis with detergent.

5.2.8 Assay for haemoglobin

10 x 10^6 cells were washed with ice-cold PBS and lysed in 0.25 % Triton X-100 in PBS for 15 min. After pelleting the lysates (600 *g*; 20 min) the supernatant was decanted and 10 µl from this added to 2 ml of 5 mg/ml tetramethylbenzidine (TMB) in glacial acetic acid and a further 2 ml of 30 % hydrogen peroxide for 15 min. To find the level of haemoglobin (in pg/ml), the absorbance of the solution was measured at 600 nm and the reading interpolated on a standard curve and shown in bar charts as fold increase compared to haemoglobin levels in untreated control K562 cells.

As an alternative TMB staining (microscopic) protocol, TMB solution, freshly prepared as described above was used with 1 X 10^6 cells (in saline) at a ratio of cells to TMB solution of 1:2 for 30 min in the dark and stopped by adding 0.2 ml of saline. Five hundred cells were counted (at the 48 h time point of the experiment) and the number of TMB-stained cells indicating the presence of heme containing globin tetrameric forms, noted. Hemin at a

concentration of 20 μM was used as a positive control for induction of erythroid differentiation of K562 cells.

5.2.9 Statistical Analysis

Statistical analyses carried out included unpaired *t* test and one-way analysis of variance (ANOVA). This was carried out using GraphPad Prism software, version 6 (GraphPad Software, SanDiego, CA). Differences giving *P* values <0.05 were considered statistically significant.

5.3 Results

5.3.1 MVs can be released from K562 cells by sublytic complement and inhibited by interfering with lipid rafts, and by using inhibitors of calpain and Rho-associated coiled-coil containing protein kinase (ROCK).

The pathways leading to MV biogenesis have not yet been fully elucidated but certain factors stimulating MV release have been known for some time. In most cases MV release is accompanied by a sharp (approximately 5-fold) rise in intracellular calcium concentrations, $[Ca^{2+}]_i$ from an average in a resting cell of 100 nM to 400-500 nM upon stimulation. The rise in $[Ca^{2+}]_i$ in cells may arise from intracellular stores or from the extracellular environment. The way intracellular calcium levels may rise from an extracellular influx usually involves stimulation of a calcium channel or by means of cell surface pores such as calcium ionophore or as created by sublytic complement activation upon a cell resulting in a membrane attack complex (MAC). In the presence of an extracellular source of calcium, given that the intracellular environment is maintained at a low concentration, calcium will flood the cytosol. This in turn sets off the process of microvesiculation. As a result, the enzymes that maintain the asymmetry of the lipid bilayer are no longer able to do so, resulting in the exposition of PS as well as the activation of calpain which cleaves the actin cytoskeleton resulting in a small blebbing of the plasma membrane.

When THP-1 promonocytic cells were stimulated with a sublytic concentration of complement (5 % normal human serum) this resulted in a significant rise of released MVs (an over 1000-fold increase) (Fig. 5.3). That this is due to sublytic complement was shown previously by using

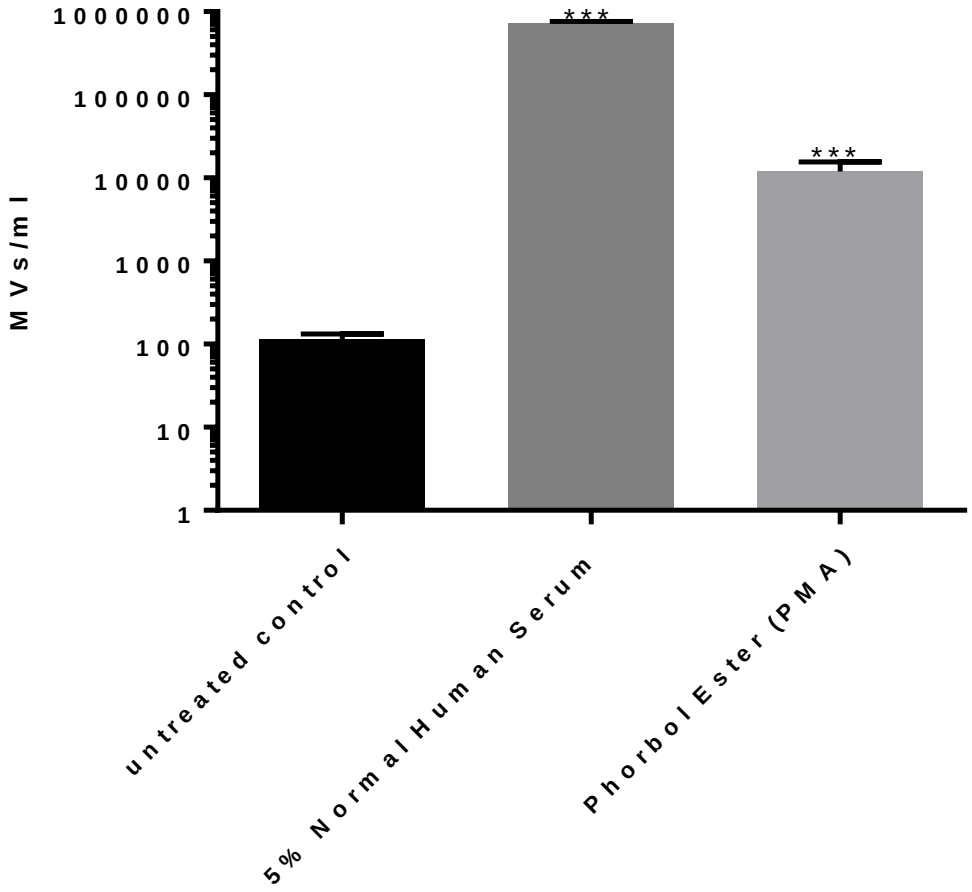


Fig. 5.3 Promonocytic Leukaemia cells release MVs upon stimulation with sublytic complement and a phorbol ester. THP-1 promonocytes were treated with 5% NHS (normal human serum) as a source of sublytic complement to release MVs as measured on the Guava EasyCyte. PMA (1 nM) which also stimulates macrophage terminal differentiation also stimulated a release of MVs from THP-1 cells but at a five-fold lower level.

heat-inactivated NHS as a source of complement proteins (Ansa-Addo, E. et al., 2010, Stratton, D., et al., 2014) as well as by removal of one of the

terminal complement proteins, C9 (Ansa-Addo et al., 2010 and Antwi-Baffour et al., 2010). These MVs showed the characteristic, tapering forward/side scatter dot blot (Figure 5.4). Using NHS (complement) as a positive control for stimulation MV release the effect of the protein kinase C activator, PMA (phorbol 12-myristate 13-acetate) was tested and found to increase MV release 100-fold compared to untreated control.

It was next decided to test a range of likely (unknown) or previously implied inhibitors of MV release. To begin with, EGTA was tested as a chelator of extracellular calcium. The Rho-associated coiled-coil containing protein kinase (ROCK) was also used, Y27632 as well as the calpain inhibitor, calpeptin. In addition, methyl β-cyclodextrin was also tested as a depletor of cholesterol as well as chlorpromazine, a known inhibitor of clathrin-mediated endocytosis (Fig. 5.5).

All reagents tested, excluding chlorpromazine, significantly reduced MV release from K562 cells. This was useful information because as researchers in the MV field it provides a useful range of inhibitors of microvesiculation, beyond calpeptin. This is not only useful in providing pharmacological agents that can potentially inhibit cancer-promoting MV release as well as a wider range of agents to choose from regarding drug resistance but also a range of drugs that to achieve such inhibition in a targeted manner may be incorporated into targeting MVs that could deliver such drugs specifically to tumour cells.

The type of cell that could be considered as a source of targeting MVs is the THP-1 monocyte. This would be for two reasons, firstly that it has been used before by our group to demonstrate membrane fusion with the parent cells (THP-1) and secondly that monocytes are chemoattracted to tumour sites by a range of chemokines including CXCL12 (SDF-1α). I found that AML-M5, acute monocytic leukaemia cells (THP-1) produce more MVs that peripheral blood monocytes (Fig. 5.6) and for this reason decided for the sake of these initial *in vitro* experiments to use THP-1 cell-derived MVs as drug delivery vehicles. Obviously, this work, if to be furthered would have the

potential of using the patient's own monocyte MVs, as a very desirable form of personalised medicine.

This thesis has focused on the use of microvesicles (MVs) but the other type of vesicle, the exosome (which together with apoptotic bodies) encompasses what is termed Extracellular Vesicles, would also potentially make a useful drug delivery vehicle. Exosomes are much more homogeneous in size than the larger MVs, but their smaller volume may well limit their usefulness. Comparing MVs and exosomes it was decided to compare their PS exposition which has been shown to be greater on MVs even though some authors have challenged this lately. As perhaps such variation may be cell-specific, PS exposition on monocyte MVs versus exosomes was compared. THP-1 monocyte MVs were shown to stain positively for Annexin V-FITC and for the monocyte marker, CD14 (Fig. 5.7). As the more desirable exosome or MV drug delivery vehicle, in terms of personalised medicine, would be a patient-derived monocyte, and because PS exposition may be important for involvement in the fusion process with recipient cell as shown before (Ansa-Addo, E., 2010), the degree of Annexin V-FITC binding was compared between monocyte-derived MVs and exosomes (Fig. 5.7 and Fig. 5.8). In accordance with previous data from the Cellular and Molecular Immunology Research Centre, it was clearly found that monocyte-derived MVs expose PS to a far greater degree than monocyte-derived exosomes (Fig. 5.6). The implication that PS plays a role in MV-target cell fusion is that in previous work from this lab, blocking of MV exposed PS with unlabelled annexin, limited significantly the degree of lipid mixing observed (dequenching assay using octadecylrhodamine labelled MVs). In this sense it would be preferable to use MVs as opposed to exosomes from monocytes to initiate any drug delivery.

Carrying on the comparison between monocytic leukemic cell derived EMVs (exosomes and microvesicles) electron microscopy showed morphologically and in terms of size there to be no difference (Figs. 5.9 and Fig. 5.10).

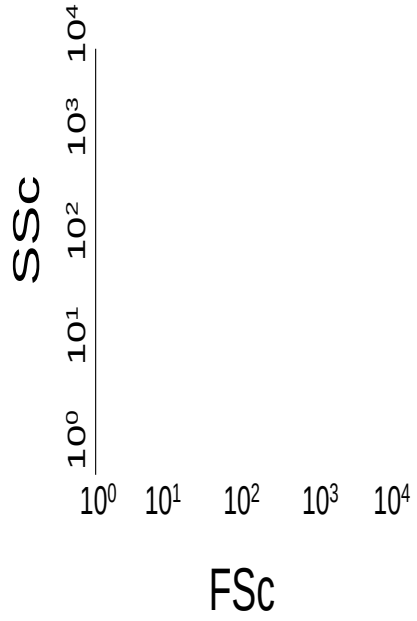


Fig. 5.4 Forward/Side Scatter plot for MVs released from THP-1 cells. A typical dot plot for size (forward scatter) versus granularity (side scatter) as obtained for THP-1 MVs showing a typical tapering shaped plot.

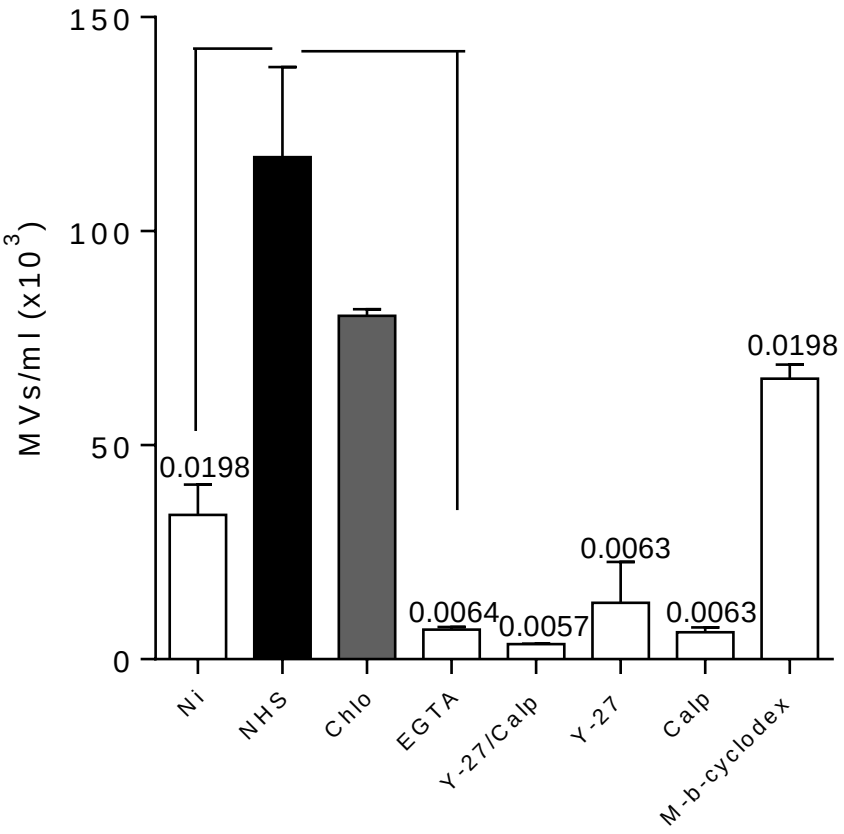


Fig. 5.5 MV release from K562 erythroleukaemic cells stimulated by sublytic complement can be inhibited by a range of pharmacological inhibitors. K562 cells were stimulated with 5% NHS for 20 min after pre-treatment for 30 min with chlorpromazine, EGTA, Y-27632, calpeptin, Y-27632 combined with calpeptin and methyl Beta Cyclodextrin and chlorpromazine. MV levels were measured using the Guava EasyCyte. The results are presented as mean ± Standard Deviation.

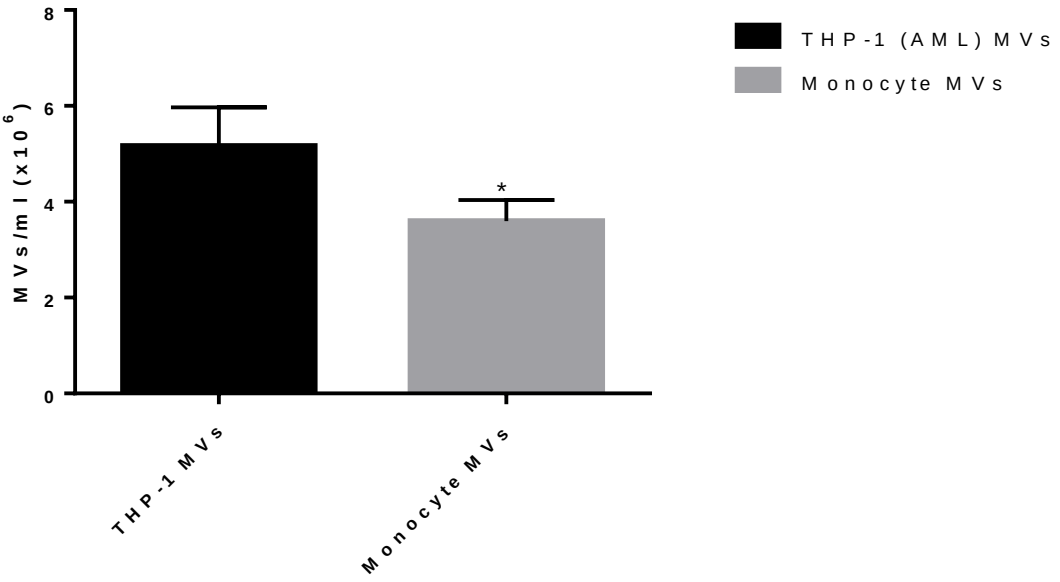


Fig. 5.6 Promonocytic leukaemia cell line (THP-1) releases more MVs than primary monocytes. An equivalent number ($3x10^6$/ml) of promonocytic leukaemia cells, THP-1 and primary peripheral blood monocytes were treated with sublytic complement and resulting levels of MVs recorded.

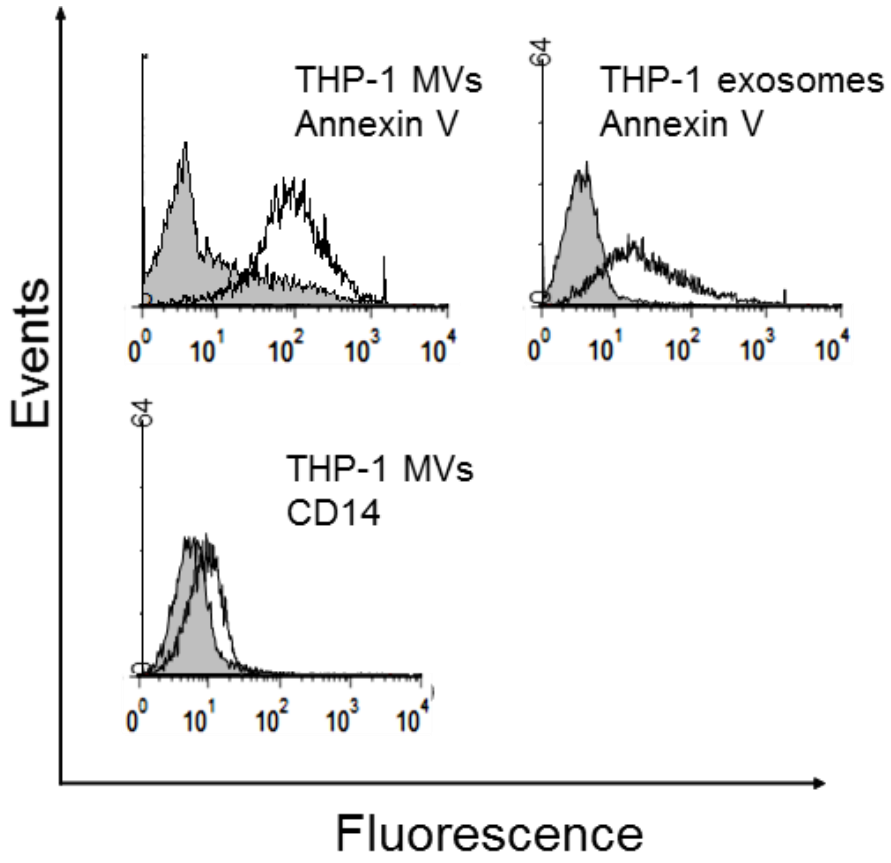


Fig. 5.7 Microvesicles released from THP-1, monocytic leukaemia cells show exposition of phosphatidylserine and are positive for CD14. THP-1 MVs were labelled with annexin V-FITC as were exosomes after isolation from cells. THP-1 MVs were also identified by positive staining with anti-CD14 (the Lipopolysaccharide receptor). Analysis was on the Guava EasyCyte flow cytometer.

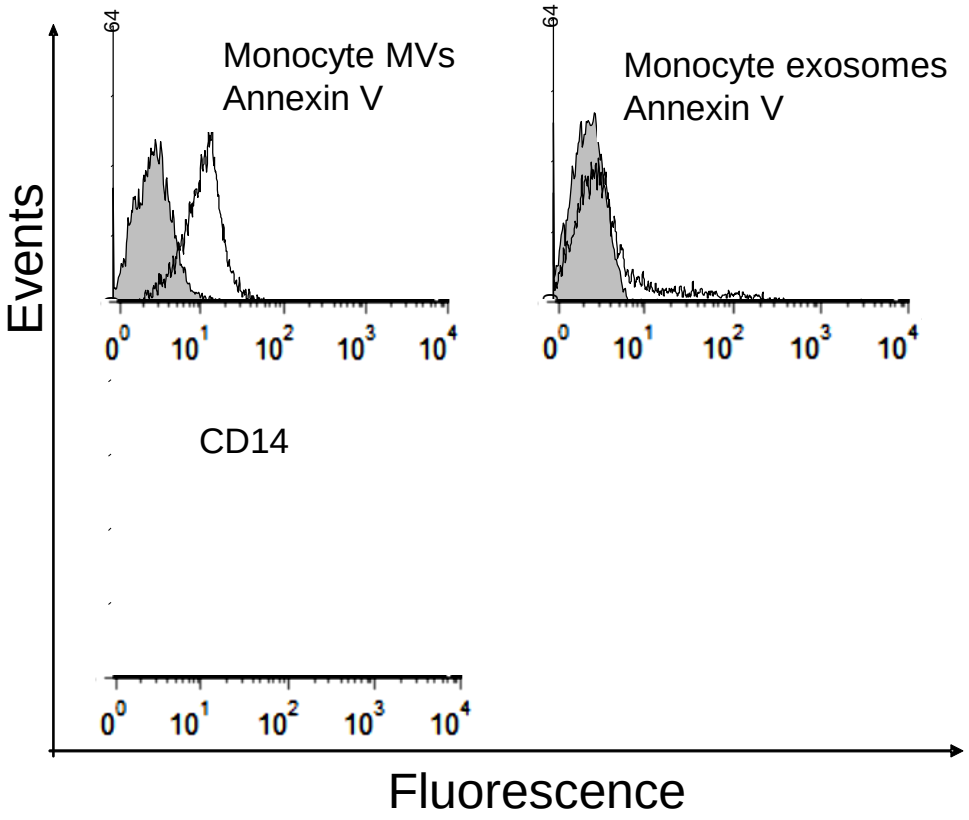


Fig. 5.8 Microvesicles released from peripheral blood primary monocytic cells show exposition of phosphatidylserine and express CD63. Peripheral blood primary monocyte MVs were labelled with annexin V-FITC as were exosomes after isolation from cells. Monocytic MVs were also identified by positive staining with anti-CD14 (the Lipopolysaccharide receptor). Analysis was on the Guava EasyCyte flow cytometer.

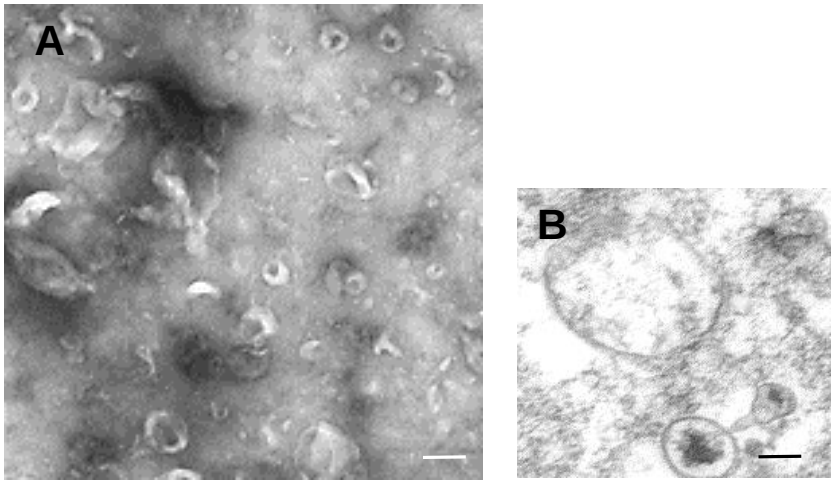


Fig. 5.9 Transmission electron microscopy of THP-1 monocyte exosomes (A) and microvesicles (B). Bar, 100 nm. Exosomes and microvesicles were isolated from THP-1, acute monocytic leukaemia cells and prepared for electron microscopy as described in Materials and Methods and viewed on a JEOL JEM-1400Plus Transmission Electron Microscope.

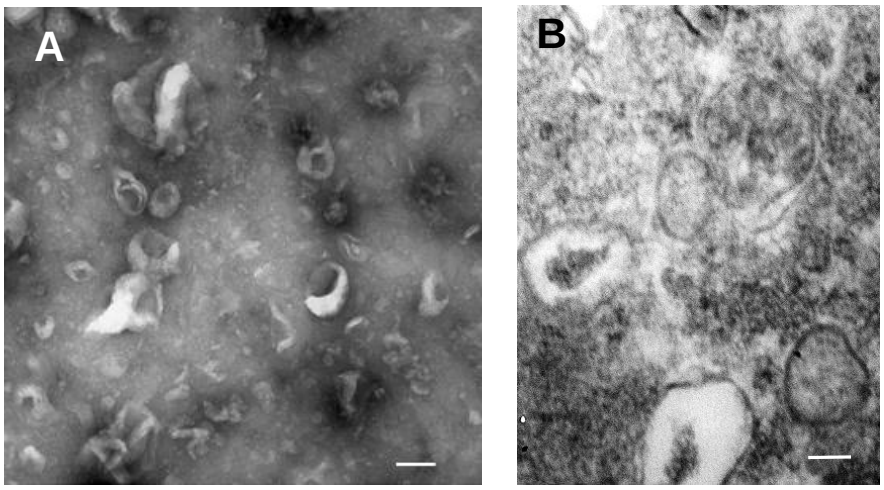


Fig. 5.10 Transmission electron microscopy of primary peripheral blood monocyte exosomes (A) and microvesicles (B). Bar, 100 nm. Exosomes and microvesicles were isolated from peripheral blood monocytic cells and prepared for electron microscopy as described in Materials and Methods and viewed on a JEOL JEM-1400Plus Transmission Electron Microscope.

5.3.2 THP-1 MVs fuse/hemi-fuse with K562 erythroleukaemic cells

To be able to use MVs as drug delivery vehicles with the intention of delivering chemotherapeutic drugs to tumour cells in the case of erythroleukaemia, it was considered paramount to establish whether MV derived from monocytic cells (whether monocytic leukemic cells, e.g. THP-1) or from peripheral blood monocytes are capable of fusion with acceptor K562 erythroleukaemic cells. The simple assay of choice was that of lipid mixing (or dodecylrhodamine, R18 dequenching). A perhaps more convincing method of establishing membrane fusion would be Fluorescence Resonance Energy Transfer (or FRET); the other problem is that lipid mixing may not tell us whether the MV-cell membrane fusion is in fact hemi-fusion. Electron microscopy was also used to show that the membrane of the R18-labelled MVs was indeed intact (5.11A) and flow cytometry was used to show that the MVs released from cells are indeed labelled with R18 (Figure 5.11B). It was then possible to mix the R18 labelled THP-1 monocyte-derived MVs with unlabelled recipient K562 cells. Figure 5.11C shows a significant increase in fluorescence, within 2 min, indicating fusion or hemifusion. That transfer of label had not occurred spontaneously without any degree of fusion/hemifusion, was confirmed by repeating the experiment at 4°C, whereupon no R18 was transferred to the recipient K562 cells. Several experiments were also included to indicate any factors that were important to mediate the subsequent fusion. It was thought that a degree of protein-protein interaction might be needed as a prerequisite to membrane fusion so surface protein was removed from the MVs by a gentle treatment with trypsin as described before (Ansa-Addo E.A. et al., 2010). Some protein interaction did indeed seem to be needed as surface proteins did indeed seem to be needed for lipid mixing. Phosphatidylserine on the MV surface was also shown to be needed by blocking membrane fusion with annexin V. Finally, increasing the amount of recipient cell membrane made available by increasing the number of cells, also increased fusion.

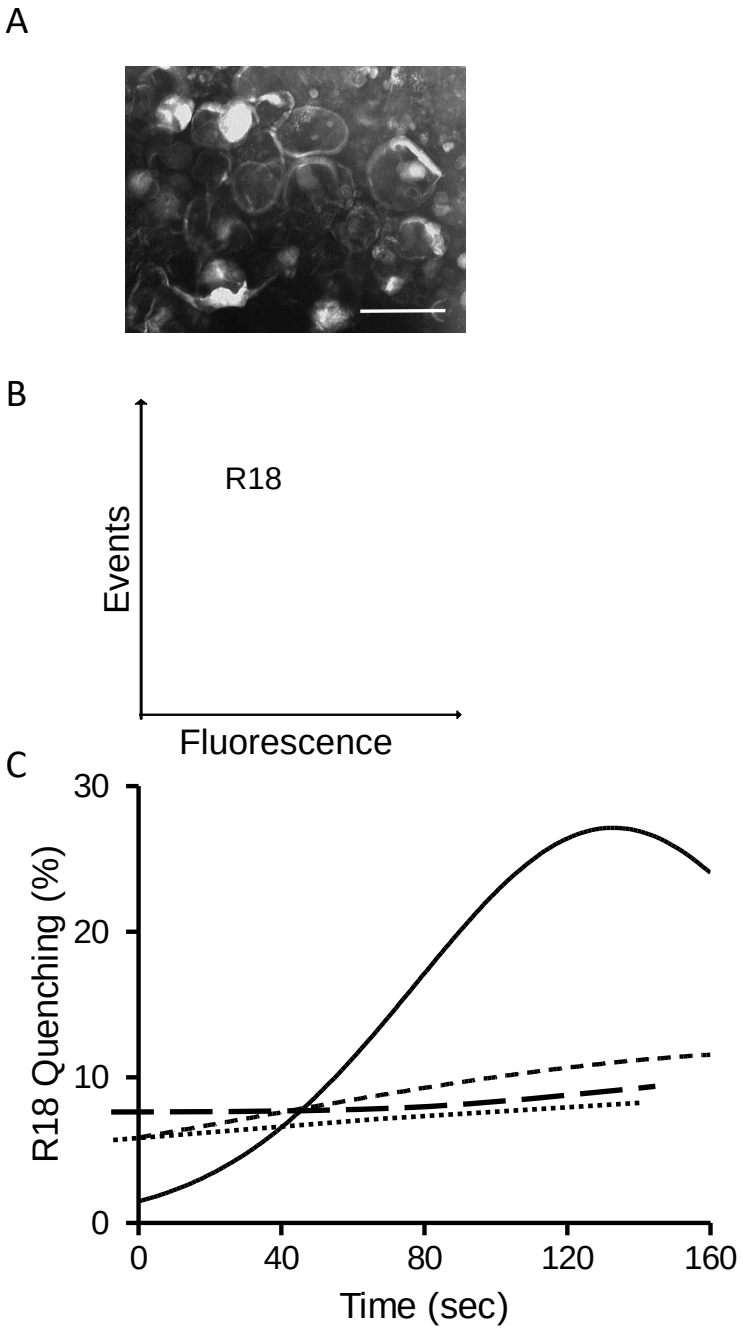


Fig. 5.11 Octadecylrhodamine-labelled THP-1 MVs fuse/hemifuse with K562 erythroleukaemia cells. R18 labelled MVs remain intact after labelling. Bar, 250 nm (A). Flow cytometry shows that THP-1 MVs are labelled with R18 (B). Lipid mixing assay shows marked increase in fluorescence indicating fusion/hemifusion with K562 membrane, within 120s of mixing MVs and cells. No fusion occurs are 4°C or when surface protein removed from the MVs or when phosphatidylserine on the MV surface was blocked with annexin V (dotted lines), (C).

Researchers at the Cellular and Molecular Immunology Research Centre have previously shown that a chemotherapeutic drug can be deliverable via MVs (Jorfi et al., 2015). In that case the drug was an alexa fluor 488-labelled methotrexate. In this thesis I have shown that THP-1 promonocytic MVs can fuse or hemi-fuse with K562 cells. The eventual aim was to see whether MVs loaded with a drug derived from liquorice, called β-Gly, which has been shown to be effective in either inducing apoptosis or limiting the proliferation of a range of cancer cells, could do so against the erythroleukaemic cell line, K562. To this aim I first wanted to confirm that β-Gly has an effect on K562 cells and to make a rudimentary analysis of the nature of this process and to then and try the drug loaded in MVs to see whether the concept of using THP-1 MVs as drug delivery vehicles has any credence. In effect this preliminary study is an attempted proof of concept.

5.3.3 β-Gly limits the proliferation of K562 cells

Firstly, 10 µM of β-Gly was used to see its effect on the proliferation of K562 cells in culture. By day 5 in culture it was very apparent that β-Gly has significantly limited proliferation compared to control and that this effect had started with increasing effect from day 3 (Figure 5.12). To see whether this effect was dose-dependent, β-glycyrretinic acid was used at increasing doses from 1 to 5 and 10 µM β-Gly. As is shown in Figure 5.13, the dose-dependent reduction in K562 cell number began to be dose dependent from day 3 but was only really significant by day 5. In conducting these studies on the capacity of beta-glycyrretinic acid to limit proliferation of K562 cells, the possibility arose that unlike some studies which had reported an increase in apoptosis, as a possible explanation for the decrease in cell numbers upon

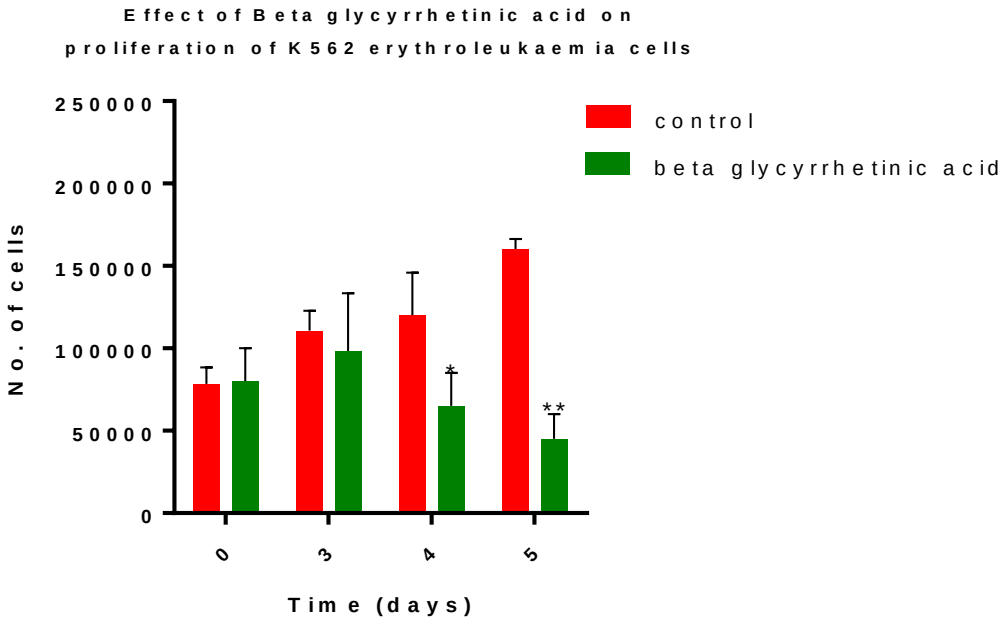


Fig. 5.12 β-glycyrrhetinic acid limits proliferation of K562 cells grown in culture. K562 cells were seeded at 80,000 cells per well. Control wells were left untreated and cell counts obtained on days 3, 4 and 5. B-Gly was added at 10 µM and beginning on day 3 began to show a decrease in proliferation which became magnified on days 4 and 5. The results represented are from 5 experiments, taking the standard error of the mean from triplicate samples.

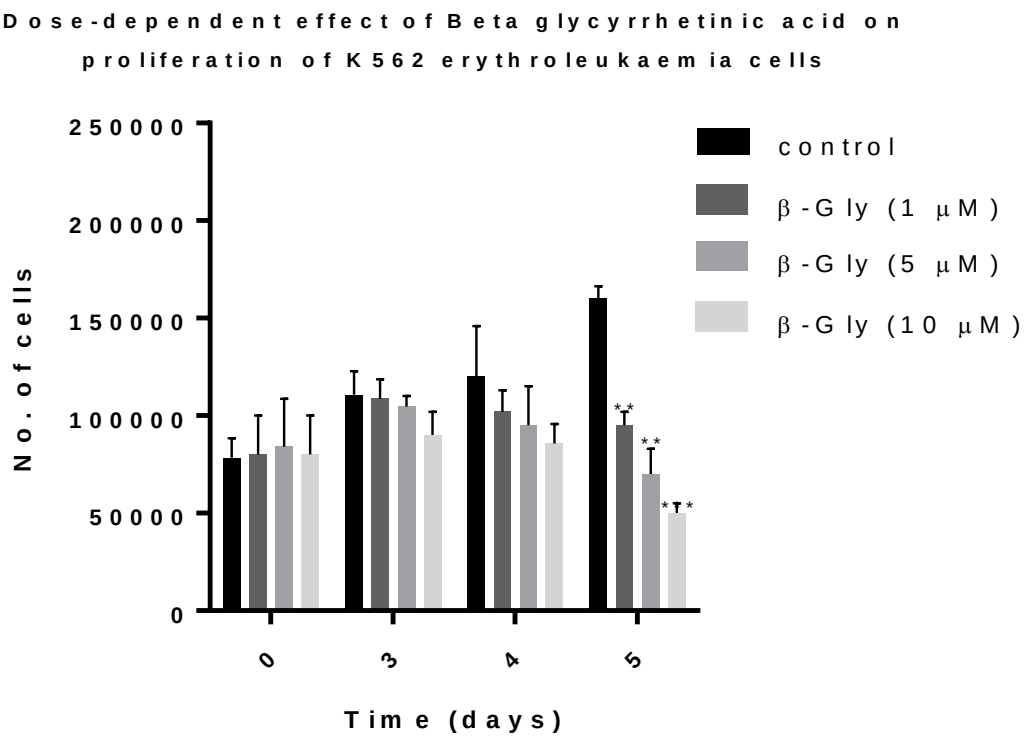


Fig. 5.13 β-Gly limits the proliferation of K562 erythroleukaemia cells in a dose-dependent manner. K562 cells in culture were treated with increasing doses of β-Gly (1, 5 and 10 μM) and cell numbers monitored over 5 days. By day 3 the highest dose (10 μM) of β-Gly already showed a reduction of cell numbers and that this was a dose-dependent effect was very apparent by day 5 where 1 μM had decreased the cell count by 40% compared to control, 5 μM by 55% and 10 μM by 70%. The error bars represent standard error of the mean, S.E.M. from 3 experiments in triplicate.

treating with beta-glycyrretinic acid, it was apparent from assessing the viability of the K562 cells (which remained high throughout the experiments, data not shown) that the observed reductions in cell number may have arisen because of an effect on limiting cell proliferation, rather than of inducing apoptosis.

5.3.4 β-Gly increase the doubling time of K562 cells but does not induce apoptosis

To give a cursory assessment of the observations made, K562 cell doubling time was firstly determined in the presence of 1, 5 and 10 µM β-Gly and then levels of apoptosis determined. As shown in Figure 5.14, with increasing doses of β-Gly, the doubling time of K562 cells increased, from 25 h in the absence of β-Gly to 28 h with 1 µM, 31 h with 5 µM and 33 h with 10 µM. The experiments to determine whether in this system, β-glycyrretinic acid was potentially limiting cell numbers by inducing apoptosis were similarly set up, with 1, 5 and 10 µM β-Gly. After 5 days, apoptosis was measured, by determining the percentage of cells stained with annexin V, as a measure of phosphatidylserine exposition (to indicate cells in early apoptosis) and also additionally permeable to the nuclear dye, 7-actinomycin D, 7-AAD, (as a measure of late apoptosis. These experiments showed that all K562 cells, even after 5 days in culture whether treated with 10 µM beta-glycyrretinic acid or not, were largely viable, the levels of early apoptotic cells typically varying between 5-10%; no late apoptotic (AnV+ 7-AAD+ cells were detected (Figure 5.15).

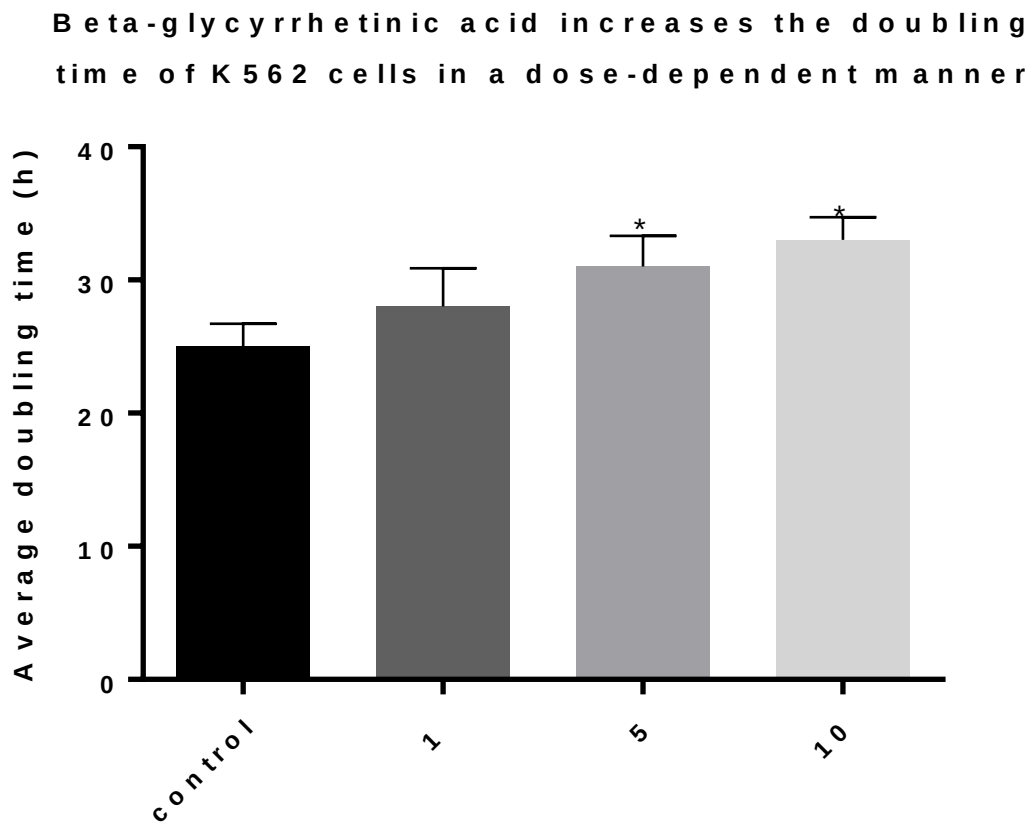


Fig. 5.14 K562 cells treated with beta-glycyrretinic acid have an increased doubling time. Cells (80,000/well) were treated with 1, 5 or 10 µM beta-glycyrretinic acid over 5 days and the average doubling time calculated.

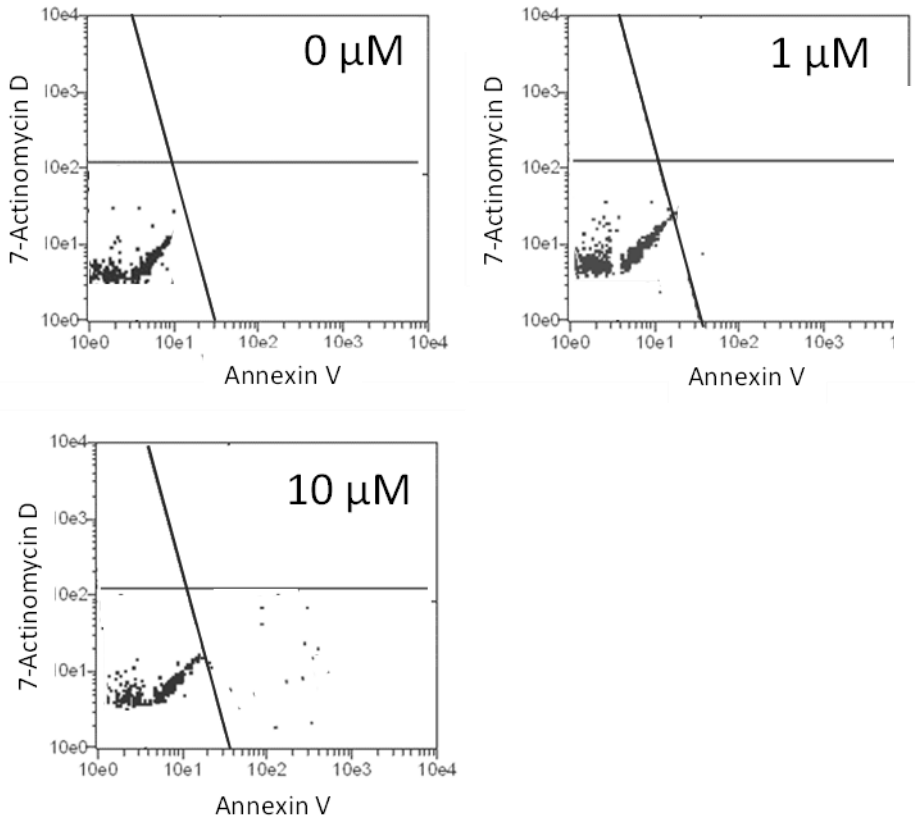


Fig. 5.15 K562 cells treated with increasing concentrations of β-Gly do not undergo apoptosis. K562 cells were treated with 1, and 10 µM β-Gly over the course of a 5-day experiment. They were then recovered and the degree of apoptosis assessed using a Guava Nexin assay on a Guava Easycyte flow cytometer. Cells in the bottom left quadrant are viable, not staining with annexin V (as a measure of phosphatidylserine exposition. Cells in the bottom right quadrant (of which there are barely any) have stained positive for AnV, indicating PS exposition and early apoptosis. Cells which have stained for AnV and the membrane permeant dye, 7-AAD are considered late apoptotic (top right quadrant) and any cells in the top right quadrant are considered dead.

Having shown β-Gly to reduce cell numbers of K562 cells in culture, the aim was to see if β-Gly-loaded MVs could induce a similar effect on K562 cells. As mentioned above, this lab had previously demonstrated methotrexate (MTX) directly in MVs released from cells treated with the drug by both monitoring the presence of fluorescent MTX and by directly measuring it and determining its concentration by HPLC (Jorfi et al., 2015). In that case it was in fact found that the drug was present at a 10-fold lower concentration than given to the parent cell. Although it was not possible to measure β-Gly directly in MVs, an experiment was devised to see the effect on K562 cells of THP-1 MVs isolated from THP-1 cells given 100 μM β-Gly. By carefully designing an experiment that included drug-free MVs isolated from healthy donor cells, the intention was to imply that any reduction in cell numbers on delivery to K562 cells was due to the drug within the MVs. As shown in Figure 5.16, by day 4 and 5 there was a significant reduction in cell numbers following treatment with β-Gly (10 μM) as before but now also with MVs isolated from THP-1 cells pre-treated with a high dose of β-Gly (100 μM). 100-fold lower concentrations of β-Gly could be used with equal efficacy when MV release was inhibited, in this case by treating the K562 cells with Y27632. By way of controls, resveratrol was found as expected to decrease cell numbers and jacalin to be mitogenic, significantly increasing K562 cell numbers. Whilst there was some inhibition of proliferation when K562 cells were treated with MVs isolated from healthy, untreated THP-1 cells, the reductions were not significant. Looking at the levels of apoptosis (Figure 5.17) and associated light micrographs (Figures 5.18 and 5.19) it was clear that neither β-Gly alone nor β-Gly-loaded MVs induced significant apoptosis of K562 cells. Jacalin which was mitogenic also induced no apoptosis whereas resveratrol did. Interestingly MVs *per se* from healthy cells do not induce apoptosis but those isolated from heat-treated THP-1 cells did, probably due to the transfer of caspase(s) as suggested by the reduced induction of apoptosis in the presence of the pan-caspase inhibitor, Z-VAD-FMK.

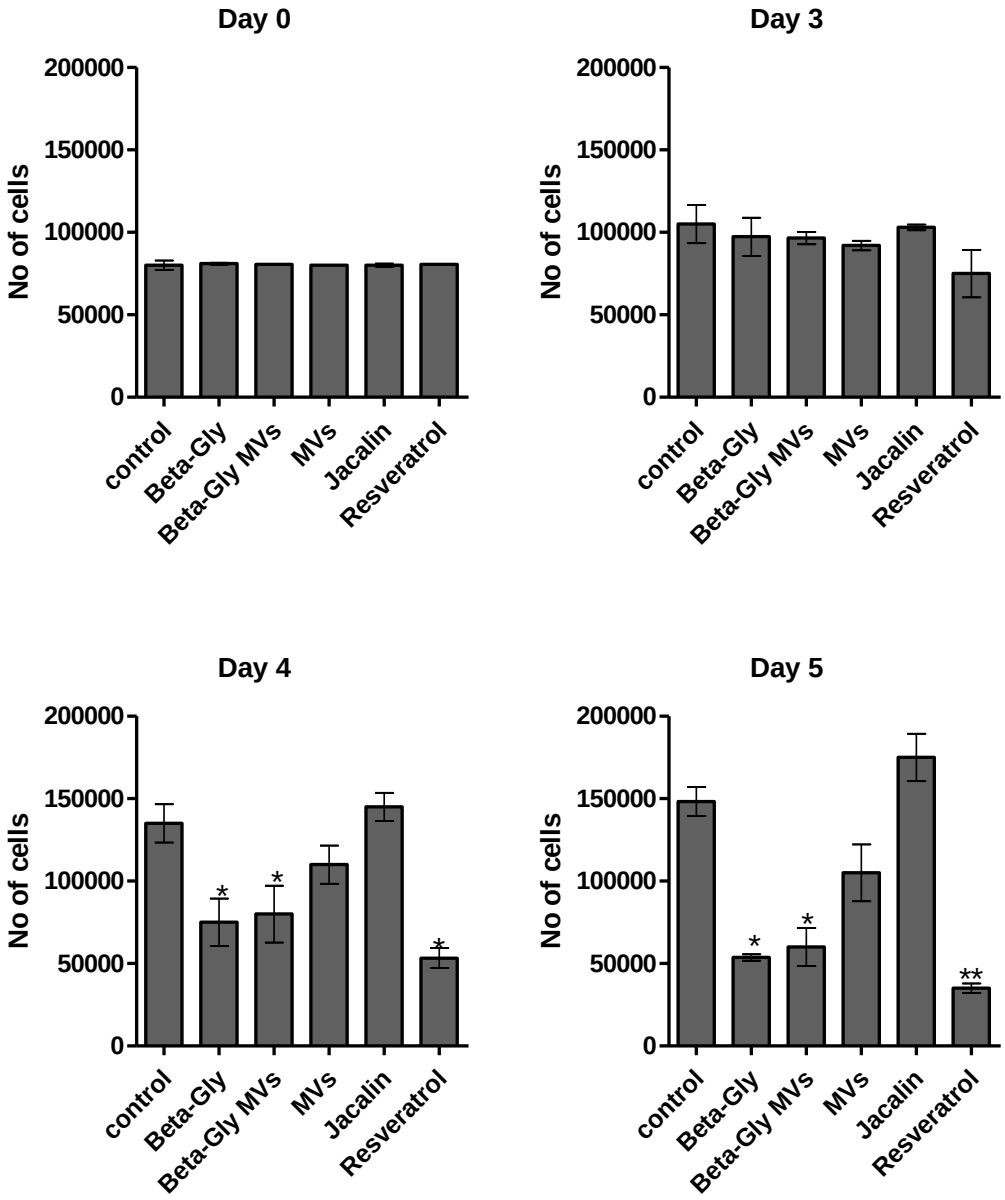


Fig. 5.16 β-Gly-loaded MVs limit proliferation of K562 cells and this is enhanced by inhibiting MV release. **By the 4th day in culture, K562 numbers, compared to control, untreated cells began to be reduced in the presence of β-Gly (10 μM) and resveratrol (40 μM). By contrast, MVs alone also reduced cell numbers by day 4 and 5, but not significantly. Jacalin, a lectin which is mitogenic for K562 cells increased cell numbers by days 4 and 5.**

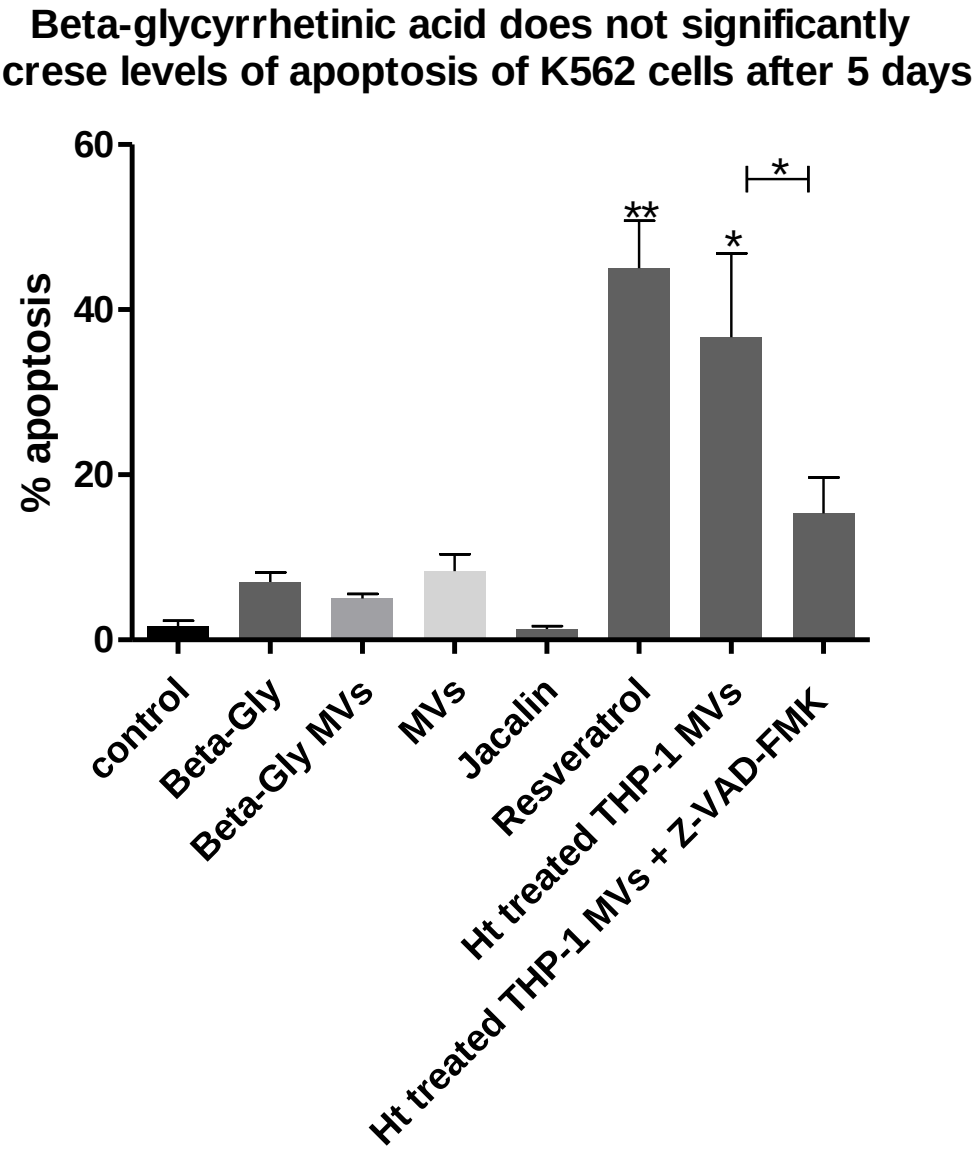


Fig. 5.17 β-Gly-loaded MVs whilst limiting proliferation of K562 cells do not induce their apoptosis. **Neither β-Gly nor β-Gly-loaded MVs induces any significant apoptosis even after 5 days of treatment. Jacalin which is mitogenic for K562 also induced no apoptosis, in contrast to resveratrol which induced 45% apoptosis by day 5. Z-VAD-FMK, the cell permeable pan-caspase inhibitor did inhibit MV-mediated apoptosis. Z-VAD-FMK, the cell permeable pan-caspase inhibitor did inhibit MV-mediated apoptosis.**

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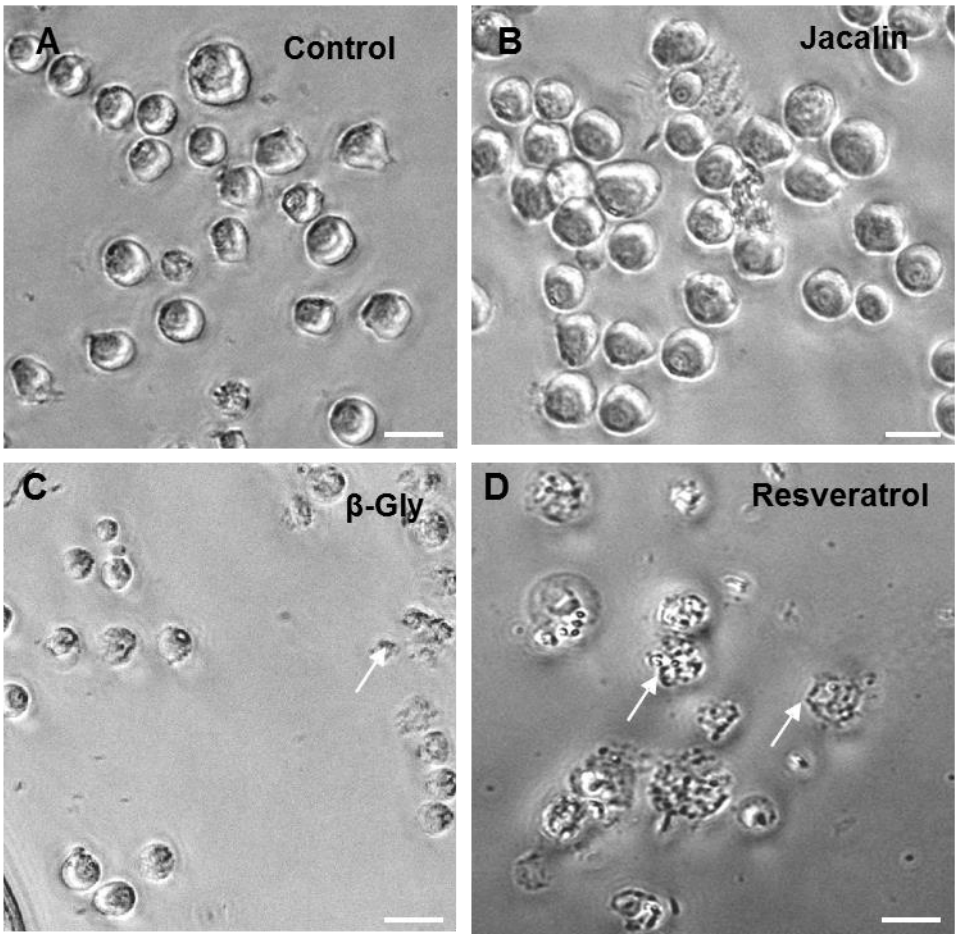


Fig. 5.18 Cell morphology (K562) after 72 h in culture following treatment with β-Gly. **Cell images were acquired using an inverted microscope from Nikon, Japan (TS100). Control, untreated cells are shown in (A) as rounded, uniform-sized cells with a smooth plasma membrane in (B) cells have been treated with 15 μM jacalin, a lectin mitogenic for K562 cells. K562 cells were treated with the liquorice-derived B-Gly at 10 μM (C) and with the apoptosis-inducing Resveratrol (40 μM), (D). Arrows indicate apoptotic cells. These results are representative of 3 independent pools. Bar is 20 μm.**

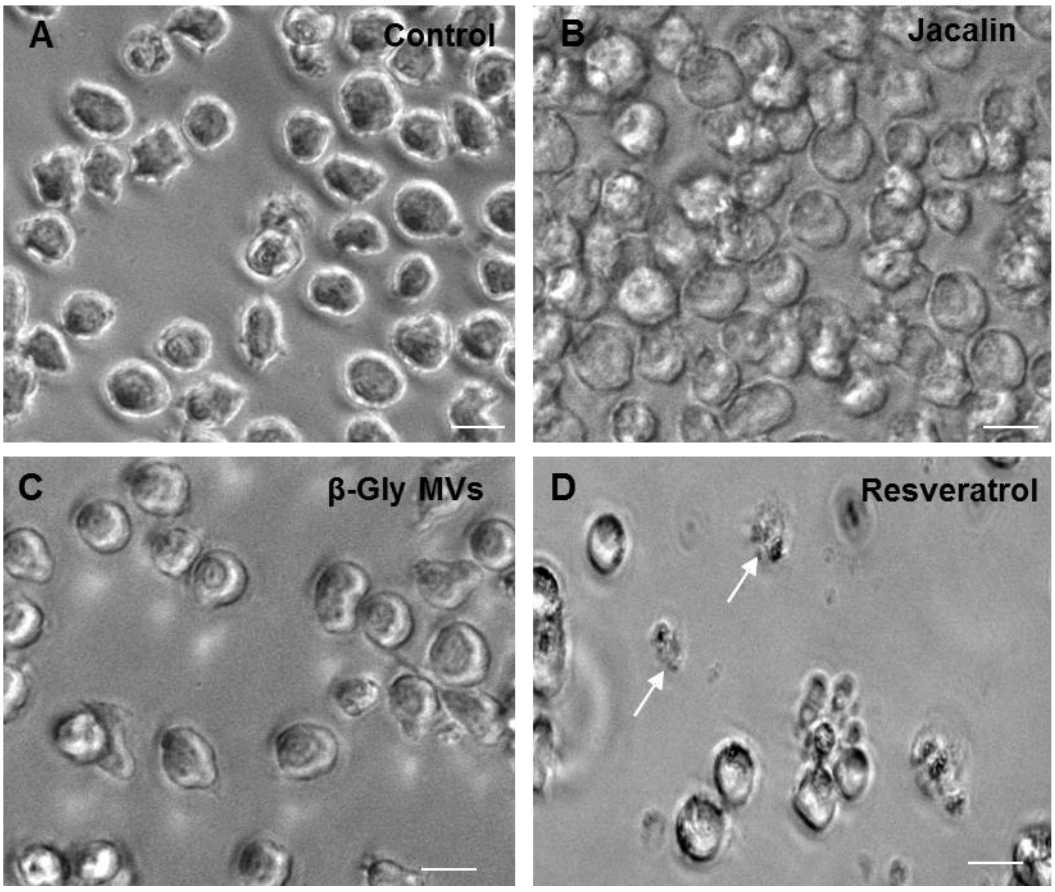


Fig. 5.19 Cell morphology (K562) after 72 h in culture following treatment with β-Gly-loaded MVs. Cell images were acquired using an inverted microscope from Nikon, Japan (TS100). Control, untreated cells are shown in (A) as rounded, uniform-sized cells with a smooth plasma membrane in (B) cells have been treated with 15 μM jacalin, a lectin mitogenic for K562 cells. K562 cells were treated with the MVs loaded with liquorice-derived B-Gly at 10 μM (C) and with the apoptosis-inducing Resveratrol (40 μM), (D). Arrows indicate apoptotic cells. These results are representative of 5 independent experiments. Bar is 20 μm.

5.3.5 β-Gly caused K562 cells to exit the cell cycle at G0/G1 and initiates erythroid differentiation

As apoptosis did not appear to have been induced by β-Gly, whether given directly or via MVs, it was supposed that some effect on the cell cycle may be observed. To that end cell cycle analysis was carried out using the Guava® Cell Cycle Assay for the Guava EasyCyte. Using this assay, it was possible to measure G0/G1, S, and G2/M phase distributions for treated and control K562 cells. This established that indeed B-Gly, whether given alone or via MVs, increased the % cells in G0/G1 from 33 to 43% and 35 to 44% respectively and reduced the % cells in G2/M from 20 to 9% and 19 to 9% respectively (Figure 5.20). As this exit from the life cycle was likely to mean that the cells were entering a state of differentiation this was briefly investigated. K562 cells are able to differentiate along an erythroid megakaryocytic pathway. As could be seen from Figures 5.18 and 5.19, under no conditions could an enlarged cell morphology be seen, so it was decided to focus a possible differentiation along the erythroid lineage. The assay chosen to assess such differentiation was that for measuring increases in fetal haemoglobin (HbF) using benzidine staining. Figure 5.21 shows that after 48 h of treatment of K562 cells with βGA or βGA-MVs, there is a distinct and clear increase in the number of cells staining positive with TMB (tetramethylbenzidine) when treated with 10 μM βGA (18%) compared to untreated control (7.7%) and positive control, 10 μM hemin-treated cells. βGA-MV-treated cells (with 15% positive TMB staining cells) compared to cells treated with MVs alone (10% staining) also therefore showed a significant increase.

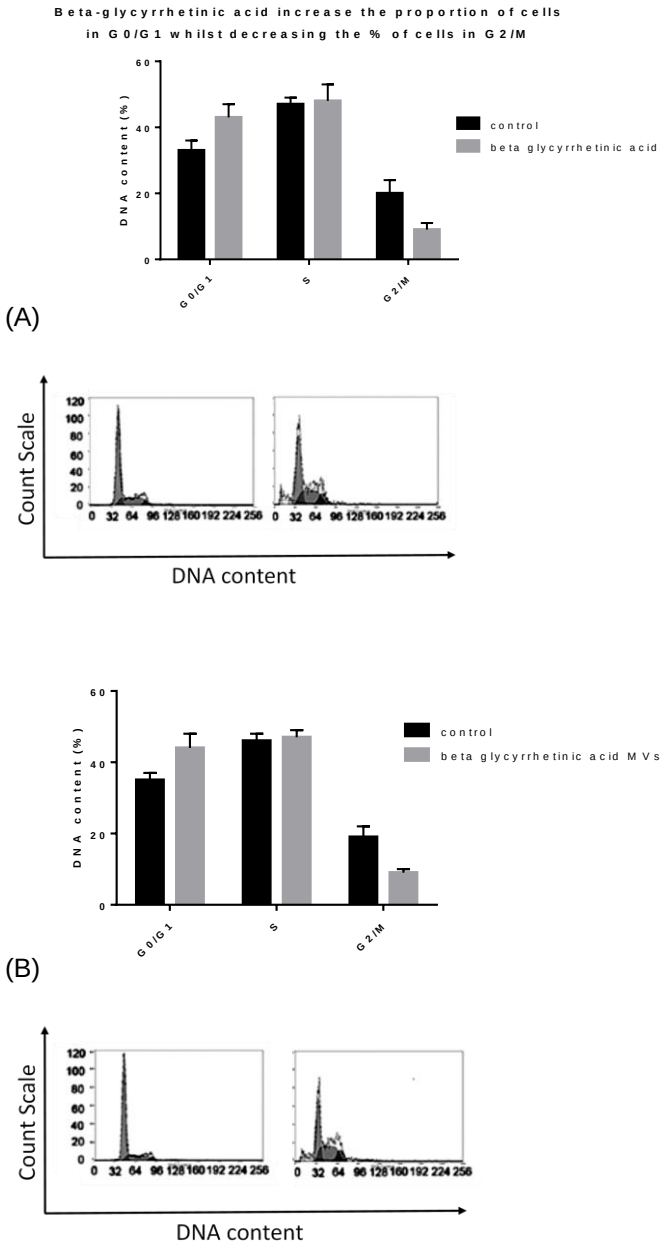


Fig. 5.20 Cell cycle analysis shows that β-Gly induces cell cycle arrest of K562 cells in G0/G1 even when delivered in MVs. K562 cells were treated with 10 µM β-Gly and with 30 µg of MVs from THP-1 cells loaded with 100 µM beta-glycyrrhetinic acid. After 72 h in culture DNA analysis was carried out using a cell cycle analysis assay (Guava® Cell Cycle Assay). It was found that both β-Gly (A) and MVs loaded with β-Gly (B) increased the percentage of K562 cells in G0/G1 phases whilst there was a concomitant decrease in the percentage of cells in G2/M.

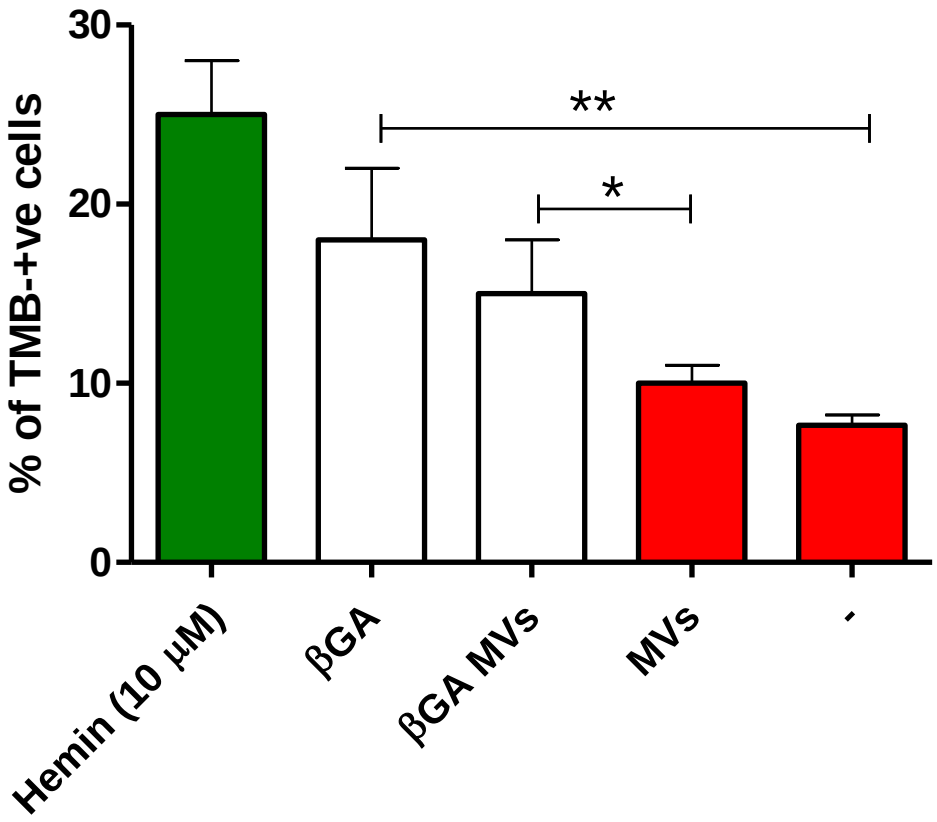


Fig. 5.21 Benzidine staining (% TMB-positive cells) as a measure or erythroid differentiation of K562 cells. K562 cells were treated with Hemin as a positive control or βGA or βGA-MVs and as a negative control with MVs alone or untreated. βGA significantly increased the number of TMB-positive cells after 48 h in culture, as did βGA-MVs compared to MVs alone.

5.4 Discussion

β-Gly derived from liquorice is widely used in Traditional Chinese Medicine as a drug described as possessing anti-inflammatory activity, inducing various cytokines and in having anti-viral activity. Colleagues at the Cellular and Molecular Immunology Research Centre first became interested in B-Gly from the point of view of its described ability to inhibit the classical pathway of complement at the level of complement C2 (Kores et al., 1997). Although the mechanism was not elucidated it was suggested that this specific inhibition might be due the ability of B-Gly to bind C2. It was during these studies that the group observed inhibitory effects on cellular proliferation in that particular work using the acute monocytic leukaemia cell line (representing AML M5) THP-1. In the meantime, many groups have shown the inhibitory effects on cell growth of cancer cells, and much research has been carried and to improve activity by structural modification and understanding the biological and pharmacological activities of B-Gly (Kang et al., 2014); several derivatives of β-Gly have been synthesized with improved anti-cancer activities (Chadalapaka et al., 2008 and Gao et al., 2012). The aim of this study was to see the effect of B-Gly on the chronic myelogenous leukaemia (CML) cell line, K562. As noted and in surveying the recent literature, it is clear that β-Gly has an effect on cellular proliferation of cancer cells. There are conflicts in the literature however regarding the mechanism(s) of such inhibition, to some extent depending on the cancer cells studied. Essentially the limiting of proliferation has been described as being due to an induction of apoptosis or to cell cycle arrest. In this chapter, proliferation, apoptosis, cell morphology, and cell cycle analysis were performed. In addition, the prospect of using MVs as drug delivery vehicles for chemotherapeutic drugs, in this case β-Gly was investigated. Lipid mixing assays were employed to estimate if there was any degree of membrane fusion between MVs and target cell membranes. This showed that octadecylrhodamine-labelled THP-1 monocyte-derived MVs underwent lipid mixing, indicating a degree of fusion or hemifusion with target K562 cells, within 120s of mixing target cells and MVs. Having indicated a likely fusion,

this of course does not exclude the possibility that MVs are perhaps taken up by a form of endocytosis, such as macropinocytosis, which could also potentially allow for delivery of drugs into cells. However, endocytosis was not investigated as part of this thesis, but is planned for future studies.

Looking at the growth curves of K562 cells with β-Gly versus controls it could be seen that over the course of the experiments, up to 5 days, any decline in numbers was not due to depletion of nutrients or build-up of waste products or overcrowding of cells, as in the control, untreated wells, the cell numbers increased up to 5 days. Treated and untreated cells grew at a similar rate up to day 3 whereupon a dose-dependent decline for β-Gly treated cells was observed, leading to a 2/3 reduction in cell numbers by day 5 and an increase in doubling time from 25 h to 33 h. The inhibition of cell proliferation was enhanced in the presence of Y27632 which was shown to inhibit MV release and therefore as shown (Jorfi et al., 2015) to limit drug efflux by MV release. The exact mechanism for this reduction in cellular proliferation is still unclear but this study has certainly begun to reveal a likely mechanism of action. β-Gly was not found to induce any significant increase in apoptosis even after 5 days but did cause the K562 cells to exit the cell cycle at G0/G1. K562 is a highly undifferentiated human erythroleukaemic cell line which can be differentiated into cells with an erythroid, myeloid, or megakaryocyte phenotype by a range of stimuli including Ara-C and hemin inducing a change along the erythroid lineage and the phorbol ester PMA along the megakaryocytic lineage (Leary et al., 1987; Sutherland et al., 1986). Although the cellular morphology after treating with βGA and βGA-MVs did not suggest differentiation to the larger megakaryocytic cell type, and the fetal haemoglobin assay suggested a differentiation to an erythroid lineage, this is far from conclusive as no markers representing differentiation to either lineage was looked at.

In other work by Liu and co-workers it was interestingly found that β-Gly had a dose-dependent anti-proliferative effect of the human acute monocytic leukaemia cells, HL-60 (AML-M2 cells). They also found that β-Glycyrrhetinic induced apoptosis but only at comparatively much higher

doses of at least 80 μM (Liu,D et al, 2007). In other work β-Gly was also found to induce apoptosis of human ovarian and breast cancer cells, but once more only at high concentrations. (Haghshenas, V et al, 2014; Sharma et al, 2012).

Although the described experiments suggest that differentiation of K562 cells is likely to be due to β-Gly, it should be noted that there is a small if insignificant degree of fetal haemoglobin (HbF) production (indicated as TMB-stained cells) when THP-1 MVs alone are given to K562 cells, suggesting that one cannot exclude that MVs themselves may be inducing a degree of differentiation. Indeed, in previous studies at CMIRC, it was found that surface proteins on THP-1 monocytic MVs carry TGF-β1 (in a latent complex) and that differentiation of THP-1 cells themselves was attributed to this although it was also speculated that microRNAs also carried within the MVs may also be playing a role in the cells' terminal differentiation (Ansa-Addo, E. et al., 2010) and as was also shown later (Ismail et al., 2013). In yet another study looking at the differentiation of K562 cells this time towards megakaryocytes, it was found that hedgehog morphogens found in MVs from stimulated T cells induced such differentiation (Martinez et al., 2006). An interesting avenue of future research would be to establish whether B-Gly itself can stimulate MV release from cells, especially if MVs are shown to have any role in the differentiation of K562 or other cells. Certainly, in cells where B-Gly induces apoptosis, it might be expected to see a degree of MV release as early apoptosis is associated with microvesiculation (Inal et al., 2012). Preliminary work in the Inal lab (unpublished data, personal communication) suggests that β-Gly does induce MV release from K562 cells at the concentrations used in this study (10 μM).

Amongst the limitation of this part of the study, there are several. Firstly, the process of getting drug into the MV has relied upon getting it first into the cell from which the MV is derived and therefore on using very high concentrations. It was not possible in the case of β-Gly to be sure that it was in the resulting MV and there was some reliance on previous work with

methotrexate (MTX) which was shown to be in the MVs, by having used Alexa Fluor-labelled MTX. It therefore could not be confirmed that it had been delivered to the recipient cells. However, it was possible to see rather than an induction of apoptosis that the β-Gly MVs induced a differentiation of erythroleukaemia cells, K562.

6. General Discussion

6.1 The lack of standardised isolation methodology of MVs and exosomes and the need for improved technology

Microvesicles are shed from all cells in the body. There are fewer MVs shed from skeletal muscle cells however and MV release is increased in tumour cells. MVs are constitutively released but in increased numbers upon stimulation for example in early apoptosis, necrosis or in infection and resulting increases in intracellular calcium. Microvesicles (MVs) also termed ectosomes or microparticles and exosomes make up EMVs (exosomes and MVs) released from most human cells. Today MV and exosome release is not thought of as a mere process to remove surface proteins (such as deposited complement proteins), and the process has gained immense interest with regard various immune responses and in intercellular communication. MVs are released directly from the plasma membrane whilst membrane blebs and pseudopodia are formed in a process called ectocytosis; exosomes however are released as a result of an endocytic process and are released by exocytosis in which a multivesicular body (MVB) releases its cargo of exosomes.

The problems involved in isolating microvesicles (MVs) and exosomes are numerous which is why the elucidation of a procedure to isolate these populations at high yield and with a high degree of purity has proved elusive for some time. The desired improvements over the standard methodologies involving differential centrifugation should ideally provide a better degree of purity of samples, and increased yield even from small cellular samples. Importantly any improved methods should provide samples that are less affected by the shear forces involved in high speed centrifugation. The procedures should also require a minimum of steps and be quick to perform and cost effective.

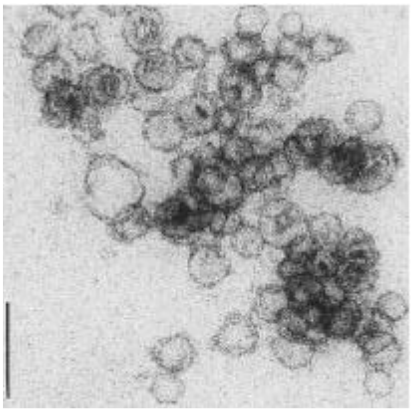


Fig. 6.1 Transmission Electron Microscopy of exosomes. The exosomes show evidence of clumping (Heijnen et al., 1999). (Bar = 100nm).

The purification and analysis of exosomes and MVs is particularly important in studies of their biological properties and as intercellular communicative vectors in their varying effects on target cells, especially when considering them as therapeutic vehicles carrying and delivering miRNA, siRNA or proteins to target cells. In the introduction to this thesis the biogenesis of MVs was described in detail including their release from the plasma membrane forming blebs initially, especially in cells undergoing early apoptosis. These stressed cells also form pseudopodia and the release of MVs takes about 20 min (Cestari et al., 2012; Stratton et al., 2015b). This is in contrast to exosome release which can take up to 3 h and is initiated from an endocytic event. Whilst exosomes range in size from 30 to 100 nm, MVs have a wider size range, from 100 nm to 1 μm in diameter, although it is believed that MVs below 100 nm in diameter also exist (Harrison, personal communication; ISEV meeting, April 2012, Gothenburg). There is thus no clean size distinction between MVs and exosomes and to compound any separation according to size there is the added problem of exosomes forming clumps and other clusters as shown in Fig. 6.1, and thus overlapping in size with the larger MVs.

6.2 Development of a reverse filtration method for isolating MVs and exosomes

With these problems of no clear size differential between exosomes and MVs, it was felt that it would not really be possible to optimise differential

centrifugation to yield pure populations and that cross contamination would remain very likely of both MVs and exosomes. This was indeed shown to be the case when looking at an MV sample prepared by differential centrifugation. This sample after filtering through a Millipore filter of 0.22 µm pore size revealed, upon analysis by transmission electron microscopy, that the sample which should not have had any exosomes because at 0.1 µm in diameter they would have passed into the filtrate was in fact found in the MV sample on the filter as shown in Fig. 6.2, indicating a clumping of the exosomes within the original sample. The other major concern with differential centrifugation for isolation of both exosomes and MVs is that the large centrifugal forces 1.5 to 2 h for MVs and 18 h for exosomes (at 100,000 *g*) are likely to be damaging potentially altering vesicle composition, especially at the surface and therefore potentially modifying their capacity to interact with recipient cells.

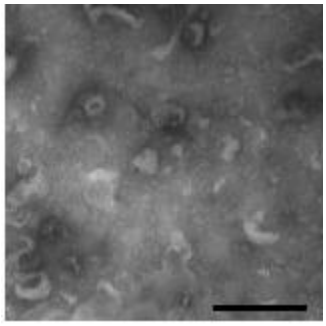


Fig. 6.2. Negative staining of differentially centrifuged MVs after filtering through a 0.22 µm filter. The distinct cup-shaped morphology of exosomes (whilst an artefactual effect of the preparation of the sample for electron microscopy does indicate exosomes) and therefore points to exosomal contamination of a MV preparation. Bar = 250nm.

6.3 Advantages of MV isolation using sucrose gradient or filtration versus differential centrifugation

MVs and exosomes have a similar composition that depends on the cell they come from. In THP1 cell derived MVs and exosomes we observed that exosomes are enriched in LAMP1 and CD63 (LAMP3) while MVs are enriched in annexin V and CD11b. Vesicles recovered from the filter binds to annexinV better that differentially centrifuged MVs. FSC Vs. SSC of SGP and filter recovered MVs resembles that described for MVs in different studies. There are many proteins as we observed that are not common to MVs and exosomes and hence identifying the protein by mass spectrometry may provide a marker that can be used to distinguish THP-1 cell derived MVs from exosomes (some of them already given). The filter recovery protocol at the time may not give absolute purification, however given the time it can be optimized to give better purification and separation of different sized MVs. A proteomic profile of both MVs and exosomes is highly recommended that will give us an insight into their composition and functions. It may also help us to answer whether MVs and exosomes share any biological function?

All the results cumulatively indicate that a better purification of MVs can be obtained using simple methods like filter recovery and/or sucrose gradient centrifugation. These methods can further be modified to isolate different sized MVs. One can optimize the speed, time or concentration of the gradient to have fractions of different sized MVs. Different sized MVs may also be obtained using different pore sized filters. For example, sets of MVs differing by 100 nm in size can be obtained using 1 µm, 0.9 µm, 0.8 and so on to 0.1 µm Millipore filters. The optimal method, which will require considerable development would be to isolate 100% pure MV or exosomes preparations and would involve using inhibitors and preferably by silencing the genes involved in the various secretion pathways.

By virtue of the simultaneous isolation of MVs and exosomes using these methods it became interesting to compare the two populations. This was

carried out simply by cytokine microarrays and even with the limited number of proteins analysed, it was possible to see that many proteins were not present in both MVs and exosomes and that it would definitely be worth carrying out a global comparison of protein between the two vesicle types with a view to finding distinguishing markers, especially for MVs.

Part of the work presented has compared the MVs and exosomes isolated by differential centrifugation with the MVs isolated by 'reverse filtration' and from sucrose density centrifugation, which is much less 'harsh,' in terms of shearing forces. In addition to exosomal contamination of MV isolates (as shown in this thesis and by others), other potential contaminants, which are of major concern, and more than likely to co-purify especially during ultracentrifugation, and include cell debris such as nucleic acid fragments (as was demonstrated in Chapter 1) but also fragments of the lipid bilayer from the plasma membrane and polymerised proteins. This becomes a major concern as the Extracellular Community is now rapidly moving into the area of using exosomes and MVs as therapeutic vesicles even in personalised therapies for example using exosomes and MVs derived from stem cells (Rani et al., 2015). This was particularly noticeable in the vesicle sample compared by agarose gel electrophoresis for contaminating nuclei acid where two very high molecular weight bands were detected in the differentially centrifuged MV sample; neither of these bands was observed in the MVs isolated by sucrose gradient (SGP) nor in the exosome isolate. This observation, of contaminating genomic DNA in MV samples, is of paramount importance because differential centrifugation is still the most common isolation method for MVs and to reiterate must be of major concern as we move into an era of using MVs and exosomes in therapy and other clinical applications.

When SDS-PAGE was used to analyse the protein profile of the various samples it was found that the number of proteins in the MV preparation isolated by differential centrifugation was greater than in the samples isolated by filtration. The most likely reason for this is that, following on from the observations of contaminating DNA that other cell debris including

possible exosomes were also isolated. The less likely possibility is that upon filtration of supernatants from prior low speed centrifugation (to remove larger cellular material and cell debris) through the 0.22 µm pore size filter, that MVs smaller than 0.2 µm diameter had been lost in the filtrate and that it was mostly these MVs that carried the missing proteins. However, considering the size range of MVs from 0.1 µm to 1 µm, the likelihood that the majority of the proteins concerned are carried by MVs smaller than 0.2 µm is very unlikely. Furthermore, several proteins which are not that abundant in differentially centrifuged MVs are in fact absent from exosome preparations and MVs recovered by filtration. Since the majority of cell debris is likely to have been removed by two centrifugations at 4,000 *g* for 30 min each, any debris small enough to be still in the supernatant will then definitely get carried over into either the MV or exosome isolates. Some of this debris which may be smaller than MVs or even exosomes may polymerise or otherwise aggregate into larger pieces that may indeed contaminate MVs and/or exosome isolates. The data presented in this thesis, including the analysis of filter recovery after sonication, by SDS-PAGE and agarose gel electrophoresis as well as ELISA for a surface-bound cytokine, TGF-β1, supports the concept that light sonication of the supernatant (SN) after low speed (2 x 4, 000 *g* centrifugations for 30 min each) breaks up such contaminating matter or clumped exosomes, to yield higher purity samples.

6.4 Analysis of plasma membrane-bound TGF-β1 on MVs and exosomes

This study also looked at the expression of TGF-β1 on MVs isolated by differential centrifugation compared with that on MVs isolated by sucrose density gradient centrifugation. This pleiotropic cytokine has numerous, sometimes opposing functions for example promoting cellular proliferation or inhibiting it, depending on the cell type. TGF-β1 is involved in tissue repair and healing during inflammation and by controlling T cell division is also involved in regulating inflammation. Work at CMIRC has implicated TGF-β1 on MVs in the terminal differentiation of monocytes (Ansa-Addo, et al.,

2010), in enhancing the invasion of cells by the intracellular protozoan parasite, *Trypanosoma* cruzi (Cestari et al., 2012) and in inducing Epithelial Mesenchymal Transition of prostate cancer cells (Haidery, A. PhD thesis, 2014). Although there were higher levels of TGF-β1 detected by ELISA in centrifuged MVs, this could not be related to the harshness of the isolation procedure as it was realised that the centrifugation process could have merely concentrated cellular TGF-β1 and that the level detected in filtered MVs could represent the true level of the cytokine on MVs. Another possibility which was tested was a comparison of the exosome and microvesicular levels of TGF-β1. This would be an important analysis as there was a chance of much reduced levels detected in exosomes because unlike MVs which bud directly off from the surface plasma membrane where TGF-β1 is tethered to the ECM in a latent complex, exosomes might be expected to carry lower levels as the exosomes are derived from intraluminal budding from an early endosome, following an endocytic event and released upon MVB fusion with the plasma membrane. The acquisition of lysosomal markers such as LAMP-1 by exosomes suggests further possibilities for loss of membrane-bound TGF-β1 and indeed unlike other cytokines such as FGF-1 and galectin-3 (which are found in the intravesicular space because detectable levels were greatly enhanced upon lysis of MVs) and which are present at similar levels in MVs and exosomes, TGF-β1 was found at four-fold higher concentrations on MVs (1 ng/ml) compared to 0.25 ng/ml on exosomes.

It has been proposed that MVs may carry certain cytokines that lack a signal peptide by a process termed ‘non-conventional protein export’ after a series of publications eloquently summarised in a review by Nickel (Nickel, 2005). One such candidate cytokine was detected in the cytokine array presented in Chapter 3, namely macrophage migration inhibitory factor (MIF) but others were shown in the lab and include galectin-3, and fibroblast growth factor-1 (FGF-1) (Inal, personal communication and Ansa-Addo, 2010). IL-1α is another cytokine, like TGF-β1 with pleiotropic effects and like TGF-β1 is bound to the cell surface (Orjalo et al., 2009) and unlike IL-1β, is rarely

secreted and can function either as an uncleaved precursor protein (pIL-1α) (Apte et al., 2006) or upon proteolytic cleavage, as a processed protein, which is released as a result of cellular injury and which can induce apoptosis. IL-1α is also involved in other immune reactions including in inflammation and haematopoiesis.

6.4 Use of the triterpenoid liquorice derivative, β-Gly to limit proliferation of erythroleukaemia, K562 cells: potential new 'differentiation therapy'

The use of β-Gly, a derivative from liquorice, as an inhibitory drug in various cancers and leukaemia's has come to the fore in recent years (Ali et al., 2016). As part of the work presented in chapter 5 of this thesis the activity of β-Gly was tested against the erythroleukaemia cell line, K562. The reason β-Gly was chosen for this study in itself was unexpected and relates to another published function of β-Gly, namely that of its capacity to inhibit complement C2 (Kroes et al., 1997), presumed to be due to its binding to C2 (Kroes et al., 1997) thus rendering it unable to bind to C4b to make the C3 convertase complex. In unpublished work, colleagues at CMIRC had been testing a previously reported hypothesis that the complement protein Factor B (specifically the Bb fragment) which is closely related to complement C2 (39% identity at the amino acid level), may function as a growth factor for B cells (Ambrus et al., 1991 and Petera et al., 1988). To see if complement C2 as the related Factor B share a similar 'growth factor-like' property, but this time on monocytic cell lines and K562, which release complement C2 after 4 days in culture, β-Gly was incorporated to abrogate any growth factor-like properties of complement C2. This work showed that β-Gly was drastically diminishing proliferation of THP-1 (acute monocytic leukaemia cells) and of K562 (erythroleukaemia cells). Initially it was not apparent if this effect was due to alteration of the cell cycle or induction of apoptosis and from the literature nothing could be gleaned as the proposed mechanisms

leading to inhibition of proliferation seemed to vary between different tumour cell types and researchers.

Experiments were carried out over the course of 5 to 6 days in culture and whereas during this period K562 cell numbers increased 23 to 25-fold, in the presence of 10 μM of β-Gly, increase in cell numbers was at least halved with only a 10-fold increase. A dose-dependent effect was also noted. The inhibition of proliferation began to be apparent from the third day after addition of β-Gly and became significant, compared to control, 48 h later, on day 5. The inhibitory effect observed could be explained by induction of apoptosis, stimulation of cellular differentiation or if cell division had been slowed down. There certainly was an increase in doubling time, a dose-dependent effect, based on an increasing concentration of β-Gly. If the interphase part of the cell cycle is prolonged, as might be expected, there would be a reduced frequency of division. This would result in some enlarged cells (Prescott, 1976), which was not observed, suggesting a reduction of growth rate for the β-Gly treated cells. In addition, it was found that β-Gly did not induce apoptosis even by the end of the experiment. This was in contrast to the initial findings of Gao et al also with K562 cells (Gao et al, 2012) but later confirmed (Gao et al, 2013). Furthermore, β-Gly had induced an exit from the cell cycle at G0/G1 with an associated increase in fetal haemoglobin suggesting differentiation towards an erythroid lineage.

In previous work using the leukemic acute monocytic THP-1 cell line (AML-M5) it was shown that THP-1-derived MVs began to limit the increase in cell numbers from 3 days post addition of MVs (30 μg protein equivalent) compared to untreated control, resulting in significant reductions in cell numbers by day 5. It was suggested at the time (Ansa-Addo et al, 2010) that this was due to TGF-β1 on the MVs themselves and that they were inducing terminal monocyte-to-macrophage differentiation with increases in the markers CD11b and CD14. These effects could be reversed by using a blocking anti-TGF-β1 antibody or by using the antagonist of TGF-β1 signalling, SB431542. These studies bring about the question as to whether any of the effects noted on K562 brought about by β-Gly, including increased

doubling time due to a prolonged cell cycle/reduced frequency of division, and likely cell cycle exit and differentiation could in fact be due to a β-Gly-mediated stimulation of MV release. To that effect, it has been shown that β-Gly does in fact increase MV release from K562 cells (Inal, J., personal communication), although at the doses used there was no dose-dependent effect noted. If β-Gly does indeed promote MV biogenesis in K562 cells, there are several pharmacological inhibitors of microvesiculation, such as calpeptin (Jorfi et al., 2015) which could be added together with β-Gly to confirm any effect of MVs released. Any such stimulation of MV release could be quantified and then any inhibition of MV release and associated effects potentially reversed by restoring the equivalent of externally sourced K562 MVs. It would be particularly interesting to establish whether prolonged, stimulation of MV release by K562 cells can initiate differentiation, whilst inducing cell cycle arrest, and therefore act as a possible differentiation therapy as is used for monocytic leukaemia (all trans retinoic acid, ATRA) (Manzotti et al., 2015). There is a precedent in the literature for this approach as already in 2009 using the same cell line, K562, it was described that induction of differentiation of these cells using miR-34a resulted in an induction of apoptosis by the mitochondrial pathway which resulted in a reduction of cellular proliferation (Navarro et al, 2009). By limiting MV release in this case with Y276342 it was possible to achieve an equal limitation of K562 proliferation, now with a 100-fold lower concentration of β-Gly (0.1 μM).

6.5 Preliminary testing of MV-based delivery of β-Gly

To test the possibility of MV-delivered β-Gly, MVs were collected from monocytes that had been loaded with β-Gly. The reason chosen for using monocyte MVs is that monocytes themselves are attracted by chemotaxis to tumours. At this stage THP-1 MVs were used but for transferral to the clinic, peripheral blood monocyte-derived MVs could equally well be used. The use of MVs as opposed to exosomes was also preferred because of their larger

size. It was also shown that the MVs undergo a degree of lipid mixing, whether fusion of hemifusion, suggesting delivery of microvesicular contents. Cells were loaded with β-Gly, which as a lipophilic drug, enters cells by passive diffusion (Goldman, 2002). It was not possible to prove that the β-Gly was actually within the MVs as had been shown by CMIRC researchers for the chemotherapeutic drug, methotrexate (Jorfi et al., 2015), but it was encouraging that β-Gly-loaded MVs were almost as effective in reducing cell numbers as β-Gly alone and that MVs on their own only limited proliferation to a non-significant level.

The phagocytosis assays of MVs and exosomes were carried out as a follow-up to previous work carried out at CMIRC in which it was found that MVs could inhibit the phagocytosis of apoptotic bodies (Antwi-Baffour, 2010). In this case however exosomes (which have a lower exposition of PS on the outer leaflet of the lipid bilayer) were tested. As expected this showed that exosomes were not as effective as MVs in inhibiting macrophage-mediated phagocytosis of apoptotic bodies.

6.6 Future work: MV-based health assessment as early biomarker of disease, health, and development of MVs as personalised therapeutic vehicles

Having performed much developmental research on MV and exosome isolation, a filtration protocol was used to evaluate plasma levels of MVs in the general population. Although a small sample was used, it was found that age did not affect plasma MV levels. It was also implied that smokers may have reduced levels of plasma MV levels, which was backed by inhibition of MV release in cell culture by nicotine (which is anti-apoptotic). It was also found that fasting increased plasma MV levels. There are many described health benefits of fasting reported in the scientific literature and whether this could be connected to increased plasma MV levels is open to speculation. Rather than concentrations of MVs, it may be their specific cargo that may have health benefits of otherwise. As intercellular communicative vectors

MVs may deliver membrane proteins, or cytokines lacking signal peptides (such as galectins and fibroblast growth factors), carried within the vesicle as well as other surface bound cytokines in inactive pro-forms such as TGF-β1 and IL-1α, MVs may also deliver microRNAs (miRNAs) and long non-coding RNAs (lnRNAs) thereby affecting gene expression in target cells (Inal et al., 2012; 2013). In terms of a MV-based monitoring of well-being, it would also be important to assess the effect of increased levels of stress hormones cortisol and adrenaline on MV biogenesis. In other work, leptin the 'satiety hormone' which by inhibiting hunger can regulate energy balance is of interest as it can also be found in MVs, in fact adipocyte MVs (Aoki et al, 2010). In view of the current obesity epidemic and association of leptin resistance (with obesity) (Lee et al. 2001), and if leptin represents a 'hidden reservoir' of the hormone, it might be wise to make such MV-based measurements of this hormone.

A likely promising avenue of future research may therefore involve the analysis of not only MVs from fasting individuals, but also from those doing high intensity physical activity for whom there are well documented health benefits. This work is already being carried out by colleagues at CMIRC with external collaborators with the view to see whether there are any beneficial effects of applying such MVs from donors after intense exercise (Frühbeis et al., 2015) to a range of disease situations.

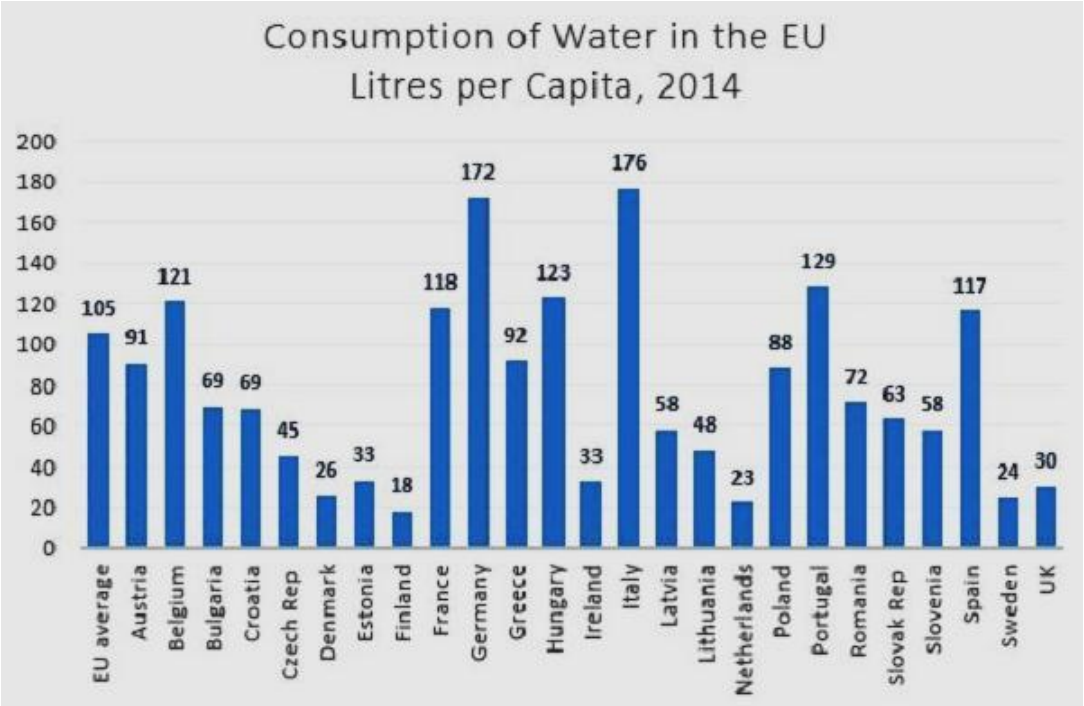
Overall as part of a programme of therapeutic lifestyle changes (TLC) with the aim of reducing disease and of inhibiting the activation of cancer epigenomes, monitoring of MVs may become a significant tool in the years to come.

MVs could also be used as vehicles carrying a very specific 'loaded' cargo and that where necessary such MVs could be manipulated with particular targeting features to deliver them to a specific cellular target. Such targeting was first performed in seminal work by Matthew Wood and his team in Oxford where mouse dendritic cell MVs were engineered with a specific peptide that targeted the MVs carrying the ability to silence a gene involved in Alzheimer's to the glial cells in the mouse brain. This work is ongoing. In

other work, liposomes have been targeted to liver hepatocytes by having a surface bound β-Gly (Li et al., 2012; Cong et al., 2016; Gumpricht et al., 2005), the same drug that was used in this thesis to inhibit erythroleukaemic K562 cell proliferation. In this case liposomes carrying a chemotherapeutic are targeted to the hepatocytes as they express receptors that can mediate the specific uptake of the β-Gly expressing liposomes (Ishida et al, 1993). A similar adaptation could be envisaged for microvesicles which have a longer half-life in the body than liposomes.

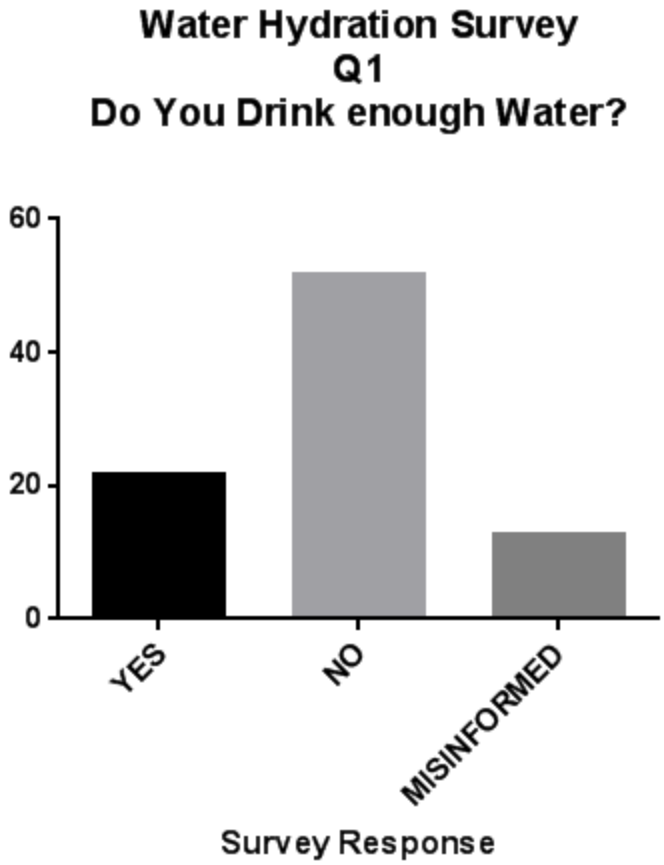
6.6.1 Hydration, plasma osmolality, plasma MVs and health (as part of Therapeutic Lifestyle Changes)

Adequate hydration ('water wellness') in the Western world is a major concern for the health of the population as recently highlighted in a US study (Brooks et al, 2017) which found in over 8,000 participants inadequate hydration to affect just under 30% of the population. Adequate hydration in this study was defined by a urine osmolality ≥ 800 mOsm/kg. An earlier study in the UK found 59.4% of people to drink only one serving of water per day and 81% to drink up to two servings per day (Jequier et al, 2010). Figures from the European Federation of Bottled waters show that this lags far behind other European countries as shown below.



http://www.efbw.org

In my own small survey of n= 166, I found more than 50% of the population was dehydrated. This data mirrored the study by the National Hydration Council (http://www.naturalhydrationcouncil.org.uk/press/water-consumption-rises-as-brits-more-health-aware/)



When the medical community refers to hydration, it is referring to adequate hydration with water as a 'nutrient,' which can be obtained from food just as much as fluids (including water) that are drunk. In terms of a recommended intake this is impossible to define as it is affected by an individual's gender, body mass, age, physical activity and the particular climate in which they live In Europe the European Food Safety Authority suggests a 2.5 L intake of water for

men and 2.0 L for women every day through a combination of food (accounting for 20-30% of the intake) and fluid (accounting for 70-80% of the intake).

The Kantar World Panel Food and Drink Usage panel is a single source purchase and consumption panel comprising of 11,000 individuals recording their total intake of food and drink (both tap and bottled water) for four one-week periods every year. This data has shown a 17 % increase in consumption (from 6 billion to 7.1 billion servings of tap water) in 2015. The intake of bottled water has increased by 9 % and tea and coffee remain the most popular drinks in the UK with the equivalent of 312 servings per person in the UK in 2015 (https://www.kantarworldpanel.com/global). The statistics also show that in the 20 years from 1995 decisions on drinks consumption has doubled from 11% that were motivated by health considerations, the top 4 choices being dairy drinks, fruit juices, tap and bottled water (https://www.kantarworldpanel.com/global).

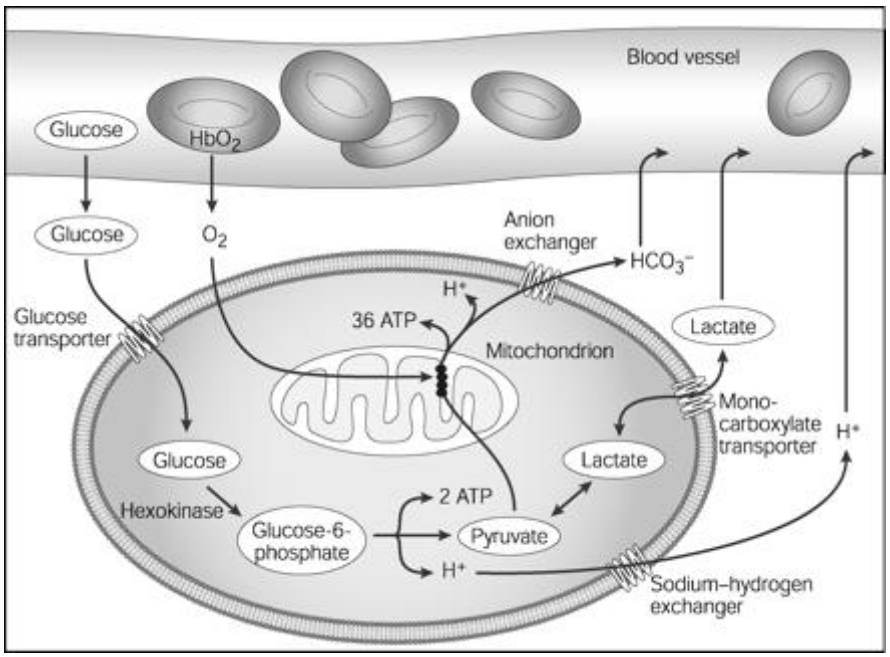
Dehydration occurs when as little as 1-2% of body mass is lost (for example 0.7 kg of body weight for a 70 kg person) (Kleiner, 1999) and can lead to a host of diseases, including kidney stones, cancers of the colon, breast and urinary tract (Michaud et al, 1999).

Dehydration causes an increase in plasma osmolality. In terms of plasma MVs measured as was performed in this study, plasma osmolality (or osmolarity) was not considered. This may be important in measuring MVs, which may in turn be isolated and screened for miRNA as early diagnostic markers of cancer, or simply as I propose as markers of general health and wellbeing. In terms of MVs being released from cells under stress, members of the Cellular and Molecular Immunology Research Centre (CMIRC) have previously shown MVs to package calcium (Stratton et al, 2015). The effect that dehydration and a decrease in levels of intracellular water may have on the capacity of cells to release microvesicles is also hitherto unknown.

Water is a universal buffer and as recently reviewed (Brown et al, 2012), the kidneys play a major role in maintaining a control over body fluid and balance

of acid-base. This homeostasis is necessary for human health and aquaporins (Agre, 2006), specifically aquaporin 2 (AQP2) plays an important role in this process. Water is needed for every function in the body, including digestion, circulation, absorption of nutrients and to enable the body to flush out toxins and in a state of dehydration, results in a diminished immune system and a myriad of physical problems (Popkin et al, 2010), indeed adequate hydration reducing risk of bowel, breast prostate and urinary tract cancers as well as of kidney stones (39% reduction) and of coronary heart disease (46% in men and 59% in women) (www.rch.org).

The so-called 'Warburg effect' describes how malignant tumours develop an acidic extracellular pH by the accumulation of lactic acid, a hallmark of tumour cells, and alkaline intracellular pH (Garber, 2004). It may be of key importance given the increasing role exosomes and MVs have been shown to play in tumour growth and spread, especially as it was recently shown that low pH can increase exosome release (as well as uptake (Parolini et al 2009; Federicci et al 2010)). This may be due to the increase of intracellular calcium (Kato et al, 2007), which is known to increase exosome and MV release (Inal et al, 2012).



Aerobic glycolysis as first described by Otto Warburg (Gatenby, 2004: Garber, 2004).

Solid tumours thus having an acidic pH because of increased fermentation metabolism, result in increased invasion and metastasis (Stetler-Stevenson et al, 1993) probably due to H^+ diffusing into the neighbouring tumour microenvironment, causing tissue remodelling and thus allowing metastasis. Tumour invasion does not occur in areas with normal pH. (Estrella et al, 2013).

Previous work has shown the importance of pH in the tumour microenvironment and its baring on tumour growth and metastasis. This work has shown that injection of alkaline buffers into the tumour microenvironment can shrink a tumour and inhibit metastasis (Robey et al, 2009; Silva et al., 2009). More recently it also was found that tumour cells undergo enhanced proliferation and are increasingly invasive in acidic environments (Estrella et al., 2013). There has recently been a massive promotion of alkaline water and of the health benefits of an alkaline diet, with barely any research to prove this hypothesis either way. Last year a comprehensive review of the scientific literature showed no evidence in support or to refute the claimed benefits of alkaline water for treating cancer or of acidic diet promoting cancer (Fenton et al, 2016), so this should be an important aim for future clinical studies within the context of acid-base homeostasis.

6.6.2 Aloe Vera, cannabinol and bromelain: plant products for MV delivery in cancer

Aloe-emodin (AE) is the main component of the anthraquinones found in Aloe Vera. There are numerous studies showing AE to have apoptotic and anti-proliferative effects on tumour cells as well as in a host of other disease conditions (Radha et al, 2014). In view of the finding in this thesis that Beta-glycyrrhetinic acid, derived from liquorice, in microvesicles has anti-proliferative properties on erythroleukaemia cells, it is interesting to note that other plant products such as AE have also been used within lipid nanoparticles (Chen et al, 2015; Wu et al, 2017). In other work, cannabinoids have been used successfully against cancer cells inhibiting proliferation and angiogenesis (Thapa et al, 2011) but also against melanoma cells (Armstrong et al, 2015) and

have even been encapsulated in so-called microparticles which in fact are biodegradable polymeric microparticles prepared using a solvent evaporation technique on an oil-in-water emulsion (Hernán Pérez de la Ossa et al, 2013). Bromelain, from the stems and fruits of pineapple (*Ananas cosmosus*) is another potential plant product with anti-cancer properties for example being synergistically active in conjunction with cisplatin against MDA-MBA-231 breast cancer cells (Pauzi et al, 2017). This has been used in bromelain-functionalized multiple-wall lipid-core nanocapsules (Oliveira et al, 2017) and that could also be delivered potentially via microvesicles or artificially synthesized microparticles. It should be noted that since the work presented in this thesis it has become apparent that cancer drugs can be loaded directly into microvesicles (Saari et al, 2015) bypassing the need to collect microvesicles carrying a drug that has been released from parent cells themselves loaded with chemotherapeutic drugs.

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