Microparticles isolated from multiple myeloma patients are indicative of tumor burden, disease progression and treatment unresponsiveness

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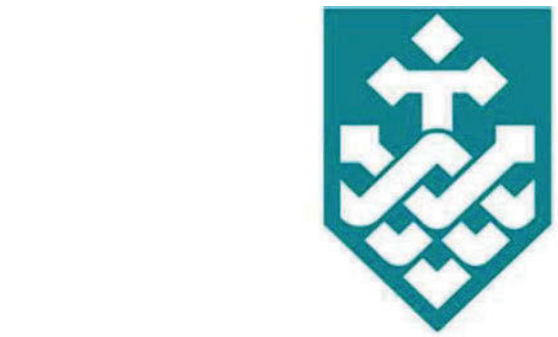

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Sabna Rajeev Krishnan certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Sabna Rajeev Krishnan
Date:

Dedication

"The tears break out ceaselessly from my red-veined eyes, for my love has become an unendurable torment. Yet who is there to listen to my pain?!
No voice of sympathy, but only the cruel melody of the soft, tinkling bell around the bull's neck, as again and again, it shakes off the buzzing fly - In this winter's midnight, with its rain and wind and scattering spray."
-Sangham Period Poetry (3rd Century BC – 4th Century AD)

*When a life departs, a **new living** is conceived….*

Perhaps, completely analogical, with all the geometrical measures and the same enigmatic intricacies, completely beyond the mediators..

And with a gestation; seemingly very relative.

The baby eyes…. same as the auspicious …reluctant to the brightness and the sobs which find solace in only the closest known warmth, the chosen ones….

A life where literal words or norms are just abstract and mere meaningless.. all too similar …

A life that feeds and dictate over the chosen warmth

A life that is vulnerable to think beyond its own needs, its own realm

A life that is too naïve to not yearn the world to stop and fix the chaos

Again all too similar…

*Yet, so distinct.. being born to a thick silence…unlike the auspicious one.. or a heart-wrenching scream of being forlorn… so distinct with the **'look backs'** and **resent** of the origin!*

*Too isolated with the urge to run back in time and pause it **before** the inevitable..*

Wishing to see, feel and absorb like before, the delineation, the warmth and the sheer joy of being the one and only one..

And there I am, this melancholic child, suspended in the continuum… stunted and deficient…breathing only through the riveting answers such as this…..

You taught me how important is to be a better human being each passing day…. That compassion as well empathy is mere way of life to align one on that very path. You left reinstating the same spirit and I witness the numerable lives you touched in your incredible journey. I yearn to live up to your unfaltering stand on any matter.

You are my reason, where I learned everything if not all..

You seeing this meant everything to me…

This is for you acha…

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Multiple myeloma patients of NSW Health Pathology and healthy volunteers: Huge thanks for your consent for this study. This is a footstep each of you leaves behind and I hope this leads to many more.

Ami, Arun Prasanth, Arun Anand thank you for keeping a much nicer version of myself within you!. Aj, your words may find my pieces again, magician!. My teachers, especially Dr. M Madhavikutty, who believed that I am capable of something special, may be this is a piece of that cake!, Thank you!.

My family, Jeev and Rishi, you both define me and this has been an incredible journey for ***US***, ***THANK YOU!.*** Amma - I stay inspired by your selflessness, incessant motivation, far sight and service driven life. Hope this brings a smile in this most difficult time of our life. Acha.. You don't have this just because you never went by the conventions and ***this is yours***…. ***TRULY*** I shall just hold it for you…

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List of Abbreviations

°C	Degree celcius
ABC transporters	adenosine triphosptase binding cluster transporters
ASCT	Autologous stem cell transplant
B2M	beta 2 microglobulin
BCRP	breast cancer resistance protein
BM	Bone marrow
BM-MSC	bone marrow-mesenchymal stromal cells
BMSC	bone marrow stromal cells
CAM-DR	cell adhesion mediated drug resistance
CD	cluster of differenciation
CpG	5'—C—phosphate—G—3'
CR	complete remission
CRP	C-reactive protein
CS	chondroitin sulphate
CyBorD	cyclophosphamide, bortezomib, dexamethasone
D-PACE	Dexamethasone along with platinol, adriamycin, cyclophosphamid
DSS	Durie -Salmon Staging
ECL	enhanced chemoluminescence
ECM	extra cellular matrix
ECM	extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
GST	glutathione S transferases
HGF	hepatocyte growth factor
HMDS	hexamethyldisilazane

hr	hour
HS	heparan sulphate
HUVEC	human umbilical cord endothelial cells
ICTP	type 1 carboxy terminal cross-linked telopeptide
IGF1	insulin like growth factor 1
IgH	immunoglobulin heavy chain
IL21	interleukin 21
IL6	interleukin -6
iMiDs	immunomodulatory drugs/agents
IMWG	International myeloma working group
ISS	International staging system
JAK/STAT3	Janus kinase/signal transducer and activation of transcription 3
LCDD	light chain deposition disease
LDH	lactate dehydrogenase
LRP	lung resistance related protein
M proteins	monoclonal proteins
MAFB	*MAF* oncogene homolog B
MAPK	mitogen-activated-protein-kinase
MCF-7	Michigan Cancer Foundation-7
MCF-7/Dx	Michigan Cancer Foundation-7/doxorubicin resistant
MCP-1	monocyte chemoattractant protein
MDR	multidrug resistance
MGUS	Monoclonal Gammopathy of Undetermined Significance
min	Minute
mir	microRNAS

MM	Multiple myeloma
MMPs	matrix metalloproteases
MPs	microparticles
MRD	minimal residual disease
MRP1	multidrug resistance protein 1
MVP	major vault protein
N-CAM	neural adhesion molecule
NBD	nucleotide binding domain
NF kB	nuclear factor kB
P-gp	Permeability glycoprotein
P13/AKT	phosphatidyl inositol 3 kinase/protein kinase B
PARP	poly- (ADP-ribose) polymerase
PARP	Poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PCAP	P300-CBP-associated factor
PCs	Plasma cells
PD	progressive disease
PE	phosphatidylethanolamine
PEI	Polyethylenimine
PFP	platelet free plasma
PICP	type 1 carboxy terminal propeptide
PR	partial remission
PS	phosphatidyl serine
PYK2	proline-rich tyrosine kinase 2
RANK	receptor activator of nuclear factor kB

RANKL	receptor activator of nuclear factor kB ligand
RNAs	ribonucleic acids
ROCK	Rho-associated, coiled-coil containing protein kinase’
S-IL6-R	soluble interleukin 6 receptor
SDF-1	stromal cell derived factor 1
SEM	scanning electron microscopy
siRNA	short interfering RNA
SM	sphingomyelin
SNP	single nucleotide polymorphism
TF	Tissue factor
TIMP-3	tissue inhibitor of metalloproteinase-3
TK	thymidine kinase
TMD	trans memrane domain
TNF-α	tumor necrosis factor-alpha
Topo II	topoisomerase II
TRAF3	tumour necrosis factor-receptor associated factor 3
uPA	urokinase type plasminogen activator
VAD	vincristine adriamycin and dexamethasone
VCAM 1	vascular cell adhesion molecule
VDJ	variable diversity joining
VEGF	vascular endothelial growth factor
VLA-4	Very late antigen-4

Publications and Provisional Patent

Rajeev Krishnan, S., Luk. F., Brown RD., Suen H., Kwan YL and Bebawy M (2016) Isolation of human $CD138^{+}$ microparticles from the plasma of patients with multiple myeloma patients. Neoplasia

Rajeev Krishnan, S., Luk. F., Jaiswal R., Brown RD and Bebawy M (2016) Multiple myeloma and persistence of drug resistance in the age of novel drugs. International Journal of Oncology

Rajeev Krishnan, S and Bebawy M Systemic signatures in multiple myeloma and their role in clinical management- under embargo

Rajeev Krishnan, S and Bebawy M Protocols used in the isolation, identification, validation and phenotyping of microparticles for use in clinical analysis- under embargo

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This thesis contains commercially sensitive information, which is granted a provisional patent **(UTS disclosure number-DISC-2016-029)**. Executed non-disclosure agreements are provided in the appendix.

Other publications

Ritu Jaiswal, Michael S. Johnson, Deep Pokharel, Rajeev Krishnan S, and Mary Bebawy Microparticles shed from multidrug resistant breast cancer cells provide a parallel survival pathway through immune evasion – (in press) BMC cancer journal.

Conferences Presentations

Conference proceedings publications

Rajeev Krishnan S, Luk F, Brown RD, Suen H, Kwan YL, Bebawy M. Microparticles as novel prognostic markers in Multiple Myeloma [abstract].In: proceedings of 2015 annual meeting of American Association of Cancer Research; 2015 April 18-22 – 23; Philadelphia (PA):AACR;2015.Abstract nr 5306

Multiple myeloma: A novel tailor-made therapeutic management [abstract].In: proceedings of American Association of Cancer Research, Special Conference on Hematologic Malignancies: Translating Discoveries to Novel Therapies; 2014 Sept 20 – 23; Philadelphia (PA):AACR;2014.Abstract nr B45

Rajeev Krishnan S, Luk F, Brown RD, Kwan YL, Bebawy M.A novel personalised therapeutic management in multiple myeloma [abstract].In: proceedings of American Association of Cancer Research, Drug Sensitivity and Resistance: Improving Cancer Therapy Conference; 2014 June 18-21; Orlando (FL):AACR;2014.Abstract nr B52

Oral Presentations

Rajeev Krishnan S, Luk F, Brown RD, Kwan YL, Bebawy M. ***"A Novel Pathway and Therapeutic Strategy for the Treatment and Prognosis of Multidrug Resistant Cancers"*** Cancer Drug Discovery& Preclinical development, Wednesday 17 September 2014 - Thursday 18 September 2014, London, UK.

Rajeev Krishnan S, Luk F, Brown RD, Kwan YL, Bebawy M. *"Multiple Myeloma-Microparticles as novel prognostic markers"* Australasia Extracellular Vesicles Conference, Cairns, Pullman Cairns International, November 20 & 21st 2014.

Rajeev Krishnan S, Luk F, Brown RD, Kwan YL, Bebawy M. *"Multiple Myeloma-Microparticles as novel prognostic markers"* **Research in Progress Meeting**, **Auditorium, research and education centre, St George Hospital, Ground floor, 4 – 10 South St, Kogarah Sydney, November 10th, 2016**

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Poster Presentations

Rajeev Krishnan S, Luk F, Brown RD, Kwan YL, Bebawy M. ***"Personalized Therapeutic Management in Multiple Myeloma"*** International Society for Extracellular Vesicles - April 17-20, 2013, Park Plaza Hotel, Boston, MA, USA

American Association of Cancer Research - June 18-21, 2014, Hyatt Grand Cypress, Orlando, FL, USA Rajeev Krishnan S, Luk F, Brown RD, Kwan YL, Bebawy M. ***"A Novel Personalized Therapeutic Management in Multiple Myeloma"*** American Association of Cancer Research - June 18-21, 2014, Hyatt Grand Cypress, Orlando, FL, USA.

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Australian Society of Biophysics: Membrane Transporter Satellite Meeting: Membrane Transporters & Channels & Their Role In Human Disease -6th December 2012, University of New South Wales, Sydney, AUS

Workshops

Uniquest Research commercialisation Workshop- 5&6 June, 2012, Radisson resort, Goldcoast, Australia.

Successful Innovation Workshop, 5th & 6th September 2012, National Innovation centre, Australian Technology Park, Eveleigh, Sydney, Australia.

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Awards and Scholarships

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Abstract

Multiple Myeloma **(**MM) is a progressive malignancy of bone-marrow plasma cells. Treatment typically involves combination chemotherapy, which forms part of a continuing cycle of treatment, remission and relapse corresponding to the evolution multiple drug resistance (MDR). There are currently no procedures available that allow for a direct, non-invasive, real time monitoring of the development of MDR in MM. Although bone marrow biopsy can directly test for the presence MDR markers on malignant plasma cells, this procedure is highly invasive, does not allow for routine assessment and fails to capture the patchy, multi-site tumor infiltrates characteristic of MM. An ideal test would directly measure markers of MDR expressed in MM cells during routine follow up, be non-invasive and representative of multi-site tumors as well allow for simultaneous comparative analysis of tumor burden.

Microparticles (MPs) are 0.1- to 1.0-μm membrane vesicles, and contain the cellular substances of their originating cell. Microparticles, are spontaneously shed from tumor cells; they carry resistance proteins and nucleic acids from their originating cell; and (iii) can confer MDR within cancer cell populations. The overarching aim of this study was to investigate the prognostic potential of MPs in MM patients. For this purpose, we characterized the morphology, phenotype and quantitated the level of non-platelet derived MPs in the peripheral blood of MM patients across all clinical states and healthy volunteers after informed consent**.** MPs were isolated from patient blood samples by ultracentrifugation and phenotyped for the presence of the plasma cell marker CD138, the MDR protein P-glycoprotein (P-gp), the stem cell marker, CD34 and for phosphatidylserine (PS) exposure and quantitated using BD TruCount^{TM} beads.

We observed significantly greater levels of total MP and $CD138^+$ MP counts in MM patients relative to healthy volunteers. The levels of these MPs were shown to correspond to tumor burden in MM patients. We also detected the presence of P-gp on MPs isolated from MM patients. Specifically, we identified a number of MP subtypes including a 'dual positive' ($CD138^-$ $CD34^+$ $P\text{-}gp^+$) population, the levels of which corresponded to aggressive and active disease (N=1). We also identified an evolving shift in the dominance of MP subtypes with disease progression. This research describes a simple blood test where by the presence of MDR can be serially monitored through 'liquid biopsy'. This thesis introduces new insights into the utility of biomarkers and the molecular mechanisms contributing to disease progression, MDR and treatment failure in MM.

Chapter 1

Multiple myeloma and Persistence of Drug Resistance in the Age of Novel Drugs (Review)

Multiple myeloma and persistence of drug resistance in the age of novel drugs (Review)

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Abstract. Multiple myeloma (MM) is a mature B cell neoplasm that results in multi-organ failure. The median age of onset, diverse clinical manifestations, heterogeneous survival rate, clonal evolution, intrinsic and acquired drug resistance have impact on the therapeutic management of the disease. Specifically, the emergence of multidrug resistance (MDR) during the course of treatment contributes significantly to treatment failure. The introduction of the immunomodulatory agents and proteasome inhibitors has seen an increase in overall patient survival, however, for the majority of patients, relapse remains inevitable with evidence that these agents, like the conventional chemotherapeutics are also subject to the development of MDR. Clinical management of patients with MM is currently compromised by lack of a suitable procedure to monitor the development of clinical drug resistance in individual patients. The current MM prognostic measures fail to pick the clonotypic tumor cells overexpressing drug efflux pumps, and invasive biopsy is insufficient in detecting sporadic tumors in the skeletal system. This review summarizes the challenges associated with treating the complex disease spectrum of myeloma, with an emphasis on the role of deleterious multidrug resistant clones orchestrating relapse.

Contents

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Abbreviations: MM, multiple myeloma; BM, bone marrow; PCs, plasma cells; M proteins, monoclonal proteins; IMiDs, immunomodulatory agents; ABC transporters, adenosine triphosphate-binding cluster transporters

Key words: multiple myeloma, plasma cells, combination therapy, multidrug resistant clones, ABC transporters, relapse, personalized management

1. Introduction

Multiple myeloma (MM) is the second most prevalent hematological malignancy worldwide, with a median onset of 60 years of age (1-6). MM is currently incurable, albeit clinically manageable and typically manifests with an accumulation of terminally differentiated monoclonal plasma cells (PCs) in the bone marrow (3). It is distinguished from solitary plasmacytoma by the presence of aberrant PCs at numerous skeletal sites (7,8).

MM can be 'secretory' or 'non-secretory' depending on the serum/urine levels of secreted monoclonal immunoglobulin. 'Secretory MM' is characterized by the presence of abnormal levels of monoclonal proteins (M-protein) or paraproteins in circulation and urine. 'Non-secretory' MM accounts for 1% of all MM cases and lacks the hallmark of increased serum or urine M-protein or paraprotein. Consequently, the diagnosis of non-secretory MM depends rather on an increase in tumor burden and evidence of end organ damage (9,10). The complex spectrum of physiological impairment attributed to MM include lytic bone lesions, osteoporosis, compression fractures, bone pain and ultimately patient immobility. The abundance of malignant monoclonal PCs also severely compromises patient immunity and hematopoiesis (11).

The inclusion of immunomodulatory drugs (IMiDs) as part of high dose chemotherapy together with systemic and cytogenetic prognostic markers have improved patient survival in MM. Thalidomide, and its derivatives are currently approved for use across all phases of MM therapy. These drugs possess immunomodulatory, anti-angiogenic, anti-inflammatory and anti-proliferative capacity (12). Over the past few decades, a

30-40% complete response rate and an increase in median survival of 4-5 years have been achieved with these drugs in combination with auto-transplants in younger *de novo* patients (13).

Most MM patients respond successfully to initial induction therapy, however, all the patients eventually relapse, forcing a review of the treatment regimen (14). A significant contributor to treatment failure leading to clinical relapse is the emergence of multi-drug resistance (MDR) (15). MDR is the phenomenon whereby the cancer cells become resistant to a wide variety of structurally and functionally unrelated drugs following exposure to a single chemotherapeutic agent (16-18). Existing measures for assessing the clinical state of MM patients include serum markers [immunoglobulins, β_2-microglobulin (B_2M), free light chain assays, creatinine, C-reactive protein (CRP) and thymidine kinase] followed by confirmation with invasive bone marrow biopsy. However, these do not offer a direct measurement of the presence or the evolution of proteins responsible for drug resistance on malignant PCs. MM is characterized by the presence of multiple clones with differing degrees of drug sensitivity at the time of diagnosis. Consequently, despite complex chemotherapeutic regimes (19), therapeutic response is unpredictable and extremely variable with MM patients. Furthermore, bone marrow biopsy cannot assess the patchy tumor infiltrates in multiple sites associated with MM and provides an indirect measure of tumor burden distributed throughout the skeletal system. This impacts the quality of life for the patient and translates to highly heterogeneous patient survival rates ranging from a few weeks to more than 10 years (20).

Aside from the significant physical and emotional costs associated with the emergence of MDR and subsequent relapse, there are also significant financial costs incurred with the management of MDR. The drugs used at relapse are typically novel, costly and with associated side effects. The estimated cost of an effective melphalan, prednisone and velcade regimen approximates $119,102 (US), while a novel superior regimen utilizing melphalan and prednisone combined with lenalidomide maintenance can reach as high as $248,358 (US) (21). Consequently, MM remains one of the most costly cancers to treat when total treatment costs are considered (21-24).

Here, we review the factors limiting the successful treatment outcome in the complex multiple myeloma clinical setting. We focus on the persistent issue of drug resistant clones in MM and the major role played by ATP-binding cassette (ABC) transporters along with other resistance mechanisms in relapse in the era of novel therapeutics.

2. Normal plasma cell characteristics

MM is a hematological malignancy characterized by the accumulation of aberrant PCs in the bone marrow (25). PCs are terminally differentiated activated B cells retained in the G1 phase of the cell cycle (26). PCs express surface markers that are reflective of their elaborate maturation and differentiation process. PCs typically can be distinguished from naïve B cells by the lack of CD10, CD19 and CD20 expression on their surface (27). Two specific surface antigens on PCs are CD38 and CD138 (28-31). CD38 is an ectoenzyme important in signal transduction, cell adhesion and calcium signaling, and is expressed across all PC developmental stages (28). CD138 is a *trans* membrane proteoglycan that facilitates cell binding, cell signaling, cell-cell and cell-extracellular matrix interactions (32). Amongst the typical markers expressed on PCs, CD45 is considered an early PC marker (plasma blasts) (27). According to the maturation stages, PCs are grouped into plasma blasts ($CD138^-$ $CD45^{++}$), early PC's ($CD138^+$ $CD45^+$) and mature PC's ($CD138^{++}$ $CD45^-$ or weak CD45 expression) based on the antigen expression on their surface (5).

PCs are prime mediators of the adaptive immune response (5,26). The development of a normal B cell starts in the bone marrow (BM) and matures following migration into the peripheral lymphatic organs. The maturation process is aided by antigen exposure, dendritic co-stimulatory signals and somatic mutations that ultimately result in high affinity antibody production (3). B cells with high affinity antigen receptors further differentiate into memory cells and plasma blasts. Eventually, highly efficient PCs that survive these processes (long-lived PCs) migrate back to the bone marrow and localize in 'niches', which aid in the further differentiation and longevity of the immune response (3,33-35).

3. Pre-malignant plasma cell characteristics - monoclonal gammopathy of undetermined significance

Monoclonal Gammopathy of Undetermined Significance (MGUS) is a benign condition that can precede malignant transformation to MM (36). Clinically, MGUS is characterized by excessive PC growth whilst retaining a stable M-protein profile (37). Serum M-protein levels of <3g/dl, small amounts of monoclonal light chains in urine, the absence of end organ damage, absence of lytic bone lesions, anemia and hypocalcaemia define the pre-malignant condition MGUS (38). The rate of transition from MGUS to MM is ~1%/year (36,38).

4. Malignant plasma cell characteristics

The exact cause of malignant transformation of PCs remains unknown. However, ras mutations are absent in pre- malignant MGUS and are observed in MM (39). It has been suggested that the myeloma clone arises from a pre-switched B cell (40), preconditioned as a result of prior exposure to certain triggers (i.e. viruses, chemicals and radiation). Other reasons proposed are an incompetent immune system, age and a family history of lymphato-hematopoietic cancer (36).

In malignant cells, the genotype is aberrant with frequent chromosomal deletions or hyperdiploidy (chromosomes 3,5,7,9,11,15,19 and 21) that results in abnormal functions of cell cycle regulatory genes (*cyclin D1*, *D2* and *D3*) (41). Malignant PCs also present with aberrant phenotypes at diagnosis. Surface markers such as CD56, CD117 and CD20 are found in decreasing order of expression on aberrant PCs. Isolated strong CD56 expression is common in MM and can be used to distinguish MM from MGUS, while $CD56^-$ phenotype is said to be associated with a high risk subtype with chromosomal abnormality [t(11;14)] in terms of survival (42-44). Malignant PCs also display an increased expression of various adhesion molecules compared to non-malignant PCs. Fibronectin receptor, very late antigen 4 (VLA-4), the lympho-

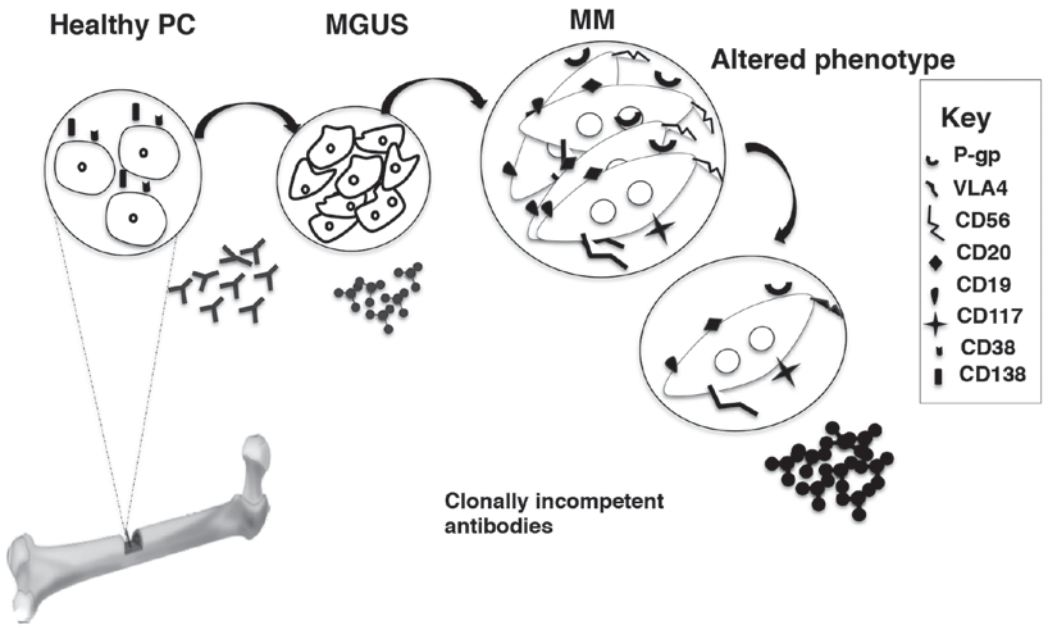

Figure 1. Malignant transformation of PCs: terminally differentiated healthy PCs reside in the bone marrow and typically express CD138 and CD38. Healthy PCs are major components of humoral response. The pre-malignant condition monoclonal gammopathy of undetermined significance (MGUS) is characterized by the presence serum M-protein and monoclonal light chains in serum and/or urine, however, patients remain well. In the malignant phase, PCs have aneuploidy, altered surface expression and patients experience high serum/urine levels of M or paraproteins along with other classic MM manifestations. In relapse, the MM initiating cells or 'side population' cells have immature B cell phenotype than the mature PCs. Major signaling pathways are aberrant resulting in the clonotypic MM cells. Clonotypic MM cells are highly proliferative and over express various MDR pumps (P-gp, MRP1 and BCRP) and early B cell marker on their surface and transporters such as vault proteins (LRP).

cyte homing receptor CD44 and neural cell adhesion molecule (N-CAM and CD56) are abundantly expressed on malignant PCs (45). In contrast, VLA-5, the laminin receptor VLA-6, and the vitronectin receptor CD51 are weakly expressed (45). In advanced MM, mature PCs escape the bone marrow niche and are found in circulation (46). Interestingly, only 50% of the circulating population expresses CD138, which is widely considered to be an exclusive mature PC marker of hematopoietic origin (27,47). Consequently, circulating PCs are also classified according to the presence or absence of CD138 apart from the maturation based classification of normal PCs mentioned above (27). The circulating $CD138^-$ PCs are thought to be plasma blasts as they express CD45, CD20, CD19 and human leukocyte antigen (HLA)-class II and more actively proliferating (5,47,48) (Fig. 1).

5. Multiple myeloma

Diagnosis. MM is diagnosed when M-protein or paraprotein exceeds 3 g/dl in serum or urine (49), when there are 10-15% aberrant PCs in the bone marrow and by the presence of skeletal lesions (5,37,50). An abnormal ratio of serum κ and λ free light chains above the normal range of κ/λ of 0.26-1.65 provides an alternative criterion if the M-protein status is not conclusive (9,10,50). In the case of 'non-secretory myeloma', ≥10% baseline clonal bone marrow PC provides the main criterion of diagnosis with renal and skeletal manifestations of MM (9,50,51).

Other clinical manifestations alone or in combination are also considered at diagnosis. These include elevated calcium levels or hypercalcemia >11.5 mg/dl/>2.65 mmol/l indicating defective bone physiology, renal insufficiency signified by creatinine >2 mg/dl/177 μmol/l or more, anemic hemoglobin levels of <10 g/dl or 2 g/dl < normal levels or hemoglobin <12.5 mmol/l or 1.25 mmol/l < normal levels, and bone lesions or pain (9). International uniform response criteria by International Myeloma Working Group recommends that amyloidosis and/or systemic light chain deposition disease (LCDD) should be correspondingly categorized as 'myeloma with documented amyloidosis' or 'myeloma with documented LCDD', requiring confirmation through bone marrow biopsy to ascertain the existence of ≥30% PCs and/or myeloma-related bone disease. Following diagnosis, MM patients are usually placed on induction therapy with conventional or novel agents followed by autologous stem cell transplant depending on eligibility of each patient (52). Response to treatment is subsequently evaluated through regular monitoring of serum and urine M-protein levels by immune fixation and confirmed by periodic bone marrow aspiration (53).

Staging criteria. MM is a highly heterogeneous disease with respect to survival and clinical manifestations (54), hence it is difficult to accommodate every criterion in one staging system (55). In 2005, the International Myeloma Working Group established the International Staging System (ISS) for MM (Table I) (54). Until 2005, MM staging predominantly relied on the Durie-Salmon Staging (DSS) system, which was established in 1975 (56). DSS correlates various biochemical factors with tumor burden for staging of malignancy. This makes it difficult to achieve consensus across various laboratories (57). The advantage of ISS is that it is a statistical model, which emphasizes the duration of survival based on

the measure of two parameters, B_2M and serum albumin (55). ISS uses B_2M as a measure of the rate of myeloma growth with serum albumin indicative of tumor burden (54,55,58). Since its launch, ISS has been validated, is statistically easier to assess and is more robust compared to the DSS system (55,59).

Genotype and multiple myeloma. Myeloma, unlike other hematological malignancies, is uniquely characterized by intricate cytogenetic and molecular genetic abnormalities resonant of epithelial tumors (60). A *de novo* patient usually presents hyperdiploid with multiple trisomies or hypodiploid with one of several types of immunoglobulin heavy chain (IgH) translocations (61). The importance of cytogenetic markers and gene profiling on therapeutic decision making is becoming increasingly evident in MM (62).

Chromosomal abnormalities associated with immunoglobulin heavy chain translocations result in abnormal gene regulation in MM (63). Cell cycle regulatory genes are impaired in MM and the dysregulation of *cyclin D1*, *D2* or *D3* is considered to be an initial oncogenic pathway in MM and MGUS (64). 25% of IgH translocations in MM directly affect *cyclin D1* (11q13), *cyclin D2* t(4;14), *cyclin D3* (6p21) or musculoaponeurotic fibrosarcoma (*MAF*) oncogene (c-*MAF*, 16q23 or *MAF* oncogene homolog B (MAFB), 20q11 (41,64). The recurrent translocations associated with MM are t(4;14)(p16;q32), and t(14;16) (q32;q23) which are correlated with a negative prognosis (61). Myeloma patients frequently present with chromosomal deletions of 13q14 and 17p13 (63). Several other genetic components such as tumor suppressor genes (p53, phosphatase and tension homolog-PTEN), retinoblastoma protein-Rb protein) and transcription factor, myelocytomatosis viral oncogene homolog (*c-myc*) also show abnormalities in MM, however, the exact origin of these genetic and epigenetic changes in the course of MM pathogenesis is not known yet (39).

Recently, the role of short non-coding RNAs (19-25 bp) in MM has been examined (65). A small number of microRNAs (miRNAs) are implicated in MM pathogenesis (65). Pichiorri *et al* demonstrated distinct miRNA profiles for malignant PCs (MGUS and MM) compared to those of normal PCs. In MGUS, *miR-21* and *miR-106b~25* clusters with oncogenic function are upregulated with *miR-21* blocking apoptosis (66). *miR-106b~25* has been shown to regulate pro-apoptotic genes and play a role in pathogenesis (67). It is believed that *miR-21* and *miR-106b~25* potentially initiate the lymphoproliferative transformation of PCs by hindering apoptosis, promoting survival of malignant cells and predisposing to secondary genetic abnormalities, leading to malignancy (65). Compared to the normal PCs, *miR-32*, *miR-17~92*, *miR-21*, *miR-106~2*, *miR-181a* and *miR-181b* are upregulated in MM. *miR-15a* and *miR-16-1* are implicated in regulating tumor proliferation in MM that are located in 13q14.3 which coincides with a frequent deletion in MGUS and MM cohort (65,68,69).

Disease presentation

Systemic monoclonal protein (M-protein or paraprotein). Monoclonal protein (M-protein or paraprotein) production, is a salient feature of secretory MM (70). Based on immunoglobulin heavy chain structure, MM is classified into IgG, IgD and IgE subtypes of which IgG MM is most common (11).

Paraproteinemia and an associated hyperviscosity syndrome, arising from elevated systemic M-protein levels are typically associated with MM (11,71). Approximately 25% of MM patients present with paraproteinuria resulting in renal insufficiency, while ~50% have renal failure (11,37,50,72) resulting from direct damage and blockage to the kidney (73). Other MM associated renal complications include, myeloma cast nephropathy, amyloidosis, fibrillary glomerulonephritis, immunotactoid glomerular nephritis and light chain deposition disease (72).

Immune incapacity. MM patients are immunocompromized due to the defective hematopoiesis and the aberrant PCs producing clonally incompetent M-proteins. This is in addition to the gradual reduction in immune competence coinciding with late middle age (40). Yaccoby *et al* proposed limited mobility in the aged population resulting in reduced exposure to antigens as the potential reason for the reduced differentiation rate of the memory B-lymphocytes to PCs (74,75).

The manipulative tumor cells strategically elude the immune watch and facilitate tumor survival. One such mechanism is the phenomenon of 'trogocytosis' in which the surface antigen exchange occurs in lymphocytes creating unique cell phenotypes with specific function (76). The immune synapse facilitates unique cell types to maintain intracellular signaling in T cell subsets and aid in tumor-induced immune suppression (77). The phenomenon of trogocytosis is more common in MM compared to other mature B cell malignancies and T cells are more proficient in acquiring antigens from malignant PCs (78). Impaired immune system in MM patients also leads to recurrent infections with a life-changing impact on patients and care givers (79).

Microenvironment-dependent disease manifestations. One of the characteristic features of MM is the tendency of aberrant PCs to be confined to the bone marrow. Malignant PCs favor a microenvironment analogous to normal long-lived PCs (3,74,75) and tend to migrate to peripheral blood only in the terminal stage of the disease (3,45,74,75). These malignant PCs evolve 'autocrine growth supporting loops' at this terminal stage which facilitate microenvironment independent survival (35). The adhesion of MM cell with bone marrow stromal cell orchestrates homing via adhesion to the endothelium, invasion through the sub-endothelial membrane, and chemotactic migration within the bone marrow stroma (35,45) (Fig. 2).

Aberrant PC interaction with bone marrow stromal cells (BMSCs) and extra cellular matrix (ECM), subsequently alter the normal microenvironment to tumor advantage (80,81). Cytokines such as interleukin 6 (IL6), vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), insulin-like growth factor 1 (IGF1) support the growth of MM cells (82,83). Along with IL6 and IGF1, IL21 promote the tumor survival while VEGF plays a role in MM cell migration with stromal cell derived factor-1α (SDF-1α) (84-87). The initial binding between MM cells and bone marrow stromal cells is mediated via adhesion receptor integrins (integrin α4β1, VLA4), through their ligands [vascular cell adhesion molecule 1 (VCAM1)] (88,89). The binding, further, upregulates cytokine and/or chemokine release from

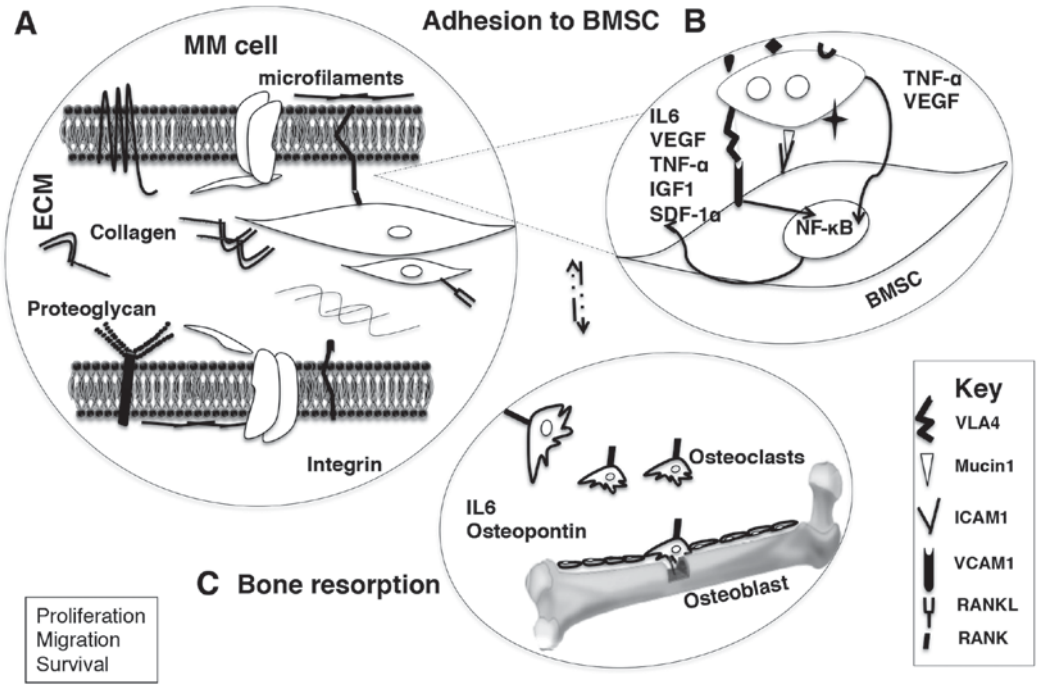

Figure 2. Microenvironment-dependent factors in MM: (A) Aberrant PCs homing to the microenvironment is mediated by integrin mediated adhesion to extracellular matrix (ECM) and bone marrow stromal cells (BMSCs). (B) The growth, survival and migration of an aberrant PC is cytokine mediated and facilitated by the adhesion of aberrant PC to the BMSC. In BMSCs, this contact triggers the cell signaling pathway and nuclear factor-κB and subsequent secretion of various adhesion molecules/cytokines on both cells. (C) Adhesion to the BMSC is also involved in the bone resorption. MM cells stimulate RANKL expression on BMSCs and reduce osteoprotegerin expression to promote osteoclastogenesis. Adhesion of MM cells to osteoclasts follows with the over production of osteopontin and IL6, supplementing MM cell growth and survival.

stromal cells to the microenvironment (Fig. 2A). In addition, the transcription factor nuclear factor-κB (NF-κB) plays a significant role in the initiation of various cell-signaling pathways in MM cell and BMSC following the adhesion (84,90). The adhesion of MM cell to stroma triggers NF-κB and mitogen-activated-protein kinase (MAPK) signaling cascade in BMSC, which in turn results in a change in phenotype of MM, and BMSC with co-expression of adhesion molecules. Subsequently, cytokines secreted from MM cells trigger inflammatory cytokine production and NF-κB activation in BMSC (IL6, TNF-α and VEGF). The inflammatory cytokines from BMSCs trigger signaling pathways in MM cells (MAPK, phosphatidyl inositol 3 kinase/protein kinase B (P13/AKT), Janus kinase/signal transducer and activation of transcription 3 (JAK/STAT3) pathways which enhance proliferation, cell cycle modulation and tumor survival via activation of antiapoptotic signals (91-93) (Fig. 2B).

Osteolytic lesions, compromise mobility, can result in spinal cord compression and moderate to severe nerve damage in MM. In fact, morbidity and mortality in MM is mostly associated with osteolytic lesions (80,81). Abe *et al* (81) demonstrated that peripheral blood mononuclear cell-derived osteoclasts enhance MM cell survival and growth in primary MM, as well as MM cell lines than stromal cells (75,80,81). Receptor activator of nuclear factor κB (RANK) on the surface of osteoclasts and the ligand (RANKL) expressed on the BMSC activate the osteoclasts while osteoprotegerin on BMSCs a decoy ligand of RANK prevents RANK-RANKL communication (89). Manipulative MM cells stimulate RANKL expression on BMSCs simultaneously reduce osteoprotegerin expression which accordingly promotes osteoclastogenesis. Consequent adhesion of MM cells to osteoclasts enhances the production of osteopontin and IL6, which augments MM cell growth and survival (88,89) (Fig. 2C).

6. Therapy

Treatment of MM typically involves combination chemotherapy including cyclophosphamide or melphalan, a steroid (dexamethasone or prednisolone), a novel agent [e.g. proteasome inhibitor, immunomodulatory drug (IMiDs)] and may be followed by autologous stem cell transplant depending on the age at diagnosis (2). Treatment of progressive MM consists of induction, maintenance and supportive regimens (50). In patients below 65 years of age, autologous stem cell transplant (ASCT) is considered (13). In many cases a single autologous stem cell transplant can result in progression-free survival in comparison with chemotherapy alone (94).

The IMiDs and the proteasome inhibitors (e.g. bortezomib and carfilzomib) have provided significant improvements in survival and quality of life in MM (95). IMiDs are structural and functional analogs of thalidomide that have potent immunomodulatory properties, anti-myeloma activity and better tolerability profiles (96). Thalidomide was the first immunomodulatory agent approved for use in MM. It is highly active against MM, however, is limited by considerable toxicity, particularly in older patients (97). Lenalidomide, an analog of thalidomide, possesses more potent activity with less toxicity and consequently is preferred for use across phases of MM treatment (98).

Thalidomide monotherapy when used for induction therapy produces a low response rate of ~35% (99,100). In the context

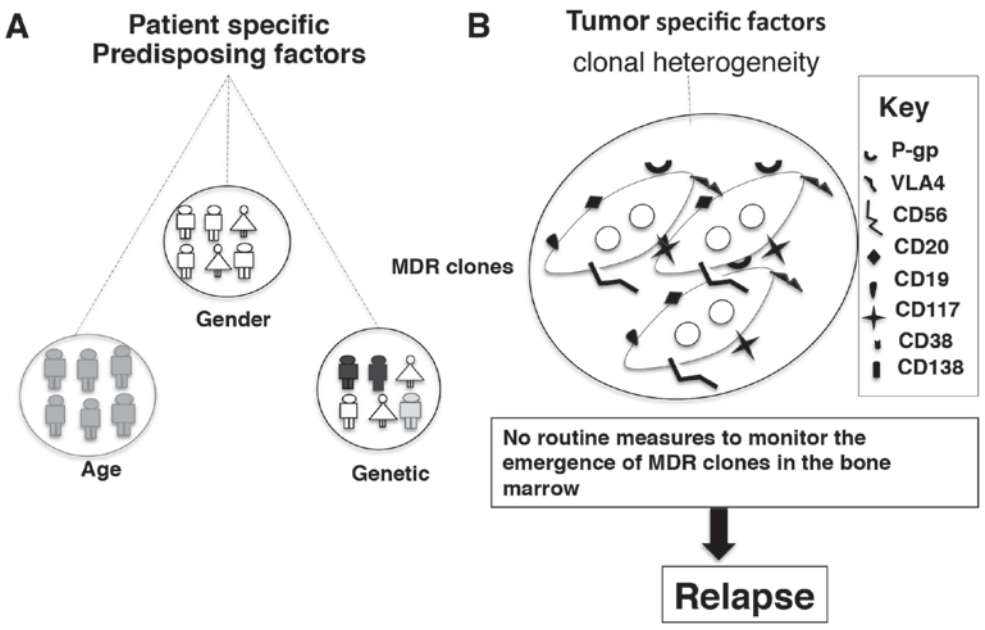

Figure 3. Patient and tumor specific factors impacting on predisposition and treatment in MM: (A) The demographic (age, gender and genetics) variables cause relative pre-disposition of MM in patients. The incidence of MM increases with age and MM manifestations mimic ageing symptoms. MM is more common in males than females. African Americans are more predisposed for MM in comparison with Caucasians or Asians. (B) Myeloma is incurable despite most patients responding to initial high-dose induction therapy and the introduction of novel class of drugs (proteasome inhibitors, IMiDs). The eventual relapse is a result of vigorous changes in MM biology and the development of drug resistance during the course of treatment. Currently, the emergence of MDR^+ MM cells is overlooked in the clinical setting.

of relapsed disease, thalidomide monotherapy results in a median event-free survival of 6-12 months and median overall survival of 14 months (101). Thalidomide's combination with dexamethasone improves the rate to 60-75% and is associated with a high incidence of grade 3-4 toxicity (102-104). For relapsed MM, the addition of an alkylating agent (cyclophosphamide or melphalan) further increases the response rate to 75-80% (105,106). In comparison with the response rates achieved using novel agents such as bortezomib or lenalidomide, thalidomide monotherapy is not superlative. In addition, combination of thalidomide with cytotoxic agents such as doxorubicin or cyclophosphamide, improves the response rate and quality of response further. Consequently, a three-combination regimen is more commonly used when thalidomide induction is considered (104). However, for consolidation/maintenance therapy, the impact of thalidomide on therapeutic outcome remains unclear. Results obtained from the British Myeloma Research Council Myeloma IX study demonstrates that thalidomide is associated with shorter post-relapse survival suggesting that thalidomide maintenance may induce drug resistance compromising duration of response and survival especially in patients with high risk genotype [t(4;14), t(14,16), t(14,20), 1q21amp, del(17p)] (107,108).

Other novel agents like thalidomide derivatives (lenalidomide) and proteasome inhibitor (bortezomib) combination chemotherapy increases the overall response rate to 90% or above (109-112).

A complete remission or complete response (CR) in MM is clinically defined as negative serum and urine immunofixation, no plasmacytoma and ≤5% PCs in bone marrow for at least 2 months (113), whereas partial response is stated by >50% reduction of serum M-protein and >90% of Bence Jones protein (113,114).

Defining clinical relapse. The malignant PCs enter a static phase with typically lower levels of proliferative markers such as thymidine kinase, high sensitive CRP marking the remission status of MM patient after successful induction therapy (115-117). However, MM cells eventually overcome this passive phase and become aggressive within a short space of time (118). This complex process is said to include loss of immune regulation, clonal evolution, cytokine deviance, oncogene stimulation and/or tumor suppressor gene anomaly (118). The mechanisms underlying initiation, a prolonged asymptomatic stage, progression and aggressive transformation of PCs are not yet clear (118). The failure of the current chemotherapeutic regimen to eliminate the malignant clone in MM is considered to be one of the major causes of consecutive relapse (118). Relapse from a complete response is clinically defined by the reappearance of the serum or urine M-protein (paraproteinemia), ≥5% bone marrow PCs, new lytic bone lesions and/or soft tissue plasmacytomas, an increase in the size of residual bone lesions and/or the development of hypercalcaemia (corrected serum calcium >11.5 mg/dl) not attributed to another cause (114,119).

7. Patient-related predisposing factors complicating diagnosis and treatment in MM

Patient age and gender. The incidence and risk of developing MM increases with age, with predominantly 80% of affected patients being above the age of 60 (1,5,120). The classic disease manifestations in MM such as anemia, bone pain and associated fracture and renal involvement imitate the complications associated with ageing process (36). Consequently, patients discount the warning signals, which results in delayed diagnosis, which severely compromises the accessible therapeutic

decisions for the elderly patients. Myeloma is more common in men than women for reasons yet unknown (5) (Fig. 3A).

Ethnicity. The incidence MM is lowest among those of Asian descent, is intermediate in Caucasians and is highest in African Americans (25,121,122). Various independent studies have suggested that there may be a greater genetic predisposition to MGUS in Africans and African Americans than in Caucasians (123). Although the reason for this genetic pre-disposition is not known, a small number of studies have revealed that the variation in the prevalence of immunoglobulin subtypes and the overexpression of either κ or λ free light chain ratios in different races may contribute to the differential cytogenetic susceptibility between races (123). The presence of a rare deletion of 193 bp in the long arm of the pseudogene [poly(ADP-ribose) polymerase-allele B] of chromosome 13 (negative prognosis in MM) is more frequent in African Americans than Caucasians (69). Although the etiology of MM remains unknown, a family history of hematological disorders, either alone or combined with exposure to certain viruses, radiation and chemicals, is a proposed risk factor (36) (Fig. 3A).

8. Tumor and treatment-associated factors complicating treatment

Clonal evolution. Numerous studies have confirmed the presence of tumor-initiating cells (stem cells) in the bone marrow and their role in disease relapse (48,124). The primary bone marrow contain a small population of clonotypic B cells with an immature phenotype ($CD138^-$) known as 'side population' or MM initiating cells with stem-cell characteristics besides the malignant 'main population' (48). These cells contain more quiescent cells than 'main population' cells in cell cycle analysis. The MM stem cells or 'side population' (SP) cells are enriched source of cancer stem cells and characteristically show low staining of Hoechst 33342 dye, have high clonogenic potential and possess self renewal capacity (48,125). The SP cells contain hypermutated Ig genes, overexpress members of the ABC transporter family such as permeability-glycoprotein (P-gp), multi drug resistance-related protein 1 (MRP1) and breast cancer related protein (BCRP) much like the stem cells (126). The self-renewal capacity of the clonotypic MM cells is mainly attributed to the abnormal signaling pathways found in MM such as Hedgehog, Notch and Wnt signaling pathways (126).

The overexpression of drug efflux pumps is known to compromise the treatment outcome in MM (124). As mentioned, the side population has high expression of MDR proteins. The inability of chemotherapeutics to eradicate MM clones is a major limitation in MM management and a major cause of relapse (127). The detrimental MM clone is persistent during the remission phase and possess high proliferating potential once activated (118). The presence of drug efflux pumps further adds to the deleterious potential of the aforementioned MM clone and cause inevitable relapse (124) (Fig. 3B).

Multidrug resistance. Primary or acquired drug resistance is a major obstacle in MM therapy. In the past, conventional chemotherapeutic treatment of MM, was primarily focused on alkylator and corticosteroid based regimens (VAD regimen-vincristine, adriamycin or doxorubicin, dexamethasone) (128). The current therapeutic regimen includes IMiDs, proteasome inhibitors to improve outcome in MM patients. However, overexpression of MDR genes, topoisomerases and glutathione transferases mediate drug resistance in MM and many cancers (129). Cell adhesion mediated drug resistance (CAM-DR) and overexpression of anti-apoptotic proteins are typical resistance mechanisms also contributing to relapse in MM (130,131).

Topoisomerase II. Topoisomerase II (topo II) is a 170-173 kDa homodimeric protein involved in DNA replication, recombination and gene transcription (132,133). Topo II is an ideal drug target and anthracyclins (doxorubicin), anthracenediones (mitoxantrone) and intercalating agents (acridines) are the main topoisomerase inhibitors used in MM therapy. These drugs interact with topo II to form a temporary complex, which prevents chromosome segregation and DNA synthesis (129). Point mutations in essential domains of the malignant PCs modify the drug target topo II by epigenetic changes such as hypermethylation at the CpG. Island of promoter region affecting the gene expression (129). Structural changes to topo II (α to β) also contribute to drug resistance to topo II inhibitors used in MM therapy (129). The sub-cellular localization of topo II is also crucial in determining the drug effectiveness and is governed by the adhesion molecule-mediated resistance mechanism in MM (134). Turner *et al* demonstrated that tumor density plays a role in topo II resistance in such a way that in high density MM tumors, majority of the topo II is transported away from the DNA to the cytoplasm and the drugs fail to form cleavable complexes resulting in poor therapeutic outcome (135).

Glutathione transferases. Glutathione (γ-glutamylcysteinylglycine) is a tripeptide thiol present throughout the mammalian organ system specifically in the liver and kidney. Physiologically, glutathione plays a critical role in clearance of xenobiotics, harmful radiations and free radicals (129,136). Glutathione transferases (GST) are a family of detoxification enzymes catalyzing the non-covalent or covalent conjugation of glutathione with the diverse detrimental electrophilic compounds. GSTs also sequester toxic compounds and protect the cells from the oxidative stress through inherent organic peroxidase activity. The cytosolic and microsomal GST forms in humans are differentiated as GST-π, -α and -μ of which GST-π form is the most common enzyme. The conjugation with glutathione makes the toxic compounds water soluble facilitating an easy expulsion from the cells. In the malignant status, this effective detoxification mechanism also becomes unfavorable. Active GSTs are either increased in the cell or the expression levels of the isozymes are altered to protect the tumor by catalyzing the toxic chemotherapeutics (136,137). Alkylating agents, melphalan and cyclophosphamide used in myeloma therapy are inactivated by GST catalysis resulting in poor therapeutic outcome (129). In addition, high percentage of co-expression of GST-π (82%) with P-gp which is another class of MDR protein (72%) in MM relapse is reported by Petrini *et al* (138). This implicates co-operation of two distinct MDR pathways in coordinating poor therapeutic response.

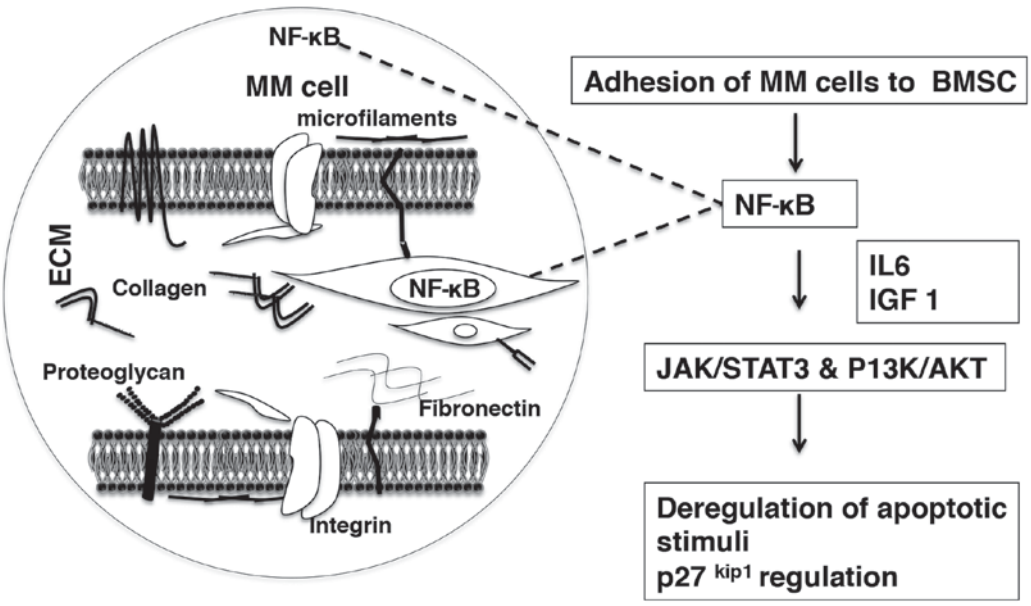

Figure 4. Microenvironment-mediated drug resistance in MM. The adhesion of MM cells to fibronectin in the extracellular matrix (ECM) enhances MM cell survival and growth. The adhesion triggers the deregulation of apoptotic stimuli and facilitates MM growth and survival through NF-κB pathway activation. The alteration of drug target (topo II), cytokine (IL6 and IGF1) mediated upregulation of cell signaling (JAK/STAT3 and PI3K/AKT) cascades also play a major role in initial drug resistance in MM.

Microenvironment-mediated drug resistance. Apart from microenvironment-mediated pathogenesis mentioned above, components of the bone marrow microenvironment contribute to treatment unresponsiveness in MM (139,140). The microenvironment related resistance mechanisms could be classified as integrin mediated adhesion to ECM (fibronectin) disrupting the apoptotic stimuli through cytokine-mediated upregulation of cell signaling and caspase mediated apoptotic cascade in MM cell. Microenvironment-dependent drug resistance in MM is considered as a bonus mechanism in MM cells by which the drug resistant cells are selected early on during initial therapy and they later acquire more explicit drug resistance during the course of chemotherapy (141).

CAM-DR. CAM-DR is induced following the interaction of malignant PCs to the ECM (141). Aberrant PCs express a variety of cell adhesion molecules, which function as cell-to-cell and cell-ECM through counter receptors. Fibronectin mediated adhesion has been shown to increase the tolerance of MM cell line (RPMI-8226) to chemotherapeutic agents and the induction of drug resistance in MM cells by suppressing apoptosis (142). Integrin molecules such as the VLA4, VLA5 and their respective receptors govern this resistance mechanism. The integrin molecules act as extrinsic factors eliciting intracellular response through focal adhesion points that stimulate signaling pathways and cytoskeletal modification (141). Damiano *et al* (142) demonstrated that the initial integrin mediated adhesion to fibronectin enhances MM cell survival and protects against apoptotic stimuli from doxorubicin and melphalan aiding tumor survival (141). The mechanism of CAM-DR can also be through blocking a specific element of the caspase mediated apoptotic pathway as shown by Shain and Dalton (141) in MM cell line RPMI-8226. The study showed direct inhibition of mitoxanthrone-induced caspase-3 and -7 cleavage. Once adhered to fibronectin, the cancer cells use the microenvironment in a number of ways to develop *de novo* drug resistance such as overexpression of cell cycle regulatory protein ($p27^{Kip1}$), alterations to drug target and by facilitating integrin mediated cell signaling and cytoskeletal reorganization (Fig. 4).

Cytokine-mediated drug resistance. The MM cell-BM microenvironment cytokines regulate apoptosis and MM cell survival through their participation in P13K/AKT and JAK/STAT3 signaling pathways (84). Novel and conventional chemotherapeutics in MM target the caspase-mediated apoptosis pathways. Caspase-8/3 mediated death receptor pathway (IMiDs, melphalan) and caspase-9/3 mediated mitochondrial intrinsic pathway (dexamethasone) follow subsequent poly-(ADP-ribose) polymerase (PARP) cleavage resulting in apoptotic death of MM cells (92,143-145). The proteasome inhibitor class (bortezomib) targets both caspase-8/3 and caspase-9/3 pathways (146). The IL6 mediated activation of JAK/STAT3 signaling cascade results in upregulation of myeloid cell leukemia sequence 1 (MCL1) and B cell lymphoma/leukemia family (Bcl-XL) leading to dexamethasone resistance (147). P13K/AKT signaling and NF-κB activation in MM cells are coordinated by IL6 and IGF1 by inducing inhibitors of drug-induced apoptosis resulting in treatment unresponsiveness and eventual survival of the tumor (148,149) (Fig. 5).

In conclusion, the MM cell-ECM interactions are a foundation for the *de novo* resistance to chemotherapeutics and thus pave the path for more mutative transformations or acquisition of classical MDR mechanisms during the course of treatment (141,142,150) (Figs. 4 and 5).

Drug efflux. Cancer cells often develop cross-resistance (to a large variety of chemically and pharmacologically unrelated drugs leading to the phenomenon of multiple (or multi-drug)

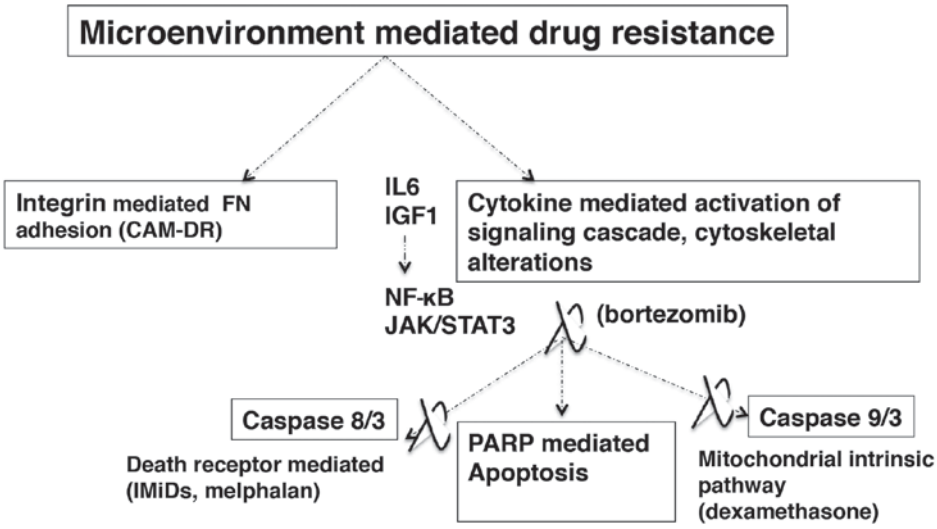

Figure 5. Microenvironment-mediated drug resistance pathways in MM: cell adhesion-mediated drug resistance and cytokine-mediated cell signaling cascade activation contribute to treatment failure in both conventional and novel therapies. Fibronectin-mediated adhesion to the ECM components trigger the cell cycle regulatory proteins ($p27^{kip1}$) limit therapeutic success with doxorubicin. Death receptor-mediated apoptotic (caspase-8/3-IMiDs, melphalan) and mitochondrial intrinsic pathways (caspase-9/3, dexamethasone) and proteasome inhibitor targets both these pathways. Cytokine (IL6 and IGF1) activate NF-κB and JAK/STAT3 pathways, disrupting the apoptotic death of tumor cells.

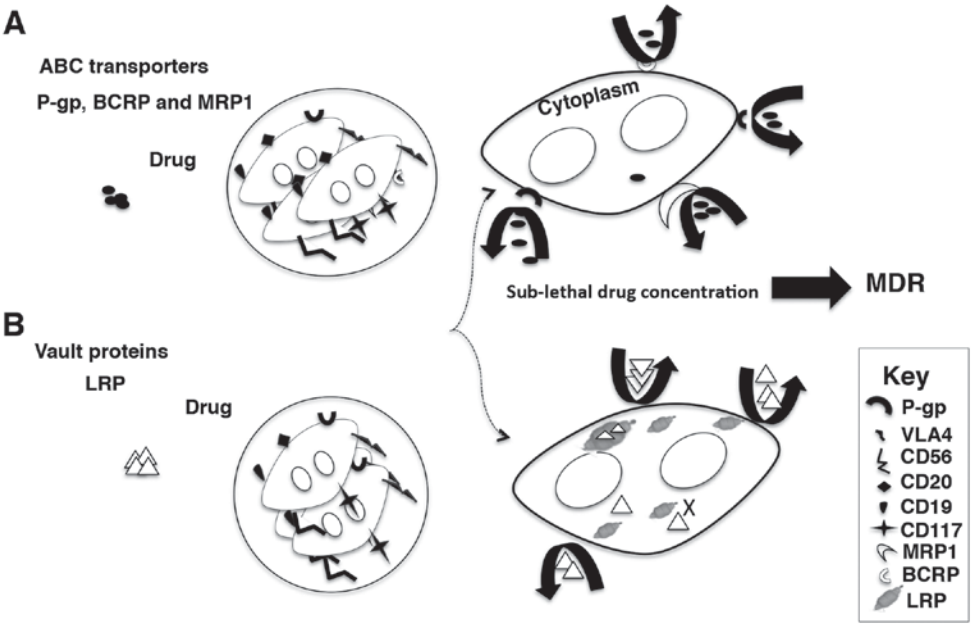

Figure 6. MDR proteins contributing to MM relapse: (A) The MDR^+ MM clones characteristically overexpress the ABC transporters and the vault proteins similar to the stem cell populations. ABC tranporters maintain intracellular sub-lethal concentration of the drug resulting in relapse. (B) Vault proteins (LRP), efflux out or sequester its substrate drugs and prevent the entry to the nucleus, which result in treatment failure and MM relapse. In addition, the transporters cause high systemic drug concentration causing serious side effects in patients with renal insufficiency.

resistance (MDR) (17). MDR is mainly attributed to the overexpression of ATP-dependent efflux transporters belonging to the ABC superfamily (17) (Fig. 6). The overexpression of the ATP binding cassette (ABC) transporters on the plasma membranes of malignant PCs contribute to MDR in MM. P-gp, MRP1 lung resistance protein (LRP) and BCRP are all members of the ABC superfamily of membrane transporters and mediate MDR in MM through their drug efflux capacity (130).

In MM therapy, maximal response rates and improved survival is achieved through combination thalidomide therapy. Combination chemotherapy is however compromised by the overexpression of the multidrug transporters (P-gp, MRP1, BCRP and LRP) on malignant cells, which maintain intracellular drug accumulation deficits in resistant cells. Although there is no current evidence to suggest that thalidomide itself is a substrate of the these drug efflux pumps, the drugs used in combination as part of the recommended regimens are themselves substrates of one or more of these efflux transporters (Table II) (151-166). This contributes to compromised therapeutic effects, reduced rates of response and overall survival of the tumor.

Table I. Current International Staging System (ISS) in multiple myeloma.

ISS stage	Criteria	Median survival in months
I	Serum β_2-microglobulin <3.5 mg/l Serum albumin ≥3.5 g/dl	62
II	Neither stage I nor stage III Or (i) Serum β_2-microglobulin <3.5 mg/l and serum albumin <3.5 g/dl (ii) Serum β_2-microglobulin 3.5 to <5.5 mg/l irrespective of serum albumin level	44
III	Serum β_2-microglobulin ≥5.5 mg/l	29

Table II. Chemotherapeutics used in MM are substrates of MDR proteins.

Drugs	P-gp	MRP1	BCRP	LRP	References
Melphalan	+	+	-	+	(152-154)
Lenalidomide	+	-	-	a	(155-157)
Bortezomib	+	+	-	a	(158,159)
Thalidomide	-	-	a	a	(160)
Prednisone/prednisolone	+	-	a	a	(161,162)
Doxorubicine	+	+	+	+	(163,165,166)
Idarubicin	+	+	-	a	(164,167)

[a]Insufficient published data to comment.

P-gp. The *ABCB1* gene located on the long arm of chromosome 7 encodes 170 kDa P-gp (17). It is a cell surface protein distributed throughout the human body and is typically found in pharmacological interfaces protecting the cell from carcinogen or xenobiotic influx (17). It has been established that an excessive amount of *ABCB1* leads to MDR in many cancers (17). P-gp mediated drug efflux sustains a sub-lethal amount of drug concentration in the intracellular environment enabling the cancerous cell to evade the toxic chemotherapeutic insult resulting in the eventual survival (17).

The main components of conventional induction regimen in MM, the vinca-alkaloid (melphalan), anthracyclines (doxorubicin, daunarubicin) are common substrates of ABC transporters such as P-gp or MRP1 (Table II). Chemotherapeutic resistance in MM patients is frequently associated with the overexpression of P-gp (167). At least 5% of cases of untreated MM presents with P-gp which can compromise the induction therapy outcome significantly (168). In addition, the circulating B cells or the 'side population' in MM express P-gp comprising the resident MDR clone, which leads to MM relapse (169). Nuessler *et al* also reported that 33% of patients at relapse or progressive MM are positive for functional P-gp (170).

Several *in vitro* and *in vivo* pharmacogenomic and pharmacogenetic studies have revealed genetic polymorphisms of the ABC transport proteins as clinical MDR facilitators in MM. These polymorphisms show diverse function and manifestation across different ethnicities and patient cohorts (171-173). Fifty single nucleotide polymorphisms (SNPs) and 3 insertion/deletion polymorphisms have been identified for P-gp. Of these, three (rs1045642, rs2032582 and rs1128503) were found to have potential therapeutic impact in MM (171,172), though, only rs1045642 ($C_{3435}T$) showed correlation to the overall survival in MM. Minimal linkage disequilibrium was shown for the other two SNPs (171). It is believed that rs1045642 may alter the substrate specificity and influence therapy in MM (173). However, the statistical comparison within the MM patient group of Northern Irish ethnicity showed that although rs1045642 had an influence on overall survival, it was insignificant in statistical comparisons between healthy controls and MM patients (173). Another study involving 115 post-transplant MM patients investigating $C_{3435}T$ polymorphism reported that C/T and T/T genotypes showed a longer overall survival than C/C genotype under dexamethasone, adriamycin (doxorubicin) and vincristine (VAD) treatment regimen (174).

MRP1. MRP1 is a 190 kDa protein coded by the *ABCC1* gene and located on the plasma membrane of both normal and malignant cells (175). Specifically, in a cell, MRP1 transports multiple organic anions (some glutathione conjugates) protecting against oxidative stress and is reliant on the intercellular glutathione levels for anthracycline transport (176-178). MRP1 gene overexpression results in clinical MDR in patients treated with natural agents such as anthracyclines and vinca-alkaloids (175,179). Abbaszadegan *et al* reported frequent detectable MRP1 mRNA in MM (100%) (167). MRP^{+ve} cells have been shown to accumulate lower amounts

of drug relative to P-gp, potentially due to their dependence on glutathione metabolism (130,179). The presence of the polymorphism, MRP1/R723Q (p.Arg723Gln) results in changes in the physico-chemical properties (size and polarity) of the protein, and this structural change significantly increase time to progression, progression-free survival and overall survival in a group of MM patients treated with velcade and pegylated liposomal doxorubicin (178). It is postulated that the variance in capacity of MRP1/R723Q isoform in trafficking and expression may be the cause of the antitumor effect of anthracyclins in this study (178). The MRP1 expression and its prognostic significance in MM or in cancer in general is comparatively less studied than P-gp (180).

BCRP. Breast cancer resistance protein is another ABC transporter family member with a molecular weight of 72 kDa typically expressed at pharmacological barriers (181,182). Structurally, BCRP encoded by the *ABCG2* gene, consists of a single nucleotide binding domain (NBD) and one *trans* membrane domain (TMD). Consequently, BCRP requires at least two NBDs to function as a drug efflux pump and usually exists as an oligomer (183). BCRP was initially described in MCF-7/AdVrp human MDR breast cancer cell line that did not express P-gp or MRP1 (184,185). In MM, BCRP shows impaired function and is not associated with drug resistance in *de novo* patients (184). However, BCRP is closely associated with the compounding problem of clonogenic potential of MM cells leading to relapse. The 'MM stem cells' or 'side population' (Hoechst 33342 low staining) have higher BCRP mRNA levels and functional activity compared to the rest of the MM cells (main population) (186). Functional BCRP expression in MM is inversely proportional to promoter methylation in *ABCG2* gene in such a way that unmethylated promoter site results in moderate or high BCRP (*ABCG2*) expression (187). Numerous polymorphisms for BCRP have been reported in literature (V12M, Q141K, F208S, S248P, F431L, S441N and F489L) however they have not been linked to MM yet (188).

Major vault protein (LRP). LRP is a 110-kDa protein expressed in the kidneys, adrenal glands, heart, lungs, muscles, thyroid, prostate, bone marrow and testis. Most vaults are complex ribonucleoprotein particles comprising two large molecular weight proteins and a small RNA in addition to the 110 kDa LRP. They are mostly present in cytoplasm, with a small fraction present in the nuclear membrane and nuclear pore complex (189). They are assumed to translocate substances across the nucleus and cytoplasm and are said to be involved in MDR (130,190). Raaijmakers *et al* reported the prevalence of LRP in untreated MM patients (153). This study established the relevance of LRP as an independent predictor in comparison with current markers (PC labeling index, serum B_2M or lactate dehydrogenase level) for therapeutic response and survival in MM patients treated with melphalan (melphalan and prednisone) (153). Thus, screening for LRP prior to treatment to identify the positive population is recommended in therapeutic design in *de novo* MM to circumvent LRP mediated drug resistance (153). There are currently more than 100 polymorphisms identified for LRP (191). LRP expression rather than polymorphic state have been correlated with therapeutic response (192-195).

Circumvention of MDR. In the past few decades, substantial research has focused on the development and trial of agents, which can reverse the drug efflux capacity of ABC transporters, in particular P-gp in cancer (15,130,196,197). Indeed, the pharmacological inhibition of P-gp activity has been a major focus in many MM clinical studies (198). In an attempt to circumvent acquired MDR, several inhibitors have been used to improve treatment outcome of patients with MM (16,199-201). The cyclosporin A reversal effect has been evident in phase II studies with MM and acute myeloid leukemia, although phase III clinical trials failed to give the expected response in progression-free survival and overall survival (197). Since the initial successful clinical trials, verapamil and cyclosporin were combined with vincristine, adriamycin and dexamethasone (VAD) in MM, however, these have had disappointing results mainly due to lack of improved efficacy or dose related toxicity (15,196,197).

In conclusion, management of MM relies on combination therapy and different drug resistance mechanisms, topo IIα and GST-π-dependent resistance, specifically the drug efflux pathways pose a significant challenge in MM clinical setting. Conservative regime in MM, are mostly substrates of ABC-transporters, topo IIα and GST-π-dependent resistance mechanisms (170). Recent studies have reported that the novel agents are also substrates of ABC transporters specifically P-gp (154,157).

9. Discussion

Herein, we explored the relevant innate and acquired challenges associated with the therapeutic management of MM including the role of MDR in therapeutic failure. Many cases of MM with late middle age onset fail to be accurately diagnosed early as recurrent infections, tiredness and bone/joint pain is often associated with normal ageing-related complications.

MM is currently an incurable and chronic disease, with 'non-secretory myeloma' exclusively dependent on frequent bone marrow aspiration for the assessment of molecular, cytogenetic markers including aberrant PC population, and categorizing complete response. Secretory myeloma is partially dependent on bone marrow aspiration for the confirmation of the clinical status (9). This is largely because the malignancy is restricted to the bone marrow and is rarely seen in peripheral blood (202). Current risk stratification in MM is also primarily dependent on cytogenetic markers and is assessed using invasive bone marrow biopsy. Nevertheless, the BM biopsy does not provide a sensitive assessment of genetic abnormalities in multiple tumor sites throughout the skeletal system of MM patients. Therefore, even invasive biopsy is not comprehensive in risk profiling patients with MM.

The current ISS, although, presents with distinct advantages over its predecessors, the precise indication of the higher ISS stage (stage III) is inconclusive in terms of whether it suggests tumor burden/aggressiveness or the level of end-organ damage or both (55). There are several reliable systemic markers present for prognosis, like B_2M, M-protein, however these markers are insufficient in gauging the transition from the indolent phase of MM to an aggressive disease state (118). In the case of 'non-secretory myeloma', diagnosis and prognosis are further limited as it lacks the typical hallmark of the disease.

T cells proficiently acquire antigens from MM cells over any other cell type and create novel cell types through trogocytosis (76,77). It is not understood clearly if the novel T regulatory cell types provide new ligands for receptors and regulate signaling pathways, however, this mechanism enables the malignant PCs to effectively evade the immune system recognition and thereby stimulate tumor growth (203).

We have very little understanding regarding the intricate cycle of dormant and malignant phase of PCs in MM or in other words how PCs escape the plateau phase in the remission status and become aggressive again in relapse. This phenomenon underlines the fact that even aggressive therapy is not successful in eliminating the neoplastic origin of MM (118). As discussed, the MM stem cell population (SP cells) and circulating $CD138^-$ PCs are said to have an aggressive proliferative and dissemination capacity (5,27). In addition, they characteristically have self-renewal potential and overexpress the ABC transporters on their surface (124). The persevering MM clone with MDR phenotype potentially lead to treatment failure and currently this aspect is not routinely monitored in the clinical setting. Another complicating aspect of MM is the high heterogeneity in survival amongst patients. MDR phenotype, genetics of MM, including specific IgH translocations and individual immune profiles are potential players with a role in the disparity in survival amongst MM patients. Present systemic markers do not assist greatly in risk stratification, thus, it is more reliant on the cytogenetic markers in this aspect. Therefore, inclusion of more systemic markers, alone or in combination that would aid in early detection, tailor an individualized approach to optimize a prognostic surveillance at diagnosis and after primary surgery is highly recommended in MM (204-207).

The derivatives of thalidomide (IMiDs) have improved overall survival and have increased the cost of treatment significantly. However, in a phase 1 clinical trial conducted in 2011, involving 21 patients with refractive myeloma who were treated with lenalidomide and temsirolimus (mTOR pathway inhibitor-CCI-779), a high concentration of the drug was detected in the blood causing toxicity. The patients experienced unusual side effects such as electrolyte imbalance, rashes, fatigue, and neutropenia. Further investigation of the pharmacokinetic profiles of CCI-779 and lenalidomide suggested a drug-drug interaction, hinting that the disposition of CCI-779 is arguably mediated by CYP3A4/5 and P-gp (208-210). The clinical trial assessed toxicity or adverse effects and response to treatment by serum and urine M-protein quantification every four weeks. There was only limited documented clinical evidence suggesting lenalidomide and P-gp interaction and this possibility was investigated through *in vitro* studies to determine whether lenalidomide can be transported by P-gp. The *in vitro* studies proved that lenalidomide is actively transported by P-gp and this effect was reversed by CCI-779 and verapamil. In addition, *ABCB1* silencing RNA or short interfering RNA (siRNA) knockdown studies *in vitro* also showed more lenalidomide uptake, supporting lenalidomide and P-gp drug-drug interaction (154).

In light of emerging studies that these novel drugs that have been incorporated to MM therapeutic management are substrates of ABC transporters, the situation warrants a re-evaluation of the manipulative power of MM cells (154). It is evident that opting for more aggressive chemotherapy has brought some promise of prolonging remission and survival in MM. However, this recent study serves as a reminder of aggressive chemotherapy pitfalls of side effects, toxicity and eventual development of MDR phenotype in patients (211). More importantly out of the innate MM complications contributing to treatment failure, MDR is an element that can be modulated and targeted, which, therefore invites specific attention.

The role of polymorphisms in *ABCB1* and *ABCC1* in both the predisposition to disease and the therapeutic outcome of MM have in recent years been studied extensively. The three most common MDR1 SNPs include 2677G>T/A in exon 21 (RefSNP ID: rs2032582), 3435C>T in exon 26 (RefSNP ID: rs1045642) and 1236C>T (RefSNP ID: rs1128503) in exon 12. The 2677G>T/A polymorphism translated into an amino acid exchange from Ala to Ser or Thr at codon 893, affecting the intracellular region of P-gp between *trans* membrane 10 and 11 (212). Both 3435C>T and 1236C>T are synonymous SNPs. The 3435C>T mutation results in a change from cytosine to thymine that translates to isoleucine. It is found in the second ATP binding domain, located between the Q-loop and the second signature motif on the intracellular side of the protein. This SNP is associated with altered MDR1 expression (213). 1236C>T affects the intracellular region of P-gp between the first A-loop and Walker A motif (212) and translates into a glycine residue. These three polymorphisms are and comprise the most common MDR1 haplotype (212). microRNAs, *-miR-15a*, *miR-16-1*, and *miR-17-92* are also shown to play a role in the heterogeneity in the clinical outcome of MM (65,68).

In terms of the therapeutic outcome, Buda *et al* investigated the prognostic role of MDR1 in the outcome of 115 MM patients treated with DAV (dexamethasone, doxorubicin and vincristine) followed by autologous stem cell transplant. This study showed that the $C_{3435}T$ polymorphism was prognostic with patients with the C/T and T/T genotypes demonstrating a longer overall survival compared to those with C/C genotype. The same polymorphism was again found to be associated with a longer time to progression and progression-free survival in relapsed and/or refractory MM patients treated with pegylated liposomal doxorubicin in combination with bortezomib (178). The T allele in SNP G2677T/A is likewise associated with a better response to DAV (214) and a better overall survival in MM (215).

The single-nucleotide polymorphism in MRP1 (rs4148356, R723Q) has also been shown to impact on the clinical outcomes of MM patients (178). The MRP1 mutation Arg723Gln has an effect on the protein expression and trafficking, significantly reducing MRP1-mediated resistance to a wide spectrum of drugs. The presence of R723Q results in extended time to progression, progression-free survival and overall survival in MM patients. This has been ascribed to the differential ability of the isoform in trafficking glutathione and/or regulating its expression (178). It is currently unknown whether polymorphisms of BCRP play a role in MM treatment outcome (216).

The integrin mediated (CAM-DR) drug resistance mechanism is considered to enable MM cells to survive the initial drug toxicity, which in the course of therapy aids in selective expression of classical drug resistance pathways such as ABC-transporter overexpression in MM cells (65,68,142).

Current measures of therapeutic response rely on invasive bone marrow biopsy, immunofixation, serum protein electrophoresis, quantitation, measurement of free light chain and CT/MRI scans (217). A full blood count, biochemistry screen, B_2M and light chain assays are other prominent systemic markers along with radiology used for staging, diagnosis and monitoring in MM (1,58). None of the above markers, however, provide a direct assessment of the emergence of MDR or detect the expression and evolution of resistance markers, polymorphic variants of resistance markers or nucleic acid signatures, which may contribute to disease progression and individual therapeutic responsiveness.

10. Conclusion

Cancer biology in general is an intricate process, especially in MM, where individual immunological and tumor profiles change dynamically during the course of treatment. Despite our knowledge of the MM landscape, the intrinsic challenge of heterogeneity provides a significant complication in the management of MM, necessitating individualized analysis of MM pathogenesis and routine monitoring of evolution of drug resistance.

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SRK conducted all the research and prepared the manuscript. MB designed and supervised the research, provided conceptual advice and revised the manuscript. RB provided topic orientatio. RJ assisted in manuscript revision. All authors reviewed and approved the final version of the manuscript.

Chapter 2

2. Systemic signatures in multiple myeloma and their role in clinical management

Running Title: Systemic signatures myeloma

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Abbreviations : multiple myeloma (MM), microparticles (MP), plasma cells (PCs). Multidrug resistance (MDR) ATP- binding cassette transporters (ABC transporters)

2.1 Abstract

Multiple myeloma (MM) is a plasma cell neoplasm mainly restricted to the bone marrow, which localises at multiple sites within the axial skeleton (1). The median age of diagnosis of MM is 60 years and is characterized by heterogeneity in survival and therapeutic outcome amongst patients. MM is presently incurable, albeit, clinically manageable. The emergence of multidrug resistance (MDR) is a major mechanism contributing to MM relapse and treatment failure. Existing systemic biomarkers aid in prognosis and therapeutic decision making at diagnosis however, risk stratification in MM is heavily reliant on plasma cell genotyping in comparison to the systemic markers. The available systemic markers do not allow for the measure of minimal residual disease (MRD) or to gauge the duration of remission. This makes disease management heavily reliant on invasive bone marrow biopsy. We have previously described the role of cancer cell derived extracellular vesicles (i.e. microparticles) in the transfer and dissemination of deleterious traits such as MDR and increased metastatic capacity in cancer. Microparticles (MPs) are ubiquitously shed from all cells in the body and play a role in normal cellular processes as well as in disease pathology. MPs provide systemic 'surrogate' markers for cells localised within inaccessible compartments such as the bone marrow. This review focuses on the potential role of MPs in providing a novel clinical biomarker for the therapeutic management of MM.

2.2 Introduction

Multiple myeloma (MM) is a plasma cell neoplasm, which is mainly restricted to the bone marrow. It is commonly diagnosed in the late middle age and accounts for approximately 10% of hematologic cancers (2). The clinical manifestations of MM include immunological impairment, renal insufficiency, osteolytic lesions, increased tendency for bone fractures, bone pain and moderate to severe nerve damage (3-5). In classical MM, aberrant plasma cells (PCs) produce clonally incompetent immunoglobulins, M-proteins or paraproteins (6), which accumulate in the periphery and are routinely used as disease diagnostic markers (7). Plasma cells are mature B cells and typically carry antigens on their surface reflecting their distinct stages of maturation and which can be used as markers to distinguish them from other B cells (8, 9). Surface expression of CD38 and CD138 (Syndecan 1) on PCs represent a mature phenotype with CD138 arguably representing the most unique mature PC marker (10). CD138 is a transmembrane heparan proteoglycan, which plays a role in the adhesion and cell-to-cell cross talk between PCs and the bone marrow. CD138 is important in promoting angiogenesis, tumour growth, dissemination and drug resistance in MM (11-15).

The therapeutic management of MM typically involves the use of combination chemotherapy, which may be followed by autologous stem cell transplantation depending on the age and treatment outcome in patients. Agents typically used include; cyclophosphamide or melphalan, steroids (i.e dexamethasone or prednisolone) and novel agents (e.g proteasome inhibitor, immunomodulatory (IMiDs). Usually, an increase in paraprotein levels together with other associated symptoms such as bone pain and /or anaemia initiate further clinical investigations including bone marrow aspiration to diagnose and stage disease (16).

During the course of treatment the response achieved during induction therapy is crucial to achieving optimal results in the long term (17, 18). Induction therapy in MM involves high dose chemotherapy with conventional drugs (melphalan, doxorubicin) or novel chemotherapeutic agents (IMiDs or proteasome inhibitors) depending on the individual circumstances (ie age, co-morbidities, tumor burden, disease stage etc.) (19). A maintenance regimen typically follows once therapeutic response is achieved (i.e partial, very good partial or complete as per IMWG criteria). This typically consists of novel agents with a steroid (20). In the event of a relapse, various the extent and duration of remission, evidence of toxicity and presence of drug resistance are important considerations in determining the follow-up regimen (18, 21). Inherent or acquired multidrug resistance (MDR) is a major obstacle in the successful management of MM and is associated with a poor prognosis and compromised patient survival (22). Although, not routinely tested for, the presence of MDR constitutes a 'high risk' prognosis.

Risk-adapted therapy' is an overarching and emerging strategy designed to accommodate factors contributing to treatment failure at the level of the individual patient. Chng *et al* describes the presence of the 17p13 deletion as a poor prognostic marker, however is not predictive of therapeutic response status to any specific drugs. Likewise, absence and down regulation of expression of cereblon protein (a component of E3 ubiquitin ligase complex that is involved in auto-ubiquitination of respective substrates expression which forms the biding target of thalidomide) has shown prognostic potential in patients using Thalidomide. Thalidomide forms a complex with cereblon and the drugs tumericidal and teratogenic activity is dependent on the presence or absence of this protein) has been proposed as a prospective predictive marker of

IMiD resistance (23) whereas tumour necrosis factor-receptor associated factor 3 (TRAF3) deletion/mutation was shown to be predictive of bortezomib response in some patients (24, 25). In saying this, these markers are not of prognostic significance as they gauge only the therapeutic response to discrete drug classes (18). The research on patients developing resistance against therapy is ongoing and the availability of a robust panel of predictive markers, which would allow for individualized approaches to treatment in cancer management, has many advantages (26, 27). For instance, tailored strategies would minimize unnecessary exposure to toxic drugs which may not be ideal and allow for informed decision making regarding continuation of treatment in advanced disease (28).

At diagnosis, clinical prognostic markers typically measure PC proliferation and tumour burden using the PC labelling index. The available systemic markers are effective in gauging tumour burden and in defining stage of disease (29-32). However, their use in risk stratification or predicting risk of relapse is very limited (18, 33, 34). Attempts to identify biomarkers with this capacity are ongoing.

Microparicles (MPs) are submicron extracellular membrane vesicles (0.1 to 1 μm in diameter) released from the plasma membranes of most cell types upon cellular activation or during apoptosis (35). Our lab discovered that MPs provide a non-genetic mechanism for the intercellular transfer of functional resistance proteins and nucleic acids in cancer cell populations (36-38). This novel pathway effectively confers the transfer, dissemination as well as dominance of multi-drug resistance (MDR) and metastatic capacity within a matter of hours (36, 38-43). The detection of high levels of circulating cancer-derived microvesicles across many malignancies such as gastric,

lung, colorectal, lymphomas and mucinous adenocarcinomas (breast and pancreatic) have defined them as potential biomarkers of diagnostic and prognostic significance (44-47). Our recent study has shown that elevated plasma cell derived MPs are present in MM patients and are of prognostic significance in individual patients (48). Consequently, MPs provide "surrogate markers" of disease burden in cancers such as myeloma localised in poorly accessible tissues (36, 48-51).

Treatment failure, manifests as a cancer recurrence or 'relapse' of disease and remains a significant limitation in cancer chemotherapeutic success. There is a need for timely and predictive clinical tests, which would support existing approaches to predict risk of relapse before it manifests clinically with increased tumour burden. This review examines the current literature in this context. We focus our review on the role of MDR proteins in MM relapse and for the first time discuss this in the context of recently identified MP biomarkers including CD138 and CD34 in disease pathophysiology, progression and treatment failure.

2.2 Multidrug resistance in multiple myeloma

Therapeutic response in MM is highly heterogeneous, with patients experiencing cycles of treatment, remission and relapse corresponding to the evolution of drug resistance. This obviously translates to significant variability in survival and a compromised quality of life. The major contributor towards this treatment unresponsiveness is a phenomenon known as multi-drug resistance mediated by ATP binding cassette (ABC) transporter family. ABC transporters (e.g.: P-glycoprotein or P-gp) are responsible for the phenomenon of multidrug resistance (MDR) in which resistance to one

chemotherapeutic results in the tumor being cross-resistant to many diverse and unrelated chemotherapeutics. ABC transporter family members efflux their substrate anti-cancer drugs resulting in sub-optimal dosage in tumor cells (52). P-glycoprotein (P-gp) is the most recognized and studied member of ABC transporter family and is a 170 kDa transmembrane protein encoded by human *ABCB1* gene mainly expressed in pharmacological barriers (53). ABC transporters typically protect the cells by effluxing toxins out of the cell in an ATP dependent manner. P-gp and other members of the ABC transporter family such as multidrug resistance associated protein-1 (MRP1) and breast cancer resistance protein (BCRP) exhibit similar albeit unique substrate repertoires and function in the same manner.

ABC transporters and drug resistance in multiple myeloma

Chemoresistance in MM patients is correlated with the overexpression of members of the ATP Binding Cassette Superfamily of membrane transporters (ABC transporters), most commonly P-gp (54). At least 5% of *de novo* MM cases present with P-gp overexpression in heparinised marrow aspirates, which can significantly compromise induction therapy outcome (55). Likewise, during the course of therapy, P-gp expression in MM cells in bone marrow increases, which leads to MM relapse (56). In support of this, the study by Nuessler *et al* reported that 33% of patients at relapse or progressive MM express functional P-gp in the bone marrow aspirate (57). The expression of other ABC transporters such as multidrug resistance related protein 1 (MRP1) and its prognostic significance in MM has also been studied although not as extensively as P-gp (58). MRP1 (encoded by the *ABCC1* gene) over-expression results in clinical MDR in patients treated with naturally derived chemotherapeutics (i.e anthracyclines and vinca-alkaloids) although unlike P-gp, MRP1's function is dependent on glutathione metabolism (59, 60). In the context of MM, Abbaszadegan *et*

al reported comparable levels of *ABCC1* transcripts in haematopoietic cells from bone marrow and peripheral blood of MM and normal subjects (54). Lung Resistance Protein (LRP) is a 110-kDa protein expressed in the kidneys, adrenal glands, heart, lungs, muscles, thyroid, prostate, bone marrow and testis. LRP is major vault protein (100kDa) comprised of a complex of ribonucleoprotein particles along with small RNAs (61). LRP is mainly localised within the cytoplasm, although a small fraction is present in the nuclear membrane and nuclear pore complex (62). LRP is believed to have a role in translocating substances across the nucleus and cytoplasm and is involved in MDR (63, 64). Raaijmaker's *et al* reported on the prevalence of LRP in *de novo* MM patients (65) and established LRP's relevance as an independent predictor of therapeutic response and survival in MM patients treated with conventional therapy (melphalan, prednisone)(65).

As previously reviewed by us in (66) both conventional agents as well as novel agents are substrates of ABC transporter proteins. The clinical management of MM is heavily reliant on combination therapy and the wide range of structurally and functionally unrelated drug substrates impede successful treatment in MM (Figure 2.1). The novel agents, IMiDs (thalidomide derivatives) and proteasome inhibitors, have improved overall patient survival significantly, compared to the conventional agents (21, 67, 68). However, treating MM with novel agents also adds significantly to the global healthcare financial burden (69). In this scenario, personalized approaches to treatment not only have the potential to improve survival but they are also cost effective (66).

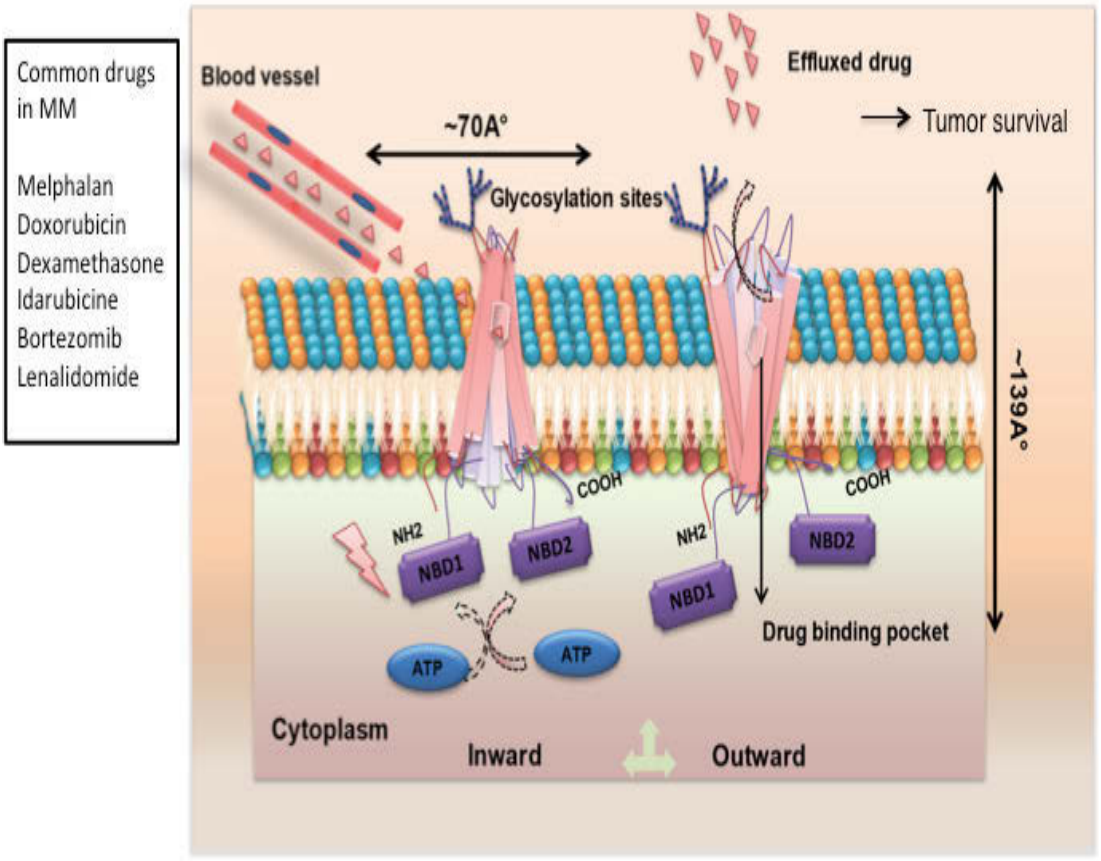

Figure 2.1. Schematic diagram of P-gp mediated drug efflux in MM. MM therapy involves combination therapy and almost all of the drugs in the repertoire are substrates of P-gp. The drug is effluxed out from the cell in an active (ATP dependent) manner to maintain a sub-lethal concentration of drug and this eventually results in tumor survival.

Clonotypic B cells which share same variable diversity joining (VDJ) arrangements and/or MM stem cells known are known as the ‘side population’ cells. ‘Side population’ cells have been shown to play a role in MM relapse and its incurability (67, 70, 71). Also, a small group of cells, which typically lack CD138 as well express stem cell characteristics are known as clonogenic cells and therapies fail to eradicate these niche cells (72). The role of CD138 in MM pathophysiology is further discussed in section 2.3.These cells typically express the ABC transporters on their surface with their presence evident by low intracellular accumulation of Hoechst 33342 dye (71) The ‘side population’ is known to be phenotypically different to mature PCs expressing early B cell markers and said to possess self-renewal capacity (67). Failure of chemotherapeutics to eradicate the clonogenic cells and/or ‘side population’ is one of the major reasons for unsuccessful therapeutic outcome in MM (67, 73). Therefore, frequent monitoring of clonogenic cell characteristics is necessary to proactively minimize relapse (55, 70, 74).

Currently, there is no clinical diagnostic tool available to directly monitor the emergence of drug resistance during the course of therapy and there is an understated need for this aspect in MM’s clinical setting. Existing tests provide limited information in this regard as they are indirect, providing measurements of tumor burden only, not the presence or evolution of drug resistance protein. In bone marrow biopsy, the sampling is restricted to discrete sampling points and is not representative of spread throughout the axial skeleton. Specifically, existing tests provide a measure of aberrant plasma cells and their morphology and consequently tumor burden rather than the presence of MDR.

2.3 CD138 and its significance in myeloma pathophysiology

CD138 (Syndecan 1) is arguably the most unique marker for mature PCs and therefore important in detecting PCs in MM (10). CD138 is a type 1 transmembrane heparan proteoglycan that facilitates interactions of PCs with extracellular matrix (ECM) and homing of PCs in the bone marrow (15, 75). CD138 mediates MM cell interaction with type 1 collagen and cell to cell adhesion along with a regulated interaction with growth factors in the bone marrow microenvironment (76). However, in the malignant state CD138 becomes a significant player in MM progression with its dynamic capacity to convert into a soluble effector molecule (15, 76).

Structurally, CD138 possesses a highly conserved cytoplasmic region at the –COOH terminus and an extracellular domain (ectodomain) at the –NH2 terminus bearing heparan sulphate (HS) or chondroitin sulphate (CS) chains (77). The short (28-34 amino acids) cytoplasmic region consists of a single variable segment flanked by two constant regions and adhere to intracellular ligands such as kinases or structural proteins (78-80) The ectodomain acts as a classical co-receptor for growth factors and a range of biomolecules such as cytokines, proteases and cell adhesion molecules for PCs (15, 75, 81). In an unstimulated state, syndecan 1 or CD138 binds to ECM components, adhesion molecules, proteins involved in lipid metabolism, proteinases and proteinase inhibitors via the HS and CS chains (78, 79). The interaction of the ectodomain with growth factors are further influenced by size and heterogeneity of HS and CS chains on CD138 ectodomain which in turn affects MM cell behaviour (76) (Figure 2.2 A).

The heparan sulphate bearing ectodomain of CD138 is shed as a whole by proteolytic

sheddases (also secretases or convertases) as a response to physiological stimuli such as chemotactic peptides, cytokines, calcium ionophores (Figure 2.2B) (80). The cleaving occurs at the juxtamembrane domain and is specifically thought to be at a dibasic region (Lys-Arg) closer to the outer leaf of the plasma membrane (80). The resultant soluble effector molecule maintains the binding capacity of their surface predecessors by means of intact HS or CS chains (15, 82). Numerous developmental and or pathophysiological events like wound healing and cancer biology are indeed affected by ectodomain shedding of CD138 triggering various intracellular pathways (82). The proteolytic cleavage is closely associated with the outer surface of the cell and is regulated by tissue inhibitor of metalloproteinase-3 (TIMP-3). Moreover, agents triggering cellular stress response via receptor activation (thrombin, plasmin) can accelerate the ectodomain cleavage and the mechanisms involved in accelerated, to that of constitutive shedding are distinct (82). Fitzgerald *et al*, 2000 demonstrated the existence of distinct mechanism as TIMP-3 and hydroxamate inhibit accelerated shedding whereas constitutive shedding is unaffected by TIMP-3 and requires approximately 10 fold higher hydroxamate for inhibition (82).

In MM, the shed CD138 accumulates in the fibrotic region of the bone marrow as well as systemically in the circulatory system (15, 83, 84). In the context of biomarker status, systemically shed CD138 is associated with a negative prognosis in MM (85, 86). Specifically, systemic CD138 was shown to be an independent prognostic indicator and with correlation to β_2M, creatinine, serum, urinary M protein and S-IL6-R in the Nordic myeloma group study (86). Another study showed higher soluble CD138 was correlated to tumor mass in MM (Dhodapkar *et al*, 1997) (85, 86)). CD138 also has a role in promoting tumour vascularisation, thereby stimulating angiogenesis and supporting

tumor growth as well as dissemination in MM (11-15) (Figure 2.2C). Khotskaya *et al* showed that silencing CD138 by RNA interference *in vitro,* resulted in cell death in MM cells (human MM cell lines, RPMI 8226, CAG (cell line established from the bone marrow aspirate of an MM patient), indicating CD138's role in MM pathogenesis (12). In the same study, CD138 was silenced in a mouse xenograft model and the cells also showed low levels of vascular endothelial growth factor (VEGF) expression. Subsequently, fewer, smaller and less invasive subcutaneous tumors were formed (12).

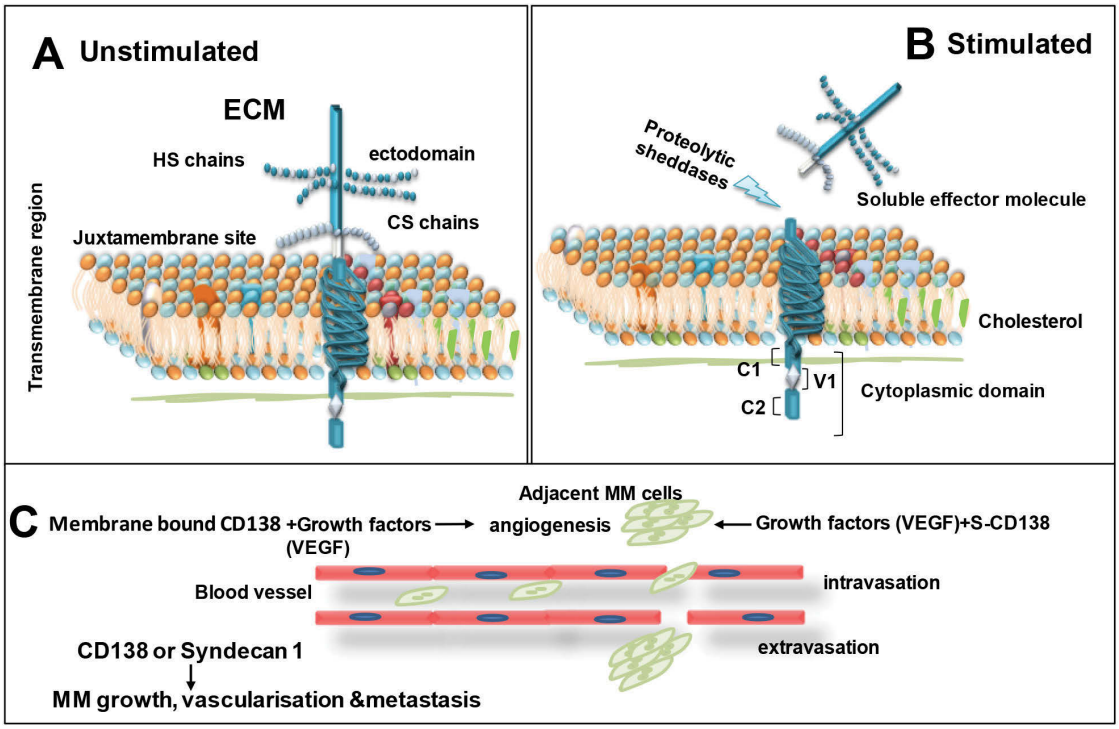

Figure 2.2 Diagrammatic representation of the role of CD138 in MM pathology: (A & B) CD138 is a versatile transmembrane heparan proteoglycan with a cytoplasmic region (a variable region (V1) and constant region (C1& C2 on either side, a transmembrane region and an extracellular region (ectodomain). At resting state, CD138 acts as a co-receptor to a wide variety of growth factors in the extracellular matrix (ECM), promoting tumor growth and metastasis. **(B)** Upon stimulation, the ectodomain is cleaved at the juxtamembrane site (by proteolytic sheddases) as soluble effector molecule and competes for the same ligands. **(C)** Both membrane bound and soluble effector molecule interact with growth factors in the extra cellular matrix initiate angiogenesis and thereby invasion, migration and metastasis of the tumor.

Seidel *et al.* demonstrated that hepatocyte growth factor (HGF) produced by MM cells is believed to have a role in bone resorption in the microenvironment and is associated with negative prognosis. The soluble CD138 effector molecule and HGF formed a complex, subsequently increasing HGF's half-life in BM (87). This demonstrates the supplementary role of shed syndecan 1 in MM pathology by influencing factors like cytokines in the BM microenvironment.

2.4 Current systemic markers used in the clinical management of myeloma

In MM, various genetic and systemic markers are established. Systemic markers provide a minimally invasive approach relative to plasma cell genotyping or the use of the plasma cell labelling index, as the latter requires bone marrow sampling. Current systemic markers (β_2M, M protein, light chains, thymidine kinase, lactate dehydrogenase) aid in prognosis as well staging and govern therapeutic decision-making (Figure 2.3). These markers provide essential biochemical measures of stage and disease progression, renal function, tumour burden, bone physiology, therapeutic outcome and the presence of inflammation commonly associated with malignancy (8) (Figure 2.3)**.** The main systemic markers and their pathophysiological significance are discussed below.

Stage, tumor burden and disease progression: Thymidine kinase (TK) is a phosphor-transferase involved in the DNA salvage pathway and specifically catalyses phosphorylation of deoxythymidine. Serum levels of thymidine kinase (TK) are an indirect measure of plasma cell proliferation (88, 89). Numerous retrospective studies

support the prognostic significance of systemic TK levels in MM patients (88-90). Brown *et al*, observed that serum TK levels > 11 U/l as associated with shorter survival rates indicating its significance as a marker of tumor endurance (89). However, the predictive power of serum TK levels is dependent on the regimens used. It provides significant prognosis with single agent melphalan however is not useful in combination therapy (89).

Likewise, elevated levels of β-2-microglobulin, M or paraprotein, light chains and acute phase proteins (inflammatory markers) are indirect systemic signatures indicative of tumor burden that is discussed in following paragraphs.

β-2-microglobulin (β_2M): β_2M is a low molecular weight (12 kDa) cytoplasmic membrane protein expressed on the surface of all nucleated cells with the exception of red blood cells. It is shed systemically post cell death or following membrane remodelling (91). β_2M is associated with major histocompatibility complex class 1 heavy chain and is a member of the immunoglobulin gene superfamily (92, 93). An elevated level of β_2M is one of the most significant prognostic measures in MM at diagnosis (94). β_2M levels indicate tumour burden and renal function. The current International Staging System (ISS) uses serum levels of β_2M and serum albumin for staging and risk stratification together with patient genotype (32). B2M is only useful in the case of symptomatic myeloma and cannot be used to gauge the transition between benign monoclonal gammopathy of undetermined significance (MGUS), asymptomatic smoldering myeloma and MM (95). β_2M can be a misleading marker of tumor load when it comes to individual patients. A recent study showed that 5 of 6 patients with stage II MM and 5 of 11 patients with stage III MM showed normal β_2M levels. This

means 58.8% of patients with substantial infiltration in the bone marrow showed false-negative β2-microglobulin levels (96).

Paraprotein monoclonal or M protein: Systemic M protein is a hallmark of secretory MM and severely impairs immune capacity of patients as a consequence of its clonal incompetence (19). Paraprotein levels are routinely monitored in secretory MM with an elevated level indicating progressive disease (7). Although, M protein (>3g/dl) in blood is used to differentiate between MGUS and MM, quantitative levels of M protein are not an exclusive marker in MM (97).

Free light chains: The ratio of free light chains (κ/λ) (0.26-1.65 mg/dL) of the monoclonal immunoglobulin is a reliable prognostic indicator in MM especially in non-secretory MM, where the classic M protein secretion is lacking and is an indirect measure of clonality. Nevertheless, serum free light chain assays have limitations including sample dilution anomalies, calibration problems and limits of detection, which may result in erroneous inference of clinical significance (98).

Acute phase proteins as a measure of tumor burden: An acute phase response is typically associated with MM (99, 100). IL6 has a significant role in B cell differentiation; especially in the final differentiation of B cells to mature plasma cells. Together with IL-6, IL-2 IL-1β and soluble IL-6 receptor (S-IL-6R) have also been shown to affect survival of myeloma patients (101, 102). A number of studies have indicated that targeting the IL6 pathway inhibits myeloma growth through inhibition of the nuclear factor kappa B and/or Janus kinase signaling pathways (103, 104). IL6 also protects MM cells against dexamethasone-induced apoptosis by activating protein

tyrosine phosphatase (99). Stromal cell derived factor (SDF)-1 alpha upregulates IL6 secretion in MM, which ensures MM growth, survival and migration (104). Likewise, glycoprotein-130 (gp130), a subunit of IL6 receptor family, is also present in its soluble effector form in circulation. This inhibits the growth of MM cells through its association with IL6 and s-IL6-R. Thus, a ratio of s-IL6-R to gp130 is of prognostic significance (101). IL6 also plays a major role in bone resorption by myeloma cells. IL6 activates osteoclasts, promotes defective bone physiology and provides a measure of the extent of bone disease in MM patients. C-reactive protein levels are regulated by IL6 and provide an indirect marker of IL6 levels and tumor burden. However, CRP levels do not correspond with markers including TK or labeling index (105). Another disadvantage of IL-6 as a systemic marker of MM is its ubiquitous nature with respect to inflammation and is not specific to MM (106).

Aberrant cellular respiration: Typical of malignant cells, myeloma cells are characterized by aberrant glycolysis (107). Elevated levels of lactate dehydrogenase (LDH), an enzyme involved in anaerobic cellular metabolism is a prognostic measure in MM (107). High levels of LDH (≥300 IU/L) have been shown to correlate with lower overall survival and failure to respond to conventional MM therapy (67). Dimopoulos *et al.,* 1991 showed that only 20% of patients with high LDH levels responded to treatment compared to 57% patients with low LDH levels (107). However, LDH's potential to help in risk stratification of patients with respect to novel agents including immunomodulators and proteasome inhibitors remains to be investigated.

Defective bone physiology: MM is typically associated with defective bone physiology. This is monitored by the use of systemic markers of bone formation and

bone degradation clinically. The rate of bone formation is measured indirectly with serum alkaline phosphatase and type 1 carboxy terminal propeptide, type 1 collagen biosynthesis marker (PICP). Similarly, bone resorption is marked by collagen breakdown products such as type 1 carboxy terminal cross-linked telopeptide (ICTP), > 5.0 μg/l) (108-110). PICP and ICTP levels are only indicative of defective bone physiology in MM.

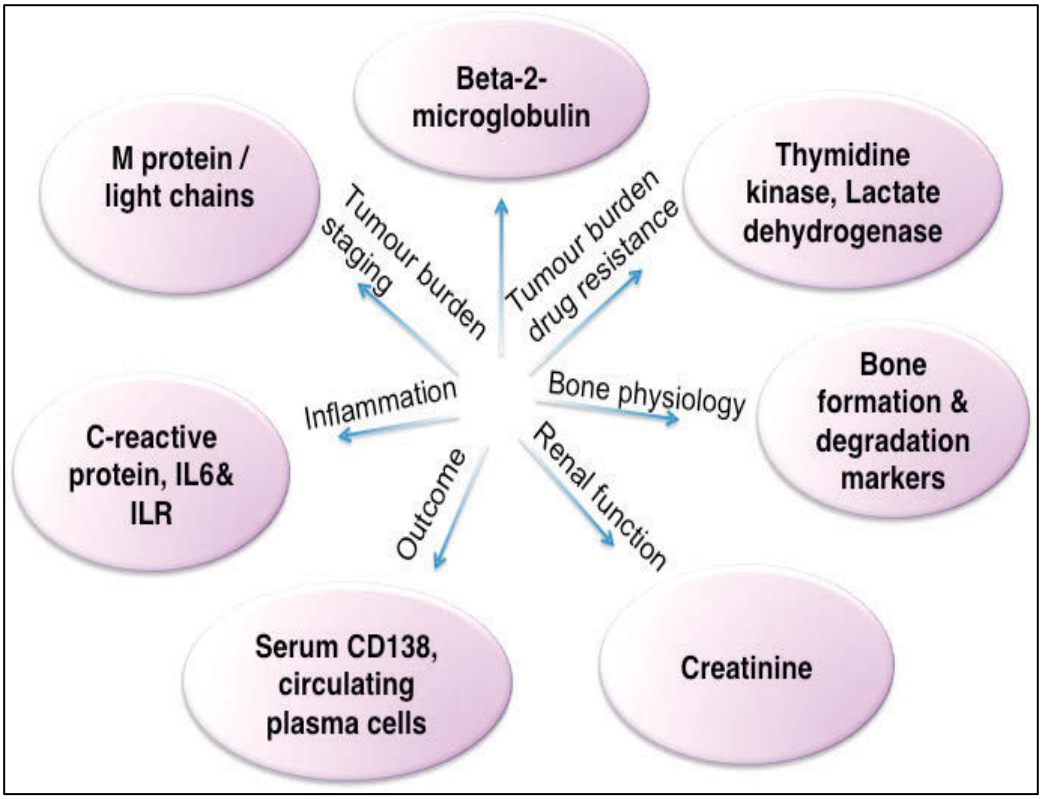

Figure 2.3. Systemic markers in MM: Current systemic markers and what they indicate assisting clinicians in patient counselling at diagnosis also during the course of treatment. Acute phase proteins indicate inflammation and tumor mass whereas M protein and β_2M specifically assist in staging. High level of serum creatinine indicates renal failure and collagen formation and degradation products PICP and ICTP indicate defective bone physiology. Soluble effector molecule of CD138 (S-CD138) and circulating PCs indicate poor outcome.

2.5 Membrane vesiculation and microparticle formation

Our earlier work discovered that microparticles (MPs), submicron-sized vesicles (i) are spontaneously shed from tumor cells; (ii) carry functional resistance proteins including P-gp and nucleic acids from their originating cell; and (iii) can confer MDR and increased metastatic capacity within cancer cell populations (36, 38, 48, 111-115). We also recently showed that plasma cell derived MPs are elevated in MM patients relative to normal volunteers and provide a systemic measure of plasma cell burden in individual MM patients (48). We discuss the MP biogenesis and their potential as 'surrogate markers' for tumours compartmentally confined such as MM.

The initial mention of membrane derived vesicles occurred in 1946 when Chargaff and West described a factor sedimented by high-speed centrifugation in the case history of a female patient with Hemophilia-like bleeding disorder (116). Years later, in 1967 Wolf described the presence of a "dust" like phenomenon around activated blood platelets which were later characterised as membrane derived vesicles (117). Since these initial observations, there has been a significant amount of research done to decipher the biology, function and physiological relevance of MPs (35, 118-131).

MP formation is a natural physiological process that involves cytoskeletal disruption. MP biogenesis is inducted by the disruption of the phospholipid asymmetry maintained across outer and inner leaflets of plasma membrane (35, 132). The fluid plasma membrane maintains an asymmetric distribution of lipids in the resting unstimulated state (133) (Figure 2.4A) The bilayer inner leaflet comprises of aminophospholipids i.e phosphatidyl serine (PS) and phosphatidylethanolamine (PE) whereas the outer leaflet has more phosphatidylcholine (PC) and sphingomyelin (SM) (134). Membrane

remodelling and loss of asymmetry occurs in response to various stimuli such as cytokines, serine proteases, growth factors, thrombin or mechanical stress (35, 118, 125, 135-137).

Three phospholipid transporters govern the asymmetric lipid distribution across the bilayer, namely, an inwardly directed 'flippase' or aminophospholipid translocase (PS & PE), an outwardly directed 'floppase' and a bidirectional 'scramblase' (138-140). The floppase and scramblase are not active in a resting cell while the flippase is "on" maintaining the asymmetric lipid distribution throughout the bilayer (138). Upon stimulation by a range of inducers, floppase specifically transfers PS from inner leaflet to the outer in an ATP dependent manner disrupting the bilayer asymmetry (139) (Figure 2.4B). The non–specific scramblase is dependent on a continued increase in cytosolic Ca^{2+} which facilitates the randomised bidirectional transport of phospholipids (139). The sustained Ca^{2+} inflow switches "off" flippase and floppase that subsequently exposes PS on the plasma membrane surface. In addition, constant high cytosolic Ca^{2+} activates non-lysosomal protein cleaving cysteine proteinase - calpain, which triggers cytoskeletal disruption and remodelling (141). The temporary imbalance in the lipid distribution across the membrane bilayer and Ca^{2+} dependent proteolysis of the cytoskeleton via calpain results in membrane budding and MP release (142) (Figure 2.4B & C).

Microparticles can also arise during apoptosis, the process of which is different as cystein protease enzymes (caspases) mediate membrane vesiculation in this case(137). This process is mainly synchronized by the caspase mediated Rho effector protein 'Rho-associated, coiled-coil containing protein kinase' (ROCK1) as well as thrombin

and tumor necrosis factor-α (136). ROCK1 complex is cleaved during apoptosis generating spasm and subsequent vesiculation and MP release (124, 143, 144). Some research groups use the term microvesicles to refer the same population denoted as microparticles here and the collective term being used in general is extracellular vesicles. The term extracellular vesicles encompass all of the vesicle population.

Microparticles are nevertheless also formed via other mechanisms such as localised hydrophilic pore formation in the lipid bilayer resulting in subsequent membrane disruption and blebbing. The hydrophilic pores enable transport of PS to the outer layer of the membrane leaflet (143, 145). Some research groups use the term microvesicles to refer the same.

MPs characteristically express the procoagulant phospholipid, phosphatidylserine (PS) on their surface and are differentiated from other extracellular vesicles such as exosomes (40-100 nm) and apoptotic bodies (>1 μm) by virtue of their size, morphology, phenotype, composition and origin (36, 146). The expression of procoagulant phospholipids on platelet derived MPs are of significance in MM as they contribute to the hypercoagulable state observed and increases the risk of venous thromboembolism (147, 148). Flow cytometric analysis is typically used to phenotype MPs, which exhibit various cell surface markers that define their cellular origin (35, 36).

2.6 Significance of extracellular vesicles

Systemically released MPs contain membrane as well as cytoplasmic proteins, lipids, nucleic acids and various components from the parent cell (125). MPs play significant roles in intercellular communication, blood coagulation, immunity and host-tumour interactions across many pathologies including cancer (125). MPs have been implicated specifically in vascular biology, inflammatory disease states such as cerebral malaria, and is known to transfer deleterious traits such as multidrug resistance, metastatic capacity and immune evasion in cancer (36, 149, 150). Disease states such as cancer, vasculitis, arthritis, autoimmune disorders and AIDS are associated with elevated MP numbers in circulation relative to healthy subjects (51, 125, 151, 152), (153).

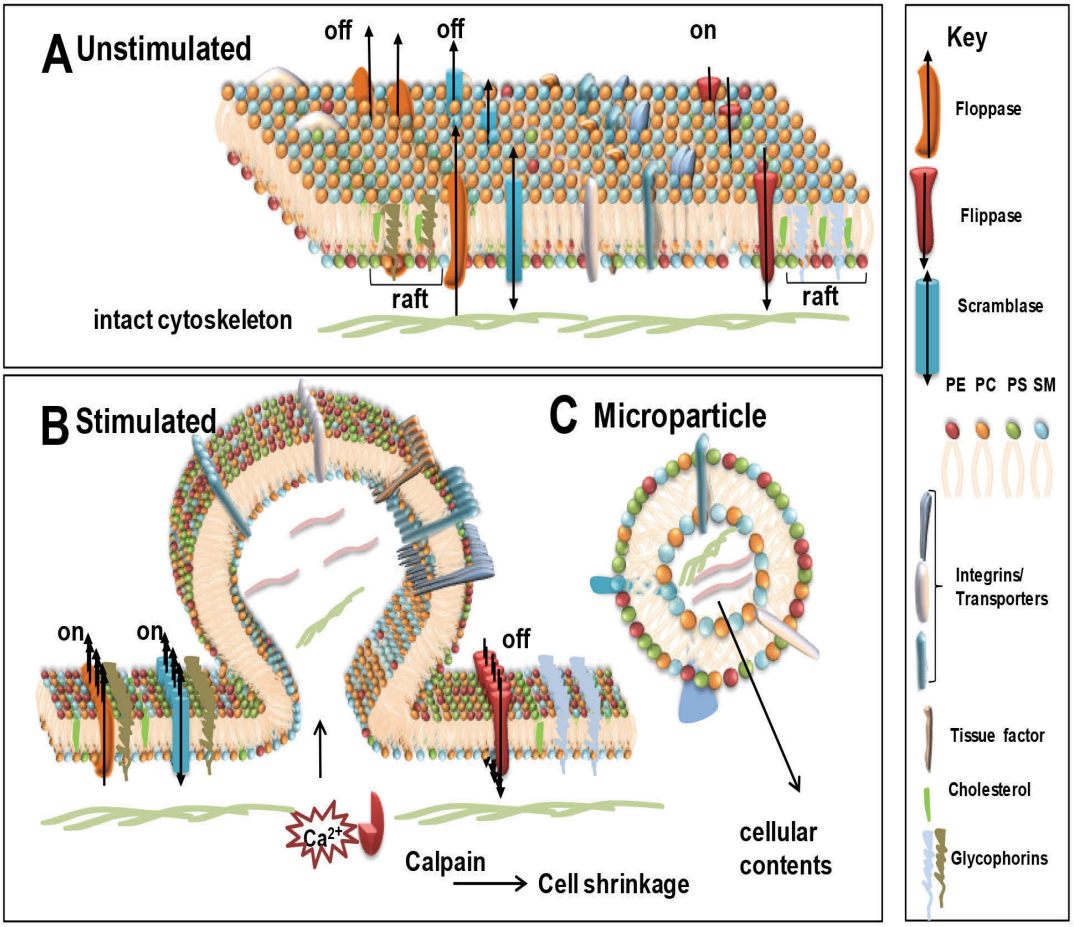
A Unstimulated
off
off
on
raft
raft
intact cytoskeleton
B Stimulated
C Microparticle
on
on
off
Ca2+
Calpain
Cell shrinkage
cellular contents
Key
Floppase
Flippase
Scramblase
PE PC PS SM
Integrins/ Transporters
Tissue factor
Cholesterol
Glycophorins

Figure 2.4 Schematic representation of membrane vesiculation: (A) Membrane asymmetry in an unstimulated cell: The plasma membrane lipid bilayer harbors, tranmembrane proteins, integrins, and ion channels. The membrane proteins are segregated laterally in lipid rafts. In the unstimulated state, the outer layer of plasmamembrane is enriched with phosphatidyl choline (PC) and sphingomyelin (SM) whereas the inner layer is enriched by phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS). The inward directed phosholipid pump flippase is active at this stage and maintains membrane asymmetry of the lipid bilayer. **(B) Disruption of cytoskeleton and membrane vesiculation.** Membrane vesiculation occurs as a response to various stimuli and as a result of cytoskeletal disruption during membrane remodelling. An influx of calcium ions in the cytosol activates cytosolic enzyme calpain disrupting the cytoskeleton, switches on floppase (outward phospholipid pump) and scramblase disrupting the (bidirectional) on the membrane. Floppase redirects PS to the outer leaflet causing exposure of PS - a hallmark of MPs. MPs take over cytoskeletal elements, nucleic acids, proteins, lipids and carry the surface markers from the cell of origin resulting in cell shrinkage. Segregation of lipids and proteins into lipid rafts enable specific packaging of contents while membrane vesiculation resulting in diversity in MPs even if the origin is same. **(C) Diagrammatic representation of a microparticle.** Micropaticles on the surface carries the hallmark of their cells of origin, membrane transporters, tissue factors and exposed phosphatidyl serine (PS). On the inside, MPs harbour selectively augmented cellular contents such as bioactive proteins, lipids and nucleic acids.

2.6 Microparticle physiology in cancer

The presence of microparticles in cancer was first described by Friend C *et al* in 1978 as "rare pleomorphic membrane line particles ranging broadly in size between 400 and 1200 A^0 "in the electron microscopy analysis of cells derived from Hodgkin's disease of a male patient (154). Cancer cells are known to have defective Ca^{2+} homeostasis affecting various cellular mechanisms such as proliferation, motility and apoptosis (155). The role of defective plasma membrane Ca^{2+} channels in cancer has been a focus of cancer biology research. As mentioned above, a continuous increase in cytosolic Ca^{2+} concentration results in membrane remodelling and MP biogenesis. In addition MPs contain cargo required for membrane remodelling such as phospholipid transporters (156), bioactive lipids and functional proteins (157). Moreover, thrombosis is a classic manifestation of cancer and is associated with disease progression (158). MPs typically carry procoagulant phospholipids such as PS and tissue factors (TF) on their surface, which facilitate the coagulation cascade (159). Tissue factor bearing membrane vesicles have the ability to interact with diverse cell types such as platelets, endothelial and stromal cells causing thrombosis at the site of interaction (160). MM patients are at high risk of thrombosis (49, 148, 161, 162) and in this context MPs provide a negatively charged platform for coagulation factors to accumulate resulting in subsequent activation of the coagulation cascade (163-165).

We were the first to report that MPs shed from malignant cells had the capacity to ensure the transfer of deleterious cancer cell traits to recipient cells leading to trait dominance within the recipient cell population (38, 39, 43). We observed that MPs isolated from MDR cells had a remarkable capacity to "re-template" the transcriptional

and protein landscape of drug responsive recipient cells to ensure the dissemination of MDR within a cancer cell population (38, 39, 43). We also reported on the mechanism for the observed rapid transcriptional response in recipient cells to occur via the selective packaging of unique RNA species in MPs from the donor cancer cells (166). Furthermore, we demonstrated that MP mediated MDR transfer is accompanied by the transfer of an interaction complex comprising resistance proteins (P-gp), CD44 and cytoskeletal binding proteins. We have also shown that MPs can confer biomechanical changes in recipient cells once again transferring donor cell traits to recipient cell populations (167) (115) (Figure 2.5). Many studies have described a myriad of roles that MPs play in cancer and haematological malignancies such as MM.

a) Tumor survival

Boing *et al* reported the presence of caspase 3 enriched platelet MPs in stored platelet concentrates and in the plasma of healthy humans (168). Surprisingly the parental platelet cells did not have any detectable caspase 3. Caspase 3 is an important enzyme in the apoptotic pathway and it is hypothesised that the cells escape programmed death by packaging caspase 3 into these submicron vesicles, effectively sequestering the enzyme within the shed cargo. This was further validated when cells accumulated caspase 3 and subsequently underwent apoptosis upon inhibition of MP release (168, 169). Moreover, MPs sequester chemotherapeutic drugs and reduce the free available drug concentration that would otherwise act on the cancer cell (112). This mechanism affectively confers a parallel and alternative pathway of drug resistance (112).

b) Immune evasion

Immunosurveillance describes the processes by which cells of the immune system look for and recognize foreign pathogens in the body. Invasion and colonization of tissue by foreign cells is normally contained by a spontaneous response by both the innate and adaptive immune systems. At times however this stringent control fails and immune escape or immunoevasion can occur through various mechanisms, such as reduced immune recognition, increased resistance to attack by immune cells or the development of an immunosuppressive microenvironment (170).

A particularly interesting and emerging area in cell biology is the role of extracellular vesicles in immunosurveillance immunoevasion. Certainly, there is emerging evidence that extracellular vesicles can be implicated in facilitating immunoevasion. For instance, the differentiation of monocytes to antigen-presenting cells has been shown to be inhibited upon fusion of MPs with monocytes (171, 172). Likewise MPs which expose latent membrane protein, an immune-suppressing antigen, can inhibit leukocyte proliferation(173). MPs are also secreted by monocytes/macrophages and these MPs contribute to modulating the epithelium. Specifically, macrophage derived MPs shuttle functional micro-RNA's into breast epithelial cell, providing an intercellular pathway for cell migration (174).

In particular, complement channel mediated immune response mechanism is impaired in cancer. The complement proteins, which are, located on the plasma membrane (C1-C14), of which, the terminal complement complexes or membrane attack complex (C5-C9) induce a higher cellular concentration of Ca^{2+} and in proportion to their functional

pore size. This disrupts the osmotic gradient of cell membrane and thereby causing complement mediated cell lysis (175). However, it has been shown that human squamous cell carcinoma cells release membrane bound vesicles enriched with the terminal compliment complex. This results in survival of these malignant cells from complement induced lysis and an efficient mechanism to escape the immune watch (176). It is reported that human colorectal cancer cells evade immune surveillance induced T-cell apoptosis by releasing Fas ligand-bearing and tumor necrosis factor–related apoptosis-inducing ligand bearing microvesicles both *in vitro* and *in vivo*(177, 178). Similarly, cancer derived MPs suppress antigen-presenting cells and also cancer cells themselves are capable of fusing with non-malignant MPs, hijack their content and thus evade immune surveillance (45, 179). We recently reported that breast cancer derived MPs can alter the phenotype and functionality of immune cells (THP-1 macrophages) and thereby facilitate immune evasion. MDR^+ breast cancer derived MPs have been shown stimulate the release of pro-inflammatory cytokines, render macrophage incapacity and stimulate the engulfment of THP-1 macrophages by MDR^+ breast cancer cells. This is a novel MP-mediated mechanism contributing to the establishment of the pre-metastatic niche and which may facilitate metastatic disease (180).

The immune system in MM is severely impaired due to the defective haematopoiesis and clonally incompetent antibodies produced by aberrant PCs. This results in recurrent infections in MM patients with a life changing impact on patients and care-givers (79). MM cells deliberately evade the immune watch consequently aiding in tumor survival through various mechanisms. The phenomenon of ‘trogocytosis’ or ‘nibbling’ is one such mechanism in MM pathology, which is more prominent in MM compared to other

plasma cell dyscrasias (78). Trogocytosis is a phenomenon by which surface antigen exchange occurs in lymphocytes creating unique cell phenotypes with exclusive function (76). Interestingly T cells are more proficient in acquiring antigens from PCs than B cells (78). Trogocytosis enables tumor-induced immune suppression by the formation of immune synapses and subsequent unique cell types, which regulate intracellular signalling in T cell subsets (77). Interestingly, there has been no in depth studies yet to investigate if there is any connection between trogocytosis and MP mediated trait transfer across cell types.

c) Extracellular matrix degradation

The extracellular matrix (ECM) is a major player in MM pathophysiology (181, 182). Interactions of malignant PCs through bioactive molecules like cytokines and growth factors with the ECM components such as stromal cells, fibronectin orchestrate MM progression and tumor survival (15, 183-186). In a study, which compared non-active MM and active MM (187), Vacca *et al*, showed that MM progression is associated with the secretion of matrix metalloproteases (MMP-2) in the bone marrow biopsy samples. The increased secretion of matrix metalloprotease is believed to assist in the medullary and extramedullary dissemination of MM (187). In terms of ECM components and MPs, it is known that MPs contain matrix metalloproteases (MMPs), its precursors and urokinase type plasminogen activator (uPA). Ginestra *et al* described an MP mechanism by which MMPs and proteins such as plasmin disrupt the ECM structure and facilitate tumor survival (188). The study compared the ascites across benign ovarian lesions, ovarian carcinomas and endometrial carcinoma. The patients with malignant status had higher membrane bound vesicle counts compared to the benign condition. In addition,

the vesicles from ovarian cancer patients contained active MMPs (MMP-2)(188). Subsequently, Graves *et al* confirmed this finding through the inhibition of MMP-2, MMP-9 and uPA which rendered microvesicles incompetent to support invasive tumor growth in ovarian cancer (189).

d) Vascularisation, invasion and migration leading to metastasis

High risk of venous thromboembolism is associated with solid and hematological malignancies (187, 190). MM progression has been shown to be accompanied with increased bone marrow neovascularization as demonstrated by elevated levels of fibroblast growth factor 2 and factor VIII+ microvessel area (187). Procoagulant MPs also promote angiogenesis by releasing various growth factors such as VEGF and IL6 (11, 14, 191). Our lab demonstrated that the *miR-503* is down regulated in recipient cells post co-culture with drug resistant cell derived MPs in breast cancer cells (39). Interestingly, *miR-503* is inversely related to metastatic ability as demonstrated by the wound healing migration assays and invasion assays. This study also established that proline-rich tyrosine kinase 2 (PYK2) was upregulated in the recipient cells and the recipient cells showed increased invasive and migratory capacity. This particular phenotype was reversible by tyrphostin A9 a pharmacological inhibitor of PYK2 phosphorylation. This study highlighted a significant aspect of the presence of effector molecules (PYK2 protein and transcripts) in MP cargo, which regulate the crucial downstream components in the cellular pathways. Besides the capacity of MPs to directly transfer of deleterious factors, this demonstrated that MP cargo can also include the transfer of arbitrator regulators in cancer. This eventually results in the reprogramming of the proteomic and/ or transcriptional landscape of the recipient cells

further expanding the role of MPs in cancer. We also demonstrated that intercellular transfer of P-gp in recipient cells was also dependent on the presence of cytoskeletal motor proteins ezrin and moesin while P-gp functionality is dependent on the presence of all three components of ERM complex (ezrin, radixin and moesin) proteins in recipient cells (192). Our recent work also showed that MPs shed from MDR^+ cells influence the biomechanical properties of recipient cells. Specifically, the study compared the stiffness (measured as Young's modulus) of drug-sensitive MCF-7 spheroids and their MDR counterparts (Dx spheroids and MDR acquired MCF-7 spheroids post co-culture with Dx-MPs) using optical coherent elastography (167). An increase on stiffness in MCF-7 cells after co-culture with MDR^+ leukemic and breast cancer cell derived MPs was observed. The study further silenced P-gp, CD44 as well ERM proteins and observed a significant decrease in stiffness post silencing. This confirmed the role of P-gp and CD44 in particular, in affecting the Young's modulus of MDR cancer cells, suggesting that their transfer by MPs plays a role in the observed increase in the stiffness of recipient cells. This is an interesting observation as stiffness is a biomechanical property of tissue which has implications in cellular function, including adherence, motility, and invasion (193, 194). Stiffness as measured by Young's modulus is a distinctive parameter of malignancy as malignant cells are known to be on average softer (lower Young's modulus) than non-malignant cells (193, 194). The pathological transformation from a systematized cytoskeletal network to an unbalanced state may have a consequence on their biomechanical properties (195, 196). However, the distinct mechanism by which this biomechanical transformation occurs in oncogenesis is not clearly identified yet.

e) Microparticle cargo.

Micro RNAs (*miRNAs*) are non- coding short RNAs (~ 22 bp) known to regulate gene expression at a post-transcriptional level (197, 198). *miRNAs* also have significant potential as prognostic markers aiding in early detection of malignancies such as colorectal cancer (*miR 21)*(199). In non-small–cell lung carcinoma, *miR -324a* levels indicated poor survival while plasma *miR-21*, *miR-494* and *miR-1973* are disease state indicators in Hodgkin lymphoma (200).

We previously showed that the RNA cargo of MPs include messenger RNA (mRNA), functional microRNA(37, 38, 43, 47, 201), as well as *many* different kinds of noncoding RNA species beyond miRNA (43, 44, 156, 202) (identified by us through unbiased surveys of microparticle RNA). These include vault RNAs as well as their derivatives, pseudogenes, tRNA fragments, and retrotransposon-derived small RNAs (44, 203, 204). The RNA content of a microparticle does not necessarily reflect the total RNA population of the cell from which it is derived; in other words, many RNA species are selectively packaged into the microparticle, suggesting their functions are destined to be performed outside the donor cell (43, 156).

miRNAs have been shown to be implicated in MM pathogenesis(149, 205). More specifically, a comparative study of miRNA profiles between pre-malignant/malignant plasma cells (MGUS and MM) and normal $CD138^{+}$bone marrow plasma cells showed that *miR-21* and oncogenic *miR-106b~25* (specifically, *miR 93*, *miR -106b* and *miR-25)* clusters were up-regulated in MGUS. *miR-106b~25* oncogenic cluster appeared to regulate pro-apoptotic genes and play a role in disease progression in this study using

miRNA microarrays and quantitative RT-PCR targeting 3^{1} –UTR of P300-CBP-associated factor (PCAF) which regulates P^{53} expression (206) (207). The lymphoproliferative transformation of plasma cells in MGUS is believed to be initiated by *miR-21* and *miR-106b~25* through their effects on hindering apoptosis, promoting survival and predisposing the benign condition to secondary genetic abnormalities, leading to the malignant state of MM (205). In the context of the malignant plasma cells, *miR-32, miR 17~92, miR-21*, *miR-106~2, miR-181a* and *miR-181b* were shown to be up-regulated relative to normal cells. Roccaro *et al* demonstrated that *miR-15a* and *miR-16-1,* which are presumed as tumor suppressors are implicated in regulating tumor proliferation in MM. Importantly, *miR-15a* and *miR-16-1* are located on the q arm of chromosome 13 and 13q14.3 is a frequent deletion in MGUS and MM cohort (205, 208, 209).

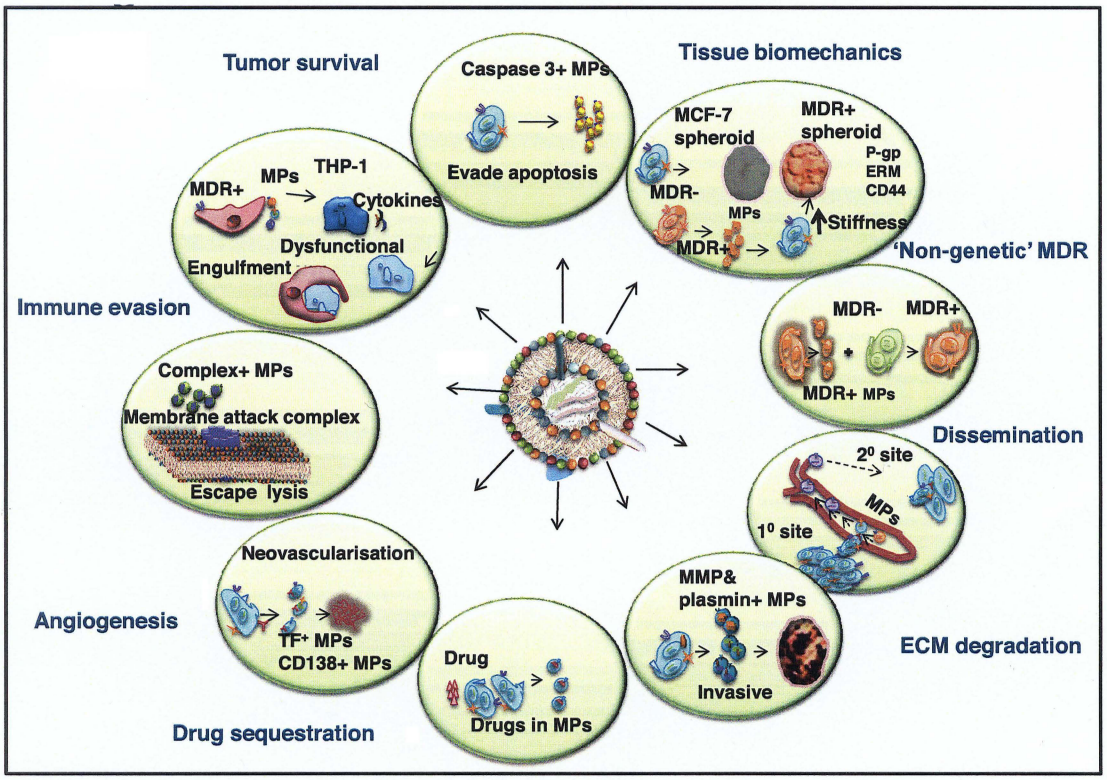

Figure 2.5 Extracellular vesicles in cancer: Extracellular vesicles (EVs) are emerging as substantial players in cancer biology. The myriad of roles played by EVs is graphically summarized here. EVs act as support system for cancer dissemination and mediators of tumor survival.

2.8 Extracellular vesicles in myeloma

Benameur et al., 2013, previously showed the presence of MPs in MM in the 5T2MM mouse model of myeloma. This study demonstrated that elevated levels of MPs were detected in late stage disease relative to early stage (210). This study was focussed on a C57BL/KaLwRij mouse model of MM, whereby MPs $CD138^+$ MPs were enumerated in early stage MM and compared to that of late stage disease (210)**.** The circulating MP number and phenotypic subtypes such as endothelial, platelet, procoagulant, RBC and WBC derived MP levels in the late stage MM (10-12 wks) were significantly higher compared to the control cohort. However, the MP count in the early stage of MM (6 wks) was significantly lower than the control groups across all the sub-types. MP count in the bone marrow was also elevated in late stage MM and expressed $CD138^+$ as demonstrated by transmission electron microscopy.

Increased risk of venous thromboembolism is a complication associated with MM pathology and it manifests in the initial induction therapy phase itself for many patients (159). MP associated TF activity is commonly observed in cancer and in MM, MPs also support hypercoagulable state and TF bearing MPs can interact with activated platelets (159, 163). Platelet MPs and their clinical role in thromboembolic risk is recently the focus of investigation especially with the introduction of IMiDs in MM(49). Auwerda *et al.* reported the higher incidence of platelet derived MP-tissue factor activity in *de novo* MM patients to that of healthy volunteers. (159). The study showed that in MM, TF activity associated with platelet derived MPs is considerably higher in *de novo* MM cohort than healthy volunteers. The platelet MP count was also shown to decline in response to treatment in MM patients (159).

Microvesicles secreted from the human myeloma cell line RPMI8226 and *in vivo* have also been shown to promote angiogenesis through the transfer of oncogenic CD138 to endothelial cells (Liu *et al*, 2014). The fusion of MM microvesicles (MVs or MPs) and human umbilical vein cells EA.hy926 was studied using confocal microscopy. The study observed that the co-incubation of MM microvesicles with EA.hy926 resulted in the reprogramming of these endothelial cells. Flow cytometric analysis demonstrated that MM-MVs transferred CD138 to EA.hy926 cells. The CD138 augmented EA.hy926 cells secreted significantly higher key angiogenic regulators in MM (vascular endothelial factor (VEGF) and IL-6. This resulted in increased proliferation, invasion and formation of tubes *in vitro* and *in viv by* EA.hy926 cells. study demonstrates the significant pathophysiological role of CD138 enriched MPs as mediators of new vasculature formation aiding in pathophysiological dissemination of MM (14).

MM pathology is a presentation of the intricate network of various components in the bone marrow microenvironment. Roccaro *et al* established that BM-mesenchymal stromal cells (MSCs) release exosomes (extracellular vesicles of 50-100 nm size) and there is substantial difference between normal BM-MSC derived exosomes to that of MM BM-MSCs in terms of their content and function (211). The study demonstrated PKH67 labelled normal and MM cells were readily ingested MM BM-MSC-derived exosomes. BM-MSC-derived exosomes from MM patients induced tumor growth *in vivo* and aided in dissemination of tumor cells in an *in vivo* translational model of MM. The study further analysed the proteomic content of the normal and MM BM-MSC-derived exosomes to clarify the tumor-initiating capacity of MM-BMSC derived exosomes. The tumor suppressor gene regulator *miRNA- miR-15a* was down regulated

in MM BM-MSC derived exosomes compared to normal BM-MSC derived exosomes indicative of a tumor-suppressive role of mesenchymal stroma cell derived exosomal *mir*-15a (208, 211). The MM BM-MSC derived exosomes had higher oncogenic protein expression, cytokines and protein kinases relative to normal BMSC -derived exosomes. The BM-MSC derived exosomes were readily ingested by MM cells which indicated a potential role of extracellular vesicles in MM biology.

In addition, Wang *et al* demonstrated that BMSC derived exosomes are crucial in communicating deleterious traits such as MM cell proliferation, migration and survival. The BMSC-derived exosomes induce resistance to bortezomib in MM cells in the 5T33 murine MM model (212). The study also validated the exchange of cytokines between BMSCs and MM cell derived exosomes by confocal microscopy. The cytokine array of the exosomes showed that BMSCs and MM cells can cross-talk and exchange cytokines through an exosome-mediated pathway. The BMSC-derived exosomes were found to be enriched with several chemotactic proteins such as stromal cell derived factor 1(SDF-1) and monocyte chemoattractant protein 1 (MCP-1) that effectively promoted *in vitro* MM cell migration. BMSC-exosomes also substantially increased the viability, proliferation and survival capacity of MM cells. Further, the effect of BMSC-exosomes on bortezomib-induced apoptosis was examined in this study. The caspase 9, 3 mediated apoptotic cascade resulting in PARP cleavage is the mechanism of bortezomib action in MM and BMSC-derived exosomes inhibited this pathway and thereby protected the MM cell. RPMI8226 MM cell line was treated with BMSC-derived exosomes obtained from MM patient bone marrow samples to validate the induced resistance to bortezomib by BMSC-derived exosomes further. The BMSC-derived

exosomes increased MM cell viability to 25% in the presence of bortezomib whereas only 9% of MM cells survived in the absence of bortezomib (212).

Harshman *et al* analyzed the proteomic content of extracellular vesicles from MM.1S and U266 MM cell lines (213). The study compared the proteome of MM 1S and U266 cell lysates to their respective extracellular vesicle population and found a significant overlap in proteomic content. This study used a novel label free approach to identify the relative abundance of proteins in the within and across these vesicles of distinct cell line origin to that of their parents. The study demonstrated that extracellular vesicles from two cell lines shared a common protein profile to a significant extent however contained a small sets of unique proteins with statistically distinct abundance. The study unravelled that MM.1S vesicles show increased abundance of HLA class II histocompatibility antigens when compared to the cell lysate. The role of this specific packaging is also described by Raposo *et al* (214). The MHC class II complexes stimulate T cells *in vitro* and specific shedding of these molecules into vesicles may be indicative of an immune evasion mechanism whereby they avoid the recognition by $CD8^+$ T cells and supporting MM cell survival.

Extracellular vesicles as a prognostic in myeloma

Circulating MPs are promising candidates for "surrogate markers" of inadequately accessible tissues such as the bone marrow compartment. MPs carry signature markers of lineage and they are selectively packaged with cellular content such as nucleic acids, micro RNAs, lipids and proteins from their cell of origin. The consideration of MP counts and molecular profile has been shown to correspond to disease pathology and/or treatment sensitivity at an individual level (48, 153, 215).

There is not much known about the role of extracellular vesicles in MM pathogenesis and their exact role in disease pathology and progression remain largely unexplored. We recently reported on the isolation, detection, morphology and numbers of systemic plasma cell derived MPs ($CD41a^-$ $CD138^+$) in MM patients (48). Microparticles were isolated by differential high-speed centrifugation, as previously described by us (36) and validated by flow cytometry for typical characteristics of size and phosphatidylserine (PS) exposure.The isolated MPs displayed a spherical and smooth morphology with a mean size of 0.1-1 μm in diameter. Platelet-free plasma was used as the starting material to ensure that contamination by platelet-derived MPs in the final preparation was minimised. Microparticles arising from plasma cells were detected using anti-CD138-APC mAb. We observed greater $CD138^+$ MP counts in MM patients relative to healthy subjects. Consistent with this, we observed greater $CD138^+$ MP counts for patients in remission (CR and PR) and with progressive disease (PD) relative to healthy volunteers. In this study, we also identified 9 patients who were in complete remission (defined using the IMWG response criteria) at the time of analysis who had greater $CD138^+$ MP counts relative to the rest of the cohort. These 56% of these patients clinically relapsed a few weeks later, demonstrating the potential for $CD138^+$ MP counts to predict the transition between remission and progressive disease before clinically used markers. We also reported on the prognostic potential for $CD138^+$ MPs in predicting 'risk of relapse' in individual patients.

We have previously investigated the potential for MPs to serve as biomarkers in gauging therapeutic outcome and MDR in cancer. Our earlier work revealed that (i) MPs are spontaneously shed from tumor cells; (ii) they carry functional resistance proteins including P-gp, MRP1 and nucleic acids from their originating cell; and (iii)

can confer MDR and increased metastatic capacity within cancer cell populations within a matter of hours (36, 38, 39, 111-115). We thus explored the clinical relevance of P-gp$^+$ MPs in MM patients. We reported the presence of numerous MP subtypes when probing for the presence of P-gp on MPs, including the presence of a dual positive MP population (CD138$^-$CD34$^+$P-gp$^+$) of 'stem cell like' origin, which was associated with an aggressive disease state. The presence and dominance of distinct MP subtypes during the course of disease demonstrates an evolving shift cell populations and phenotypes during disease progression and following treatment. In light of these studies we conclude that CD138 that is synonymous with plasma cell burden cannot be considered a 'static' biomarker throughout the full course of disease; rather it appears relevant during responsive states and diminishes in aggressive disease. This has important implications in how we define the utility of biomarkers with respect to disease progression generally.

PS is a ubiquitous marker of MPs arising from loss of phospholipid asymmetry during MP biogenesis (137). However, it is also known that PS is not an exclusive marker with expression variable within the MP population (129, 143). PS is also emerging as an important mediator in extracellular vesicle biology. A recent study showed that PS on hypoxia induced mesenchymal stem cell derived microvesicles was crucial in the internalisation of vesicles into human umbilical cord endothelial cells (HUVECs) (216) suggestive of a role in supporting angiogenesis. We observed significantly elevated numbers of PS$^+$ MPs across all MP subtypes and elevated levels were observed specifically across active disease states. The significance of the increased PS$^+$ MP event in myeloma is currently unknown and may be linked to the dissemination of malignant cells to extramedullary sites during disease progression (216) (Figure 2.6).

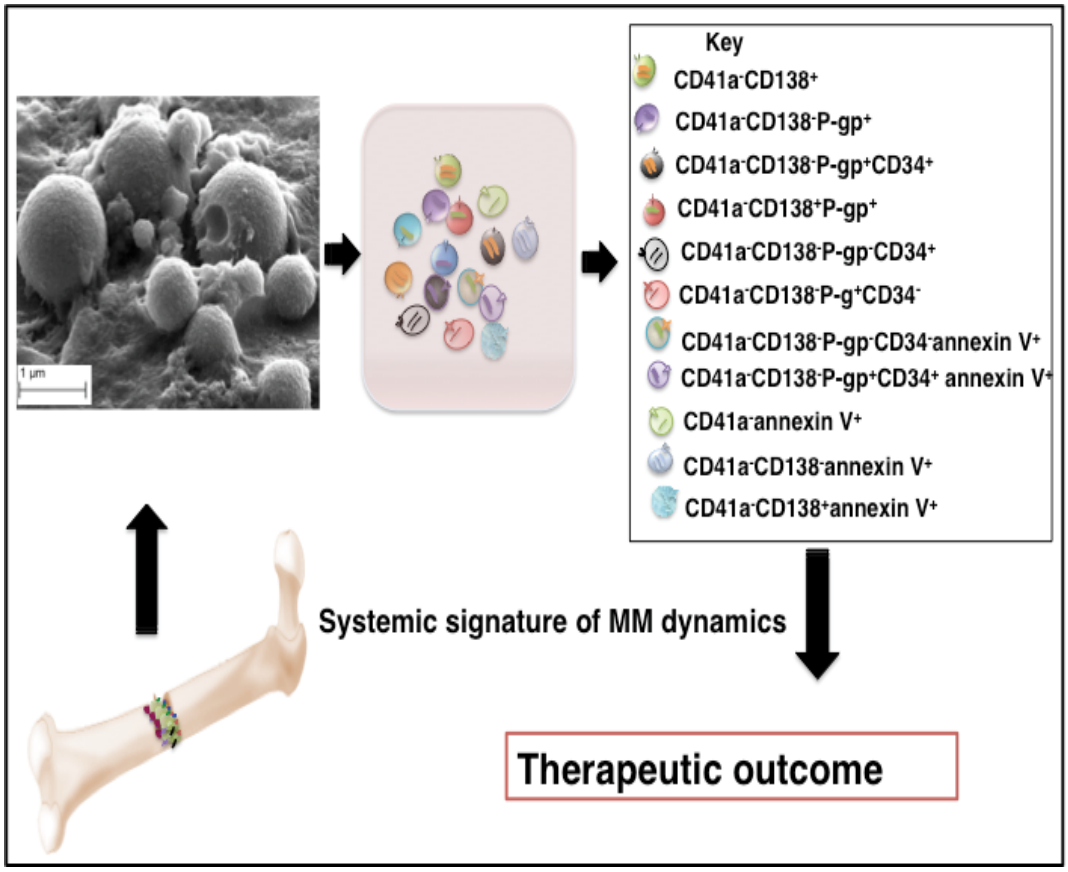

Figure 2.6 Microparticle subtypes in MM: Microparticles of 0.1-1µm diameter are systemically shed in MM. They are readily detectable based on CD138 positivity and based on MP sizing parameters. Multiple sub-sets of MPs are present in MM and they collectively or exclusively represent the evolving dynamics of MM during the course of therapy and contribute to therapeutic outcome.

2.9 Conclusion

This review has examined current systemic markers in MM and their limitations in giving substantial information in optimising desirable therapeutic outcome in patients. We discussed factors that can impact on patient responsiveness and the need for individualised approaches to maximise treatment efficacy and survival. As therapeutic response in MM is unpredictable with the evolution of drug resistance during the course of therapy, despite the use of the novel agents, we discussed the need for a systemic clinical tool which can gauge the evolution of MDR, predict risk of relapse and monitor disease progression routinely in the clinical management of myeloma. Existing clinical tools are limited as they provide an indirect measurement of tumor burden, cannot directly measure resistance protein expression routinely or non-invasively and cannot capture the patchy, multi-site tumor infiltrates associated with MM.

Our prior studies have shown that MPs are effective vectors in the transfer of MDR proteins in vitro and *in vivo*. The presence of MDR proteins within the vesicle cargo makes them potential biomarkers with prognostic potential for gauging the development of MDR in MM.

Indeed, our recent clinical studies have shown that the number, phenotypes of MPs in MM are indicative of patient response state, the emergence of MDR and disease progression. The demonstration that the emergence of P-gp^{+} MDR in MM can be detected and monitored serially by analysing MPs in patient blood samples makes a significant contribution to achieving this goal. We also discuss the need to reassess the utility of defined biomarkers during disease progression given our observations for the

diminishing presence of CD138, the classic myeloma cell marker in aggressive and/ or progressive stages of disease in MM. The existence and dominance of MP subtypes reflect the evolving and ever changing dominant cell populations during the course of disease and in response to treatment. Taken together, in defining biomarkers, careful consideration should be given in this context.

MPs are detected systemically in healthy individuals; nonetheless higher levels are indicative of cellular activation across a number of pathologies (51, 215, 217). The detection of circulating cancer-derived MPs from different cancers, has defined them as promising "surrogate" markers in compartmentalised malignancies (i.e. brain and bone) (44-47). Surface phenotyping using flow cytometric technique is the gold standard applied in MP analysis as they display various cell surface markers denoting their cellular origin (35, 36). This aligns well with the routine flow cytomteric applications used in routine haematology and in the current MM clinical setting. This seamless integration of this approach when combined with existing clinical tools provides for a thorough and systematic assessment of the complete disease landscape in individual myeloma patients. A routine and assessment and monitoring patients for the 'risk of relapse' prior to clinical manifestation has the potential to tailor treatment regimens to patient characteristics and see increases in progression free survival.

As discussed, there is currently no cure for MM making the clinical management and 'control' of the condition of utmost importance for optimising patient quality of life. The approaches to treatment myeloma are generally divided into; 'cure' where hard-hitting chemotherapy is adopted and secondly, 'control' where the emphasis is on maintaining the quality of life. The numbers and molecular profiling of MPs can

potentially assist with both. Furthermore, the individualized approach in therapeutic management of MM addresses the vast inter-individual variability limiting current generalised approaches.

In conclusion, a systemic biomarker with personalized prognostic capacity for determining the evolution of disease progression provides a relevant addition to the current repertoire of prognostic clinical tools. The ability to continuously monitor patients during the course of treatment would allow for improved patient survival as alternative treatments can be initiated promptly to prevent re-occurrence of significant tumor burden. This would certainly be useful in cases of non-secretory myeloma, which lack the classic manifestation of elevated M-protein levels. These new insights into the molecular mechanisms contributing to disease progression, MDR and treatment failure in MM and identify key biomarkers, introduce new approaches for disease state management in MM.

Author contributions

SRK performed all the experiments, data analysis and prepared the manuscript. MB designed and supervised the research, provided conceptual advice and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Aims and Hypothesis

Hypothesis: A relationship exists between clinical outcome and the levels and phenotype of non-platelet derived microparticles ($CD41a^-$), such that the levels and phenotype of microparticles can be a predictive indicator of clinical state, drug responsiveness and risk of relapse in MM patients.

In testing this hypothesis we aim to:

Aim 1: Develop a validated workflow for the isolation, detection and phenotyping of $CD138^+$ (plasma-cell-derived) microparticles from the peripheral blood of myeloma patients at diagnosis and during therapy using polychromatic cytometry.

Aim 2: To detect and enumerate the microparticles in the peripheral blood of myeloma patients (*de novo* and under active treatment) and statistically associate $CD138^+$ microparticle count to distinct clinical response states with respect to tumor burden.

Aim 3: To phenotypically characterize non-platelet derived MPs for the presence of P-gp, CD34 and the extent of phosphatidylserine (PS) exposure and to statistically compare the association of these phenotypes and levels are indicative of disease progression as well treatment unresponsiveness in myeloma. The study design used to achieve these aims is as follows.

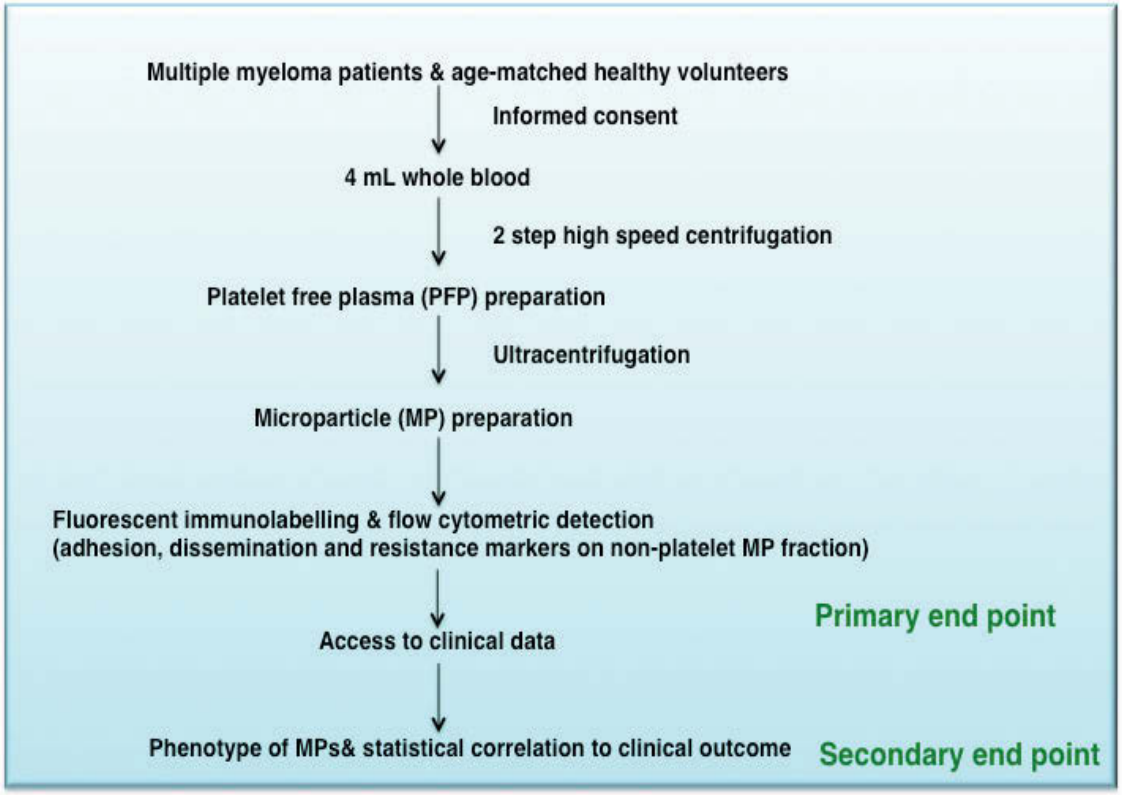

Figure 2.7 A sequential outline of the study design with primary and secondary outcomes is diagrammatically represented.

Chapter 3

3. The development of validated protocols and workflows for the isolation, quantitation and phenotyping of microparticles from myeloma patients.

Running title: Microparticle protocols and workflow

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Key words: biomarkers, extracellular vesicles, microparticles, multiple myeloma, platelet free plasma, polychromatic flow cytometry, prognosis

Abbreviations: microparticles (MPs), multiple myeloma (MM), phosphatidylserine (PS), platelet free plasma (PFP), scanning electron microscopy (SEM)

Conflict of Interest: The authors declare no conflict of interest

3.1 Abstract

Microparticles (MPs); a subtype of extracellular vesicles, are important players in the cell-cell communication in many pathologies including cancer. Multiple myeloma (MM) is the second most common hematological malignancy, a plasma cell neoplasm that is predominantly restricted to the bone marrow compartment. Flow cytometry is an integral technique used in clinical haematology and is used for cell analysis. The utility of extracellular vesicles as biomarkers of disease burden and state is gaining momentum in clinical applications. With the availability of advanced high resolution flow cytometry and improved flourochrome technology, characterizing extracellular vesicles, specifically microparticles, as part of routine diagnostics provides a viable clinical tool. Here, we provide validated methodologies and workflows for the isolation, storage, enumeration, phenotyping and visualization of MPs from clinical samples. We conclude the discussion on the application of this workflow as a component of 'liquid biopsy' with significant information on tumor burden, disease progression and treatment unresponsiveness in MM.

3.2 Introduction

Microparticles (MPs) are increasingly recognized to play important roles in cell biology with key pathophysiological consequences (1-4). In fact, they form a network for localized and long-range signaling between cells (5). In the context of MM, aberrant plasma cell clones orchestrate survival though their homotypic and heterotypic interactions with the tumor microenvironment, including the extracellular matrix, other immune cells and bone cells (6) (7). These intricacies support a complex and enigmatic survival mechanism associated with MM. We have shown that MPs contribute to this niche and play a role in disease pathology (8).

There are many techniques used in characterizing MPs currently. The major aspects of MP research interests are their cellular origin, count or levels, cargo, function and expression of proteins nucleic acids and lipids they carry. The prominent methodologies used in MP research are flow cytometric phenotyping, various microscopic techniques such as Electron, florescence and atomic microscopies and capture based assays. Electron and fluorescence confocal laser scan microscopy is used to study the morphological and membrane characteristics of MPs. Gold-labeled antibodies are also used to study the MP surface expression. These techniques while giving substantial information and confirmation on the MP characteristics are time consuming and are not applicable in clinical setting for direct detection from plasma. Capture based assays or microplate affinity assays use either antibody or annexin and is another method used in MP research. The wells are coated with the probe (antibody or annexin) and a secondary antibody is used to analyze captured MPs or using their functional property. These assays are robust and can be used to study MPs directly from plasma. Capture based assays can be modified specifically for clinical use for large number of samples however

it cannot directly measure the MP levels as it measures the total phosphate or via prothrombinase assays to quantitate MPs. In addition, morphology and size of MPs cannot be analyzed through this method (9, 10).

Fluorescence immunolabeling and flow cytometric detection are widely used to phenotype MPs, as they display various cell surface markers that indicate their cellular origin and are a direct measurement of MP characteristics of interest. The main points of interest in MP research in a clinical setting are MP phenotype, levels and cargo. Flow cytometry offers direct analysis of phenotype and levels as well a consolidation to explore the cargo. MPs typically carry exposed phosphatidylserine (PS) on their surface as a hallmark, which can be detected by immunolabeling and flow cytometric detection (3, 11). MPs are present in the plasma of healthy individuals, however elevated levels are indicative of cellular activation and disease. Elevated MP levels have been reported for conditions such as diabetes, vascular diseases, malaria and cancer (1, 4, 8, 12-14). Current improved range of flurochromes with improved signals also facilitates multicolour flow cytometric phenotyping.

The assay validated here specifically for MM uses CD138, a transmembrane proteoglycan expressed on the surface of plasma cells for phenotyping. CD138 is arguably an exclusive marker for the flow cytometric phenotyping of plasma cells (15). CD138 has a dual nature as it resides in two forms; as an integrated membrane protein and a soluble effector molecule (16). CD138 facilitates cytoskeletal organization, cell proliferation, migration and cell-extra-cellular matrix exchange (17, 18) (15). Interestingly, it has been reported that a large percentage of soluble markers detected systemically are in fact MP bound (19, 20).

We describe a robust and reproducible workflow for the detection, quantitation and phenotyping of plasma cell derived MPs from patient clinical samples. Specifically, we detail methods for sample preparation, MP isolation, MP validation from patient blood samples, immunolabeling and flow cytometric phenotypic protocols, quantitation of MP counts in patient samples, imaging MPs using scanning electron microscopy, electrophoresis and Western blot analysis. This validated workflow is translatable across other malignancies and/or chronic diseases as well.

3.3. Materials and Methods

3.3.1 Antibodies and other reagents

Annexin V450, anti-CD138-APC (clone MI15), anti-CD41a-PE (clone HIP8), anti-P-gp-FITC (clone 17F9*),* anti-CD34-PE-Cy7 *(*clone 8G12 Y7*)*, matched isotype controls, BD^{TM} CompBead anti-mouse-Ig k, Sphero^{TM} Rainbow calibration particles, 10x annexin binding buffer, and TruCount^{TM} tubes were purchased from BD Biosciences and/ or BD Pharmingen Australia/ NZ. Latex beads (0.3 μm LB3 & 1.1 μm LB11), Phosphate buffered saline, Tris buffer, Bovine Serum Albumin, skim milk, MOPS transfer buffer (20X), Transfer buffer, sample buffer and RIPA, all purchased from Invitrogen, Australia, cell lytic buffer, protease inhibitor cocktail RPMI-1640 media, Methanol were purchased from Sigma-Aldrich, Australia. Foetal bovine serum, Novex® Sharp Pre-Stained standard and 4–12% NuPAGE Bis-Tris gel were from Invitrogen, Life Technologies, Victoria, Australia. Scanning electron microscopy specific reagents were provided by the Australian Microscopy and Microanalysis Research Facility, Sydney. Anti-LRP mAb, (clone 42, was from BD Biosciences, Australia) anti-Mouse-HRP secondary antibody was purchased from Promega, Australia, enhanced chemoluminescence system was from Roche Applied Science, Australia and anti-P-gp antibody (clone C219, GeneTex, Australia).

3.3.2 Pre-analytical protocols

3.3.3 Study design and sample collection

This study was approved by the Sydney Local Health District Human Research Ethics Committee of Concord Repatriation General Hospital, Sydney, Australia, (HREC/11/CRGH/223). Blood samples were collected from MM patients and healthy subjects (older than 18 years of age) after obtaining informed consent at the Concord Repatriation General Hospital (CRGH) and Royal Prince Alfred Hospital (RPAH) blood collection centers. Healthy subjects were strictly non–cancer patients with normal hematology devoid of any cytotoxic treatment or radiotherapy of any nature in the past 5 years. Pregnancy was also an exclusion criterion for healthy subjects and for MM patients. Four milliliters of peripheral venous blood was drawn into K3 EDTA BD vacutainer tubes after standard phlebotomy procedures (9, 21). The patients and healthy subjects were de-identified, and a reference code was assigned to each sample.

3.3.4 Storage and freeze-thaw

Freezing vs. Fresh: In assessing the effects of freeze-thaw cycles on antigen detection, fresh platelet free plasma (PFP) from 4 mL whole blood was divided into 200 µl aliquots. One aliquot was analyzed immediately using anti-CD138-APC mAb, and annexin V-V450 whereas another aliquot was frozen at -80 °C for at least 24 hours. The frozen aliquot was thawed on ice, followed by MP isolation and flow cytometric detection to compare if the freezing has any impact on antigen detection.

3.3.5 MP isolation from cell lines and patient samples

The drug sensitive human acute lymphoblastic leukaemia cell line CCRF-CEM (CEM), its multidrug resistant (MDR) derivative VLB_{100} and the human myeloma cell line, OPM2 were used for validation and optimization of protocols. The CEM and VLB_{100} cell lines have been validated earlier by us as an appropriate model for study of P-gp mediated MDR *in vitro* (3). All cell lines were cultured in RPMI-1640 media supplemented with 10% heat-inactivated foetal bovine serum and maintained at 37 °C and 5% CO_2. All cells lines were tested for mycoplasma contamination routinely. MPs were isolated from confluent cell cultures by differential centrifugation as described previously (3, 22-25). Briefly, cell supernatant was collected and centrifuged at 500 × *g* for 5 min to pellet the cell population or debris. The supernatant was further centrifuged at 15,000 × *g* for 1 h at 15 °C and the pellet was re-suspended in serum free RPMI-1640. The MP suspension was further centrifuged at 2000 × *g* for 1 min to remove remaining debris. To concentrate the MP population, the supernatant was further centrifuged at 18,000 × *g* for 30 min and re-suspended in serum free RPMI-1640. MPs were validated by flow cytometry (LSRII, LSR Fortessa X-20, BD Biosciences, CA, USA) by annexin V-V450 staining as described previously (8). MP total protein content was quantified using Qubit® 2.0 Fluorometer protein assay (Invitrogen, Life Technologies) as per manufacturer's instructions.

For MP isolation from patient samples, up to 4 mL of whole blood was collected in EDTA vacutainer tubes. Within 2 hours of collection, the blood was transferred into a 10 mL polypropylene tube and centrifuged at 1,500 × g for 20 min at room temperature to obtain platelet-poor plasma (9). The platelet-poor plasma was spun at 13,000 × g for

2 min at room temperature to obtain platelet-free plasma (PFP) from the supernatant (1). The PFP was divided into 200µL aliquots, which were subjected to direct immunolabeling or ultracentrifuged at 18,890 × g, 4 ºC for 30 min to pellet the MPs (9). The supernatant was removed and the pellet resuspended in phosphate-buffered saline (PBS) or subjected to immunolabeling for flow cytometric surface phenotyping.

3.3.6 MP sample preparation for Scanning Electron Microscopy

Preparation of PEI solution: Polyethylenimine (PEI) is a cationic polymer that facilitates attachment of specimen to the coverslips. A 1% V/V working solution of PEI was prepared in MilliQ (MQ) water.

Coating Coverslips with PEI: Previously cut thermonox coverslips were rinsed briefly with 90% acetone followed by MQ water and incubated in 1% PEI for 1 hour in a 6 well plate. The coverslips were rinsed twice with MQ and subsequently dried at 60 degrees for 10 min. Adhering MPs to thermonox coverslips is described in chapter 4.

3.3.7 Validation of MP cargo

Preparation of MP and cell lysates: Spinning the cell culture supernatant for 5 min at 500g pelleted the cells. MPs were purified as described above. To the cell and or MP pellet, 1% protease inhibitor cocktail (P8340; Sigma–Aldrich) in 25 µl of cell lytic buffer was added (C2978; Sigma-Aldrich), incubated for an hour on ice with rigorous pipetting in 15 min intervals. The sample was centrifuged at 10000 x g for 10 min and the supernatant collected.

Protein quantitation: The protein content of the lysates was quantified using Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies) as per the manufacturer's instructions.

Electrophoresis and Western blotting.

25- 40 µg total protein was separated by electrophoresis using a 4–12% NuPAGE Bis-Tris gel (Invitrogen, Life Technologies) at a constant voltage of 150 V for 60 min and electroblotted onto a PVDF membrane at 30 V for 90 min. This was followed by blocking the membrane for 1-2 h using 2% skim milk in PBS and 0.05% Tween 20 (TBST). For detection of P-gp and LRP, membranes were incubated overnight at 4 degrees anti-P-gp (clone C219, GeneTex, Australia) (1:1000 dilution) and anti-LRP mAb (1:1000) (clone 42, BD, Australia/New Zealand) on a plate rocker. After washing the membranes 3 times in TBST, the membranes were incubated for 1 h using anti-Mouse-HRP secondary antibody (Promega, NSW, Australia) at a 1:10,000 dilution. Protein expression was visualized using an ECL (enhanced chemoluminescence) system (Roche Applied Science, NSW, Australia). Novex® Sharp Pre-stained standards (LC5800, Life technologies, Australia) were used as the molecular weight marker. The membranes were imaged using the luminescent image analyser LAS-3000 (Fujifilms, Brookvale, NSW, Australia).

3.4 Analytical Methods

3.4.1 Flow cytometry

Instrument calibration

A number of quality control measures were applied to ensure data validity and quality. The instrument was primed three times and milliQ water run on high flow rate for 10 minutes prior to analysis ensuring the sheath filter was free of any air bubbles. The cleaning protocol was run on high flow rate with FACSRinse and FACSClean followed by milliQ water through the fluid lines of the cytometer (5 min minimum as per manufacturer's recommendations) prior to and following each experiment. The performance of the instrument lasers was ensured prior to all experiments using rainbow calibration particles or 8 peak calibration beads and CS&T beads on low flow rate (BD Biosciences, Australia). All buffers (1x binding buffer or PBS) used for the re-suspension of the MP pellet were also run separately as controls to ascertain that the majority of events detected were from MP preparations. Compensation was achieved using BD™ CompBead anti-mouse Ig. The positive and negative control BD™ CompBead's were incubated with relevant antibodies for 30 minutes in the dark and run on the LSR FortessaX-20. The samples were acquired on low flow rate to ensure maximum resolution for the submicron vesicles during acquisition. The electronic abort rate was noted during acquisition and the samples were diluted accordingly**.**

Comparative assessment of MP resolution and gating parameters

All flow cytometric analyses were conducted using an LSRII flow cytometer (BD Biosciences) and the Cell Quest Pro analysis software/FACSDiva. As MPs vary in size (0.1 to 1 μm), latex beads of known diameter (0.3 [the lowest possible detection size in a BD LSRII] and 1.1 μm, Sigma-Aldrich) were used to define the gating parameters for MP detection. For this purpose, a 1 in 10 dilution of the beads was prepared in PBS according to the manufacturer's recommendation. The 1.1-μm beads were gently sonicated to disrupt any aggregates and incubated in PBS for 1-2 hours at room temperature to form a homogeneous solution prior to acquisition. The data acquisition was based on the forward and side scatter (size and granularity) properties of the beads, and the threshold was adjusted on side scatter to eliminate background noise. The defined MP region was applied to the MP scatter from platelet free plasma. The acquisition was based on the events falling within the gate.

3.4.2 Immunolabeling workflow.

A. Titration of antibodies

Manufactures recommended titre volumes of purchased monoclonal antibodies are ideal for cell preparations and usually not optimized for MP phenotyping. Therefore the optimal amount of antibody needed to be determined to stain the MP preparations for the assay. We used cell line MPs (VLB_{100} which over express P-gp and MCF-7 which express CD138) to determine if the manufacturer's recommended titre for anti-P-gp-FITC and anti-CD138-APC were ideal for immunolabeling MPs. For this purpose, five micrograms of MP preparation from respective cell lines was incubated with increasing volumes of anti-P-gp-FITC and CD138-APC respectively. This was followed by an

ultracentrifugation wash step for 30 min at 18,890g at 4 degrees and flow cytometric phenotyping was conducted.

B. Optimization of immunolabeling workflow

We wanted to assess the loss of MPs during ultracentrifugation wash steps as the eventual aim is to develop a clinical test, which can detect the physiological levels of MPs that might be having pathophysiological implications in patients. To achieve this we compared three immunolabeling approaches 1) Immunolabeling MP pellet (no wash step) 2) Immunolabeling with a wash step in between or Recovery of MPs after ultracentrifugation wash step 3) Direct immunolabeling of the platelet-free plasma and compared the results. In addition, MM characteristically have excess serum proteins especially at advanced/non-responsive and or progressive disease. This experiment is also aimed to show if immunolabeling the concentrated MP pellet has any consequence in flow cytometric antigen detection in terms of the response state of patients at the time of sampling. Further, the wash/ no wash vs direct immunolabeling of plasma was also conducted to determine the most suitable method, which can be smoothly translated to the clinical setting with respect to time consumption. Moreover, given MP's submicron size, to examine if stearic hindrance limits the flow cytometric phenotyping of MPs we decided to compare if sequential or simultaneous labeling has any effect on antigen detection through flow cytometry.

1) Immunolabeling MP pellet (no wash step): MP aliquots were immunolabeled sequentially, without prior or in between washing step. The percentage of the gated

population for CD138-APC and annexin V-V450 were compared across three approaches.

2) Immunolabeling with a wash step in between or Recovery after ultracentrifugation wash step: MP aliquots were immunolabeled sequentially, incubated and washed by means of an ultracentrifugation step to compare the percentage of expression in the gated population for respective antigens and also to assess the loss of MPs during the ultracentrifugation.

3) Direct immunolabeling of the platelet-free plasma: Antibodies were added directly to the PFP (50 µl), incubated in the dark for 30 minutes at room temperature, re-suspended in 500 µl PBS or 1x BB and analyzed on the LSR II and the percentage of gated population positive for respective antigens were compared across three approaches.

C. Sequential and simultaneous immunolabeling

MP aliquots were immunolabeled sequentially, and simultaneously with antibodies directed against CD138, CD41a, PS, incubated and washed using ultracentrifugation step and subsequently ran on LSRII to compare if it has any impact on the detection of antigen by flow cytometry.

3.5 Data acquisition

Platelet free plasma (PFP) was prepared as described previously (8, 9). PFP was divided into 200 µl aliquots, which were subjected to direct immunolabeling for flow cytometric surface phenotyping or MP isolation by ultracentrifugation at 18,890 × *g*, 4ºC for 30

min. The supernatant was removed and the MP pellet was immunolabeled for flow cytometric detection of surface markers (CD41a, CD138, P-gp, PS (annexin V) in technical triplicates for each patient MP count.

3.5.1 Platelet-derived MP exclusion and surface antigen detection

The human PFP sample contained a large number of platelet-derived MPs (9). Therefore, to avoid platelet-derived MP contamination, the acquisition was gated to exclude these particles using sequential gating for the platelet marker CD41a (CD41a–PE mAb [clone HIP8]). The PFP was divided into 200-µL aliquots and ultracentrifuged to pellet MPs prior to immunolabeling (8).

MP aliquots were immunolabeled with 5µl anti-CD138-APC mAb (clone MI15), 20 microliters of anti-CD41a-PE mAb (clone HIP8, all from BD Australia) and incubated for 30 minutes in the dark at room temperature and washed once in ice-cold PBS. The pellet was resuspended in 200 µl PBS at room temperature in dark and analyzed on the LSRII for surface immunophenotyping.

Five µl of anti-CD138-APC, 20 µl of anti-CD41a-PE, 5 µl of CD34-PE-Cy7, 20 µl of anti-P-gp-FITC and annexin-V450 (for measuring PS exposure) were added to the MP pellet and incubated for 30 minutes in the dark at room temperature and washed in ice-cold PBS (3, 8, 26). Relevant isotype-matched and unstained controls were ran in parallel. Platelet derived MPs were excluded during the analysis using anti-CD41a-PE in the downstream analysis.

3.5.2 Enumeration of MPs.

For the quantitation of MPs, MP preparations from 200 µl PFP were added to BD TruCount™ tubes as per the manufacturer's recommendations. The tube was slightly vortexed prior to acquisition. The number of MPs per µL plasma was calculated using the manufacturer's formula,

N = [MP/number of measured beads] × [beads per tube/volume of plasma]

The stop gate was set at a fixed number of 2000 or 10000 of TruCount™ beads during the data acquisition (8).

3.5.3 Statistical Analysis.

All statistical analyses were performed using GraphPad Prism version 6.0 or 7 for Windows (San Diego, CA, USA), and the data are presented as the mean ± SEM unless otherwise stated. P value was generated for non–parametric data by Wilcoxon matched-pairs signed rank test $P<0.0001$ (****) was considered significant.\

3.6 Results and Discussion.

3.6.1 Pre-analytical Assessment

A number of pre-analytical and analytical assessments including sample storage, immunolabeling workflow were examined to establish the optimal protocol for clinical use. Few pre-analytical variables related to blood collection in a clinical setting for this type of study were already described in detail in literature and we incorporated those methodologies in our study. As per literature, needle size of 21 G is recommended to

minimize unnecessary platelet activation during blood collection for MP study. Also, it is recommended that first few mls of blood should be discarded to avoid venepuncture related damage (9). In terms of anticoagulants; Sodium citrate, EDTA, Acid-Citrate-Dextrose and Heparin are the commonly used anticoagulants in blood collection for MP isolation. They specifically limit platelet activation during collection and plasma isolation. However, EDTA is suggested as more appropriate anticoagulant in multicentre studies as the time between collections and processing can be varied. It has been reported through independent studies that blood collected in heparin and sodium citrate tubes gave significantly higher number of annexin V positive MPs than EDTA. EDTA strongly chelate Calcium while heparin preserve extracellular Calcium and thus minimize the variation from physiological MP counts for multicenter studies (9).

3.6.2 Storage and Freeze-Thaw

We observed that MPs isolated from freeze- thawed PFP (-80^{0}C frozen) samples were significantly compromised for the detection of the CD138 antigen compared to that observed for fresh PFP samples. The absolute CD138$^+$ MP count from the fresh PFP samples was 6.2 fold greater relative to counts obtained from frozen PFP origin (*p>0.0001, n=15*) Figure 3.1A. The annexin V$^+$ absolute MP counts did not show any significant difference between the fresh and frozen PFP origin (*p=0.37, n=10*) (Figure 3.1B). The data is represented as mean+/-SEM

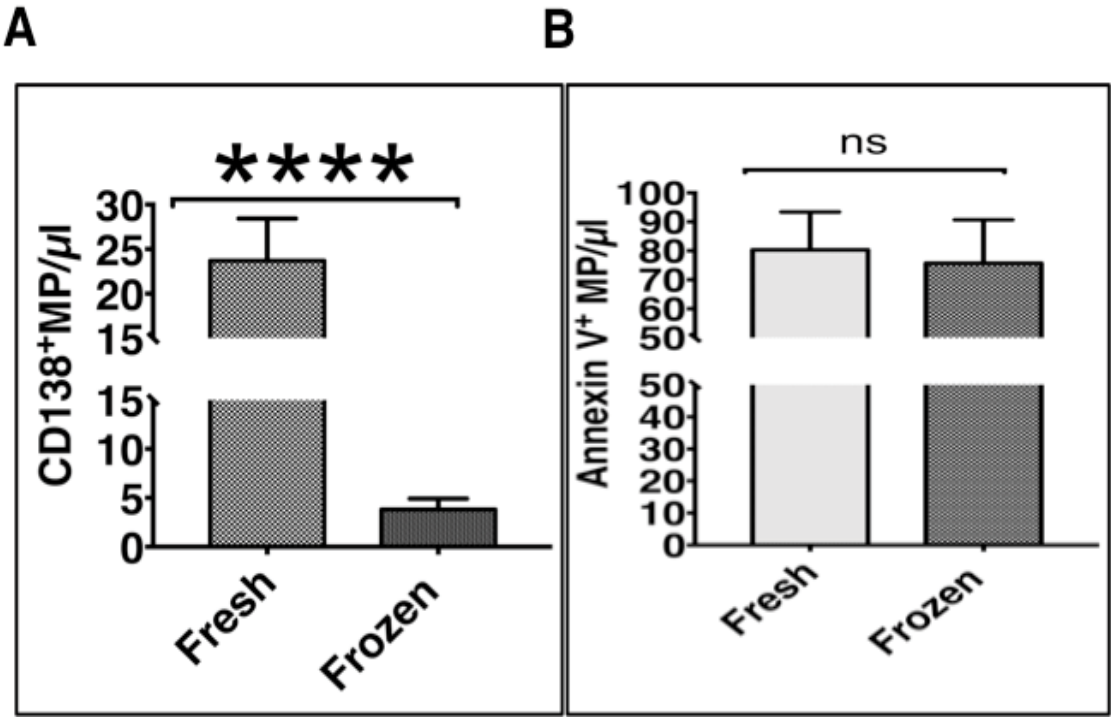

Figure 3.1 Sample storage optimization. PFP was prepared, and the sample was divided in two 200µl aliquots. One sample was immediately frozen at −80°C, whereas MPs were freshly isolated from the other portion and immunolabeled with CD138-APC and annexin V-V450. The frozen sample was thawed on ice, followed by MP isolation and fluorescence immunolabeling. The samples were analysed using TruCount^{TM} tubes on LSRII **(A)** The absolute count of CD138^{+} and (n=15) **(B)** annexin V^{+} MPs, between freshly isolated MP sample and the MPs isolated from frozen PFP is compared (n=10). Wilcoxon matched-pairs signed rank test was used to generate P values, *p<0.0001*(****). Data represents mean +/-SEM.

3.6.3 Validation of MP cargo by Western blot

Microparticles isolated from *de novo* and relapsed MM patients were probed for the presence of P-glycoprotein (P-gp) and lung resistance-related protein (LRP) by Western blot analysis. Patient samples detected positive for the presence of the MDR proteins during the course of treatment and during relapse. Figure 3.2A, B and C shows the presence of drug resistance markers from the MP preparations of patients Lane 1, P-gp - control; lane 2, P-gp^{+} control; lane 3 - 4, 30 and 40 μg, respectively MP lysate (*de novo* patient, Mar. 2013), Figure 3.2 A. Lane 1, P-gp^{+} control; lane 2, P-gp – control; lane 3, 30 μg MP lysate (patient in partial remission and during active treatment, Jul. 2013); lanes 4 -5, 30μg MP lysates (relapsed patients) respectively (during active treatment Jul. 2013). Anti-P-gp antibody, clone C219 (GeneTex, Australia) used. Figure 3.2B. We also observed the presence of LRP expression in MPs from relapsed patients (Figure 3.3C). The presence of P-gp in the patient sample in lane 3 (Figure 3.2B) was also confirmed by flow cytometry post induction therapy as shown in Figure 3.2D. MP lysate in lane 2 had LRP expression at the time of unresponsiveness to bortezomib as shown by the IgA profile of the patient (Figure 3.2E). Although, we detected cargo of resistance proteins via Western blot which was subsequently confirmed by flow cytomtetry, we specifically noted that Western blot is not a sensitive and quantitative or a feasible method for determining prognostic significance in a cohort. The optimization of basic Western blot parameters such as primary, secondary antibody dilution, incubation times and conditions were found to be highly variable for different patient samples for optimal results. Likewise, Western blot is not feasible as a routine clinical tool in terms of workflow as well. MP morphology from MM patients is discussed in detail in chapter 4.

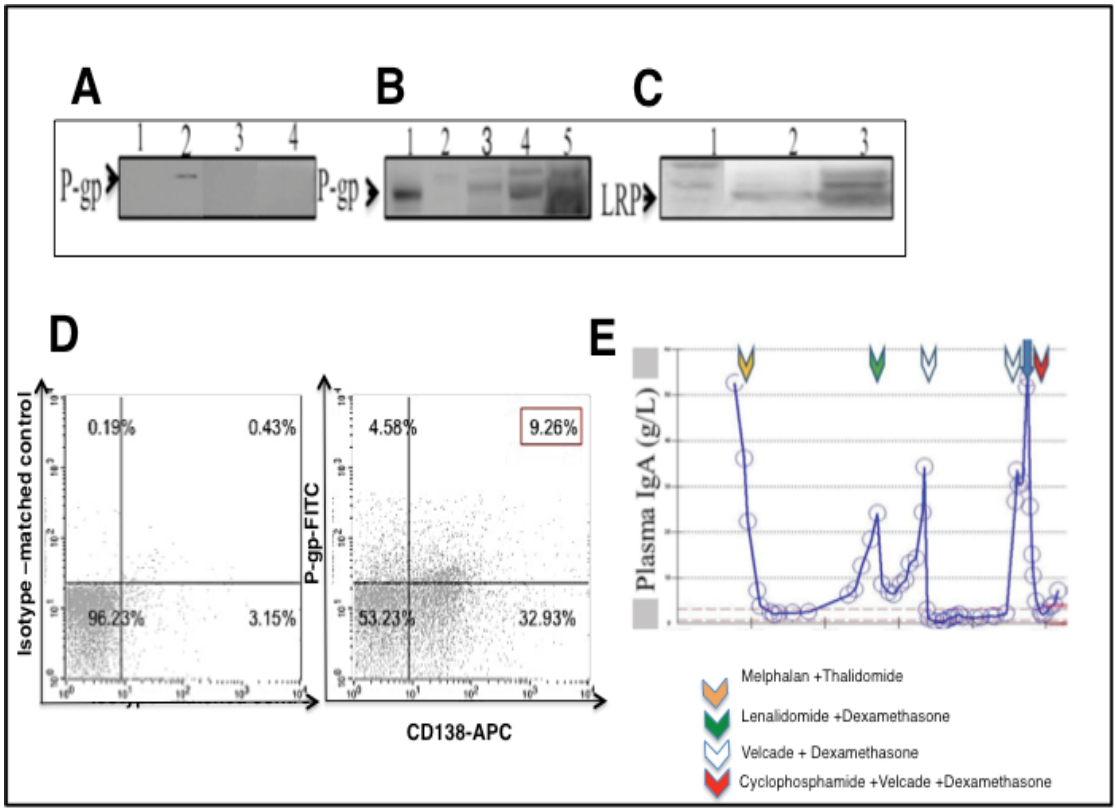
A
B
C
1 2 3 4
P-gp
1 2 3 4 5
P-gp
1 2 3
LRP
D
Isotype –matched control
0.19%
0.43%
96.23%
3.15%
P-gp-FITC
4.58%
9.26%
53.23%
32.93%
CD138-APC
E
Plasma IgA (g/L)
Melphalan +Thalidomide
Lenalidomide +Dexamethasone
Velcade + Dexamethasone
Cyclophosphamide +Velcade +Dexamethasone

Figure 3.2 Validation of MP cargo constituents by Western blot. (A) P-gp expression in MPs from MM patients. Lane 1, P-gp– control; lane 2, P-gp + control; lane 3 - 4, 30 and 40 μg, respectively microparticle lysate (*de novo* patient, Mar. 2013) **(B)** Lane 1, $P\text{-}gp^{+}$ control; lane 2, P-gp – control; lane 3, 30 μg microparticle lysate (patient in partial remission and during active treatment, Jul. 2013); lanes 4 -5, 30 μg microparticle lysates (relapsed patients) respectively (during active treatment Jul. 2013). Anti-P-gp antibody (clone C219, GeneTex, Australia) was used for detection **(C) LRP expression in MPs from MM patients.** Microparticle lysates from relapsed patients (Lane 1, protein ladder), (patients, lane 2, 3 respectively). Anti-LRP (clone 42, (BD Australia) used in 1:1000 dilution. **(D)** Surface expression and flow cytometric detection on (*de novo* patient, Mar. 2013 shown on figure 3.2A lane 3 &4 post induction therapy. P-gp expression was detected flow cytometrically also in August 2013. MCF-7 & MCF-7/Dx cell lysates were used as negative and positive controls for P-gp expression **(E)** Disease response profile of a myeloma patient over the period 2008-2014 based on IgA. LRP^{+}MPs were detected at the time of Bortezomib (Velcade) unresponsiveness.

3.7 Analytical assessment

Flow cytometry

Instrument calibration, comparative assessment of MP resolution and gating parameters.

Microparticles are intact 0.1-1 μm diameter vesicles that are shed during plasma membrane remodeling and characteristically express phosphatidylserine on their surface. MPs are differentiated from other extracellular vesicles such as exosomes (40-100 nm) and apoptotic bodies (>1 μm) by virtue of their size (3). MPs were isolated by ultracentrifugation at 18,890 ×g for 30 minutes at 4 °C and were resuspended in PBS for flow cytometric analysis. MP gate was defined using a uniform suspension of latex beads of known diameter in (0.3-1.1 μm, Sigma-Aldrich, Australia) in PBS (Figure 3.3 A and B). However, MPs have biological properties as well the latex beads and MPs differ in physical parameters. Specifically, beads typically have higher refractive indices and, consequently, lower limits of size detection by flow cytometry (9, 27). As a result, the gating parameters were set within the above-mentioned technical limitation, and the threshold was set on side scatter to avoid background noise during acquisition.

The LSRFortessa X-20 has a 100 mW 488 nm blue laser (20-50 mW). As scatter signals are collected off the blue laser, powerful laser provides more energy for excitation of flourochromes which in turn improves signal and resolution do detect and resolve small particles. Fortessa X-20 is also capable of measuring up to 14 fluorescent parameters and performance of all detectors can be monitored with the powerful built-in quality control function ideal for multicolour flow cytometry of submicron particles Figure 3.3B.

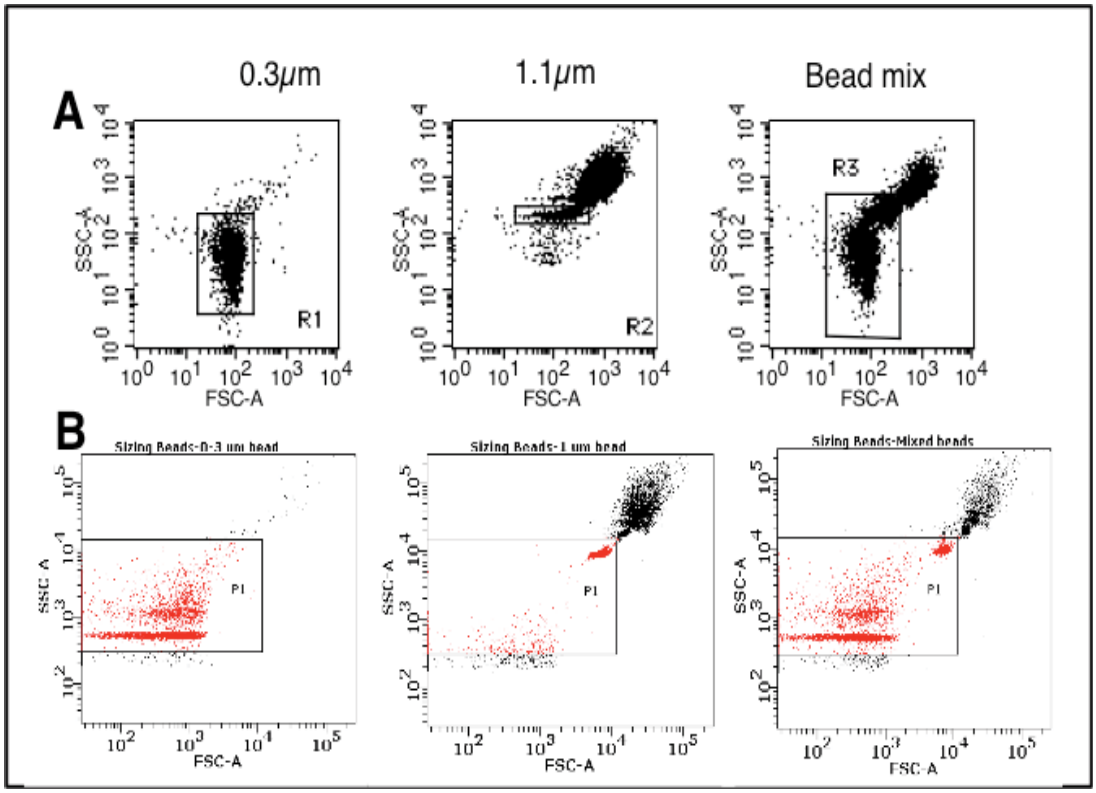

Figure 3.3 Comparative assessment of MP resolution and establishment of the MP gating parameters. (A) Latex beads of known diameter (0.3 and 1.1 µm- Sigma–Aldrich) were used to define the gate for MP analysis. R1 represents the lowest possible limit on forward scatter using 0.3 µm beads, R2 represents the upper limit of MP size using 1.1 µm beads and R3 represents the region where the MPs locate based on the lower and upper limit set by the beads. (B) The region P1 is defined by defining MP region by 0.3 and 1.1 µm latex beads on LSR Fortessa X-20. The defined MP region gate was applied to the MP scatter plot to identify the particles of interest (highlighted in red).

Immunolabeling workflow

A. Titration of antibodies

The percentage of MP staining was plotted against volume of each of the antibody as shown in figure 3.4A and B. The percentage of staining was found to increase with increasing antibody concentration until reaching plateau. For both anti-P-gp-FITC and anti-CD138-APC, we observed that the minimum concentration of antibody for optimal results was similar to the manufacturer's recommendation.

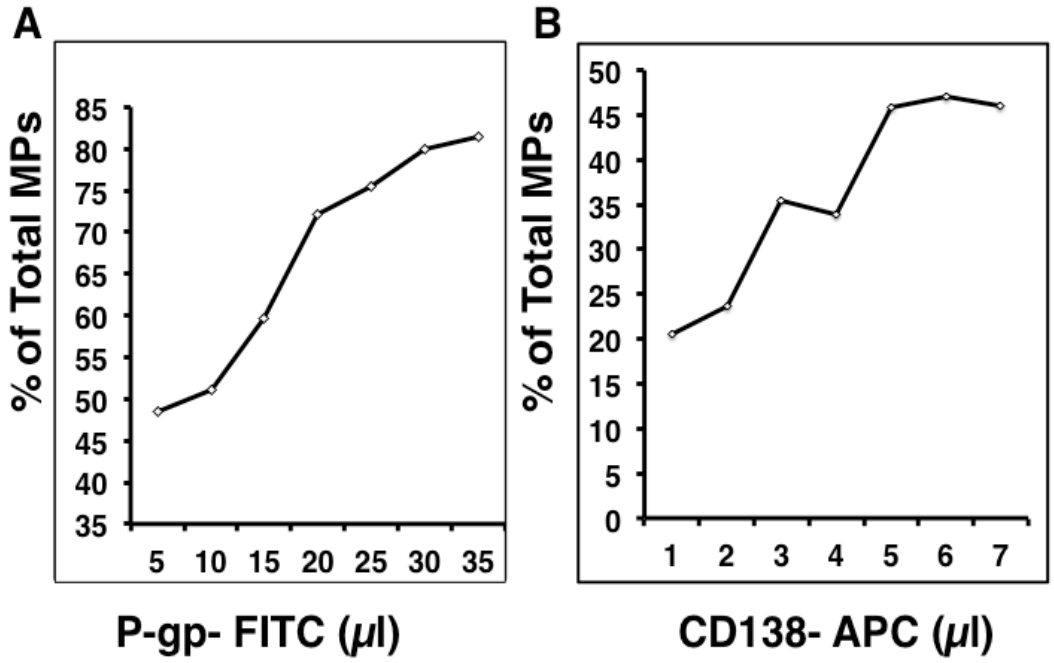

Figure 3.4 Determination of antibody concentration for optimal MP staining. To determine the optimal antibody concentration for MP immunolabeling, MPs were isolated from cell lines (VLB $_{100}$ and MCF-7) and 5 micrograms of MP pellet resuspended in RPMI without FBS were immunolabeled with increasing concentrations of **(A)** anti-P-gp-FITC and **(B)** anti-CD138-APC.

B. Comparative analysis of direct immunolabeling of PFP vs MP pellet

Three different approaches were considered in optimizing the immunolabeling of MPs. The data is expressed as percentage of gated population positive for the respective antigens and compared across three approaches. As mentioned, the methods considered included 1) MP isolation by ultracentrifugation and subsequent immunolabeling of MP pellet without a prior or post ultracentrifugation wash step. 2) Immunolabeling the MP pellet and a subsequent wash step to eliminate unbound antibodies 3) Direct immunolabeling of PFP (50 µl) (Figure 3.5).

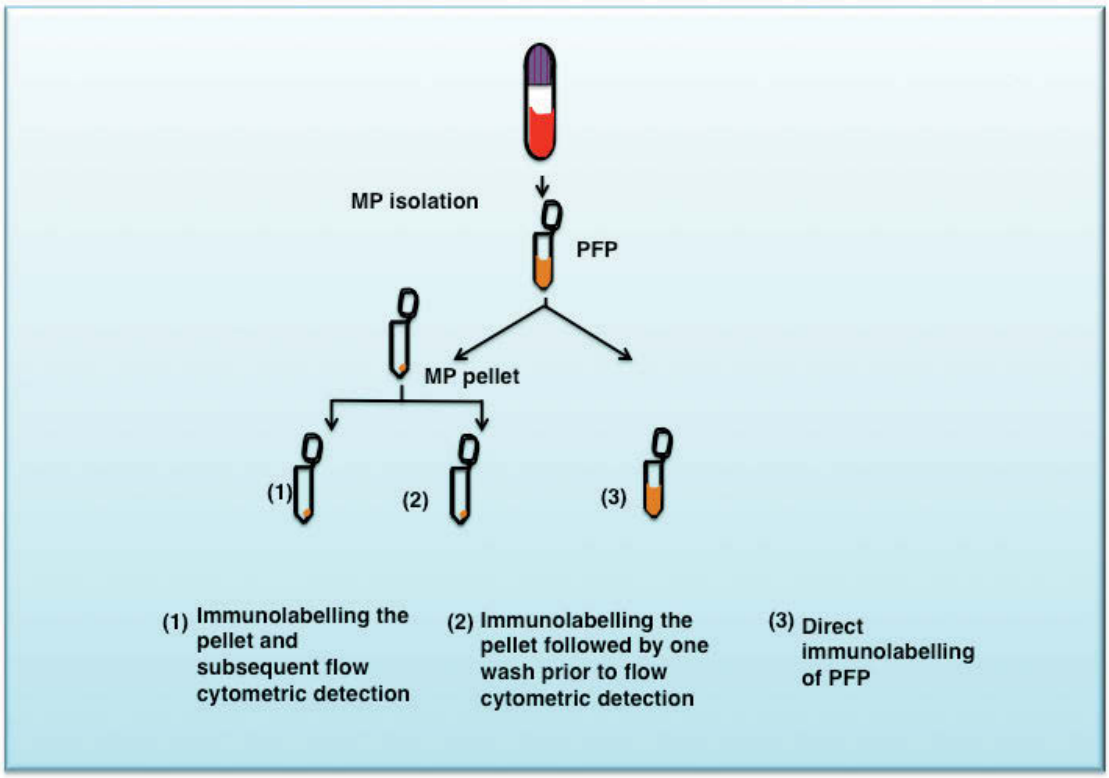

Figure 3.5 Immunolabeling workflow. Three distinct immunolabeling approaches (1) immunolabeling the MP pellet without a prior or post wash step (2) immunolabeling the MP pellet with an additional wash step post immunolabeling (3) direct immunolabeling of platelet free plasma.

The results were comparable across the three approaches. However, we noted a number of important aspects of the immunolabeling methodology which are discussed as follows along with the rationale of choosing MP isolation method for this phase of the study; 1. adaptability of the workflow in the clinical setting. 2. recovery of MPs after ultracentrifugation steps.

We also examined the three approaches to determine if there was any difference in terms of annexin positivity of MPs with respect to the above mentioned approaches. Annexin V typically measures the PS exposure on MPs and approach 1 showed 31.59% annexin V positive events in the gated population (Figure 3.6B, left panel). Approach 2 with the ultracentrifugation wash step albeit prior to annexin V addition showed 20.43% in PS exposure (Figure 3.6B, middle panel) whereas approach 3 showed a 52% in PS exposure in the gated population (Figure 3.6B, right panel).

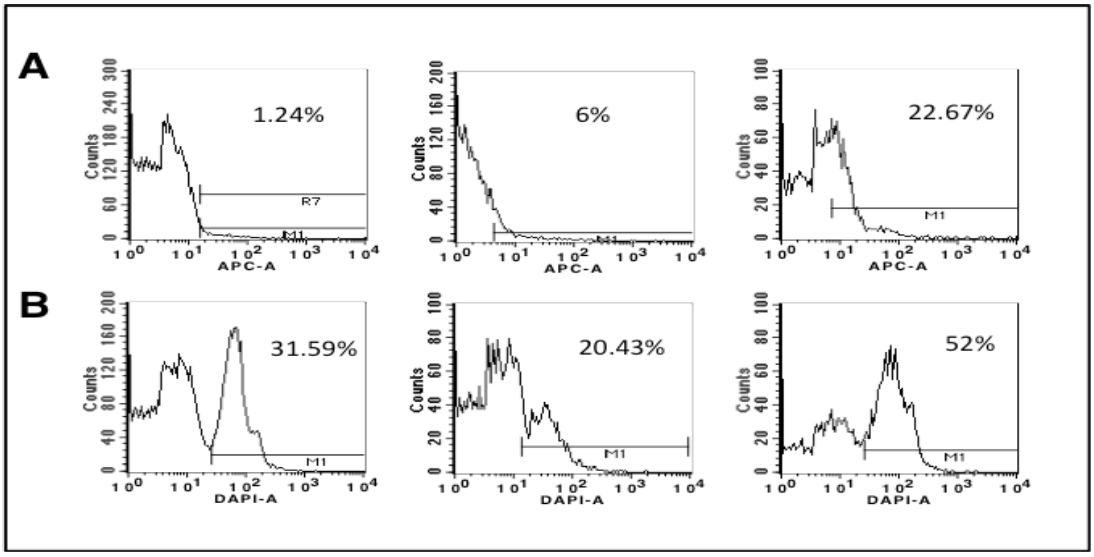

Figure 3.6 Immunolabeling optimization: PFP from an MM patient was divided into 3 portions. Two portions of PFP were ultracentrifuged at 18,890g for 30 min and MPs were isolated. MP pellets (left panel A and B) and a 50 µl of PFP sample were immunolabeled (right panel) using **(A)** anti-CD138 mAb and **(B)** annexin-V450 as per manufacturer's recommendation. One MP sample, immunolabeled with **(A)** CD138 and **(B)** annexin-V450 was subjected to an ultracentrifugation wash step and flow cytometric phenotyping was conducted (**A** and **B**, middle panel).

1. Adaptability of the workflow to a clinical setting: Direct immunolabeling of PFP gives distinct MP phenotype and minimal loss of MP population by ultracentrifugation steps. This underlines that once MP parameters are established on the flow cytometer; direct immunolabeling plasma can be used to successfully distinguish MP events from background and/or any other contaminants. This method also has the advantage of minimal loss in terms of MP count and thus the percentage of surface expression. Thus this approach will present a closer picture of the physiological levels of MPs in a patient. Further, it makes the workflow simpler for translation to a clinical setting without the need for an ultracentrifugation step to pellet the MPs. However, considering the pilot nature of our study, we opted for approach 2 for the entire study i.e; immunolabeling the pellet with a wash step (Figure 3.6A and B right panel)

2. Recovery of MPs after ultracentrifugation: The disadvantage of approach 2 was the loss of a significant number of MPs during the ultracentrifugation washing steps. However, phenotype was identified distinctly (CD138 and annexin V) compared to the isotype-matched /unstained control compared to the approach 1 Figure 3.7A and B middle panel). We used approach 2 throughout our study, as it was a preliminary study.

C. Sequential vs simultaneous antigen immunolabeling

While optimizing the conditions for polychromatic flow cytometry for surface phenotyping MPs (CD41a-PE), CD138-APC and annexin V-V450 in this case), we did not observe a difference in the sensitivity in detection between sequential (of either order) and simultaneous for antigen detection (Figure 3.7A and B, left middle and right panels). However, it was the washing step after the addition of annexin-V450, which

only had a noticeable impact on annexin V^+ events for this patient. We note that the sequential or simultaneous is irrelevant in the case of immunolabeling MPs however, if the experiment involves measurement of PS exposure, it is ideal to follow sequential labeling just in the case of annexin V. Other antibodies can be added simultaneously and after staining can be subjected to an ultracentrifugation wash step to remove unbound antibodies. The sample can be further subjected to annexin V labeling and subjected to flow cytometric phenotyping. This recommendation is made as we specifically noticed that the wash step after the addition of annexin V results in considerable reduction in annexin V^+ events (Figure 3.7C, left, middle and right panels).

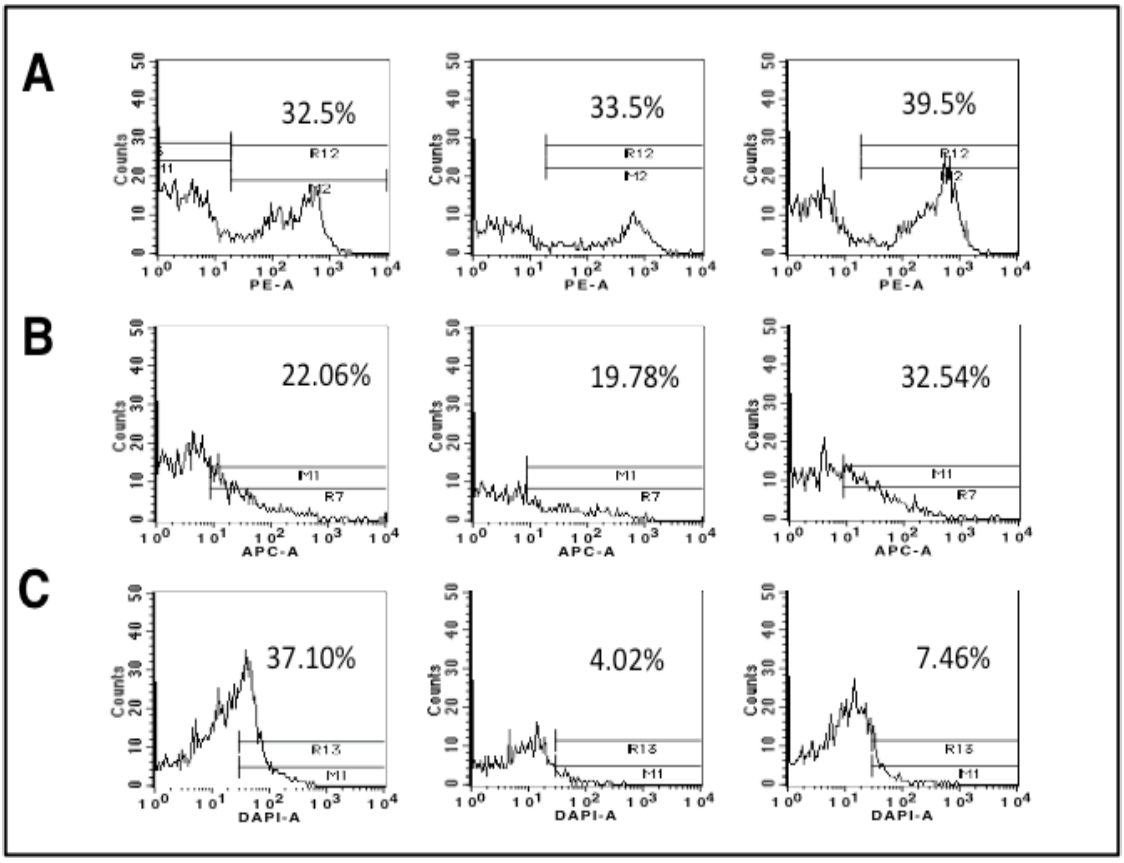

Figure 3.7 Sequential vs simultaneous immunolabeling approaches. (A) Anti-CD41a-PE, **(B)** anti-CD138-APC mAb and **(C)** annexin V-V450 were sequentially (left and middle panel respectively represent the order of sequential addition (first CD41a followed by CD138) or simultaneously (right panel) added to immunolabel MPs followed by flow cytometric phenotyping as described in detail in the methodology. The various approaches do not influence the surface expression of antigens as detected by flow cytomteric detection. However, wash step does have an impact on the MP number, which reflects on percentage of expression of respective markers.

3.8 Data acquisition

Platelet MP exclusion and surface antigen detection and enumeration

The MPs isolated from MM patients were probed for CD41a, a typical platelet surface marker, to exclude platelet-derived MPs from the analysis. To achieve this goal, MPs were isolated and dual labeled with anti-CD138-APC mAb (clone MI15) and anti-CD41a-PE mAb (clone HIP8) to quantitate the platelet-derived MPs present in the sample. MP gating parameters were applied to the MP scatter (Figure 3.8A, right panel). Platelet-derived MPs were excluded and only the $CD41a^-$ MPs were used for subsequent analysis (Figure 3.8A, right panel). The MP pellet was also immunolabeled with anti-CD138-APC mAb (clone MI15), annexin V-V450 (PS), anti-CD34-PE-Cy7, anti-P-gp-FITC to validate and confirm the origin of the MPs. Matched isotype controls were ran in parallel (Figure 3.8B and C). We detected distinct MP phenotypes from the PFP of MM patients and the absolute counts were 161/µl, 6189/µl, 31/µl and 9/µl for CD138, annexin V, P-gp and CD34 respectively (Figure 3.8C). Absolute count of MPs was calculated using TruCount ^{TM} tubes and the data is shown in (Figure 3.1).

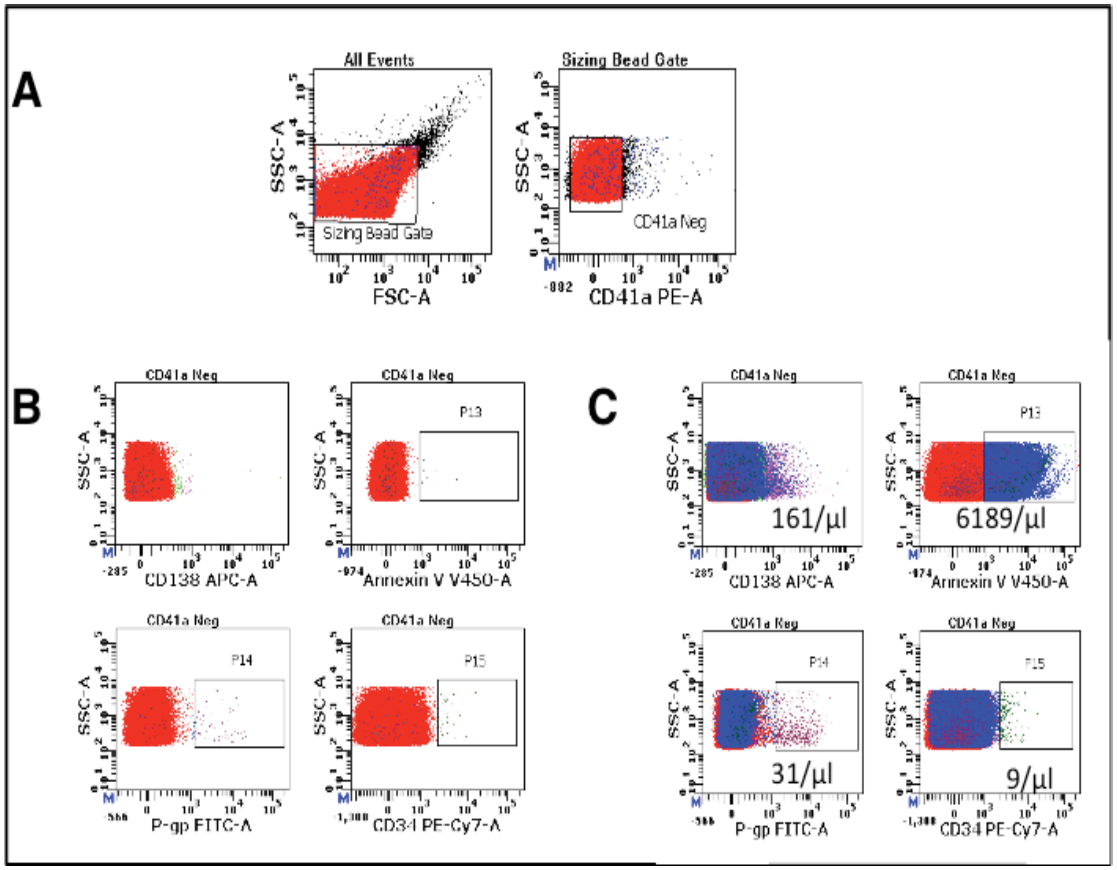

Figure 3.8 Detection of distinct MP populations from the platelet free plasma of MM patients. Monoclonal antibodies (CD41a-PE, CD138-APC, P-gp-FITC, CD34-PE-Cy7 and annexin V-V450) were used in manufacturer recommended dilutions against respective antigens. Distinct MP subtypes were identified in the peripheral blood of MM patients following flow cytometric detection described in the method section. **(A)** Gating strategy to identify the MP subpopulations based on the defined MP size gate (left panel) and platelet derived MP exclusion (right panel). **(B)** Isotype-matched controls were used to define the positive and negative populations for respective antigens. **(C)** MP subtypes in a *de novo* MM patient shown as absolute count. Data represents absolute count of MPs calculated as per manufacturer's formula as per the methods section.

3.9 General Discussion

Multiple myeloma is a chronic, incurable plasma cell neoplasm that is restricted to the bone marrow microenvironment; it is rarely detected in the periphery (6). There are two types of MM, based on the presence or absence of M or paraprotein secretion from aberrant plasma cells into the circulation, which are known as 'secretory' and 'non-secretory' MM, respectively (28). Monitoring response in secretory MM and risk stratification rely on highly invasive BM biopsy for assessing the treatment response. On the other hand, in 'non-secretory myeloma', BM biopsy remains an exclusive method for assessing the therapeutic response and making crucial therapeutic decisions (29, 30). Current prognostic tools are very limited in gauging the evolution of drug resistance during the course of therapy.

As mentioned, MPs are 'surrogate markers' in pathological conditions involving physiologically less accessible tissues, such as the endothelium (4, 13). Moreover, there is currently a focus on deciphering the roles of MPs in thrombosis and infections across various laboratories. Remarkably, periodic unresponsiveness to various novel chemotherapeutic regimens, resulting in relapse, is one of the characteristics of MM (31). Therefore, constant monitoring of the patient's therapeutic response is mandatory in the MM clinical setting (32). However, MM is restricted to the bone marrow, and the most accurate assessments of therapeutic response and decisions depend on invasive procedures. Thus, the management of MM in the clinical setting is heavily dependent on BM biopsy in aged and immunocompromized MM patients, which exposes these patients to the risk of infections, thrombosis and lasting discomfort (33).

We have found that freezing and thawing the PFP prior to the isolation of the MPs impacts the integrity of the CD138 marker on MPs. Likewise, additional ultracentrifugation steps reduced the MP count. In addition, an MP quantitation protocol was established based on TruCount^{TM} bead count as an internal control during the acquisition of the sample.

In conclusion, we have demonstrated that MM cells shed MPs into the circulation as detected by CD138 and annexin V positivity in a CD41a^{-} gated MP population (platelet MP exclusion) using flow cytometry with the outlined workflow. This methodology enabled us to characterize the MP phenotypes further. Our correlative analysis of MP data with respective clinical information suggested that MPs could be prospective candidates for a minimally invasive prognostic marker in MM. This novel prognostic can be used to evaluate MM patients routinely and can be used to predict the "risk of relapse" prior to the clinical manifestation of symptomatic relapse. The methodology provides a means to measure the emergence of drug resistance, an evolving shift of cancer populations during the course of disease and possibly the emergence of a 'stem cell like' population, which contributes to treatment unresponsiveness. This is in support of the 'cancer stem cell theory' contributing to the incurability of cancer. This novel prognostic thus offers a considerable advantage over the current prominent systemic markers in MM (M protein or paraprotein levels or beta-2-microglobulin, light chain ratio etc.). Current systemic markers are also limited in therapeutic risk stratification in terms of predicting duration of remission to specific therapies (34). Therapeutic risk stratification is a vital component of therapeutic management in MM clinical setting mainly due to the high heterogeneity in survival (35). Risk grouping correspondingly helps in appropriate counselling of newly diagnosed patients, selection of most suitable

chemotherapeutic regimen with minimal side effects and an ideal therapeutic outcome with respect to quality therapeutic interventions. Further, risk grouping orientates clinical research constantly identifying the gaps and limitations of specific therapies in terms of outcomes (35). The method validated here can potentially be used to assess the clinical correlation of MP numbers and their molecular cargo and have the prospective to be readily integrated to the 'liquid biopsy' concept in MM or any hematological clinical setting. 'liquid biopsy' is expected to give significant insight into the 'genetic landscape' of all the cancerous patches and a more comprehensive assessment of the tumor microenvironment systemically.

Author contributions:

SRK prepared the manuscript and conducted all the optimization experiments. MB designed and supervised the research, provided conceptual advice and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Chapter 4

Isolation of human CD138^{+} microparticles from the plasma of patients with multiple myeloma

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Isolation of Human CD138 $^{+}$ Microparticles from the Plasma of Patients with Multiple Myeloma[1]

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Abstract

The confinement of multiple myeloma (MM) to the bone marrow microenvironment requires an invasive bone marrow biopsy to monitor the malignant compartment. The existing clinical tools used to determine treatment response and tumor relapse are limited in sensitivity mainly because they indirectly measure tumor burden inside the bone marrow and fail to capture the patchy, multisite tumor infiltrates associated with MM. Microparticles (MPs) are 0.1- to 1.0-μm membrane vesicles, which contain the cellular content of their originating cell. MPs are functional mediators and convey prothrombotic, promalignant, proresistance, and proinflammatory messages, establishing intercellular cross talk and bypassing the need for direct cell-cell contact in many pathologies. In this study, we analyzed plasma cell–derived MPs (CD138 $^{+}$) from deidentified MM patients ($n = 64$) and normal subjects ($n = 18$) using flow cytometry. The morphology and size of the MPs were further analyzed using scanning electron microscopy. Our study shows the proof of a systemic signature of MPs in MM patients. We observed that the levels of MPs were significantly elevated in MM corresponding to the tumor burden. We provide the first evidence for the presence of MPs in the peripheral blood of MM patients with potential applications in personalized MM clinical monitoring.

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Introduction

Recent advances in therapy for patients with multiple myeloma (MM) using novel agents such as immunomodulatory drugs and proteasome inhibitors have prolonged patient survival by an average of 3 to 4 years [1]. Despite these advances, there is currently no means to foresee impending relapse before the onset of clinical manifestations, which results in the deterioration of the condition and requires a review of the patient's treatment regimen [2].

Relapse may be due to inherent genetic factors related to the malignant clone or to the microenvironment. Monitoring for minimal residual disease has involved flow cytometry or molecular methods of patient blood samples and bone marrow aspirates to identify high-risk groups [3]. However, there is a significant limitation in measuring the duration of achieved remission and impending relapse before the clinical manifestation in the current MM clinical setting. Consideration of the impact and significance of membrane budding in MM has had little attention. The physiological plasticity of the plasma membrane leads to membrane budding, which results in the systemic release of submicron (0.1-1.0μm) fragments called microparticles (MPs). These vesicles are shed in response to various stimuli during cellular activation and apoptosis and are also involved in intercellular cross talk [4–6]. MPs are detected systemically in healthy individuals; nevertheless, elevated levels are indicative of cellular activation and are common in diseases including diabetes, inflammation, vascular disease, and cancer [7–9]. Physiologically, MPs participate in cell signaling and also the exchange of proteins and nucleic acids between distinct cell types. Their

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significance in disease pathology and their ability to act as "surrogate markers" of disease activity in poorly accessible tissues have been widely documented [6,8,10,11]. Flow cytometric analysis is routinely used to phenotype MPs, as they display various cell surface markers that define their cellular origin [4,6]. MPs characteristically express phosphatidylserine (PS) on their surface and are differentiated from other extracellular vesicles such as exosomes (40-100 nm) and apoptotic bodies (>1 μm) by virtue of their size and phenotype [6,12].

Platelet-derived MPs and their role in thromboembolic risk have generated much interest and initiated specific studies in plasma cell (PC) dyscrasias like MM [10]. The introduction of immunomodulatory drugs in MM therapy and their association with an increased risk of venous thromboembolic episodes have initiated interest in MPs in MM [10]. However, non–platelet-derived MPs ($CD41a^-$) in MM and their clinical significance have remained unstudied.

Among the various surface markers that are reflective of the elaborate maturation and differentiation process in PCs, CD138 (a transmembrane heparan sulfate proteoglycan) is expressed on the surface of mature PCs [13]. CD138 acts as a classical co-receptor for growth factors, angiogenic factors, and small signaling molecules like chemokines [14–16]. $CD138^+/CD45^-$ represents the phenotype of mature PCs in bone marrow, and only $CD138^+$ is considered to be an exclusive marker for flow cytometric phenotyping of PCs [17,18]. The present study is designed to detect and enumerate the submicron vesicles in the peripheral blood of MM patients (*de novo* and under active treatment). As the peripheral blood of MM patients contains platelet-derived MPs, we used CD41a which is a typical platelet marker to exclude platelet-derived MPs from our population of interest. Furthermore, we investigated the correlation between number of $CD138^+$ MPs and distinct clinical states. This study describes for the first time the isolation and characterization of $CD138^+/CD41a^-$ MPs in the plasma of MM patients. We demonstrate that the levels and phenotype of MPs are indicative of the disease state and therapeutic outcome in MM patients. This evidence suggests that MPs found in the blood could provide a novel prognostic means to monitor the malignant cells in MM.

Materials and Methods

Antibodies and Reagents

Annexin V-V450 (BD Horizon, cat. no.560506), anti–CD138-APC (clone MI15; cat. no. 347193), anti–CD41a-PE (clone HIP8; cat. no. 555467), TruCount tubes (cat. no. 340334), and matched isotype controls were purchased from BD Biosciences Australia/New Zealand. Latex beads of diameter 0.3 μm (cat. no. LB3) and 1.1 μm (cat. no. LB11) were from Sigma-Aldrich, Australia. Australian Microscopy and Microanalysis Research Facility at the Australian center provided all consumables for electron microscopy. Myeloma cell line OPM2 was kindly provided by Royal Prince Alfred Hospital Haematology, Sydney, Australia, and was tested for mycoplasma at University of Technology Sydney before use.

Study Design and Patient Selection Criteria

This study was approved by Sydney Local Health District Human Research Ethics Committee (HREC) of Concord Repatriation General Hospital (CRGH) (HREC/11/CRGH/223-CH62/6/2011-150), Royal Prince Alfred Hospital HREC (SSA/12/RPAH/10), and Human Research Ethics Committee at University of Technology Sydney (2012-004R). The blood samples were collected from MM patients and normal subjects (>18 years of age) after informed consent at the CRGH and Royal Prince Alfred Hospital blood collection centers. The subjects were de-identified (name and address) and were assigned a code for accessing clinical information. This is a preliminary study, and a predetermined sample size was not calculated because we are analyzing all samples accessible following patient consent. Thus, we are working with a fixed though unknown sample size. Normal subjects were age-matched, noncancer patients with normal hematology who presented at the hospital and were devoid of any cytotoxic treatment or radiotherapy of any nature in the past 5 years. Pregnancy was also an exclusion criterion. In total, 18 normal subjects and 64 MM subjects were assessed, which included treatment-responsive [$n = 26$ and $n = 18$ for partial remission (PR) and complete remission (CR), respectively], *de novo* ($n = 8$), and relapsed ($n = 14$) MM patients [19]. We had access to the longitudinal samples for *de novo* cohort due to regular clinical visits of this cohort for the front-line treatment, whereas the MP data from remission, progressive disease (PD), and healthy cohorts represent one point in time.

Isolation of Microparticles from MM Patient Peripheral Blood

Up to 4 ml of EDTA whole blood was centrifuged at 1500 × *g* for 20 minutes at room temperature (RT) to obtain platelet-poor plasma and followed by 13,000 × *g* for 2 minutes at RT to obtain platelet-free plasma (PFP) from the supernatant [20]. The PFP was divided into 200-μl aliquots, which were subjected to direct immunolabeling or MP isolation by ultracentrifugation at 18,890 × *g*, 4°C for 30 minutes [20]. The supernatant was removed, and the MP pellet was immunolabeled for flow cytometry. Technical triplicates were performed for each patient MP count. Biological replicates were not feasible because of the small volume of sample from each patient.

Data Acquisition and Flow Cytometric Detection of Microparticles

Flow cytometric analyses were conducted using an LSRII flow cytometer and the CellQuest Pro analysis software (BD Biosciences, Australia/New Zealand). Latex beads of 0.3 and 1.1 μm diameters were prepared and used according to the manufacturer's recommendation to define the MP gate. Compared with MPs, latex beads typically have higher refractive indices and, consequently, lower limits of size detection by flow cytometry [20,21]. As a result, the threshold in forward and side scatter was adjusted to avoid background noise during acquisition. The predefined MP gate was applied to all samples during analysis. The performance of lasers was validated before each experiment using Sphero Rainbow calibration particles (BD, Australia/New Zealand; cat. no. 559123).

Surface Protein Phenotyping of Microparticles

Microparticles were isolated and validated as previously described by us [6]. Detection of the cell surface antigens, CD138, CD41a, and Annexin V was run in parallel with relevant isotype controls and unstained controls for MP samples isolated from both MM patients and normal age-matched healthy subjects. Compensation of fluorophores was established using the setup feature in BD FACSDiva software. To detect and exclude platelet-derived MPs during the analysis, the isolated MP pellet was dual labeled using 5 μl of anti–CD138-APC and 20 μl of anti–CD41a-PE for 30 minutes at RT. MPs were pelleted at 18,890 × *g*, 4°C for 30 minutes and resuspended in 200 μl of PBS for flow cytometric analysis.

Sample Storage Optimization

In assessing the effects of sample freezing on antigen detection, PFP prepared from freshly collected blood was divided into 200-μl aliquots.

MPs were isolated from PFP before and after freezing at −80°C for >24 hours and analyzed by surface protein phenotyping.

Quantitation of Systemic Microparticles

Isolated PC-derived MPs were resuspended in 200 μl of PBS and added to BD TruCount tubes as per the manufacturer's recommendation for quantitation. The tube was vortexed gently before data acquisition. The number of MPs per μl of plasma was calculated using the manufacturer's formula: N = [MP number of measured beads] × [beads per tube/sample volume added]. The stop gate was set at a fixed number of 2000 or 10000 of TruCount beads during the data acquisition.

Microparticle Morphology

One percent polyethylenimine–coated Thermanox coverslips were prepared, and the MP suspension (in PBS) was immobilized onto the coated cover slips for 30 minutes. The coverslips were washed in 0.1 M PBS (pH 7.4) and fixed in primary fixative 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer for 30 minutes. Buffer washes were performed before the addition of secondary fixative 1% osmium tetroxide for 1 hour. The coverslips were washed 3 times for 5 minutes each with ultrapure water. A series of dehydration steps in 30% to 95% ethanol was performed for 5 minutes in each step, followed by a final wash in ultrapure ethanol twice for 10 minutes. Coverslips were left in hexamethyldisilazane for 2 minutes, following which the hexamethyldisilazane was completely removed before air-drying the sample overnight. The samples were mounted onto silver specimen stubs with double-sided carbon tape, lined with silver DAG, and coated with platinum. Scanning electron microscopy (SEM) was performed using a Zeiss Ultra Plus FESEM (Carl Zeiss, Oberkochen, Germany) at secondary electron at 10 kV. MP morphology were visualized across all clinical states: *de novo*, remission, and relapsed. OPM2 cell–derived MPs were used as controls. MP size was analyzed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD). The experiment was performed in technical duplicates for each patient sample.

Statistical Analysis

Mann-Whitney (U) test was conducted for the nonparametric data using GraphPad Prism version 6.0 for Mac (GraphPad, La Jolla, CA). The data are presented as the mean or mean ± SD and Mann-Whitney constant U as stated. The results with a predictive value of (****) $P < .0001$, (**) $P < .01$, and (*) $P < .05$ were considered significant.

Results

Detection of PC-Derived Microparticles from PFP

MPs were isolated by differential high-speed centrifugation, as we have previously described [6]. PFP was used as the starting material to ensure that contamination by platelet-derived MPs in the final preparation was minimized. MPs were validated by flow cytometry for typical characteristics of size and phosphatidylserine exposure (using Annexin V-V450) (Figure 1, *A* and *B*). The gating parameters for the MP region (R3) were defined using 0.3-μm latex beads (R1: the lowest possible limit on FS for BD LSRII) and 1.1-μm beads (R2: represents the upper limit for MPs, Figure 1*A*).

As platelet-derived MPs comprise the major population systemically, our analysis was confined to CD41a$^-$ (platelet marker) MPs. MPs arising from PCs were detected using anti–CD138-APC mAb (clone MI15). CD138 is an exclusive marker of PCs and allows for the detection of MPs originating from PCs [17].

We observed a significantly greater number of MPs from patient samples relative to healthy subjects. In the plasma of healthy volunteers, we observed that 1.5% of total MPs were Annexin V$^+$ CD138$^+$ (Figure 1*B*, *left panel*) relative to 0.09% for isotype control (data not shown). In contrast, we observed that 12. 8% of total MPs were Annexin V$^+$ CD138$^+$ (Figure 1*B*, *right panel*) relative to 0.46% for isotype control (data not shown). As PS expression is not an exclusive criterion for MPs, as not all MPs expose PS, we consequently chose to identify MPs based on their size and their phenotype.

Sample Storage Optimization

Freezing PFP before the MP isolation was shown to have significant impact on the integrity of the CD138 marker on MPs. Specifically, in the representative data, we observed that 24% of the gated population detected CD138$^+$ using fresh sample (Figure 2*A*) compared with 5.63% for the same sample after freezing (Figure 2*B*). Consequently, MP isolation was conducted using freshly isolated PFP without freezing.

Total Microparticle Numbers Increase in MM

In considering the cohort data, we observed greater numbers of total MPs in MM patients relative to healthy volunteers (Figure 3*A*). The absolute number of MPs from MM patients was on average seven-fold greater per μl relative to healthy volunteers ($U = 30$, $P < .0001$). Consistent with this, we observed greater numbers of total MPs for *de novo* patients ($U = 71.50$, $P < .0001$), patients in remission (CR: $U = 2$, $P < .0001$; PR: $U = 15$, $P < .0001$), and patients with PD ($U = 9$, $P < .0001$) relative to healthy volunteers (Figure 3*B*). We observed no significant difference in total MP counts across the different clinical states.

CD138$^+$ Microparticle Numbers Increase in MM

In considering the cohort data, we observed greater numbers of CD138$^+$ MPs in MM patients relative to healthy volunteers (Figure 4*A*). The absolute number of CD138$^+$ MPs from MM patients was on average 3.45 fold greater per μl relative to healthy volunteers ($U = 632$, $P < .01$). Consistent with this, we observed greater numbers of CD138$^+$ MPs for patients in CR/PR and with PD relative to healthy volunteers. We observed no significant difference between healthy and *de novo* patients, which we attribute to early stage of disease at diagnosis for 75% of patients examined. We measured greater, albeit insignificant, CD138$^+$ MP numbers for patients with PD relative to those in remission. MM stem cells ("side population") are known to contribute to MM relapse. These tumor-initiating cells are also phenotypically CD138$^-$ and may impact MP counts for some patients with PD [22,23]. CD138$^+$ MP numbers were greater in patients in PR ($n = 26$), in CR ($n = 18$), and with PD ($n = 14$) relative to healthy volunteers ($n = 18$). $P < .05$ (*), $P < .01$ (**) (data not shown). We observed significantly increased CD138$^+$ MP counts in CR ($U = 49$, $P < .0022$), PR ($U = 25$, $P < .0231$), and PD ($U = 23$, $P < .0018$) relative to *de novo* patients (Figure 4*B*). Furthermore, we also identified five patients who were in CR at the time of analysis who had greater CD138$^+$ MPs counts relative to the rest of the cohort (gray circles, Figure 4*B*). These same patients were found to clinically relapse a few weeks later, demonstrating the sensitivity and potential for CD138$^+$ MP numbers to predict the transition between remission and PD in the absence of clinically used markers of relapse in individual patients.

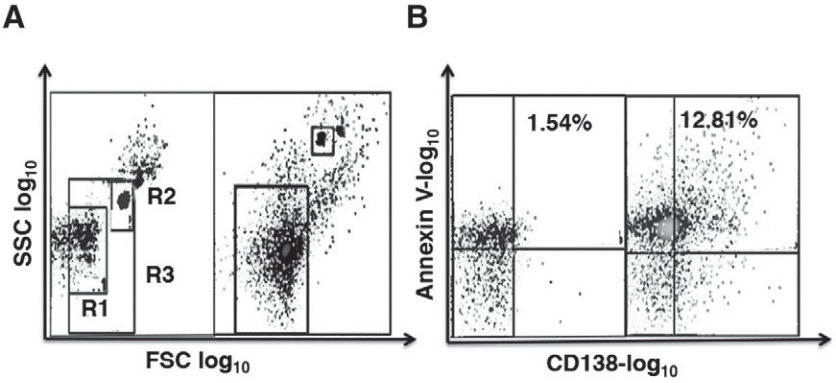

Figure 1. Detection of PC-derived MPs from PFP. Latex beads of known diameter (0.3 and 1.1 μm; Sigma-Aldrich) were used to define the size gates. (A) R3 represents the MP region based on the lower (R1) and upper limit (R2) set by the beads. The predefined size gating (R3) was applied to the patient's MP scatter plot to identify the region of interest. The total number of acquired events when the stop gate was set at 10,000 TruCount bead counts is shown on the right. (B) In the left scatter plot, MPs isolated from the PFP taken from a normal healthy volunteer with only 1.5% MPs were Annexin V^+ $CD138^+$, whereas 12.8% of MPs isolated from MM patient were Annexin V^+ $CD138^+$ as shown on the right.

$CD138^+$ Expression Correlates with Therapeutic Response in Individual Patients

We observed a significant prognostic potential for $CD138^+$ MPs in predicting "risk of relapse" and therapeutic response in individual patients. We conducted a series of assessments whereby we profiled $CD138^+$ MPs from diagnosis throughout the course of therapy for eight individual patients. Figure 5 summarizes the case of a 52-year-old female MM patient. $CD138^+$ MP count fell in accordance with response to treatment (Figure 5*A*). The patient's free kappa/lambda ratio also showed a decrease, in response to therapy, consistent with a decline in tumor burden. However, soon after, the patient developed plasmacytomas around the hip area together with bone lesions in the spine, pelvis, and femur. We observed a corresponding increase in $CD138^+$ MPs, whereas we observed no corresponding increase in paraprotein levels observed during this same period (Figure 5*B*). The patient was then placed on a thalidomide/dexamethasone maintenance regimen. This corresponded with a drop in both CD138+ MP counts and IgG levels. The patient underwent autologous stem cell transplant, $CD138^+$ MP counts increased, and IgG levels stabilized.

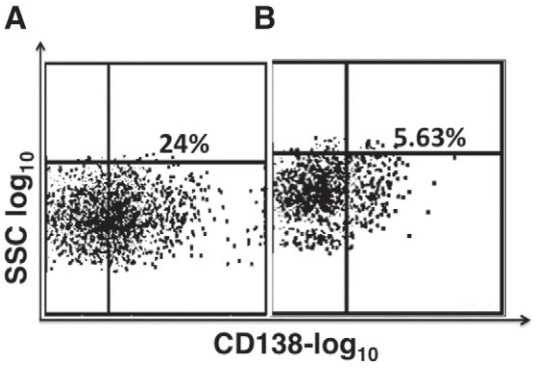

Figure 2. Sample storage optimization. PFP was prepared, and the sample was divided in two 200-μl aliquots. One sample was immediately frozen at −80°C, whereas MPs were freshly isolated from the other portion and subjected to immunolabeling. The frozen sample was thawed on ice, followed by MP isolation and fluorescence immunolabeling. (A) Twenty-four percent of MPs were $CD138^+$ for the fresh MP sample, whereas only 5.63% of MPs were $CD138^+$ when isolated from PFP post freezing/thawing (B).

Morphology and Size of the Microparticles

SEM was used to visualize the morphology of MPs across different clinical states. The morphology of MPs was compared with the OPM2 myeloma cell line–derived MPs (Figure 6*A*, *leftmost panel*). In *de novo* patients (Figure 6*A*, *second panel from left*) and patients in remission (Figure 6*A*, *third panel from left*), MPs displayed a regular spherical surface, whereas MPs isolated from relapsed patient displayed an irregular surface with the presence of vacuoles and/or craters (Figure 6*A*, *rightmost panel*). We observed no significant difference in the size across distinct clinical response states with 0.75 ± 0.16 μm, 0.83 ± 0.53 μm, and 0.87 ± 0.44 μm for the *de novo*, remission, and relapsed cases, respectively (Figure 6*B*).

Discussion

We report on the isolation and detection of non–platelet-derived ($CD41a^-$) $CD138^+$ MPs in the blood of patients with MM. Our data demonstrate that the total MP count is a predictor of the diseased state in MM relative to healthy volunteers and is independent of the

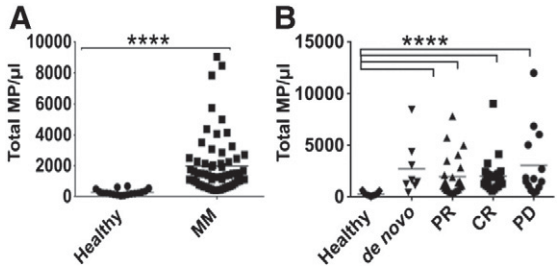

Figure 3. Total MP counts increase in MM. The total counts in MM patients and healthy subjects were compared using the manufacturer's formula. (A) Total MP numbers were significantly greater in the MM cohort relative to the healthy cohort. (B) Total MP numbers were greater in *de novo* ($n = 8$), PR ($n = 26$), CR ($n = 18$), and PD ($n = 14$) patients relative to healthy subjects ($n = 18$) [$P < .0001$ (****)].

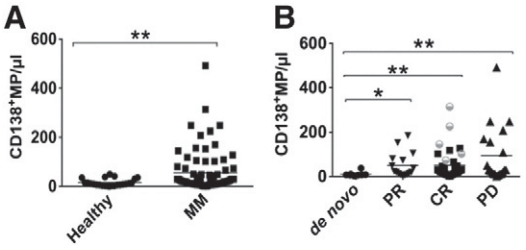

Figure 4. CD138$^+$ MP increases in MM. The CD138$^+$ MP counts in MM patients and healthy subjects were compared using the manufacturer's formula. (A) CD138$^+$ MP numbers were significantly greater in the MM cohort relative to the healthy cohort. (B) The absolute numbers of CD138$^+$ MPs in the PD, CR, and PR cohort were significantly increased compared with that of the *de novo* cohort. Gray circles represent the patients that were found to have PD in 3 to 4 weeks from sampling.

clinical state. We also report on the elevated levels of CD138$^+$ MPs in MM patients relative to healthy volunteers. We showed that the CD138$^+$ MP count was significantly higher in MM patients during the course of active therapy across the different clinical response states. Also, we show that CD138$^+$ MP count promises a sensitive assessment of disease progression and therapeutic outcome in individual MM patients. The substantial outcome of the study is the prospective of a minimally invasive, effective systemic marker for prognosis determination and prediction of therapeutic response during the course of therapy. However, this is a pilot study, and further research is required to confirm its usefulness in a clinical setting. For the same reason, we were limited with only technical replicates of samples, and biological replicates were not feasible at this stage.

Current response criteria assessment in MM rely on direct measure of disease burden via an invasive bone marrow biopsy, immunofixation, serum protein electrophoresis, quantitation, measurement of free light chain, and computed tomographic/magnetic resonance imaging scans [24]. Nonetheless, all the above markers have collectively failed to gauge the transition phase from orderly disease state to the progressive, and thus novel systemic markers which can provide for this will aid in improving the clinical management of MM.

Clinical monitoring of non-secretory MM was impeded until the introduction of the light chain assays [25]. Free light chain assays assess the free lambda, free kappa, and their ratio in the peripheral blood as non-secretory MM lacks the classic paraprotein marker [19]. Nevertheless, clinical monitoring of non-secretory MM remains limited as light chain assays have inadequacies including sample dilution anomalies, calibration problems, and limits of detection, which may result in erroneous inference of clinical significance [25].

As a stress response, the pliable plasma membrane undergoes lipid bilayer remodeling, which in turn results in systemic shedding of the MPs by most cell types [5,11]. MP levels are elevated in cancer and inflammatory conditions and have a substantial pathophysiological significance [6,11,26]. The surface molecules present on MPs identify their cellular origin. MPs mediate both long-range and short-range intercellular cross talk [4,5].

Microparticles are emerging as important "surrogate markers" of many disease states as well as of physiologically less accessible tissues, such as the endothelium [8,9]. Unresponsiveness to chemotherapeutic regimens, resulting in relapse, is one of the characteristics of MM [2]. Therefore, constant monitoring of the patient's therapeutic response is mandatory in the MM clinical setting [27]. However, the neoplastic cells of MM are mostly restricted to the bone marrow until advanced state; therefore, a systemic marker with personalized prognostic capacity for determining treatment responsiveness in MM is of significant clinical importance. Specifically, in the case of non-secretory MM where the overproduction of the classical paraprotein marker is absent, MP analysis will provide an important supporting clinical diagnostic tool. Indeed, recently, elevated levels of MPs were reported in late-stage MM than the early stage in a mouse model study [28].

CD138 mediates PC to cell adhesion and is shed from the surface of PCs to the microenvironment [29]. The molecule accumulates in

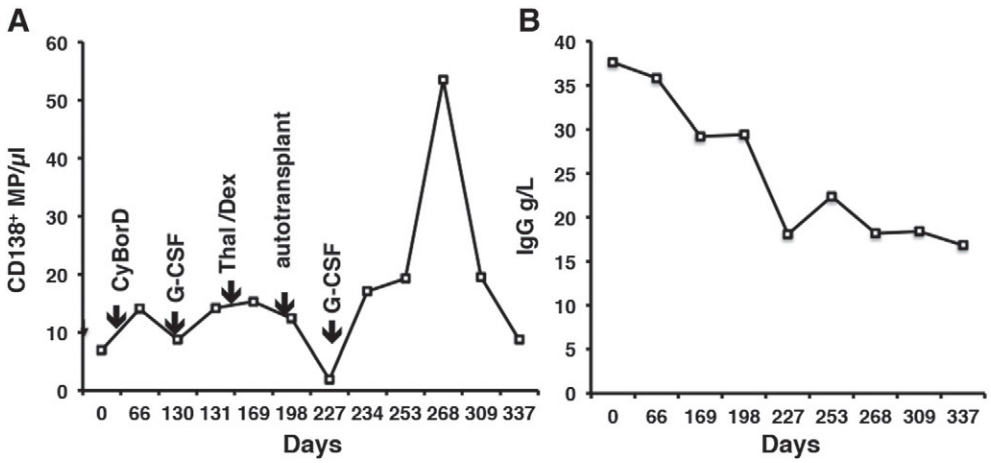

Figure 5. CD138$^+$ microparticles in a 52-year-old patient during treatment. MPs were isolated from the PFP of a 52-year-old MM patient at diagnosis and during the course of treatment. CD138$^+$ MP counts (A) were measured and profiled against IgG levels (B) during the course of clinical interventions. CD138$^+$ MP counts fell following the commencement of cyclophosphamide, bortezomib, and dexamethasone treatment (CyBorD). This corresponded to a drop in IgG levels also. The patient then underwent stem cell mobilization with G-CSF. At this point, the patient developed bone lesions and plasmacytomas. Consequently, the patient was placed on a thalidomide/dexamethasone maintenance regimen. This corresponded with a drop in both CD138$^+$ MP counts and IgG levels. The patient underwent autologous stem cell transplant, CD138$^+$MP counts increased, and IgG levels stabilized.

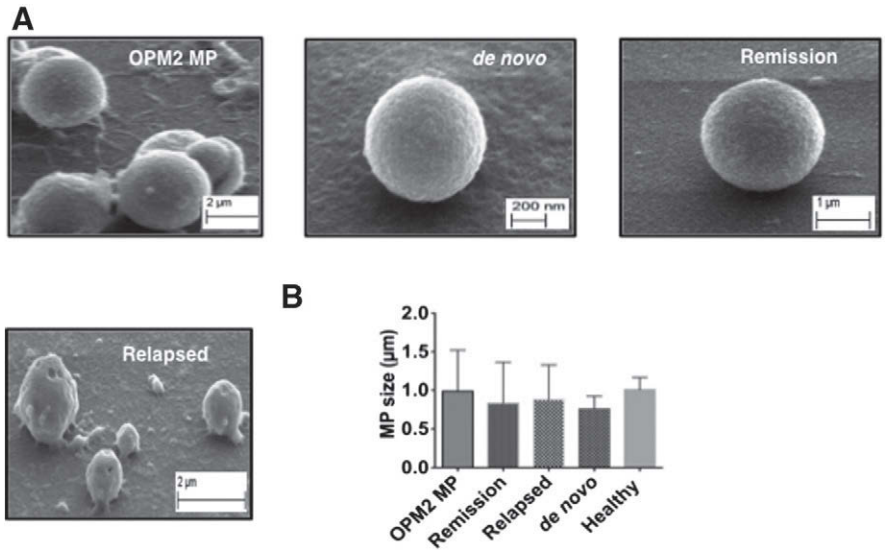

Figure 6. Morphology and size of the microparticles. SEM was used to visualize the morphology of MPs across different clinical states. (A, leftmost panel) Electron micrographs of MPs isolated from myeloma cell line OPM2 (magnification, ×5.40 K) were used as a control. (A, second and third from left panel, respectively) MPs isolated from OPM2 cells from a *de novo* patient sample (magnification, ×24.64 K) and from a patient in remission (magnification, × 19.40 K) had regular spherical surface. (A, rightmost panel) MPs isolated from a relapsed MM patient had an irregular surface with the presence of vacuoles and/or craters (magnification, ×9.18 K). (B) No significant differences in the size of MPs were observed across the different clinical states. Data are expressed as mean ± SD.

the extracellular matrix and may serve as a reservoir promoting MM proliferation and growth [30]. The loss of expression of CD138 from PCs is correlated with a negative prognosis and is associated with aggressive disease based on the *in situ* expression studies conducted on bone marrow sections taken from MM patients [29,30]. Consequently, a subpopulation of $CD138^-$ PCs in bone marrow has been reported and is identified as immature compared with $CD138^+$ [31]. According to the literature, $CD138^-$ PCs were found to be in the S phase and therefore possess higher proliferation potential [31]. We measured greater, albeit insignificant, $CD138^+$ MP numbers for patients with PD relative to those in remission. MM initiating cells ("side population") are known to contribute to MM relapse, are phenotypically $CD138^-$, and may be underestimating MP counts (derived from $CD138^-$ PCs) for patients with PD [22,23]. The transmembrane co-receptor CD138 is versatile as it can be cleaved off from PC surface to bone marrow and transform to soluble effector molecule, competing with the same ligands. However, CD138 enrichment and packaging into MPs during membrane remodeling as a response to bioactive molecules could be one alternate pathway orchestrated in MM pathogenesis and progression.

We have previously reported MPs as "submicron messengers" for the "non-genetic" transfer of drug resistance in lymphoblastic leukemic and breast cancer cell lines [6]. Furthermore, we have established that MPs selectively package cargo, which in turn contributes to the spread and acquisition of deleterious trait dominance in cancer [32–35]. This study adds to this body of research and provides support for the use of MPs as a novel prognostic for the personalized therapeutic management of MM. We have also shown that $CD138^+$ MP counts can predict the transition between remission and PD in the absence of clinically used markers of relapse in individual patients before clinical manifestations. We did not observe a significant difference in the $CD138^+$ MPs between *de novo* MM patient cohort and healthy volunteers that we attribute to the early stage of disease at diagnosis for 75% of patients examined. We also note that only eight *de novo* patients were sampled in this analysis. The study was designed to acquire any MM sample available to us at the given point of time. Therefore, we could only recruit a limited number of patients before the start of chemotherapy. The total numbers of MPs were higher in MM patients irrespective of the clinical states. We observed that $CD138^+$ MP count shows significant reduction in a positive therapeutic response in individual patients as shown in the representative case of a 52-year-old MM patient in this study. We observed reduction in $CD138^+$ MP count as a response to therapy from baseline and an increase in $CD138^+$ MP before the clinical manifestation of PD.

The scanning electron micrographs of MPs isolated from patients with MM (*de novo*, remission, and relapsed) and the OPM2 cell line showed a spherical morphology with a mean size of 0.1 to 1 µm. In contrast, MPs isolated from a relapsed patient mostly displayed an irregular morphology with the presence of vacuoles or craters. The reason for this is currently being investigated; however, it is known that in a late-stage subset of MM, PCs lose their characteristic markers harboring less differentiated B cell markers on their surface, and the subset is known as PC leukemia [36]. It is interesting to note that our earlier work has shown that MPs isolated from leukemia cells also display an irregular surface [34] similar to the systemic MP from the relapsed MM patient which may be consistent with this phenotype. By virtue of our isolation technique, the vesicle fraction is consistent, and their average size is around 0.7 µm, which exceeds the size of the exosome population as supported by our SEM data. Other contaminants like immune complexes or protein aggregates (<80 nm) were also ruled out from our homogeneous vesicle fraction by virtue of their size [34,37]. The conserved transmembrane structure of CD138 maintains the morphology and cytoskeletal organization of numerous cell types [38]. As described, cleaving of the heparan sulfate–bearing ectodomain of CD138 from PC and

subsequent accumulation of CD138 ectodomain in fibrotic regions are reported in MM. High levels of CD138 in the serum of MM patients have been described as a deleterious prognosis in MM [29]. Soluble effector molecules act as a potential reservoir for the dissemination of cancer progression, invasion, and metastasis in MM, influencing morphology and cytoskeletal organization of PCs along with the cell-extracellular matrix interactions. [30]. Thus, the loss of ectodomain from PC surface to bone marrow microenvironment in progressive MM may affect the enrichment of CD138 into MPs derived from them. This might also explain the change of the morphology of PC-derived MPs observed in our SEM images of relapsed patient.

In conclusion, we have demonstrated that there are significantly elevated levels of total systemic MPs and, specifically, $CD138^+$ MPs in MM patient peripheral blood compared with healthy volunteers. We propose a new clinical tool, which may support the existing clinical assessment. Further studies will involve thorough correlative studies to compare this methodology with existing clinical tools in the context of disease management. To the best of our knowledge, this is the first report on the isolation of non–platelet-derived MPs from MM patients and the identification of circulating $CD138^+$ MPs in patients across all MM clinical states. This clinical study provides support for a potential systemic and noninvasive prognostic biomarker of MM. This novel noninvasive clinical test supports the existing clinical tools, aiding to monitor transition between the controlled and advanced disease state in MM. The future studies will be focused into the clonal, resistance markers on MPs in MM and their role in treatment failure in tumor cells in the bone marrow during the course of MM chemotherapy. This is significant because such tests are currently lacking. There is also currently no cure for MM, and the unbiased survey of MPs we describe has the potential to identify novel targets beyond prognostication into treatment.

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Author contributions:

SRK conducted all the experiments, data analysis and literature review which resulted in this manuscript. MB designed and supervised the research, provided conceptual advice and revised the manuscript. RB provided clinical data and assisted in manuscript revision. HS accessed the clinical data. FL provided conceptual advice and guidance. YLK provided the clinical samples and clinical data. All authors reviewed the results and approved the final version of the manuscript.

Chapter 5

5. P-glycoprotein expression in microparticles is indicative of disease progression and treatment unresponsiveness in myeloma.

Running Title: P-gp in myeloma microparticles

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Abbreviations : multiple myeloma (MM), microparticles (MPs) , complete remission (CR) , progressive disease (PD), partial remission (PR), multi-drug resistance (MDR), phosphatidylserine (PS), platelet free plasma (PFP),

Conflict of Interest: The authors declare no conflict of interest

5.1 Abstract

Background: At diagnosis multiple myeloma (MM) patients present with multiple clones with differing degrees of drug sensitivity. Currently there is no biomarker that monitors the emergence of multidrug resistance (MDR) in MM. Microparticles (MPs) are submicron-sized vesicles that can confer MDR within cancer cell populations through intercellular transfer of functional resistance proteins and nucleic acids. **Methods:** Blood was collected from MM patients, age-matched healthy volunteers after informed consent and platelet free plasma was prepared (PFP). MPs were pelleted by ultracentrifugation, phenotyped and quantified with flow cytometry using Annexin-V450, CD138-APC, P-glycoprotein-FITC, CD41a-PE and CD34-PE-Cy7 using BD TruCount^{TM} beads. Platelet derived MPs were excluded using CD41a-PE in the analysis. The MP count in MM patients was compared to age-matched healthy volunteers using *Mann-Whitney U* test. **Results:** We report on the presence of P-glycoprotein (P-gp**)** on MPs isolated from MM patients. We identify MP subtypes including a 'dual positive' ($CD138^{-}$ $CD34^{+}$ $P\text{-}gp^{+}$) population of 'stem cell like origin', with levels elevated in aggressive and active disease states (N=1). We also identify an evolving shift in the dominance of these subtypes with disease progression. **Conclusion:** We present an approach with personalized prognostic capacity for determining the evolution of MDR across a number of distinct cell types (plasma cell and 'stem cell like origin') in MM. MDR can be serially monitored by analysing MPs in the context of a 'liquid biopsy'. This introduce new insights into the utility of biomarkers generally and the molecular mechanisms contributing to disease progression, MDR and treatment failure in MM.

5.2 Introduction

First-line treatment for eligible newly diagnosed multiple myeloma (MM) patients involves high dose combination chemotherapy with autologous stem cell transplantation (1). At diagnosis, multiple clones exist within MM tumors, each with differing degrees of drug sensitivity (2). The presence of these multi-clone tumors contribute to a high and unpredictable incidence of multidrug resistance (MDR) in patients and translates to significant variability in patient survival, ranging from a few weeks to more than 10 years (3, 4).

MDR is a unique type of resistance in which cancer cells, following exposure to a single agent, become cross-resistant to a broad range of drugs used in combination chemotherapy (5). Synonymous with MDR is the overexpression of proteins including: (i) plasma membrane transporters that efflux drugs from cancer cells (e.g. P-glycoprotein, also known as *ABCB1*/MDR1/P-gp, multidrug resistance protein 1 [*ABCC1*/MRP1], and breast cancer resistance protein [BCRP]) (6, 7), and (ii) proteins localized in nuclear pore complexes (8), that protect cells via intracellular redistribution of drugs (e.g. lung resistance protein (LRP). Each protein has a unique substrate repertoire despite significant overlap in substrate recognition (9).

Elevated P-gp expression has been reported for almost all haematological cancers and is correlated with a poor prognosis and compromised response to chemotherapy (5). P-gp expression has been shown to increase by up to 75% in MM patients subsequent to treatment in consecutive bone marrow samples (10). The promise of newer drugs, lenalidomide, bortezomib and carfilezomib has been hampered by reports that they are

also P-gp substrates although this remains to be confirmed (11-13). Many agents typically used in combination chemotherapy in the treatment of MM are P-gp substrates and hence existing chemotherapeutic regimens as a whole, are compromised (13).

Apart from full blood count and biochemistry screen, β-2 microglobulin (β_2M), serum protein electrophoresis, immunofixation, measurement of free light chain, bone marrow biopsy, and CT/MRI are commonly used for staging, diagnosis and disease monitoring in MM (14, 15). None of these can directly assess the emergence of MDR or detect the expression and evolution of resistance markers, polymorphic variants or nucleic acid signatures, all of which may contribute to disease progression and therapeutic response. Specifically, there is a need for minimal residual disease assessment in a peripheral blood sample by flow or molecular techniques, as all current methods rely heavily on bone marrow biopsy (16).

Microparticles (MPs) are small membrane vesicles (0.1 to 1 μm in diameter) released from the plasma membranes of most cell types (17). Circulating cancer-derived MPs have been detected in many cancers (18-20). Importantly, we reported that (20) cancer-derived MPs can confer the transfer and spread of MDR within cancer cell populations within a matter of hours (21-25). MPs do this by virtue of the presence of functional resistance proteins and nucleic acids within the vesicular cargo (21, 22, 25).

We have recently reported the presence of $CD138^+$ MPs in the plasma of MM patients (26). We observed greater $CD138^+$ MP counts in MM patients relative to healthy subjects (26). Interestingly, we observed greater $CD138^+$ MP counts for patients in

remission (complete remission, CR and partial remission, PR) and with progressive disease (PD) relative to healthy volunteers (26), however we observed no significant difference between healthy subjects and *de novo* patients. We identified 9 patients who were in complete remission (defined using the IMWG response criteria) at the time of analysis who had greater $CD138^+$ MP counts relative to the rest of the cohort. Five of those patients relapsed clinically within 4 weeks, demonstrating the potential for $CD138^+$ MP counts to predict the transition between remission and progressive disease before more conventional clinical markers. We also showed $CD138^+$ MPs provide a sensitive assessment of disease progression in individual patients in this particular study (26).

Circulating MPs are promising candidates for "surrogate markers" of difficult to access tissues such as the bone marrow compartment. MPs carry signature markers of lineage and they are selectively packaged with cellular contents such as nucleic acids, micro RNAs, lipids and proteins from their cell of origin. The combination of MP counts and molecular profile has been shown to correspond to disease pathology and/or treatment sensitivity at an individual level (26-28).

In this study, we phenotypically characterized non-platelet derived MPs for the presence of P-gp expression and showed that its presence is indicative of disease progression and treatment unresponsiveness in myeloma.

5.3 Materials and Methods

5.3.1 Reagents & Antibodies

Annexin V-V450 (BD Horizon™), anti-CD138-APC (clone MI15), anti-CD41a-PE (clone HIP8), anti-P-gp-FITC (clone 17F9*),* anti-CD34-PE-Cy7 *(*clone 8G12 Y7*),* matched isotype controls BD™ CompBead anti-mouse-Ig k, Sphero™ Rainbow calibration particles and TruCount™ tubes were from BD Biosciences Australia. Latex beads of diameter 0.3 *(cat. no. LB3)* & 1.1 *(cat. no. LB11)*µm were purchased from Sigma-Aldrich, Australia.

5.3.2 Study design and patients

This study was approved by the Sydney Local Health District (SLHD)-Human Research Ethics Committee (HREC) of Concord Repatriation General Hospital (CRGH) [(HREC/11/CRGH/223-CH62/6/2011-150], Royal Prince Alfred Hospital (RPAH) HREC (SSA/12/RPAH/10) and the University of Technology Sydney (2012-004R). Blood samples were collected from MM patients and healthy volunteers (>18 years of age) after informed consent at the CRGH and RPAH blood collection centres in accordance with the Declaration of Helsinki. The subjects were de-identified and assigned a code for accessing clinical information. Healthy volunteers were age-matched, non-cancer patients with normal hematology and devoid of any cytotoxic treatment or radiotherapy of any nature in the past 5 years. Pregnancy was also an exclusion criterion. In total, 25 normal subjects and 74 MM subjects were assessed, which included treatment responsive (*n=32, n=15* for partial remission and complete remission respectively), *de novo (n=14)* and relapsed (*n=18)* MM patients. Patient responses were determined according to IMWG guidelines (47).

5.3.3 Isolation and flow cytometric detection of MPs.

Platelet free plasma (PFP) was prepared as described previously (26, 48). PFP was divided into 200 µl aliquots, which were subjected to direct immunolabeling or MP

isolation by ultracentrifugation at 18,890 × *g*, 4ºC for 30 min. The supernatant was removed and the MP pellet was immunolabeled for flow cytometry in technical triplicates for each patient MP count. Latex beads of 0.3 and 1.1µm diameters were prepared and used according to the manufacturer's recommendation to define the MP gate and was applied to all samples during analysis (26). Flow cytometric analyses were conducted using LSRII flow cytometer/ LSR Fortessa X20 and the CellQuest Pro, FACSDiva analysis software (BD Biosciences).

5.3.4 Surface phenotyping of systemic MPs and quantitation.

Cell surface antibodies directed against CD138, CD41a, CD34, P-gp and phosphatidylserine were added to the MP pellet as previously described (25, 26, 49). Relevant isotype–matched and unstained controls were run in parallel. Platelet derived MPs were excluded during the analysis using anti-CD41a-PE. MPs were re-suspended in 500 µL PBS and quantitated using BD TruCount™ beads as previously described (26).

5.3.5 Statistical analysis

Mann-Whitney (U) test was conducted for the non-parametric data using GraphPad Prism® version 7.0 for Mac (GraphPad, La Jolla, CA, USA). The data presented as the mean and *Mann–Whitney constant U.* The results with a predictive value of (***) P<0002, *(**) P<0.01* and *(*) P<0.05* were considered significant.

5.4 Results

5.4.1 P-gp$^+$ MP numbers are elevated in *de-novo* and progressive disease MM patients.

We observed significantly greater numbers of P-gp$^+$ MPs in the total (CD41a$^-$) MP population in MM patients relative to healthy volunteers (Figure 5.1A). The absolute number of P-gp$^+$ MPs from MM patients was 5.1fold greater number per µl relative to the healthy volunteers *(U = 605, p< 0.01)*. Specifically, we observed a 5.67 *(U= 70, p< 0.01)* and 12.4 *(U= 104, p< 0.01)* fold increase in P-gp$^+$ MPs for *de novo* and progressive disease (PD), respectively, relative to the healthy volunteers (Figure 5.1B). There was no significant difference between P-gp$^+$ MPs between healthy volunteers and patients in complete (CR) or partial remission (PR) (Figure 5.1B).

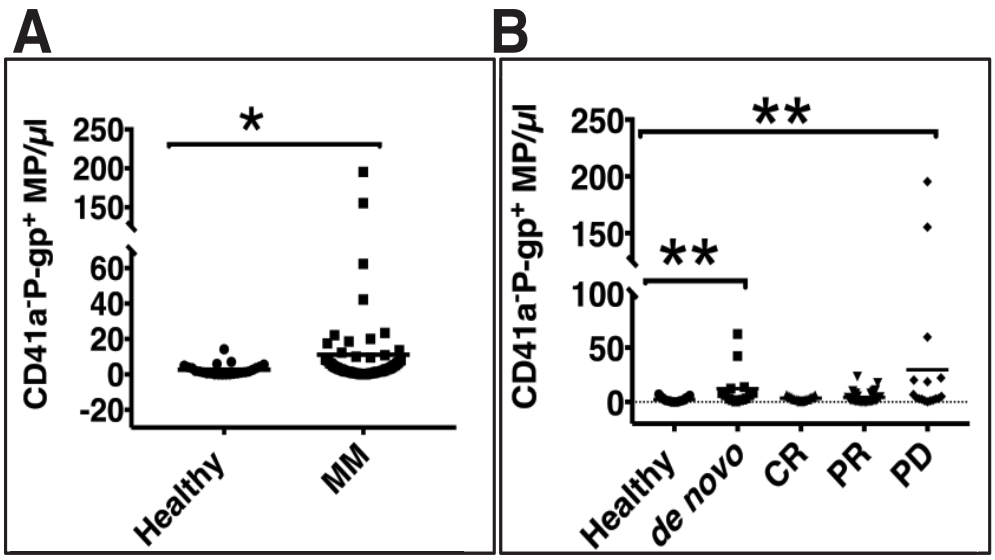

Figure 5.1 P-gp$^+$ MP increases in MM. The P-gp$^+$ MP counts in the total MP (CD41a$^-$) population in MM patients and healthy subjects were determined using TruCount™ beads **(A)** P-gp$^+$ MP counts were significantly greater in the MM patients *(n=69)* relative to healthy volunteers *(n=25)*, *p<0.01* (****). **(B)** P-gp$^+$ MP counts were greater in patients in *de novo* (*n=14*) and progressive disease (PD, *n=17*) relative to healthy volunteers, (*p<0.01* (****)). There was no significant difference in the P-gp$^+$ MP count of healthy volunteers compared to patients in partial remission (PR, *n=32*) and complete remission (CR, *n=13*). P values were generated using *Mann–Whitney U* test and the data is represented as mean.

5.4.2 CD138$^+$ MPs do not express significant levels of P-gp on their surface.

P-gp expression on the CD138$^+$ MP population in MM patients was not significantly increased compared with the healthy volunteers *(U=716, p=0.28)* (Figure 5.2A). There was no significant increase observed across *de novo*, CR, PR and PD subpopulations (Figure 5.2B) respectively relative to healthy volunteers.

In contrast, P-gp expression on the CD138$^-$ MPs showed a significant 4.5 fold increase relative to healthy volunteers *(U=553, p=0.009*, Figure 5.2C). The P-gp$^+$ CD138$^-$ MP numbers were 3.6 fold higher in the *de novo* cohort *(U=57, p=0.0003)* and 12.2 fold in PD relative to the healthy volunteers *(U=126, p=0.04)*. The absolute numbers were not significantly different in the CR *(U=151, p=0.19)* or PR *(U=195, p=0.14)* cohorts compared to the healthy volunteers. (Figure 5.2D).

We have previously shown a greater, albeit insignificant, CD138$^+$ MP count for patients with PD relative to those in remission (26). MM stem cells, which may be the tumor-initiating cells in MM, are phenotypically CD138$^-$. Consequently, MP counts for some patients with PD may be under-estimated when only the CD138$^+$ population is considered (26, 29, 30). As the overexpression of resistance markers, including P-gp, is also associated with advanced disease, the apparent lack of significance observed by us with respect P-gp$^+$CD138$^+$ MPs in this study may arise as a consequence of the proliferation of the cancer stem cell population in advanced disease. To examine this, we selected five individual patients across all disease states and phenotyped MPs for the presence P-gp, CD34 and CD138. CD34 is a transmembrane protein belonging to the CD34 family of sialomucins and is an established haematopoietic stem cell marker (31).

Although, not typically used in phenotyping plasma cells, CD34 is present on a minor subpopulation of MM stem cell clones (32). Its function as an adhesion receptor appears to be required for binding to the endothelium during extravasation in extramedullary disease (32). The selected panel of individual patients included (a) aggressive disease, patient 1 (b) progressive disease, patient 2 (c) stable disease, patient 3 (d) partial remission, patient 4 and (e) a long-term survivor in remission, patient 5. We also phenotyped MPs for the extent of phosphatidylserine (PS) using annexin V. PS is expressed preferentially on the surface of MPs of cancer cells of 'stem cell like' origin (33). PS on MPs has been recently shown to be required for interactions with vascular endothelial cells in neovascularisation and is associated with cancer progression (33).

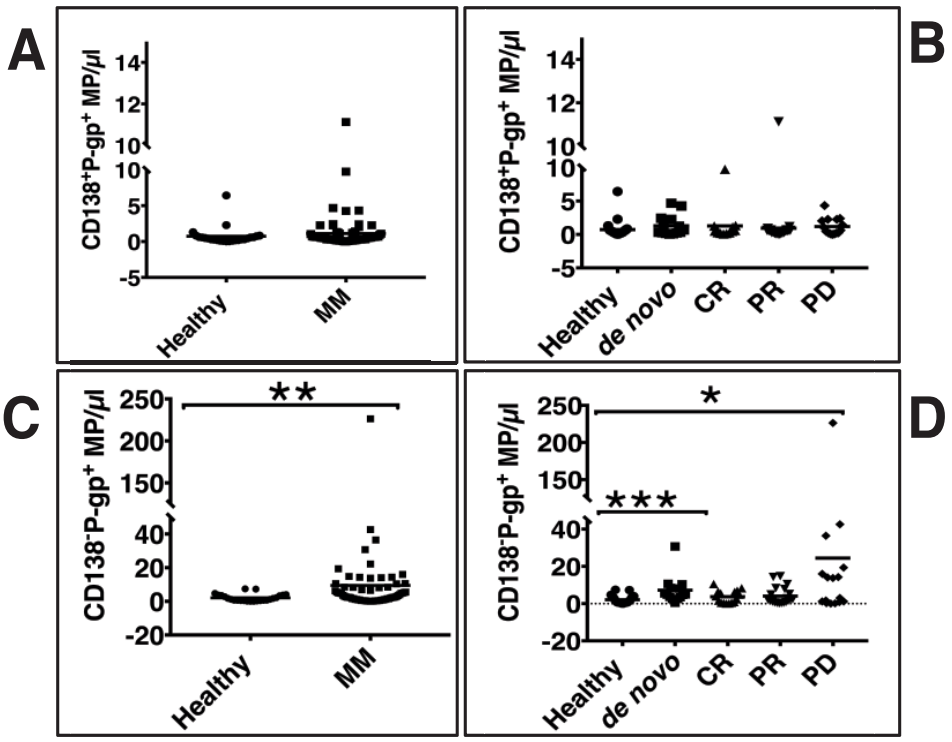

Figure 5.2 CD138$^+$ MPs do not significantly express P-gp. (A) The CD138$^+$ P-gp$^+$ MP count was elevated in MM relative to healthy volunteers however was not significant in a CD138$^+$ MP population. **(B)** Consequently, CD138$^+$ P-gp$^+$ MP count across *de novo (n=14)*, PR *(n=32),* CR *(n=13)* and PD were elevated though not significant. **(C)** The CD138$^-$ P-gp$^+$ MP count was significantly elevated in MM patients relative to healthy volunteers *(n=25)* (*p<0.01* (***)). **(D)** CD138$^-$ P-gp counts were significantly higher in *de novo* cohort *(P=0.0002 (***))* and PD *(p<0.05 (*))* however was not significant for CR, PR. P values were generated using *Mann–Whitney U* test and the data is represented as mean.

5.4.3 CASE 1: 58-year-old female patient with aggressive disease.

Figure 5.3A-C demonstrates the serial P-gp$^+$ MP count of a 58-year-old female patient (patient 1) who was diagnosed with IgG MM in September 2013 with 86% plasmacytosis in the bone marrow aspirate (0 days). At diagnosis, the P-gp$^+$ MP count was minimal. Induction therapy with cyclophosphamide, bortezomib, dexamethasone (CyBorD) commenced in September 2013 (Figure 5.3 black dot) but in November 2013 cyclophosphamide was withdrawn due to severe anaemia (Figure 5.3 pink dot). A bone marrow biopsy in December 2013 showed partial response with 46% plasmacytosis. During this time, the number of P-gp$^+$ MP was increasing steadily which was consistent with the emergence of MDR. Thalidomide was added from January-April 2014 (Figure 5.3, blue dot). A follow up biopsy showed reduced plasmacytosis of 23% in April 2014 (~days 70-80). The paraprotein increased to 38.3 g/l (progressive disease) in June-July 2014 and the treatment regimen changed to lenalidomide/dexamethasone from July-October 2014 (~100 days) (Figure 5.3 red dot). Patient 1 relapsed with a right side posterior mass along the chest wall in early February 2015 (60% plasmacytosis, around 130 days) while the M-protein level was only 18g/l at that point in time (data not shown). At this time, P-gp$^+$ MPs in PFP continued to significantly increase. Dexamethasone along with platinol, adriamycin, cyclophosphamide and etoposide (D-PACE) and melphalan added to treatment regimen at this point (Figure 5.3 green dot) and patient 1 achieved partial remission (~ day 495). She had a successful autologous stem cell transplant in July 2015. However, she relapsed soon and became unreponsive to all therapy in November 2015 and passed away in December. Rather the predominant Pgp$^+$ MP subtype was CD138$^-$ (Figure 5.3B). Figure 5.3C shows the profile of P-gp$^+$ CD138$^+$ MPs in the same patient (26).Upon examining the MP profiles for annexin V$^+$

sub populations in patient 1, we did not observe any significant difference in the levels between the $CD138^+$ and $CD138^-$ subtypes (data not shown).

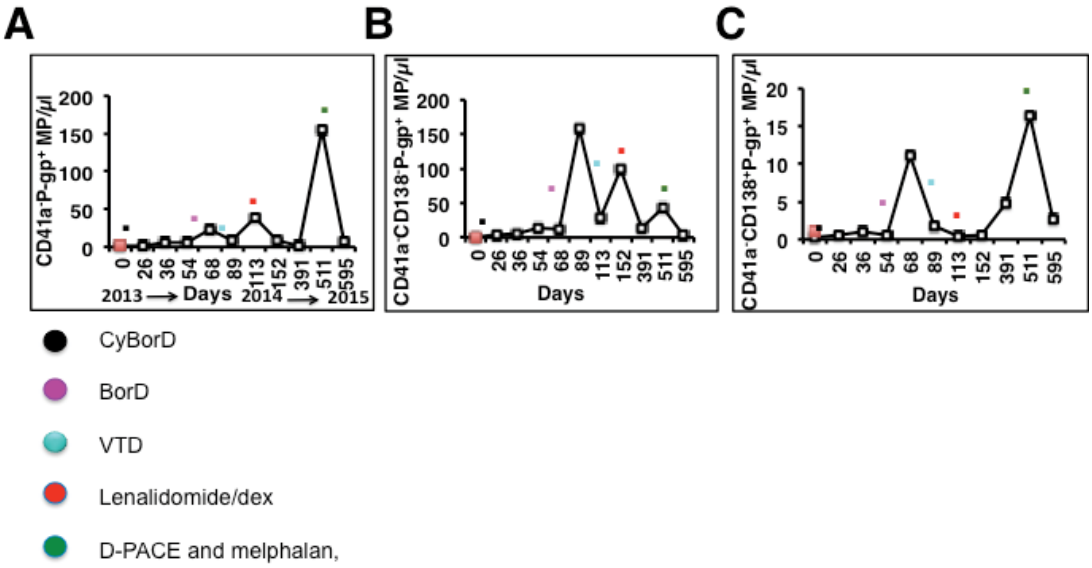

Figure 5.3 P-gp$^+$ MPs in a 58-year-old patient with aggressive disease during the course of treatment (patient 1). MPs were isolated from the PFP of patient 1 at diagnosis and during the course of treatment. The absolute P-gp$^+$ MP counts (Y-axis) and time of MP sampling post diagnosis (X-axis) are shown. **(A)** (CyBorD, black dot; BorD, pink dot; VTD, blue dot lenalidomide/dexamethasone, red dot and D-PACE and melphalan, green dot), CD41a$^-$P-gp$^+$ MP count of patient 1 is shown **(B)** Corresponding CD41a$^-$CD138$^-$ P-gp$^+$ and **(C)** CD41a$^-$CD138$^+$P-gp$^+$ MP profiles of patient 1 are shown respectively.

A blood sample was taken from patient 1 on 11th of February 2015 during progressive disease and prior to stem cell transplantation. This sample showed elevated numbers of $CD34^+$ (496.81/µl) and P-gp^+ (155.29/µl) total $CD41a^-$ MP events (Table 5.1)compared to that observed when the patient was in partial remission in May 2015 (7.33/µl and 6.31/µl for $CD34^+$ and P-gp^+, respectively). Gating parameters were established to detect $CD41a^-$ events in the context of CD138 and are shown in Figure 5.4A (supplementary figure 1). We compared the levels of $CD34^+$ and P-gp^+ MP events within $CD138^+$ (red) and $CD138^-$ (blue) MP subtypes (Figure 5.4B and C). The predominant population which was P-gp^+ and $CD34^+$ was the $CD138^-$ MP subtype (referred to as the 'dual positive' population for simplicity) Figure 5.4B, left panel, gate P1, 12.48/µl). We detected very little P-gp^+ $CD34^+$ $CD138^+$ MPs (Figure 5.4B, right panel, gate P4, 0.30/µl). We also identified additional MP sub-sets which were $CD138^-$ P-gp^+CD34^- (Figure 5.4B,left panel, gate P3, 56.45/µl) and $CD138^+$ P-gp^- $CD34^+$ MP (Figure 5.4B,right panel**,** gate P2, 28.5/µl). We did not detect MPs within the $CD138^+$ population that were solely $CD34^+$ and P-gp^+ (Figure 5.4B, right panel, gate P5 and P6).

The CD138 MP subtypes were gated and phenotyped for the presence PS exposure using annexin V. We detected the presence of annexin V^+ MPs **(**Figure 5.4C, left panel, gate P11, 5/µl) within the $CD138^+$ P-gp^+CD34^- MP population. In contrast, we did not detect annexin V positive events on $CD138^+$ P-gp^+CD34^+ MPs (Figure 5.4C, right panel, gate P12, 0 events) (Table 5.1).

In summary, this patient with an aggressive disease course demonstrated significantly elevated levels of P-gp on MPs of 'stem cell like' origin (i.e. $CD138^-$ P-gp^+ $CD34^+$). A small proportion of this population also was positive for PS.

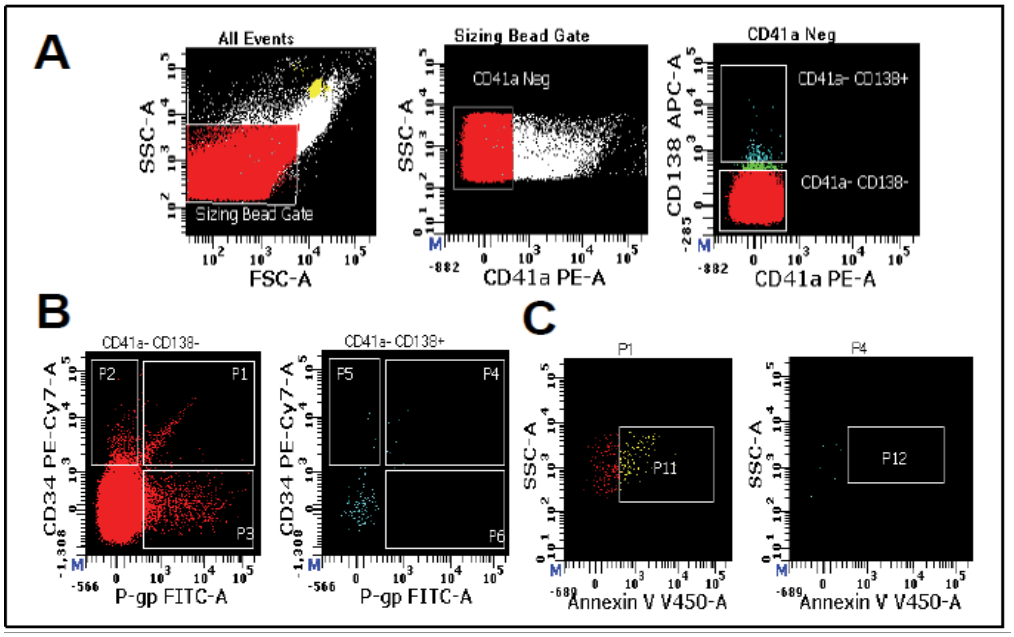

Figure 5.4 Elevated levels of 'dual positive' MPs in patient 1 with aggressive disease. The presence of P-gp and CD34 in $CD138^-$ (red events) and $CD138^+$ (blue events) MP subpopulations was established by flow cytometry in patient 1. **(A)** A sequential gating strategy using MP size gate (left panel) followed by gating for CD41a (middle panel) and CD138 (right panel) was applied to the total MP population (left panel). The $CD41a^-$ population was defined based on +/- staining for anti-$CD41a^-$ PE (middle panel). **(B)** The total population ($CD41a^-$) was gated based on CD138 -/+ staining (left panel, red events, right panel, blue events, respectively). Within this MP population, we phenotyped for $CD138^-$ $P\text{-}gp^+$ $CD34^+$ (left panel, gate P1) population and $CD138^+$ $P\text{-}gp^+$ $CD34^+$ sub-population (right panel, gate P4). **(C)** The CD138 MP subtypes (gate P1 & P4 of left and right panel respectively). The CD138 MP subtypes (gate P1 & P4 of left and right panel respectively) were gated and phenotyped for the presence PS exposure using annexin V (left panel, gate P11, yellow events) (right panel, gate P12, orange events) respectively.

5.4.4 CASE 2: 66-year-old female patient in progressive disease

A 66-year-old female patient (patient 2) was diagnosed with kappa light chain myeloma in 2014. She was enrolled and treated in a clinical trial MLN9708 (cyclophosphamide/dexamethasone) from December 2014 until March 2015, which was stopped in February 2015 due to progressive disease with a rise in kappa light chains. At the time of sampling on 5th May 2015, she was on CyBorD therapy for her progressive disease. During this time we observed $CD34^+$ (40.5/µl) and P-gp^+ MP events (60/µl) within the total ($CD41a^{-}$) MPs (Isotype–matched control; supplementary figure 2). Within this population, we detected the presence of $CD138^-$ P-gp^+ $CD34^+$ population (Figure 5.5A, left panel, gate P1, 4.6/µl) and $CD138^+$ P-gp^+ $CD34^+$ population **(**Figure 5.5A, right panel, gate P4, 0.5/µl). We also detected a sub-set of $CD138^-$ P-gp^+ $CD34^-$ MPs (Figure 5.5A, left panel, gate P3, 58.8/µl) and $CD138^+$ P-gp^- $CD34^+$ (Figure 5.5A, right panel**,** gate P6, 3/µl).

The CD138 MP subtypes were gated and phenotyped for the presence PS exposure using annexin V. We detected a minimal presence of $CD138^-$ P-gp^+CD34^+ annexin V^+ MPs **(**Figure 5.5B, left panel, gate P11, 1.1/µl) in this patient at this given point in time. $CD138^+$P-gp^+CD34^+ annexin V^+ MP levels were also minimal (Figure 5.5B, right panel, gate P12, 0.4/µl) (Table 5.1).

In summary, compared to patient 1, this patient with progressive disease demonstrated lower levels of the 'dual positive' population and showed only minimal positivity with PS.

5.4.5 CASE 3: 63-year-old male patient in stable condition

A 63-year-old male in a stable disease state (patient 3) at the time of sampling was diagnosed with IgG kappa multiple myeloma 2011 (smoldering myeloma 2008, active myeloma July 2011). The induction therapy consisted of 6 cycles of cyclophosphamide, thalidomide and dexamethasone followed by autologous stem cell transplant on 30th March 2012. The patient experienced severe peripheral neuropathy associated with thalidomide and an increase in serum paraprotein, which resulted in a treatment change to lenalidomide, and dexamethasone July 2012. At the time of sampling in May 2015, the patient was on lenalidomide and dexamethasone, zometa and aspirin.

The patient presented with $CD34^+$ (5.13/µl) and $P\text{-}gp^+$ (6.3/µl) MPs in total MPs ($CD41a^-$) at the time of sampling (Isotype–matched control; supplementary figure 2).Within this population, we detected the presence of $CD138^-$ $P\text{-}gp^+$ $CD34^+$ population (Figure 5.5C, left panel, gate P1, 4.7/µl) and $CD138^+P\text{-}gp^+CD34^+$ (Figure 5.5C, right panel, gate P4, 0.2/µl). We also found a sub-set of $CD138^-P\text{-}gp^+CD34^-$ (Figure 5.5C, left panel, gate P3 23.13/µl) and $CD138^-P\text{-}gp^-CD34^+$ (Figure 5.5C**,** left panel, gate P2, 18.54/µl). We also observed a sub-set of $CD138^+P\text{-}gp^+$ $CD34^+$ (Figure 5.5C, right panel, gate P5, 1.2/µl) $CD138^+$ $P\text{-}gp^+$ $CD34^-$ (Figure 5.5C, right panel, gate P6, 1/µl).

The CD138 MP subtypes were gated and phenotyped for the presence PS exposure using annexin V. We detected the presence of $CD138^-$ $P\text{-}gp^+CD34^+$ annexin V^+ MPs **(**Figure 5.5D, left panel, gate P11, 1.6/µl) in this patient at this given point in time and $CD138^+$ $P\text{-}gp^+$ $CD34^+$ annexin V^+ MP (Figure 5.5D, right panel, gate P12, 0.3/µl events) (Table 5.1).

In summary, the ‘dual positive’ population was present in comparable levels in patient 3 relative to patient 2. The sub-set was also not significantly enriched with PS.

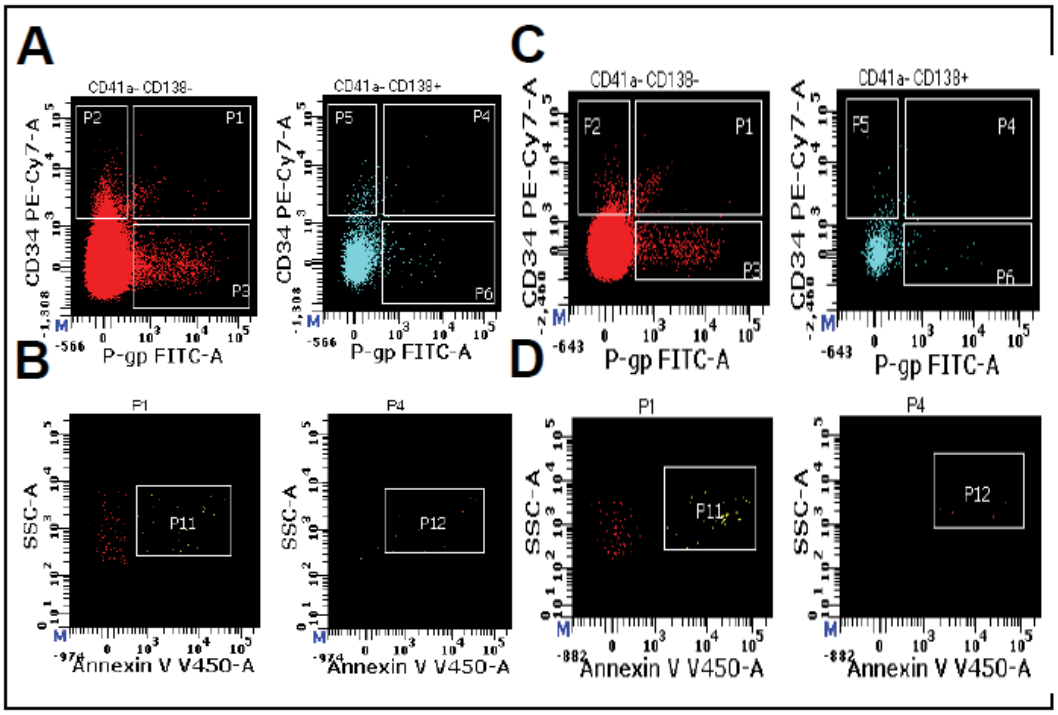

Figure 5.5 66-year-old female patient in progressive disease (patient 2) and 63-year-old male patient (patient 3) in stable condition. The presence of P-gp and CD34 in $CD138^-$ (red events) and $CD138^+$ (blue events) MP subpopulations was established by flow cytometry in patient 2 and 3. **(A)** The total population ($CD41a^-$) was gated based on CD138 -/+ staining (left panel, red events, right panel, blue events, respectively). Within this MP population, we phenotyped for $CD138^-$ $P\text{-}gp^+$ $CD34^+$ (left panel, gate P1) population and $CD138^+$ $P\text{-}gp^+$ $CD34^+$ sub-population (right panel, gate P4). **(B)** The CD138 MP subtypes (gate P1 & P4 of left and right panel respectively) were gated and phenotyped for the presence PS exposure using annexin V (left panel, gate P11, yellow events) (right panel, gate P12, orange events) respectively. **(C)** & **(D)** Likewise, profile of patient 3.

5.4.6 CASE 4: 71-year-old male patient in partial remission

A 71-year-old male (patient 4) was diagnosed on February 2014 following a biopsy of a right shoulder mass. He presented with widely disseminated skeletal disease with multiple lesions as evidenced by positron emission tomography scan. Induction therapy consisted of CyBorD treatment from April 2014. The patient achieved very good partial remission after 6 cycles and treatment was stopped at 6 cycles instead of 8 due to severe peripheral neuropathy resulting from bortezomib. The sample analyzed was taken on 12th August 2014. We observed numbers of CD34^{+} (15.13/µl) and P-gp^{+} MP events (10/µl) within the total (CD41a^{-}) MP population (Isotype–matched control; supplementary figure 3). Within this population we detected the presence of CD138^{-} P-gp^{+} CD34^{+} population (Figure 5.6A, left panel, gate P1, 7.2/µl) and CD138^{+} P-gp^{+} CD34^{+} (Figure 5.6A, right panel, gate P4, 0.5/µl). We also found a sub-set of CD138^{-} P-gp^{+} CD34^{-} (Figure 5.6A, right panel, gate P3, 36.53/µl) and CD138^{-} P-gp^{-} CD34^{+} (Figure 5.6A**,** left panel, gate P2, 63.17/µl) in this sample. We also observed a very small sub-set of CD138^{+} P-gp^{-}CD34^{+} (Figure 5.6A, right panel, gate P5, 4/µl) and CD138^{+} P-gp^{+}CD34^{-} (Figure 5.6A, right panel, gate P6, 2.2/µl).

The CD138 MP subtypes were gated and phenotyped for the presence PS exposure using annexin V. We detected the presence of CD138^{-} P-gp^{+}CD34^{+} annexin V^{+} MPs **(**Figure 5.6B, left panel, gate P11, 2.5/µl) and CD138^{+} P-gp^{+}CD34^{+} annexin V^{+} MP was negative (Figure 5.6B, right panel, gate P12, 0 events) (Table 5.1).

In summary, patient 4 demonstrated elevated albeit lower levels of the 'dual positive' population compared to that of the patient 1. The sub-set was enriched with PS however levels were lower than that detected for patient 1.

5.4.7 CASE 5: 62-year-old male patient in remission – long-term survivor

A 62-year-old male (patient 5) was diagnosed at 50 years of age with IgG kappa MM with bone marrow biopsy showing 10-15% plasma cell infiltration. His induction regimen consisted of VAD - vincristine, adriamycin (doxorubicin) and dexamethasone. This was followed by an autologous stem cell transplant in 2007, after which he remained in an unmaintained complete remission for almost three years. He experienced a relapse in 2012 with rise in serum paraprotein albeit he had no other issues. He was given thalidomide and achieved very good partial response in early 2013 with bone marrow biopsy showing only 3% plasma cell infiltration and M-protein too low to quantitate. His M protein started to increase in late 2014 and reached 17g/l in October 2014. The patient was subsequently enrolled and treated on a clinical trial (lenalidomide/dexamethasone plus or minus daratumumab) in December 2014. At the time of sampling the patient was responding very well and he eventually achieved stringent complete remission with ongoing chemotherapy. This patient is a long-term survivor (12years) with successful therapeutic interventions over a long period.

At the time of sampling on 5th May 2015, we observed numbers of CD34^{+} (5.13/µl) and P-gp^{+} MP events (6.3/µl) within the total (CD41a^{-}) MP population (Isotype–matched control; supplementary figure 3). We detected the presence of CD138^{-} P-gp^{+} CD34^{+} population (Figure 5.6C, left panel, gate P1, 2.54/µl) and CD138^{+} P-gp^{+}CD34^{+} (Figure

5.6C, right panel gate P4, 3.0/µl). We also found a sub set of $CD138^{-}$ $P\text{-}gp^{+}CD34^{-}$ (Figure 5.6C, left panel, gate P3, 52.83/µl) and $CD138^{+}$ $P\text{-}gp^{-}$ $CD34^{+}$ (Figure 5.6C**,** left panel, gate P2, 14.46/µl). We also observed a very small sub-set of $CD138^{+}$ $P\text{-}gp^{-}CD34^{+}$ (Figure 5.6C, right panel, gate P5, 4.5/µl) and $CD138^{+}$ $P\text{-}gp^{+}CD34^{-}$ (Figure 5.6C, right panel, gate P6, 2.4/µl).

The CD138 MP subtypes were gated and phenotyped for the presence PS exposure using Annexin V. We detected the presence of $CD138^{-}$ $P\text{-}gp^{+}CD34^{+}$ annexin V^{+} MPs (Figure 5.6D, left panel, gate P11, 0.5/µl) and $CD138^{+}$ $P\text{-}gp^{+}CD34^{+}$ annexin V^{+} MP (Figure 5.6D, right panel, gate P12, 1.17/ µl) (Table 5.1).

In summary, this patient demonstrated significantly reduced levels of the 'dual positive' population compared to all other disease states. We also observed barely detectable PS exposure on this population of MPs. The followup of patient 1 in remission is given in supplementary figure 4.

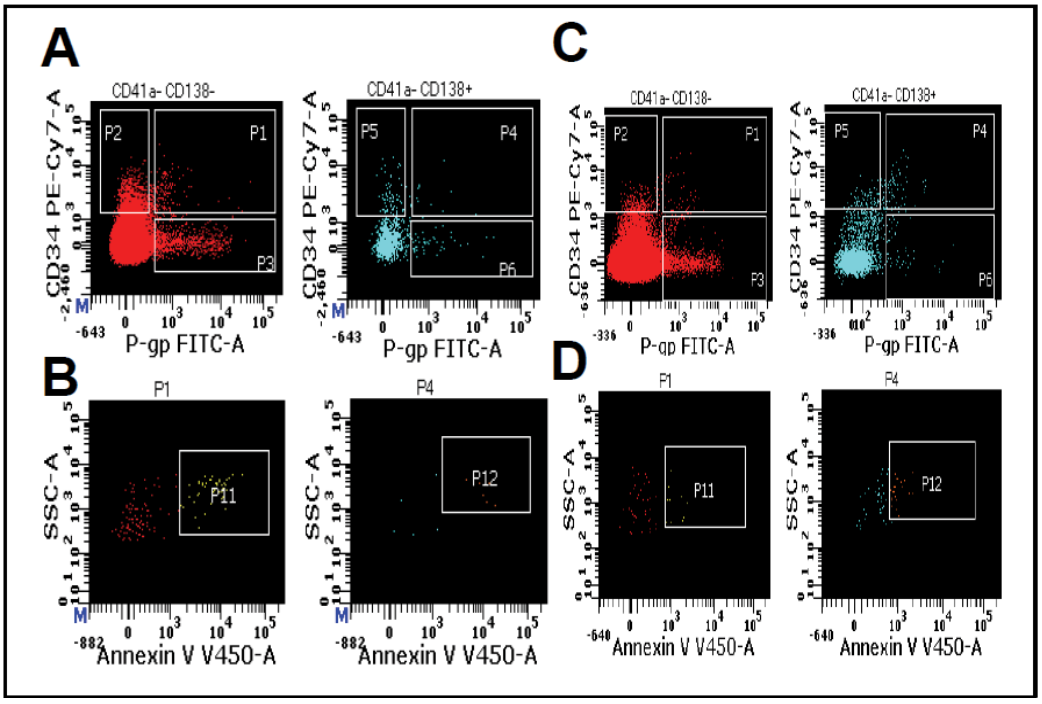

Figure 5.6 71-year-old male patient on partial remission (patient 4) and 62-year-old male patient in remission – long-term survivor (patient 5). The presence of P-gp and CD34 in $CD138^-$ (red events) and $CD138^+$ (blue events) MP subpopulations was established by flow cytometry in patient 4 and 5. **(A)** The total population ($CD41a^-$) was gated based on CD138 -/+ staining (left panel, red events, right panel, blue events, respectively). Within this MP population, we phenotyped for $CD138^-$ $P\text{-}gp^+$ $CD34^+$ (left panel, gate P1) population and $CD138^+$ $P\text{-}gp^+$ $CD34^+$ sub-population (right panel, gate P4). **(B)** The CD138 MP subtypes (gate P1 & P4 of left and right panel respectively) were gated and phenotyped for the presence PS exposure using annexin V (left panel, gate P11, yellow events) (right panel, gate P12, orange events) respectively.

Overall, we observed elevated levels of the $CD138^{-}$ $P\text{-}gp^{+}$ $CD34^{-}$ MP sub-set in MM patients in the following descending order, PD > aggressive disease > remission > PR > stable. In addition, we also observed a sub-set of $CD138^{-}$ $P\text{-}gp^{-}$ $CD34^{+}$ MPs in the following descending order PD > PR > aggressive > stable > remission in these patients (Table 5.1).

The origin of $CD138^{-}$ $P\text{-}gp^{+}$ $CD34^{-}$ and $CD138^{-}$ $P\text{-}gp^{-}$ $CD34^{+}$ MP subtypes are difficult to gauge and is currently unknown, given the possibility of CD138 shedding in malignant plasma cells and the dynamic nature of antigen expression during malignancy (29, 34). However, $CD138^{-}$ $P\text{-}gp^{+}$ $CD34^{-}$ subtype may originate from putative MM stem cells or cell types other than platelets such as endothelial cells or red blood cells. $CD138^{-}$ $P\text{-}gp^{-}$ $CD34^{+}$ sub-set appear to be 'stem cell like' although they are $P\text{-}gp^{-.}$ These cells may have, however, other ABC transporters i.e. BCRP which has also been shown to be present on cancer stem cells (30) Alternatively, we have previously shown that P-gp exists in an inside out orientation in addition to its right side out orientation and consequently may underestimate the dual positive MP population (35).

5.4.8 $Annexin^{+}$ MP represents a more aggressive state in MM

Annexin V^{+} MP subpopulations within the total ($CD41a^{-}$) MP population were significantly (5.6 fold) increased in MM patients relative to the healthy volunteers *(U=607, p=0.009)* (Figure 5.7A). Annexin V^{+} MP counts were 3.1 fold elevated in the *de novo* cohort relative to healthy volunteers *(U=80, p=004)* while the partial remission

cohort showed a 3.08 fold increase in annexin V^+MP counts *(U=249, p=0.02)* to that of healthy volunteers. The annexin V^+ MP counts were 13.9 fold higher in the progressive disease cohort relative to healthy volunteers *(U=136, p=0.02)*. We did not observe any significant difference in annexin V^+MP counts between the CR cohort and healthy volunteers (Figure 5.7B).

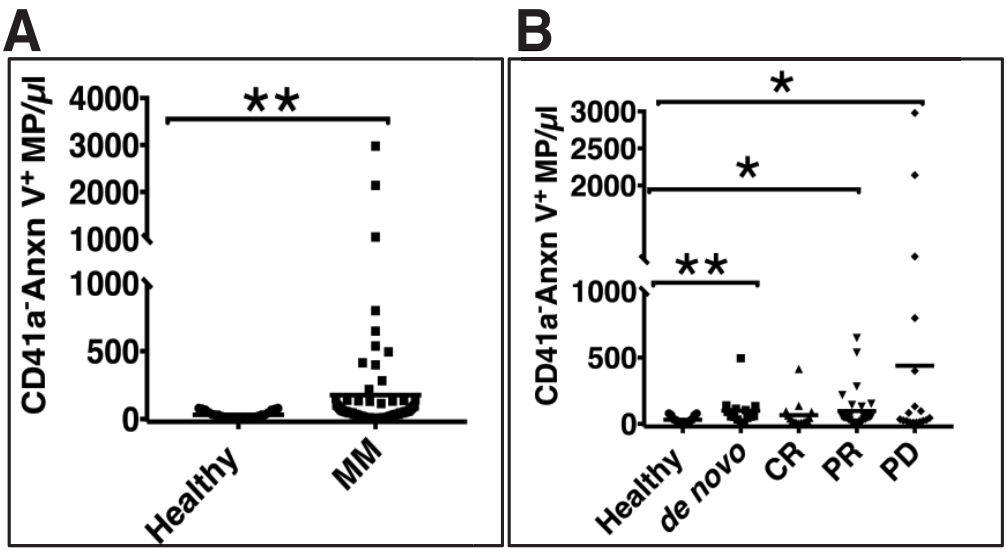

Figure 5.7 Annexin^{+} MP represents a more aggressive state in MM. The annexin V^{+} MP counts in MM patients and healthy subjects were compared using Trucount^{TM} beads. **(A)** Annexin V^{+} MP counts were significantly greater in MM patients *(n=74)* relative to healthy volunteers *(n=25) p<0.01* (****). **(B)** Annexin V^{+} MP counts were greater in *de novo* (*n=14*), partial remission (*n=31),* and progressive disease (PD, *n=18*) relative to healthy volunteers (*n=25*). No significant difference in annexin V^{+} MP counts was observed between the CR *(n=15)* and healthy volunteers. P values were generated using *Mann–Whitney U* test and the data is represented as mean (*P<0.01* (****), *P<0.05* (***)).

5.4.9 Annexin V^+ and CD138 do not co-express in progressive disease.

Figure 5.8A shows the $CD138^+$ annexin V^+ MP profile of MM patients with respect to healthy volunteers. We observed a significant 2.4 fold greater PS exposure in $CD138^+$ MP sub-set for MM patients compared to healthy volunteers *(U=452.5, p=0.006)*. In the cohort data, the *de novo* and PR cohorts showed a 3.1 *(U=82.5, p=0.005)* and 3.61 *(U=96.5, p=002)* fold increase, respectively in PS exposure to that of healthy volunteers whilst we observed no significant difference with CR or PD cohorts relative to healthy volunteers (Figure 5.8B).

Figure 5.8C shows the $CD138^-$ annexin V^+ MP profile of MM patients. We observed a significant 4.3 fold increase in PS exposure in MM patients compared to healthy volunteers in the $CD138^-$ sub-set of MPs *(U=570, P=0.009)*. In the cohort data, the *de novo* patients had a 7.73 fold higher PS exposure *(U=77, p=0.003)* on $CD138^-$ MPs compared to healthy volunteers while patients with PD had a 6.9 fold higher PS exposure *(U=94, p=0.003)* in the $CD138^-$ sub-set (Figure 5.8D) relative to healthy volunteers. We did not observe any significant difference in PS exposure for the CR and PR cohorts with respect to healthy volunteers (Figure 5.8D).

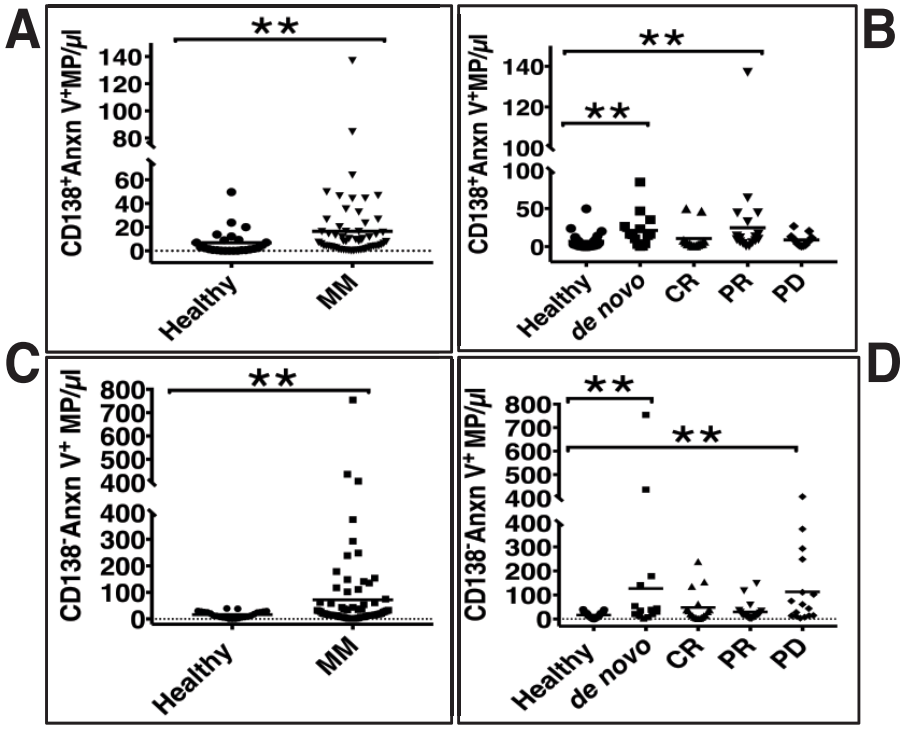

Figure 5.8 Annexin V$^+$ and CD138 do not co-express in progressive disease. (A) PS$^+$MPs in the CD138$^+$ MP sub-set in MM patients were significantly elevated in MM patients compared to the healthy volunteers *(p<0.01, (**))*. **(B)** CD138$^+$PS$^+$MP levels in the *de novo* and PR cohort were significantly higher relative to healthy volunteers *(p<0.01, (**))* while CR and PD had insignificant levels relative to healthy volunteers. **(C)** PS$^+$MPs in the CD138$^-$ MP sub-set were significantly elevated in MM patients relative to that for healthy volunteers. **(D)** CD138$^-$PS$^+$MPs were significantly higher in *de novo* and PD cohorts relative to healthy volunteers. There was no significant difference in CR and PR cohorts relative to healthy volunteers. *Mann–Whitney U* test was conducted to generate P values and the data is represented as mean (*P<0.01 (**)*).

Table 5.1: MP phenotype in MM patients with respect to the clinical response state

Patient	Age (yrs)	Gender	Response state	Total MP count ($CD41a^-$)		$CD41a^-$							
						$CD138^-$				$CD138^+$			
				$P\text{-}gp^+$ (µl)	$CD34^+$(µl)	$P\text{-}gp^+CD34^+$(µl)	$P\text{-}gp\text{-}CD34^+$(µl)	$P\text{-}gp^+CD34^-$(µl)	$P\text{-}gp^+CD34^+anxn\ V^+$(µl)	$P\text{-}gp^+CD34^+$(µl)	$P\text{-}gp^-CD34^+$(µl)	$P\text{-}gp^+CD34^-$(µl)	$P\text{-}gp^+CD34^+anxn\ V^+$(µl)
Patient 1	58	F	PD (aggressive)	155.29	496.81	12.5	28.5	56.4	5	0.3	0.3	0	0
Patient 2	66	F	PD	60	40.5	4.78	74	60	1.1	0.5	5	3	0.4
Patient 3	63	M	Stable	6.3	5.13	4.7	18.5	23	1.6	0.2	1.2	1	0.3
Patient 4	71	M	PR	10	15.13	7.2	63	36.5	2.5	0.5	4	2.2	0
Patient 5 (long-term survivor)	62	M	Remission	6.3	5.13	2.5	14.4	53	0.5	3.0	4.5	2.4	1.17

5.5 Discussion

Our previous recent study showed that $CD138^+$ MPs were elevated in MM patients across all response states compared to healthy volunteers and correspond to plasma cell burden and therapeutic response in individual patients (26). We also reported higher albeit insignificant $CD138^+$ MP counts in the progressive disease cohort compared to the remission cohort (26). Here, we expand on these initial observations and demonstrate for the first time the presence of MP subtypes in the context of P-gp expression-specifically the presence of $CD138^+$P-gp^+CD34^- and $CD138^-$P-gp^+CD34^+ MP subpopulations. We show that MM patients have higher P-gp^+ events in the total $CD41a^-$ and $CD138^-$ MP population compared to healthy volunteers, specifically in the *de novo* and PD cohorts. P-gp^+ events within the total MP population as well as within each MP subtype were shown to correspond to treatment response when levels were monitored in individual patients.

CD138 is the most useful surrogate marker for plasma cells and it is the most appropriate marker when a single marker is used (36). In this study, however, we observed that P-gp^+ MP events in MM patients were predominantly within the $CD138^-$ population. We also report on the predominance of a 'dual positive' ($CD138^-CD34^+$P-gp^+) population of '*stem cell-like'* origin, the levels of which appear to be defined by the patient's clinical status. In assessing the phenotype of the total MP population in MM patients, we also detected for the first time greater numbers of phosphatidylserine positive (PS^+) MPs relative to healthy volunteers. Specifically this was observed in the *de novo*, PR and PD cohorts in the total $CD41a^-$ MPs.

Our earlier work discovered that MPs shed from MDR cancer cells carry functional P-

gp from the cell of origin within the cargo (21, 22, 37). P-gp expression is typically induced in malignant cells following drug exposure. However it can also be inherently expressed in some cancers. This is consistent with our findings whereby we detected 35% of the *de novo* patient population to have significantly elevated P-gp$^+$ events within the total CD41a$^-$ MP population. In attempting to correlate with disease status, we did not observe any significant difference in P-gp$^+$ events within the CD41a$^-$ MP population amongst the responsive cohorts (i.e. PR and CR) relative to healthy volunteers. However, we observed significantly greater P-gp$^+$ MP events in patients with progressive disease. Within the total CD41a$^-$ MP population we identified a number of different subtypes based on the presence of CD138 and P-gp.

When we examined CD138$^+$ MPs in the context of P-gp expression we did not observe any significant difference in MM patients relative to healthy volunteers. Rather, we observed a significant increase in P-gp$^+$ MPs in the CD138$^-$ MP sub-set in MM patients relative to healthy volunteers, and its presence coinciding with disease progression. The predominance of the P-gp$^+$ CD138$^-$ phenotype may be the consequence CD138 shedding which has been previously established as occurring in aggressive disease (high serum CD138 is associated with a negative prognosis in MM)(26). Another possibility which has been previously unexplored in the context of extracellular vesicles, is the potential emergence of the 'side population' comprising the putative myeloma stem cells during the course of disease. These stem cells are typically CD138$^-$ and express high levels of functional MDR proteins such as P-gp (38-40). Lower expression of CD138 on plasma cells is indicative of an immature phenotype, poor prognosis in a number of studies and they were also found to be less sensitive to lenalidomide treatment (29, 30, 41).

Considerable inter-individual variation exists in the clinical setting and is impacted by a multitude of factors including tumor specific variables (such as histology, DNA ploidy, tumor volume, genotype), host specific variables (such as age, genotype, gender, pharmacokinetics, co-morbidities) and treatment specific variables (including treatment regimen, duration of response, minimal residual disease). This variablility contributes to the vast inter-individual differences in survival observed in MM and questions the generalized approach to disease state management. The adoption of N=1 principles in this scenario could potentially provide a paradigm shift in clinical management of MM (42, 43). We have tried to incorporate the elements of N=1concept which are gaining prominence in oncology in our case studies presented here.

We included an additional arm to this study whereby we phenotyped and quantitated MP sub-sets for the presence of CD138, P-gp and CD34 in a group of patients across five different clinical response stages. We detected a significant predominance of the 'dual positive' population (P-gp^{+} CD34^{+} CD138^{-} MPs) indicating a 'stem cell like' origin in aggressive > PR ~ PD > stable > remission patients. In contrast, the order of CD138^{+}CD34^{+}P-gp^{+} MPs were reversed i.e. remission > PD = PR > stable > aggressive. Patient 1 (aggressive) and patient 4 (PR) had a higher plasma cell labelling index (PCLI, 5.5% and 7.02% respectively) at the time of MP sampling corresponding to the predominance of 'dual positive' population. This is in contrast to levels observed in patient 1 when in partial remission in May 2015 had a PCLI of 0.5% and the 'dual positive' population at this time was 1.73/µl. PCLI data for patient 2, 3 and 5 were not available around the time of MP sampling. This data supports that MP release reflects not only tumor bulk but also disease activity.

Patient 2 (PD) and patient 3 (stable) had almost identical 'dual positive population'. Patient 3 was in a stable condition while patient 2 was already showing response after one cycle of bortezomib (as defined by a drop in light chain levels) at the time of sampling and correspondingly had less 'dual population' relative to patient's 1 and 4. Patient 5 had a barely detectable 'dual positive' MP population corresponding to the remission and long-term survivor status. This data clearly demonstrates an association between elevated levels of the 'dual positive' MP population and treatment unresponsiveness as well disease activity. These results are very promising and certainly demonstrate a consistent trend between the MP status and disease state on the *proviso* that only one patient from each disease state was examined. This study demonstrates support for N=1 approach to therapeutic monitoring in patients and also forms the essential basis for a larger scale population study in future.

PS is a ubiquitous marker of MPs arising from loss of phospholipid asymmetry during MP biogenesis (44). However, it is also known that PS is not an exclusive marker with expression being variable within the MP population (45, 46). PS is emerging as an important mediator in extracellular vesicle biology. A recent study showed that PS on hypoxia induced mesenchymal stem cell derived microvesicles was crucial in the internalisation of vesicles into human umbilical cord endothelial cells (HUVECs) (33). This may suggest a role in supporting angiogenesis. We observed significantly elevated numbers of PS^+ MPs in the total $CD41a^-$ MP population in MM patients relative to healthy volunteers. Specifically, we observed significantly elevated levels in *de novo*, PR and PD, but not in CR evidencing elevated PS^+ MP counts associated with 'active disease' states. We also observed significantly higher PS^+ events in the $CD138^-$ MP sub-set in MM patients (specifically, in *de novo* and PD). Unlike P-gp, there was significant,

PS exposure in the $CD138^+$ MP sub-set for the MM cohort relative to healthy volunteers as well and specifically for the *de novo* and PR cohort in the cohort data. Interestingly, the PD cohort however had lower PS exposure in the $CD138^+$ MP sub-set relative to that of healthy volunteers consistent with the dynamic nature of the CD138 antigen.When phenotyping the MPs from the 5 patients examined PS^+ events 'dual positive' MP popultion were greatest in aggressive > PR > stable > PD > remission patients. The significance of the increased PS^+ counts in myeloma is currently unknown and may be linked to the dissemination of malignant cells to extramedullary sites during disease progression (33).

This study taken with our earlier work identifies two distinct MP subtypes of clinical relevance. The $CD138^+$ MPs provide a marker of plasma cell burden particularly prior to the development of aggressive disease state (26). In addition, the presence of a 'dual positive' MP population ($CD138^-CD34^+P\text{-}gp^+$) of 'stem cell like' origin appears to provide a marker of disease progression and treatment responsiveness within individual patients, specifically in aggressive disease. It appears that $CD138^+$ cannot be considered a 'static' biomarker of MM disease evolution. Whereas it plays an important role as a measure of tumor burden in responsive disease, its presence on the cell surface diminishes in an aggressive disease state. This has important implications in how we define the utility of biomarkers generally. Rather biomarkers need to be considered at each stage of disease.

MPs provide a surrogate marker of cells of origin, which in the case of myeloma are predominantly confined to the bone marrow. Our findings have a number of important clinical implications. We provide evidence that MDR in patients with MM can be

detected and monitored serially by analysing MPs in blood samples in the context of a 'liquid biopsy'. We also demonstrate the presence of MDR markers across a number of distinct cell types including cells of plasma cell origin and 'stem cell like origin'. The latter appears to provide a reservoir of P-gp positive cells, the levels of which correspond to disease progression. This has important implications in the design of effective treatment strategies, included targeted approaches whereby distinct cell clones with discrete phenotypes and shifting dominance present at various times and must be considered during the design of efficacious treatment interventions.

We present a systemic biomarker with personalized prognostic capacity for determining the evolution of MDR provides a relevant addition to the current repertoire of prognostic clinical tools. The ability to continuously monitor patients for this phenotype during the course of treatment allows for optimal patient management as alternative therapies can be initiated promptly to prevent disease progression. This would be particularly useful in cases of non-secretory myeloma, which lack the classic manifestation of elevated M-protein levels and are consequently difficult to monitor. These findings introduce new insights into the molecular mechanisms contributing to disease progression, MDR and treatment failure in MM and identify key biomarkers, which can be explored in future clinical studies.

Author contributions:

SRK performed all the experiments, data analysis, literature review and prepared the manuscript. MB designed and supervised the research, provided conceptual advice and revised the manuscript. RB provided conceptual advice and assisted in manuscript revision. DJ, YLK provided the patient samples and clinical data assisted in revising manuscript. HS helped in sample logistics and accessed de-identified clinical dataAll authors reviewed the results and approved the final version of the manuscript.

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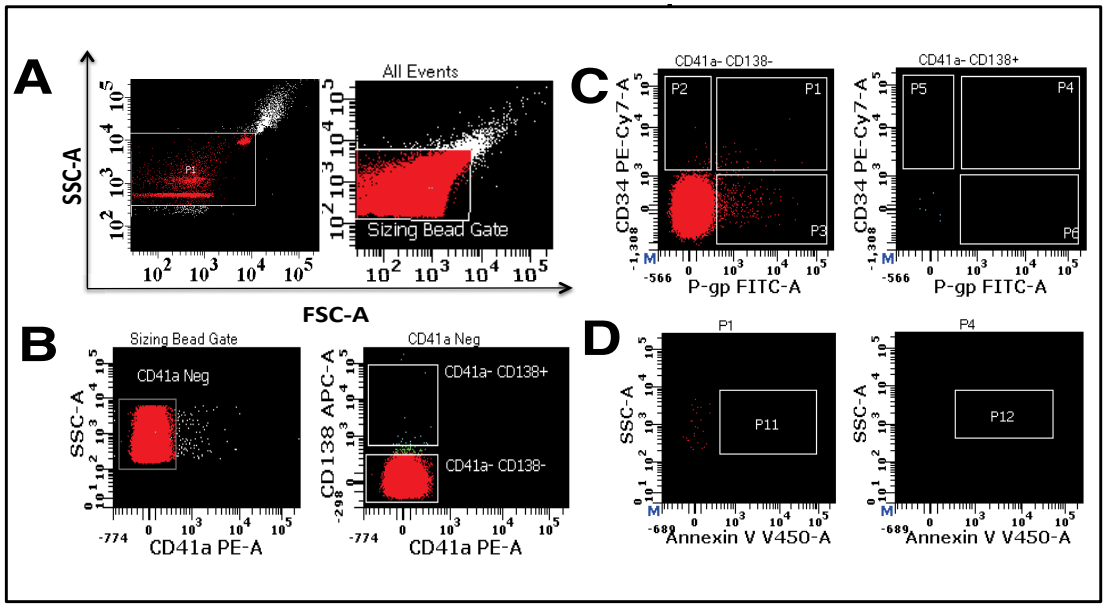
Supplementary figures for chapter 5

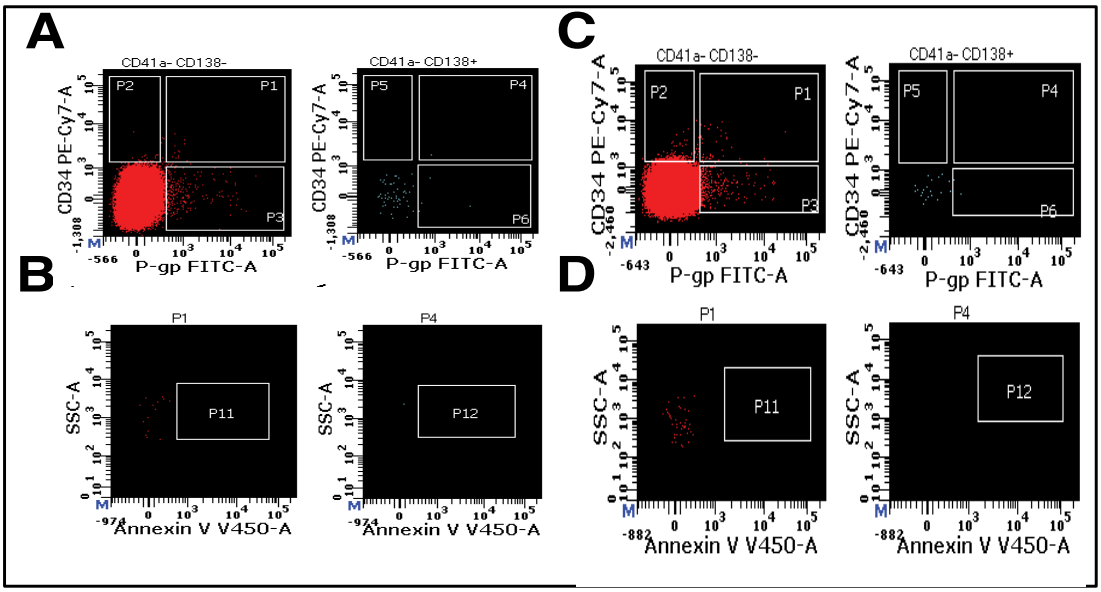
Legend to supplementary figures

Supp.fig. 5.1 Gating strategy to define parameters for +/- staining in patient 1 (aggressive disease)



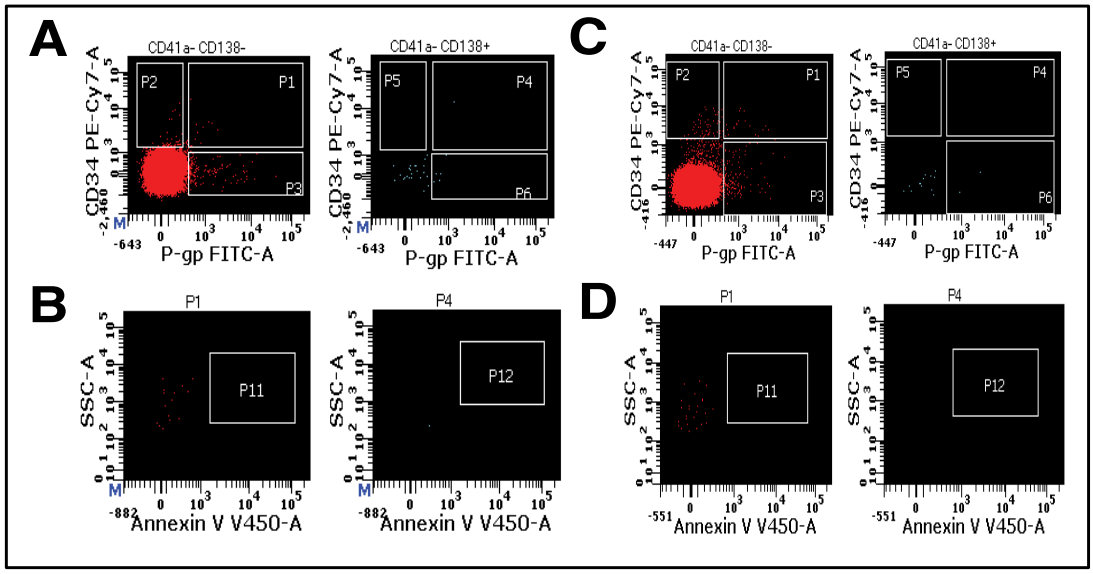
(A) Latex beads of known diameter (0.3 -1µm) were resuspended in PBS and analysed using the LSR Fortessa X20. MP size gate were defined as P1 (1A, left panel). The MP size gate was applied to the patient MP population (1A, right panel) **(B)** A sequential gating strategy using MP size gate (1B, left panel) followed by gating for CD41a and CD138 (1B, right panel) was applied to the patient MP population (1B, left panel). **(C) CD138+/- population was gated based on anti CD138^{-}APC +/-staining for patient 1**. **(C)** Isotype-matched control was used to define gating parameters for positive and negative staining for CD138^{-} P-gp^{+} CD34^{+} (1C, left panel) and CD138^{+} P-gp^{+} CD34^{+} (1C, right panel) for patient 1**(D)** Gates were defined for CD138^{-}CD34^{+}annexin V $^{+}$ (1D, left panel) and CD138^{+}CD34^{+} annexin V^{+} (1D, right panel) for patient 1.

Supp.fig. 5.2 Isotype-matched control to define parameters for +/- staining in patient 2 (PD) and 3 (stable)



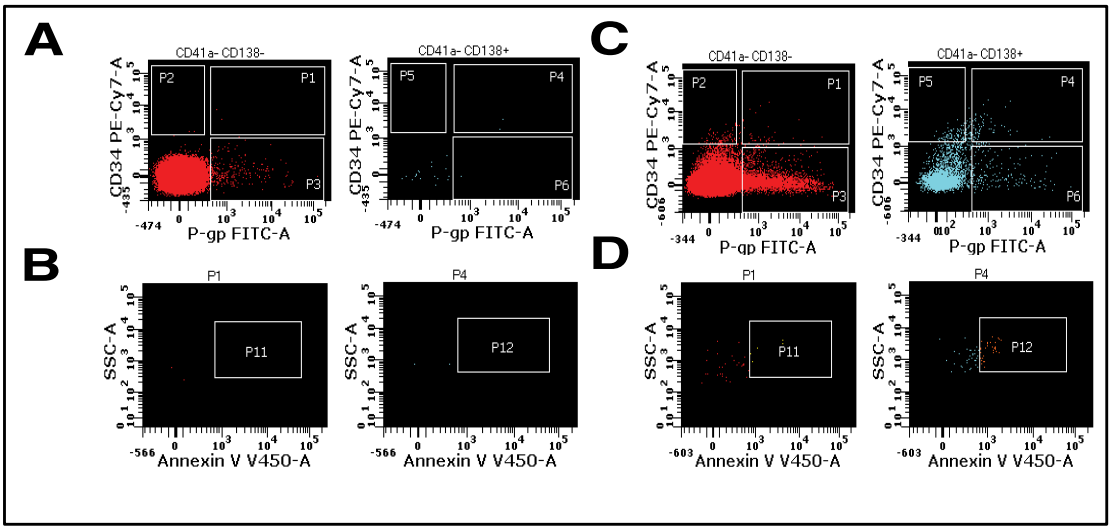
(A) CD138+/- population was gated based on anti-CD138$^-$APC +/-staining for patient 2 and 3. Isotype-matched control was used to define gating parameters for positive and negative staining for CD138$^-$ P-gp$^+$ CD34$^+$ (2A, left panel) and CD138$^+$ P-gp$^+$ CD34$^+$ (2A, right panel) for patient 2 **(B)** Gates were defined for CD138$^-$CD34$^+$ annexin V$^+$ (2B, left panel) and CD138$^+$CD34$^+$ annexin V$^+$ (2B, right panel) for patient 2 **(C)** Isotype-matched control was used to define gating parameters for positive and negative staining for CD138$^-$ P-gp$^+$ CD34$^+$ (2C, left panel) and CD138$^+$ P-gp$^+$ CD34$^+$ (2C, right panel) for patient 3 **(D)** Gates were defined for CD138$^-$CD34$^+$ annexin V$^+$ (2D, left panel) and CD138$^+$CD34$^+$ annexin V$^+$ (2D, right panel) for patient 3.

Supp.fig. 5.3 Isotype-matched control to define parameters for +/- staining in patient 4 (PR) and 5 (remission)



(A) CD138+/- population was gated based on anti CD138$^-$APC +/-staining for patient 4 and 5. Isotype-matched control was used to define gating parameters for positive and negative staining for CD138$^-$ P-gp$^+$ CD34$^+$ (3A, left panel) and CD138$^+$ P-gp$^+$ CD34$^+$ (3A, right panel) for patient 4 **(B)** Gates were defined for CD138$^-$CD34$^+$ annexin V$^+$ (3B, left panel) and CD138$^+$CD34$^+$ annexin V$^+$ (3B, right panel) for patient 4 **(C)** Isotype-matched control was used to define gating parameters for positive and negative staining for CD138$^-$ P-gp$^+$ CD34$^+$ (3C, left panel) and CD138$^+$ P-gp$^+$ CD34$^+$ (3C, right panel) for patient 5 **(D)** Gates were defined for CD138$^-$CD34$^+$ annexin V$^+$ (3D, left panel) and CD138$^+$CD34$^+$ annexin V$^+$ (3D, right panel) for patient 5.

Supp.fig. 5.4 Isotype-matched control to define parameters for +/- staining patient 1 in partial remission and 'dual positive' population in May 2015



(A) CD138+/- population was gated based on anti CD138^{-}APC +/- staining for patient 1 in PR. Isotype-matched control was used to define gating parameters for positive and negative staining for CD138^{-} P-gp^{+} CD34^{+} (4A, left panel) and CD138^{+} P-gp^{+} CD34^{+} (4A, right panel) for patient 1 in PR **(B)** Gates were defined for CD138^{-} CD34^{+} annexin V^{+} (4B, left panel) and CD138^{+}CD34^{+} annexin V^{+} (4B, right panel) for patient 1 in PR. **(C) MPs were phenotyped patient 1 in partial remission status for the presence of 'dual positive' population based on CD138 -/+ staining**. We assessed the presence of P-gp, CD34 in CD138^{-} (red events) and CD138^{+} (blue events) MP sub-sets by flow cytometry (4C, left and right panel respectively). **(D)** MPs were also phenotyped for the levels of PS enrichment using annexin V in the in CD138^{-} (4D, left panel, yellow events) and CD138^{+} subpopulation of MPs (4D, right panel, orange events).

Chapter 6

6. A Novel approach for Individualized Risk-Stratification in multiple myeloma

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Multiple myeloma (MM) is a plasma cell neoplasm, which results in cumulative physiological inefficiency for patients. The median age of diagnosis is over 60 yrs and is usually preceded by monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma, both of which do not require therapeutic interventions (1). MM is the second most common hematological malignancy after Non-Hodgkin's lymphoma and is mainly restricted to the bone marrow, which results in end-organ damage. The disease is characterized by clinical manifestations including impaired immunity, renal insufficiency, defective bone physiology and moderate to severe nerve damage (2-4). The 5-year survival rate in MM is 43% and the median survival is 7 years (5)

In Australia, approximately, 1500 people are diagnosed with myeloma every year (5, 6). MM is associated with cycles of remission and relapse. Therapeutic strategies typically involve combination chemotherapy +/- autologous stem cell transplant (ASCT). The decision of inclusion of ASCT in the regime is dependent on the age of diagnosis and the tolerance as well to the response achieved to induction therapy (7). A significant cause of treatment failure and relapse in MM is the evolution of drug resistance to multiple agents used in treatment (8). MM is one of the most costly cancers to manage as a result of the multitude of physical manifestations associated and the range of chemotherapeutic agents employed during the course of therapy. There are also costs associated with the management of adverse effects as typical agents used in MM are teratogenic (thalidomide). Overall, the management of cancer including that of MM has a huge impact on the Australian healthcare sector, with myeloma and Lymphoma accounting for $106 million in cancer healthcare costs (5, 9).

MM can be secretory or non-secretory based on the presence or absence of monoclonal proteins. In classical secretory MM, aberrant plasma cells produce clonally incompetent immunoglobulins, M proteins or paraproteins (10). M proteins accumulate in the periphery and are routinely used as a disease diagnostic whereas non-secretory disease lacks this classic manifestation, and as a result can complicate diagnosis. Additionally, the median age of onset, the presence of diverse clinical manifestations, the vast heterogeneity in survival rate, clonal evolution as well as the presence of intrinsic and acquired drug resistance also impact on the therapeutic management of both types of myeloma. MM patients at diagnosis typically have multiple clones, each with differing degrees of drug sensitivity dispersed throughout the axial skeleton (11). The presence of these multi-clone tumors contributes to a significant variability in patient survival, ranging from a few weeks to more than 10 years (12, 13).

The emergence of MDR during the course of therapy contributes significantly to treatment failure in MM. The introduction of the immunomodulatory (iMIDs) agents and proteasome inhibitors has seen an increase in overall patient survival. However, these novel drugs add significant cost to costs associated with the clinical management of MM. For the majority of patients, relapse remains inevitable as these agents, like the conventional chemotherapeutics are also subject to the development of MDR. Clinical management of patients with MM is currently compromised by lack of a suitable test to monitor the development of clinical drug resistance in individual patients. The current MM prognostic measures are not designed to focus on MDR per se or the presence of MDR proteins.

The high heterogeneity in survival amongst patients complicates disease management and underlines the need and significance for risk stratification at an individualized level. Current approaches to achieving this are based on tumor genotyping, which is of significance at diagnosis and has paved the way for the adaptation of risk-adapted therapy in MM. The Mayo stratification of myeloma and risk–adapted therapy (mSMART) working group has established guidelines on the outcome of novel therapies based on the genotype based risk grouping (14, 15). (15). Risk–adapted therapy in MM is an evolving shift towards personalized management in MM.

It is also known that an individual's immune profile of MM patient have a role in the disparity in survival amongst MM patients (16, 17). The T helper 17-T regulatory cell ratio has been shown to have a significant role in overall survival of MM patients in such a way that an imbalance in T_{h17} to T_{reg} ratio is most prominent in MM relative to MGUS or any other plasma cell dyscrasia. An increase in the ratio results in reduced overall survival in MM as it is immunosuppressive (17) Further supporting this, long-term survivors (<10 yrs) are reported to have normal T_{h17} to T_{reg} ratio (18). This also support towards a personalized approach in MM management.

The absence of a test, which allows for risk-stratification based on the presence or emergence of MDR in MM patients, is a limiting factor to optimized therapeutic management of disease. This is because MM is managed through combination therapy that is subject to MDR. MDR is caused by the overexpression of ABC transporters on the plasma membrane of resistant cancer cells. These transporters efflux a wide range of structurally and functionally unrelated drugs from the plasma membrane of cancer cell types, including malignant plasma cells. This mechanism maintains a sub-lethal drug

concentration in the cell, which in turn results in tumor cell survival, resistance and disease relapse. The emergence of MDR contributes significantly to the wide variability in myeloma survival. Other factors contributing to the heterogeneity include; tumor specific variables (such as histology, DNA ploidy, tumor volume, genotype), host specific variables (such as age, genotype, gender, pharmacokinetics, co-morbidities) and treatment specific variables (including treatment regimen, duration of response, minimal residual disease).

We have previously described the role of cancer cell derived extracellular vesicles (i.e. microparticles) in the transfer and dissemination of deleterious traits such as MDR and increased metastatic capacity in cancer. Microparticles (MPs) are cell derived extracellular vesicles ubiquitously shed from all cells in the body and play a role in normal cellular processes as well as in disease pathology. MPs are considered to provide systemic 'surrogate' markers for cells localised within inaccessible compartments such as the bone marrow. Many techniques such as high-resolution microscopy, capture based assays are used in MP research. However, flow cytometric phenotyping is the 'gold standard' technique used in MP research, as it offers a direct assessment of cellular origin, count and phenotype.

In 2009, Bebawy *et al*., discovered a 'non-genetic pathway' for the acquisition of multidrug resistance through the transfer of extracellular vesicles. This seminal paper showed that when drug sensitive acute lymphoblastic leukemic cells (CCRF-CEM) were co cultured with MPs isolated from the drug resistant variant (VLB100) cell line this results in the acquisition of functional P-gp and consequently MDR within the span

of 4 hours (19). This phenomenon was later shown to occur in other cell types and through the transfer of other resistance proteins including MRP1 (20).

Following this discovery, Bebawy's team also showed that MPs shed from malignant cells ensure the transfer of deleterious cancer cell traits to recipient cells and facilitate trait dominance within the recipient cell population (20-22). MPs isolated from MDR cells were capable of "re-templating' the transcriptional landscape of drug responsive recipient cells to ensure the transfer and acquisition of MDR within cancer cell populations (20-22). The team discovered a rapid transcriptional response in recipient cells, mediated through the selective packaging and intercellular transfer of unique RNA species (ABCB1 and ABCC1 transcripts as well as miRNA's) in MPs from the donor cancer cells (23). Recently, the same team also discovered that MPs shed from MDR cells are also capable of conferring alterations in the biomechanical properties in recipient cells (24) (25).

Gong *et al.,* also demonstrated that MPs passively and actively sequester chemotherapeutic drugs and reduce the free available drug concentration available to cancer cells (26). This mechanism effectively confers a parallel and alternative pathway of drug resistance (26). Gong *et al* also demonstrated that the MP cargo can also include the transfer of regulatory intermediates in cancer (22). This study showed that proline-rich tyrosine kinase 2 (PYK2) was up regulated in the recipient cells resulting in an increased invasive and migratory capacity in recipient cells, although the presence of PYK2 was absent in the cargo transferred. Bebawy and colleagues have also recently shown that MPs shed from MDR cancer cells are implicated in immune evasion by cancer cells (27). It is thus well established that extracellular vesicles, specifically

microparticles (MPs) have the potential to serve as biomarkers of risk of relapse given the presence of functional resistance proteins and transcripts contained within the cargo.

Other research validating the use of extracellular vesicles as a novel biomarker in MM come from the vast evidence suggesting their role in myeloma specifically. Benameur et al., 2013, showed using the 5T2MM mouse model of myeloma, elevated levels of MPs in late stage disease relative to early stage of disease (28). Likewise, platelet derived MPs and their role in thromboembolic risk has also been demonstrated in MM. MPs are shown to have role in tumor survival by blocking apoptosis, invasion and migration and neovascularization, PS support the hypercoagulable state and the active interaction of platelets and tissue factor bearing MPs have been shown in MM (29, 30). MPs derived from the human myeloma cell line RPMI8226 have been shown to promote neovascularization through the transfer of oncogenic CD138 to endothelial cells both in vitro and in vivo (31). EVs furthermore aid in MM survival via extracellular matrix degradation and immune surveillance evasion. Wang et al demonstrated that bone marrow mesenchymal stem cell derived exosomes have a role in aiding survival inducing resistance to bortezomib in the 5T33 murine MM model through BMSC-derived exosomes (32). Harshman *et al* reported a significant overlap in proteomic content between the profile of myeloma cell lines MM 1S and U266 cell lysates to their respective extracellular vesicle population (33). This study provided further evidence for the selective packaging of the cargo in these vesicles. Specifically, it showed that EVs from MM.1S vesicles show increased abundance of HLA class II histocompatibility antigens when compared to the cell lysate. Thus EVs from two cell lines although shared a common protein profile to a significant extent however contained small sets of unique proteins with statistically distinct abundance. The

extended research summarized here clearly indicates the potential of MPs as an efficient vector in facilitating tumor progression and survival in MM.

This study aims to expand on this vast body of research on the deleterious role of MPs through translational application of these findings. Current systemic markers of MM only give an indirect measure of tumor burden and do not gauge the presence of resistance proteins. There are no existing clinical tests available to assess inherent or acquired 'resistance' in MM during the course of therapy. Likewise, bone marrow sampling, which is used for confirmation of diagnosis and restaging, fails to capture the patchy, multi-site tumour infiltrates evident with the disease. The current monitoring tools are also limited in the case of non-secretory myeloma. Therapeutic intervention in relapsed MM is dependent on the obvious clinical manifestations of disease recurrence, further compromising the therapeutic management of disease.

Based on the above-mentioned strong background evidences, the understanding of MP signatures in a malignancy can potentially shed some light to the complex survival machinery. As mentioned, MM is mostly restricted to the bone marrow deep tissue making the assessment of tumor microenvironment during the course of therapy very difficult. Therefore, our hypothesis is that a relationship exists between clinical outcome and the levels as well the phenotype of non-platelet derived MPs ($CD41a^-$), such that the levels and phenotype of MPs can be a predictive indicator of clinical state, drug responsiveness and risk of relapse in MM patients.

In testing the hypothesis, our specific aims were (1) to develop a validated workflow for the isolation, detection and phenotyping of $CD138^+$ (plasma-cell-derived) MPs from the

peripheral blood of myeloma patients at diagnosis and during therapy (2) to detect and quantitate the MPs in the peripheral blood of myeloma patients (*de novo* and under active treatment) and statistically associate $CD138^{+}$ MP count with clinical response states (3) (a) to phenotypically characterize non-platelet derived MPs for the presence of P-gp, CD34 and phosphatidylserine expression (b) to demonstrate an association between MP levels and phenotype with clinical response state and disease progression.

The data presented in this thesis addresses the issue that elevated P-gp is correlated with poor prognosis and response in MM (8, 34) and that currently there is no clinical approach to monitor the emergence of MDR in real time, systemically. It is known that, P-gp expression increases by 40-75% in MM subsequent to treatment (35). Specifically, in the light of the emerging reports that the newer drugs lenalidomide and bortezomib *etc* are substrates of P-gp, the aim is to address this significant limitation in the MM clinical setting. Here, we provide "proof of principle" for a novel minimally invasive clinical test that can comprise a "liquid biopsy" approach to disease state management in MM.

Chapter 1 provides a detailed literature review published in International Oncology Journal in 2016, covers the background of our research question and analyzes the problem of multidrug resistance in MM. The complexities of the multifaceted disease are covered in the review, including MDR as a persisting problem in MM despite the introduction of novel drugs, The review discusses the absence of routine monitoring for the emergence of MDR in MM and the role of the ABC transporters in contributing to MDR in MM.

Chapter 2 is further exploring the literature about systemic prognosis and risk grouping strategies in MM and is divided in two parts. The first part of the review describes current limitations in risk-stratification strategy and risk-adapted therapy in MM. The review demonstrates that current approaches are based on the detection of cytogenetic abnormalities and is rather very limited in terms of the presence and / or persistence of drug resistance markers. The second part of chapter 2 focuses on the potential of MPs as a prognostic indicator across many cancers including MM. The discussion covers MP's potential in providing an individualized assessment in MM patients with respect to tumor burden, "risk of relapse", emergence of drug resistance and the identification of MP subtypes of clinical relevance as discovered through the research described in this thesis. The hypothesis and aims are described consequently.

Chapter 3 addresses aim 1 of this study that is to develop a validated workflow for the isolation, detection and phenotyping of $CD138^+$ (plasma-cell-derived) MPs from the peripheral blood of myeloma patients at diagnosis and during therapy. This chapter describes the development of validated workflows and protocols for MP isolation, morphological assessment, quantitation and phenotyping with the required agility to readily be adapted in the clinical diagnostic setting. Flow cytometry is a routinely used technique in current hematological setting, which enables the validated workflow readily integral to the MM clinical setting.

Chapter 4 addresses aim 2 that is to detect and quantitate the MPs in the peripheral blood of myeloma patients (*de novo* and under active treatment) and statistically associate $CD138^+$ MP count with clinical response states. This body of work was published in Neoplasia journal in 2016.

The results in this chapter report on the isolation and detection of non–platelet-derived ($CD41a^-$) $CD138^+$ MPs in the blood of patients with MM. The data demonstrates that the total MP count is significantly higher in MM cohort relative to healthy volunteer cohort and that total MP count is a predictor of the disease state. Total MP count is higher albeit statistically insignificant across all clinical response states (*de novo*, partial remission, complete remission and progressive disease. We detected greater levels of $CD138^+$ MPs in MM patients relative to healthy volunteers and significantly greater levels of $CD138^+$ MP in MM patients during the course of active therapy across the clinical response states. In the context of personalized medicine, $CD138^+$ MP count promises a sensitive assessment of disease progression and therapeutic outcome in individual MM patients (36). Morphologically, MPs isolated from *de novo* and patients in remission are spherical with a smooth surface and shared a similar morphology to the OPM2 derived MPs. The MPs from relapsed patients displayed a corrugated and irregular morphology. The size of these vesicles averaged between 0.7- 0.8 μm. We did not observe any size differences with respect to MPs across the clinical states. The chapter concludes that $CD138^+$ MPs can be detected from the peripheral blood of MM patients and their count together with phenotype can be predictive of disease status and therapeutic response.

Chapter 5 addresses aim 2 and 3 which is to phenotypically characterize non-platelet derived MPs for the presence of P-gp, CD34 and phosphatidylserine expression and to demonstrate an association between MP levels and phenotype with clinical response state and disease progression.

This chapter identifies MP subtypes of clinical relevance and their prognostic significance upon correlation with the clinical data. The results from this chapter demonstrate the presence of MP subtypes in the context of P-gp expression. Specifically, we report the presence of $CD138^{+}P\text{-}gp^{+}CD34^{-}$ and $CD138^{-}P\text{-}gp^{+}CD34^{+}$ MP subpopulations of clinical relevance in MM. We observed that, MM patients have higher $P\text{-}gp^{+}$ events in the total $CD41a^{-}$ and $CD138^{-}$ MP population compared to healthy volunteers, $P\text{-}gp^{+}$ events within the total MP population as well as within each MP subtype were shown to correspond to treatment response when levels were monitored in individual patients. There is also significantly elevated numbers of PS^{+} MPs in the total $CD41a^{-}$ MPs and other subtypes of MPs in MM patients relative to healthy volunteers. Specifically, elevated PS^{+} MP levels were detected for *de novo*, partial remission (PR) and progressive disease (PD), but not in complete remission (CR) evidencing elevated PS^{+} MP counts associated with 'active disease' states. It appears that $CD138^{+}$ cannot be considered a 'static' biomarker of MM disease evolution. CD138 appears to play an important role as a measure of tumor burden in responsive disease; its presence on the cell surface diminishes in an aggressive disease state. The data presented in this chapter has important implications in how we define the utility of biomarkers generally.

In summary, we have validated a method to identify MPs expressing CD138, annexin V, P-gp, CD34 from the PFP of MM patients, This method has been authenticated across important analytical variables, and is feasible as well as reproducible. Moreover, we substantiated protocols for immunolabeling workflow, quantitation, storage and phenotyping for relevant markers of the cell of origin. The significance of the

methodology and clinical utility as a supporting risk stratification tool is summarized in (Figure 6.1).

We would like to add that this is a preliminary study that aims to expand the patient cohort in future studies and thus limited with small number of patient samples. We only had technical replicates of samples, as biological replicates were not feasible at this stage. The time points are more scattered than definite for cross-sectional and longitudinal data. The MP parameters were set using latex beads of known diameter. However, latex beads typically have higher refractive indices relative to biological molecules such as MPs and, consequently, lower limits of size detection by flow cytometry (37, 38).

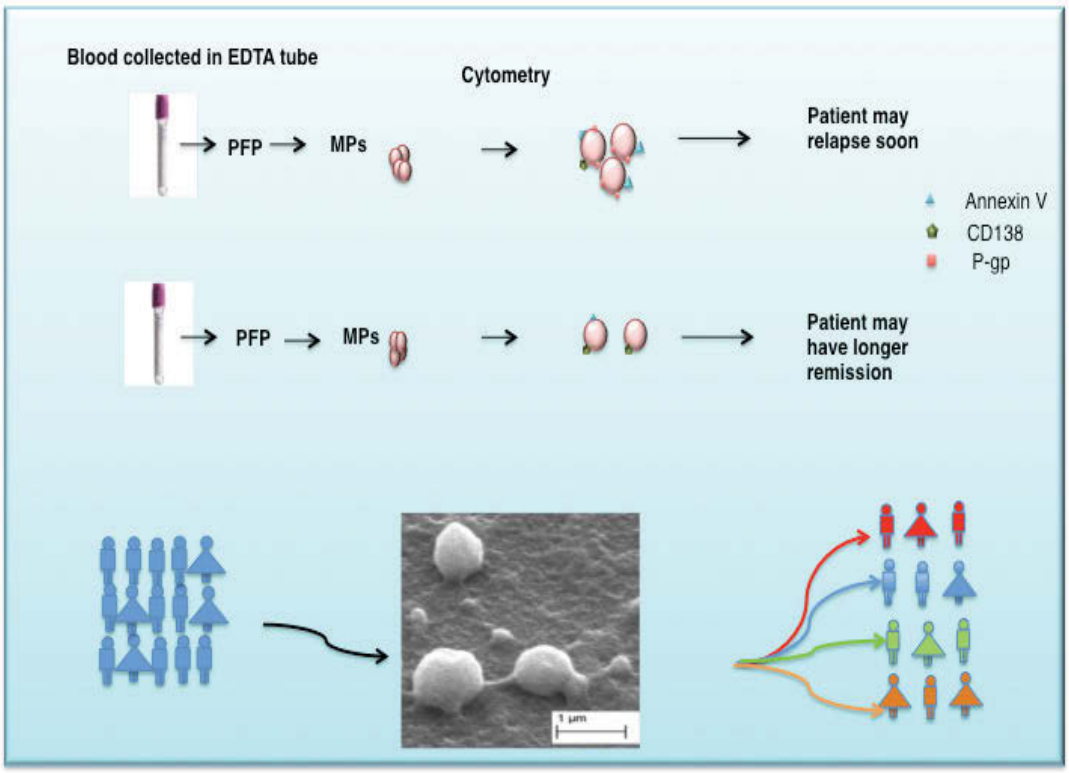

Figure 6.1 Graphical representations of prognosis and risk-stratification in MM based on MP phenotype. The validated workflow can be easily adapted into the hematology clinical setting defining MP subtypes. This information can be used as an additional personalized risk-stratification tool for MM patients based on the deleterious markers (Eg: P-gp and/or PS and/or CD34) expressed on MPs from the PFP of MM patients.

Chapter 6 covers the conclusions and future directions of this research.

Future directions

The significant outcome of this body of research is that the MPs ($CD41a^{-}CD138^{+}$) can be detected and serially monitored from the peripheral blood of MM patients at diagnosis and during treatment. MPs provide a snap shot of the disease, which is mainly restricted in the inaccessible bone marrow compartment. Further, the study identified two distinct MP subtypes of clinical relevance. $CD138^{+}$ MPs provide a marker of plasma cell burden prior to progression to an aggressive disease state. The 'dual positive' MP population ($CD138^{-}CD34^{+}P\text{-}gp^{+}$) of 'stem cell like' origin provides a marker of disease progression, treatment responsiveness and aggressive disease. $CD138^{+}$ is not a 'static' biomarker of MM disease evolution. It plays an important role as a measure of tumor burden in responsive disease; however, its presence on the cell surface appears to be diminishing in aggressive disease. This has important implications in how we define the utility of biomarkers in the clinical setting, especially in the case of targeted therapies. The predominance of $CD138^{-}CD34^{+}P\text{-}gp^{+}$ has to be read in the context of the persistent MM initiating niche or MM stem cells and/or side population, which are gaining prominence as the reason to why MM is incurable (39-44). Bebawy's team also recently reported the role of MPs in tissue biomechanics in cancer (24). There is also evidence that putative myeloma stem cell niche trigger stiffness and aid in drug resistance (45).

The Darwinian clonal evolution model of MM model identifies three distinct temporal types of tumors in MM (46, 47). These distinct types are described as a) genetically

stable b) linearly evolving and c) heterogeneous clonal mixtures with an altered dominance in clones. The results shown in this study, particularly in chapter 5 indicates that MPs provide a very sensitive picture of the "evolving shift" in the dominance of cancer progenitor cells with disease progression as evidenced by the presence of 'stem cell like origin' MPs in aggressive disease and the diminishing presence of CD138 on MP surface in progressive disease. This is corroborated with reduced PS exposure on $CD138^{+}$ subtype of MPs and the lack of co-localization of P-gp and CD138 in aggressive disease states. It is also reported that the discerning pressure of targeted therapy might be causing this altered dominance of sub-clones (11). The results from this study also supportive of this as novel therapies fail in aggressive MM. The comparison between a long term survivor who had considerable therapy free period during the course of disease (12 years of MM) and the aggressive MM (~ 2 years survival since diagnosis despite being younger than 60 yrs of age at diagnosis) showed significant differences in absolute count of MP subtypes. The comparison disclosed a massive 96 fold higher $CD41a^{-}CD34^{+}$, a 25 fold increase in $CD41a^{-}P\text{-}gp^{+}$ population, a 5 fold increase in the 'dual positive' 'stem cell like' population ($CD138^{-}$ $CD34^{+}$ $P\text{-}gp^{+}$) and a 10 fold difference in PS exposure in aggressive MM patient in comparison with the long-term survivor at the time of sampling. This clearly indicates that there is a pathway as reflected by MPs, contributing to the complexity of MM heterogeneity. This is also instructive towards an individualized, risk-adapted therapy that would be ideal to minimize the selective pressure arising from targeted therapy, which might be triggering the altered dominance of clones.

The validated workflow has a substantial promise for its incorporation into a routine, individualized assessment and treatment re-evaluation in MM clinical setting. This

research provides proof that the presence and evolution of drug resistance proteins can be detected and monitored systemically during the course of treatment. This may prove predictive of relapse and facilitate therapeutic intervention prior to onset of clinical manifestations and deterioration of the condition. MDR evolves during treatment and invasive procedures for directly assessing MDR are harrowing and expensive. This workflow provides a simple individualized and non-invasive blood test (a liquid biopsy) for the continuous monitoring of MDR in myeloma patients. This has potential to improve chemotherapeutic success, patient survival and improve quality of life.

MDR is a significant impediment to treatment across most cancers; similar approaches are applicable and translatable across other malignancies in future studies. The identification of biomarkers correlating to MDR and evolution of disease will also provide insight into the molecular mediators regulating this phenotype at cellular and clinical levels. It will also identify new protein and nucleic acid therapeutic targets for the circumvention of MDR clinically. This study is a necessary pre-requisite for larger scale clinical testing of the utility of the profiles we identify as a prognostic biomarker of MDR, in MM. Their development addresses a need associated with all cancers, but particularly MM, to identify as early as possible whether a treatment is working or not primarily to prevent unnecessary cytotoxic exposure for patients. This assay has the potential to support clinical decision-making in the context of personalized/precision/stratified medicine and consequently help in reducing healthcare costs with respect to a more rational use of drugs and lesser hospital admissions.

Author contributions

SRK performed all the experiments, data analysis, literature review and prepared the manuscript. MB designed and supervised the research, provided conceptual advice and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Appendices

Executed Non-disclosure assignments

UTS
UNIVERSITY OF TECHNOLOGY SYDNEY

STAFF IP ASSIGNMENT CONFIRMATION

THIS AGREEMENT is effective from the 20th day of September 2016

BETWEEN: Mary Bebawy ("Employee")

AND: THE UNIVERSITY OF TECHNOLOGY SYDNEY (ABN 77 257 686 961)

of PO Box 123, Broadway, Ultimo, NSW 2007 ("UTS")

BACKGROUND:

A. The Employee is the inventor of an invention titled "*Microparticle Cancer Diagnostic*", UTS disclosure reference number DISC-2016-029.
B. The Employee is employed by UTS.
C. The invention was made by the Employee in the course of his/her employment at UTS and the rights, title, and interest in the invention vests immediately in UTS by condition of their employment.
D. UTS wishes to obtain protection for the invention by applying for patents in relation to the invention ("the patent application").

THIS AGREEMENT PROVIDES:

In consideration of UTS undertaking the commercialisation of the invention in accordance with the Research-Generated Intellectual Property and Commercialisation Vice-Chancellor's Directive and the sharing of any commercialisation benefits with the Employee in accordance with UTS Intellectual Property policy:

1. The Employee confirms the assignment to UTS his/her entire right, title and interest in and to:

   (a) the invention;
   (b) the Patent Application, including the right to claim Convention priority;
   (c) any patent applications derived from or related to the invention; and,
   (d) any patent granted pursuant to the invention in Australia and all other countries of the world.

2. The Employee agrees that they will at the expense of UTS do all acts and execute all documents necessary or desirable for further assuring the title of UTS to the invention and for obtaining and securing patent and corresponding protection in Australia and all other countries of the world.

Signed Production Note: Signature removed prior to publication.)
Production Note: Signature removed prior to publication.

in the presence of:

.....

Witness Nadia Pece-Barbara

Signed for and on behalf of UTS)
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Witness

David Robson, Director

Research and Innovation Office

UTS
UNIVERSITY OF TECHNOLOGY SYDNEY

Assignment of Student Intellectual Property Agreement

University of Technology Sydney
ABN 77 257 686 961
Of 15-73 Broadway Ultimo NSW 2007
("UTS")

The Student Identified in Item 1 of the Schedule
("You")

Background:

A. You, as a student at UTS has participated and may continue to participate in the Project identified in **Item 2** of the **Schedule** in the course of Your studies which has resulted in access to the Confidential Information and the development of the Intellectual Property.

B. As such, You may be a part owner of the Intellectual Property.

C. UTS wishes to obtain protection for the invention as described in the Project identified in **Item 2** of the **Schedule** by applying for patents in relation to the invention ("the patent application").

D. UTS seeks an assignment of Your part of the Intellectual Property to it.

E. You, after seeking independent legal advice, agree to enter into this Agreement to assign your interest in the Intellectual Property to UTS.

Agreement:

Definitions

In this Agreement:

"**Confidential Information**" means all information in any form which:

(a) is provided by one of us for the purpose of the Project; and

(b) by its nature appears confidential or which either of us identifies as confidential

and includes all copies, notes or records of that information and the Intellectual Property but excludes information of the kind described in clause 3.2.

"**Intellectual Property**" means such of the following as relates to the Project: all rights resulting from intellectual activity whether capable of being protected by statute, common law or in equity and includes, but is not limited to all inventions, discoveries, innovations, technical information, prototypes, processes, improvements, patents, circuit layouts, computer programs, drawings, plans, specifications, copyright, trademarks, designs (whether registerable or not) and plant variety rights.

"**Publication**" means:

(a) a PhD thesis or other thesis written by You that encompasses any Intellectual Property;

(b) any paper or publication written by You to be published or presented that encompasses any Intellectual Property; and

(c) any document written by You that encompasses any Intellectual Property including any laboratory notebooks.

"**Studies**" means the studies that are to be undertaken by You within the confines of the Project.

"**Student**" means You.

1. Assignment of Intellectual Property

1.1 You hereby assign to UTS all of Your right, title and interest in and to:

(a) the Intellectual Property related to the invention as described in the Project identified in **Item 2** of the **Schedule** and immediately on its creation;
(b) the Patent Application, including the right to claim Convention priority;
(c) any patent applications derived from or related to the invention as described in the Project identified in **Item 2** of the **Schedule**; and,
(d) any patent granted pursuant to the invention as described in the Project identified in **Item 2** of the **Schedule** in Australia and all other countries of the world.

1.2 You retain the copyright subsisting in all Publications.

1.3 You agree to be bound by any policy of UTS relating to the Intellectual Property.

1.4 At the cost of UTS, you agree to execute all such documents and perform such acts UTS considers reasonably necessary in order to perfect the rights and powers created or intended to be created by this Agreement or to give full force and effect to, or facilitate the transactions provided for in this Agreement.

1.5 You grant to UTS a royalty free perpetual non-exclusive license throughout the world to reproduce, adapt and amend all Publications for the purpose of including any Publication, or any part of a Publication, in any patent application in relation to the Intellectual Property.

2. Consideration

2.1 In consideration of the assignment in clause 2:

(a) UTS will provide You with supervision, leave and other arrangements in accordance with UTS policies;

(b) UTS will give You access to equipment, facilities and a supervisor(s) to the extent necessary for You to properly carry out Your Studies including access to the Project; and

(c) If You are a creator or inventor, UTS agrees that You will share in net commercialisation revenues of the Intellectual Property resulting from the Project, if any, with all other joint creator/inventors in accordance with UTS policies.

2.2 You acknowledge that the Intellectual Property may have commercial potential for UTS and that in order for UTS to capitalize on that commercial potential, it requires ownership of the Intellectual Property.

2.3 You agree to carry out Your Studies under the supervision and reasonable direction of the Supervisor.

2.4 You agree to use your best endeavours to comply with any UTS lab notebook protocols.

3. Confidentiality

3.1 You must treat all Confidential Information as confidential and You must not disclose Confidential Information to any third person without UTS's written permission.

3.2 Your obligation to maintain confidentiality does not apply to information you can prove was:

(a) in the public domain otherwise than as a result of an unauthorised disclosure by one of us; or

(b) provided by a third party who has a legal right to possess and disclose the information to the recipient; or

(c) already known by You before disclosure; or

(d) developed independently of the Confidential Information.

4. Publication

4.1 Any disclosure by You of any Confidential Information or Intellectual Property must first be submitted for review and consent by UTS. Such consent will not be unreasonably withheld.

4.2 Reasonable grounds for withholding consent may include, without limitation:

(a) If the publication or other disclosure would be prejudicial to a patent application or the commercial interests of UTS; or

(b) If the publication or disclosure would be in breach of any third party restrictions imposed on UTS provided that UTS will offer You the opportunity at the time of Your request to delete, amend or revise that part of the proposed publication containing the Confidential Information and or Intellectual Property.

4.3 UTS may arrange for examiners of any thesis to enter into confidentiality agreements and You agree to provide UTS a copy of the final draft of any thesis at least 30 days prior to submission to examiners.

5. Your Warranties

You warrant to UTS that:

(a) Except as disclosed in writing by you to UTS, and except for this Agreement, you have not entered into any deed, contract, arrangement or understanding dealing in any way with the Intellectual Property or the Confidential Information.
(b) To the best of Your knowledge, the Intellectual Property does not infringe any rights of any person.

6. Acknowledgement of Legal Advice

You hereby acknowledge that UTS has, prior to the execution of this Agreement, recommended that You seek independent legal advice on the terms and conditions of this Agreement.

7. Entire Agreement

This Agreement records our entire agreement and supersedes all earlier agreements and representations and communications that may have taken place between us.

8. Variation

This Agreement may only be altered or varied in writing signed by each of the parties.

9. Execution of Counterparts

This Agreement is properly executed when:

(a) each party has executed this document; or

(b) if the parties execute separate but identical documents, when those separately executed documents are exchanged between the parties, including by mail, facsimile transmission or electronically.

10. Governing Law

This Agreement is governed by the laws of the State of New South Wales and the parties submit to the non-exclusive jurisdiction of the courts of that State.

Execution and Date

Executed as an Agreement.

Date: The date of this Agreement is the last date on which the Agreement has been signed by both parties.

Signed for and on behalf of **University of Technology Sydney**
by its duly authorised officer in the presence of:

Production Note:
Signature removed prior to publication.

Signature of witness

Nadia Pece-Barbara

Name of witness (please print)

Production Note:
Signature removed prior to publication.

Signature of authorised officer

David Robson
Director, Research and Innovation Office

8/11/16

Date

Signed by **the Student identified in Item 1 of the Schedule** in the presence of:

Production Note:
Signature removed prior to publication.

Signature of witness

Nadia Pece-Barbara

Name of witness (please print)

Production Note:
Signature removed prior to publication.

Signature of Student

SABNA RAJEEV KRISHNAN

Name of Student (please print)

01-11-2016

Date

Schedule

Assignment of Student Intellectual Property Agreement

Item 1 – Student Details (Parties) Name: Address: Student Number:	 Enter Name SABNA RAJEEV KRISHNAN Enter Address [redacted] Enter Student Number [redacted]
Item 2 – Project Details (Parties) Project Title: *"Microparticle Cancer Diagnostic"* Invention – UTS disclosure reference number DISC-2016-029 Supervisor: Mary Bebawy Faculty: Graduate School of Health, Pharmacy.	

Conference Proceedings Publications

Clinical Cancer Research
clincancerres.aacrjournals.org

doi: 10.1158/1557-3265.PMS14-B52
Clin Cancer Res February 15, 2015 21; B52

Clinical Cancer Research
clincancerres.aacrjournals.org

doi: 10.1158/1557-3265.PMS14-B52
Clin Cancer Res February 15, 2015 21; B52

Abstract B52: A novel personalized therapeutic management in multiple myeloma

Rajeev S. Krishnan[1], **Frederick Luk**[1], **R. D. Brown**[2], **Y. L. Kwan**[3], **and Mary Bebawy**[1]

[+] Author Affiliations

[-] Author Affiliations

Abstracts: AACR Precision Medicine Series: Drug Sensitivity and Resistance: Improving Cancer Therapy; June 18-21, 2014; Orlando, FL

Abstract

Introduction: Multiple Myeloma (MM) is an incurable hematological malignancy affecting plasma cells marked by highly heterogeneous survival rate. Relapse is a significant impediment to the successful treatment of MM clinically. One of the main causes for relapse is drug resistance to cancer chemotherapy. Currently risk stratification to MM sub -groups and categorization of complete response to therapy are assessed based on molecular, cytogenetic markers using bone marrow biopsy as available systemic markers are incompetent in this regard. We are exploring the clinical significance of our recent in vitro and in vivo findings of a novel non-genetic basis to MDR whereby tiny vesicles called microparticles (MPs) shed from cancer cell's surface transfer MDR phenotype intercellularly. MP isolated from the blood of patients who suffer from Multiple Myeloma will be phenotyped for resistance, adhesion and dissemination markers and assessed whether these characteristics are predictive of treatment outcome.

Materials and Methods: We have analysed 44 de-identified Multiple Myeloma patients. The platelet free plasma was ultracentrifuged, MM- derived microparticles were identified and quantified with flow cytometry using Annexin V450, CD138 APC, P-glycoprotein -FITC in BD TruCount tubes. Also platelet derived MPs were identified and excluded using CD41a PE and compared to age-matched healthy volunteers. Western blot analysis was conducted on microparticle lysate probing for the presence of Lung-resistance related protein (LRP).

Results: Plasma cell derived MPs were identified based on the CD138 expression from the peripheral blood plasma of MM patients. The number of systemic microparticles was found to be significantly higher in MM patients compared to the healthy volunteers. Also the systemic microparticles carried the drug resistance markers.

Conclusions: There are elevated numbers of microparticles in MM that potentially correlate with tumour aggressiveness and they carried MDR phenotypes systemically. Phenotyping MM –derived microparticles holds the potential for a non-invasive personalised systemic biomarker to predict therapeutic response.

Citation Format: Rajeev S. Krishnan, Frederick Luk, R. D. Brown, Y. L. Kwan, Mary Bebawy. A novel personalized therapeutic management in multiple myeloma. [abstract]. In: Proceedings of the AACR Precision Medicine Series: Drug Sensitivity and Resistance: Improving Cancer Therapy; Jun 18-21, 2014; Orlando, FL. Philadelphia (PA): AACR; Clin Cancer Res 2015;21(4 Suppl): Abstract nr B52.

Clinical Cancer Research
clincancerres.aacrjournals.org

doi: 10.1158/1557-3265.HEMMAL14-B45
Clin Cancer Res September 1, 2015 21; B45

Clinical Cancer Research
clincancerres.aacrjournals.org

doi: 10.1158/1557-3265.HEMMAL14-B45
Clin Cancer Res September 1, 2015 21; B45

Abstract B45: Multiple myeloma: A novel tailor-made therapeutic management.

Sabna Rajeev Krishnan[1], Mary Bebawy[1], Frederick Luk[1], Ross Duncan Brown[2], and Yiu Lam Kwan[3]

+ Author Affiliations

+ Author Affiliations

Abstracts: AACR Special Conference on Hematologic Malignancies: Translating Discoveries to Novel Therapies; September 20-23, 2014; Philadelphia, PA

Abstract

Introduction: Multiple Myeloma (MM) is an incurable hematological malignancy affecting plasma cells marked by highly heterogeneous survival rate. Relapse is a significant impediment to the successful treatment of MM clinically. One of the main causes for relapse in MM is the development of multidrug resistance (MDR) to cancer chemotherapy. Currently, risk stratification to MM sub-groups and categorization of complete response to therapy are assessed based on molecular, cytogenetic markers using bone marrow biopsy as available systemic markers are incompetent in this regard. We are exploring the clinical significance of our recent in vitro and in vivo findings of a novel non-genetic basis to MDR whereby tiny vesicles called microparticles (MPs) shed from cancer cell's surface transfer MDR phenotype intercellularly. Microparticles isolated from the peripheral blood of patients who suffer from Multiple Myeloma will be phenotyped for resistance, adhesion and dissemination markers. Subsequently, these parameters will be correlated clinically to assess whether these characteristics are predictive of treatment outcome.

Materials and Methods: We have analysed 46 de-identified Multiple Myeloma patients and 18 normal subjects. The platelet free plasma was ultracentrifuged, plasma cell derived microparticles were identified and quantified with flow cytometry using Annexin V450, CD 138 APC, P-glycoprotein (P-gp)-FITC in BD TruCount tubes. Also, platelet derived MPs were identified and excluded using CD41a PE and compared to age-matched normal volunteers. Western blot analysis was conducted on microparticle lysate probing for the presence of Lung-Resistance related Protein (LRP). The morphology and size of the MP fraction from MM patients were compared across distinct clinical state of de novo, remission, relapsed through scanning electron microscopy as well.

Results: Plasma cell derived MPs were identified based on the CD138 expression systemically. The number of systemic microparticles was found to be significantly higher in MM patients compared to the healthy volunteers. Multidrug resistance markers (LRP & P-gp) were expressed on MPs. The morphology of MP fraction from relapsed patient was distinct from the remission and de novo patients whereas no difference in the size was observed across the cohort.

Conclusions: There are elevated numbers of MPs in the 46 MM subjects (for all stages) compared to the 18 normal subjects, supporting elevated CD138+MP numbers in MM. Overexpression of Lung –Resistance related Protein and P-glycoprotein on CD138+MPs are of prognostic significance in MM. Thus, phenotyping plasma cell derived MPs in MM patients provides a personalized systemic biomarker to predict therapeutic response because the microparticles may be the potential 'biosignatures' of the less accessible bone marrow compartment. This would possibly unravel the role of microparticle population in disseminating deleterious messages systemically aiding in the Multiple Myeloma clone survival.

Note: This abstract was not presented at the conference.

Citation Format: Sabna Rajeev Krishnan, Mary Bebawy, Frederick Luk, Ross Duncan Brown, Yiu Lam Kwan. Multiple myeloma: A novel tailor-made therapeutic management. [abstract]. In: Proceedings of the AACR Special Conference on Hematologic Malignancies: Translating Discoveries to Novel Therapies; Sep 20-23, 2014; Philadelphia, PA. Philadelphia (PA): AACR; Clin Cancer Res 2015;21(17 Suppl):Abstract nr B45.

Cancer Research
cancerres.aacrjournals.org

doi: 10.1158/1538-7445.AM2015-5306
Cancer Res August 1, 2015 75; 5306

Cancer Research
cancerres.aacrjournals.org

doi: 10.1158/1538-7445.AM2015-5306
Cancer Res August 1, 2015 75; 5306

Abstract 5306: Microparticles as novel prognostic markers in multiple myeloma

Sabna Rajeev Krishnan[1], **Mary Bebawy**[1], **Ross Duncan Brown**[2], **Frederick Luk**[1], and **Yiulam Kwan**[3]

[+] Author Affiliations

[-] Author Affiliations

Proceedings: AACR 106th Annual Meeting 2015; April 18-22, 2015; Philadelphia, PA

Abstract

Introduction: Multiple Myeloma (MM) is an incurable hematological malignancy affecting plasma cells marked by highly heterogeneous survival rates and confinement of the disease to bone marrow (BM). Relapse is a significant impediment in the clinical setting and the development of multidrug resistance (MDR) to therapy is the main cause of relapse. Currently, risk stratification to MM sub-groups and categorization of complete response to therapy are established on molecular and cytogenetic markers using bone marrow biopsies. We are exploring the clinical significance of plasma cell derived microparticles as a novel prognostic indicator in MM. Materials and Methods: We have analysed 79 de-identified MM patients and 24 normal subjects. Platelet free plasma was centrifuged and plasma cell derived MPs were identified and quantified by flow cytometry using Annexin V450, CD138 APC, anti-P-glycoprotein (P-gp)-FITC (17F9) in BD TruCount tubes. Platelet derived MPs were excluded from the analysis using CD41a-PE. All patient samples were compared to age-matched healthy volunteers. Western blot analysis was conducted on MP lysates probing for the presence of Lung-Resistance related Protein (LRP) and P-glycoprotein (P-gp). Morphology and the size of MP fraction from MM patients were investigated using scanning electron micrographs Results: The number of systemic MPs and $CD138^{+}$MPs were found to be significantly higher in MM patient samples compared to the healthy volunteers. MDR markers (LRP & P-gp) were expressed on systemic MPs from relapsing MM patients. MPs from patients were spherical in shape and had smooth surface consistent with those isolated from the MM cell line OPM2.

Conclusions: There are elevated numbers of systemic MPs in all the 79 MM subjects (across all disease stages) compared to the healthy volunteers. The expressions of CD138 on MPs in the MM patients offer a sensitive assessment of disease progression and therapeutic outcome. Systemic MPs from MM patients carry a 'snapshot' of the less accessible bone marrow compartment and may provide a novel systemic 'biosignature' of MM progression and therapeutic outcome in the clinical setting. The MDR markers on systemic MPs may support dosage regimen and therapeutic decisions in MM clinical setting.

Acknowledgements: This project has ethical approval from Sydney Local Health District Human Research Ethics Committee (CRGH)- EC00118 # HREC/11/CRGH/223 (CH62/6/2011/150).

We would like to thank all the volunteers whom have contributed to this study.

Citation Format: Sabna Rajeev Krishnan, Mary Bebawy, Ross Duncan Brown, Frederick Luk, Yiulam Kwan. Microparticles as novel prognostic markers in multiple myeloma. [abstract]. In: Proceedings of the 106th Annual Meeting of the American Association for Cancer Research; 2015 Apr 18-22; Philadelphia, PA. Philadelphia (PA): AACR; Cancer Res 2015;75(15 Suppl):Abstract nr 5306. doi:10.1158/1538-7445.AM2015-5306

Contact: Sydney Local Health District Human Research Ethics Committee – CRGH
Concord Repatriation General Hospital (CRGH)
Concord NSW 2139
Telephone: *(02) 9767 5622* *Fax (02) 9767 6569*
Email: *ethicscrgh@email.cs.nsw.gov.au*

Our Ref: *(HREC/11/CRGH/150)*

CONCORD
REPATRIATION GENERAL
HOSPITAL

5 January 2012

Associate Professor Mary Bebawy
Graduate School of Health, School of Pharmacy
University of Technology
PO Box 123
Ultimo
NSW 2007

Dear Professor Bebawy,

Re: HREC/11/CRGH/223 CH62/6/2011- 150
Characterization of Myeloma Derived Microparticles for the Development of a Novel Diagnostic to predict treatment outcome.

Thank you for submitting the above multi-centre project for single ethical and scientific review. This project was first considered by the Sydney Local Health District Human Research Ethics Committee – CRGH at its meeting held on 27 October 2011. This Human Research Ethics Committee (HREC) has been accredited by the NSW Department of Health as a lead HREC under the model for single ethical and scientific review.

This lead HREC is constituted and operates in accordance with the National Health and Medical Research Council's *National Statement on Ethical Conduct in Human Research* and the *CPMP/ICH Note for Guidance on Good Clinical Practice.*

I am pleased to advise that the Committee has granted ethical approval of this research project.

The documents reviewed and approved include:

- National Ethics Application Form (NEAF) – submission code AU/1/DE8B04
- Methodology, Overview of Project and Timeline - undated
- Information for Participants - Master Version 3 dated 1/01/2012
- Information for Healthy Volunteers - Master Version 4 dated 1/01/2012
- Participant Consent Form – Version 1 dated 21/12/2011
- Participant Consent Form for Healthy Volunteers – Version 2 dated 21/12/2011

Other:
- Advertisement for Healthy Volunteers – CRGH Version 2 dated 3/01/2011
- Advertisement for Healthy Volunteers – RPAH Version 2 dated 3/01/2011

The HREC has provided ethical and scientific approval for the following sites:
1. Concord Repatriation General Hospital
2. Royal Prince Alfred Hospital

Please note the following conditions of approval:

1. You will immediately report anything which might warrant review of ethical approval of the project in the specified format, including unforeseen events that might affect continued ethical acceptability of the project, (including Serious Adverse Events).

2. Proposed changes to the research protocol, conduct of the research, or length of HREC approval will be provided to the HREC for review in the specified format.

3. You will notify the HREC and other participating sites, giving reasons, if the project is discontinued at a site before the expected date of completion.

4. You will provide an annual report to the HREC, and at completion of the study in the specified format.

5. You will adhere to the study protocol at all times.

HREC approval is valid for five (5) years subject to the supply of an annual progress report. The first report should be sent to the Concord Hospital Research Office by **31/01/2013.**

Should you have any queries about the HREC's consideration of your project please contact the Executive Officer - Ms Virginia Turner on (02) 9767-5622. The HREC Terms of Reference, Standard Operating Procedures, membership and standard forms are available from the website: www.sswahs.nsw.gov.au/concord/ethics .

You are reminded that this letter constitutes ethical approval only. You must not commence this research project at a site until separate authorisation from the Chief Executive or delegate of that site has been obtained.

Please forward a copy of this letter to all site investigators for submission to the relevant Research Governance Officer

We wish you every success in your research.

Please quote the above file number in all correspondence.

Yours sincerely,

Production Note:
Signature removed prior to publication.

Professor Andrew McLachlan
Chairman
SLHD Human Research Ethics Committee – CRGH

☐ ***Please complete and return a copy of this page to the Concord Hospital Research Office as acknowledgment of your acceptance of the Conditions of Ethical Approval.***

Printed Name	**Signature**	**Date**
Chief Investigator		

Sydney Local Health District Human Research Ethics Committee (CRGH).
Concord Repatriation General Hospital
Concord NSW 2139
Telephone: *(02) 9767 5622* *Fax (02) 9767 6569*
Email: *ethicscrgh@email.cs.nsw.gov.au*

Our Ref: *(CH62/6/2011-151)*

CONCORD
REPATRIATION GENERAL
HOSPITAL

5 July 2013

A/Professor Mary Bebawy
Graduate School of Health
Pharmacy
UTS
PO Box 123
BROADWAY
NSW 2007

Dear Professor Bebawy,

Re: **CH62/6/2011-150 – M Bebawy**
HREC/11/CRGH/223
Characterization of Myeloma Derived Microparticles for the Development of a Novel Diagnostic to predict treatment outcome.

Thank you for responding to the Expedited Ethical Review Panel's request regarding the proposed amendment to the above study.

The following documents have now been reviewed and approved on behalf of the Expedited Ethical Review Panel of the SLHD Human Research Ethics Committee:

Revised Study Protocol
Revised Master Participant Information Sheet – Version 3 dated 1 January 2013

This lead HREC is constituted and operates in accordance with the National Health and Medical Research Council's *National Statement on Ethical Conduct in Research Involving Humans* and the *CPMP/ICH Note for Guidance on Good Medical Practice.*

A copy of this letter must be forwarded to all Principal Investigators at every site for submission to the Research Governance Officer.

Yours sincerely,

Production Note:
Signature removed prior to publication.

Phil Sanders
Administrative Officer
SLHD Human Research Ethics Committee – CRGH

Eth: HREC Approval Letter - UTS HREC 2012-004R

Ethics Secretariat [Research.Ethics@uts.edu.au]
Sent: Monday, 13 February 2012 4:35 PM
To: Dr Mary Bebawy [Mary.Bebawy@uts.edu.au]
Cc: Sabna Rajeev Krishnan; Ethics Secretariat [Research.Ethics@uts.edu.au]

Dear Mary and Sabna,

Re: "Characterisation of myeloma derived microparticles as novel diagnostic marker to predict treatment outcome"
[External Ratification: Concord Hospital Human Research Ethics Committee HREC approval - HREC/11/CRGH/223 - CH62/6/2011-150 - 05/01/12 to 05/01/17]

At its meeting held on 07/02/12, the UTS Human Research Ethics Expedited Review Committee reviewed your application and I am pleased to inform you that your external ethics clearance has been ratified.

Your UTS clearance number is UTS HREC REF NO. 2012-004R

You should consider this your official letter of approval. If you require a hardcopy please contact the Research Ethics Officer (Research.Ethics@uts.edu.au).

Please note that the ethical conduct of research is an on-going process. The National Statement on Ethical Conduct in Research Involving Humans requires us to obtain a report about the progress of the research, and in particular about any changes to the research which may have ethical implications. This report form must be completed at least annually, and at the end of the project (if it takes more than a year). The Ethics Secretariat will contact you when it is time to complete your first report. You must also provide evidence of continued approval from the Human Research Ethics Committee you originally received approval from.

I also refer you to the AVCC guidelines relating to the storage of data, which require that data be kept for a minimum of 5 years after publication of research. However, in NSW, longer retention requirements are required for research on human subjects with potential long-term effects, research with long-term environmental effects, or research considered of national or international significance, importance, or controversy. If the data from this research project falls into one of these categories, contact University Records for advice on long-term retention.

If you have any queries about your ethics clearance, or require any amendments to your research in the future, please do not hesitate to contact the Ethics Secretariat at the Research and Innovation Office, on 02 9514 9772.

Yours sincerely,

Professor Marion Haas
Chairperson
UTS Human Research Ethics Committee

C/- Research & Innovation Office
University of Technology, Sydney
Level 14, Tower Building
Broadway NSW 2007
Ph: 02 9514 9772
Fax: 02 9514 1244

Sydney Local Health District Human Research Ethics Committee (CRGH).
Concord Repatriation General Hospital
Concord NSW 2139
Telephone: *(02) 9767 5622* *Fax (02) 9767 6569*
Email: *ethicscrgh@email.cs.nsw.gov.au*

Our Ref: *(CH62/6/2011-151)*

CONCORD
REPATRIATION GENERAL
HOSPITAL

5 July 2013

A/Professor Mary Bebawy
Graduate School of Health
Pharmacy
UTS
PO Box 123
BROADWAY
NSW 2007

Dear Professor Bebawy,

Re: **CH62/6/2011-150 – M Bebawy**
HREC/11/CRGH/223
Characterization of Myeloma Derived Microparticles for the Development of a Novel Diagnostic to predict treatment outcome.

Thank you for responding to the Expedited Ethical Review Panel's request regarding the proposed amendment to the above study.

The following documents have now been reviewed and approved on behalf of the Expedited Ethical Review Panel of the SLHD Human Research Ethics Committee:

Revised Study Protocol
Revised Master Participant Information Sheet – Version 3 dated 1 January 2013

This lead HREC is constituted and operates in accordance with the National Health and Medical Research Council's *National Statement on Ethical Conduct in Research Involving Humans* and the *CPMP/ICH Note for Guidance on Good Medical Practice.*

A copy of this letter must be forwarded to all Principal Investigators at every site for submission to the Research Governance Officer.

Yours sincerely,

Production Note:
Signature removed prior to publication.

Phil Sanders
Administrative Officer
SLHD Human Research Ethics Committee – CRGH

Institutional Biosafety Committee
Ethics Secretariat
C/O Research and Innovation Office
City Campus
Building 1 Level 14 Room 14.31
PO Box 123 Broadway
NSW 2007 Australia
T: +61 2 9514 9681
F: +61 2 9514 1244
www.uts.edu.au

UTS CRICOS PROVIDER CODE 00099F

24 July 2015

A/Prof Mary Bebawy
Pharmacy
CB07.04.53
UNIVERSITY OF TECHNOLOGY, SYDNEY

Dear Mary,

2011-19-R-C – M. Bebawy, S. Krishnan. D. Joshua, Y. Kwan, R. Brown – "Characterization of Myeloma derived Microparticles for the development of a Novel Cancer Diagnostic to predict treatment outcome"

PROJECT DETAILS	
Location	Level 6 Labs, Building 4, UTS Broadway Campus
Involves	Cytotoxins
Type of Dealing	N/A
Approval period	17/01/2012 to 17/01/2017

On behalf of the UTS Biosafety Committee, I have considered and approved the request to amend the above protocol by:

1. Changing location from Building 4 Level 7 labs (Lab 6.17 and 6.30) to 07.04.070; and
2. Removal of Prof Geoges Grau and Dr Frederick Luk from the protocol.

If you wish to make any further changes to your research, please contact the Ethics Secretariat at the Research and Innovation Office on 02 9514 9645.

Yours sincerely,

Production Note:
Signature removed prior to publication.

Dr Rosetta Martiniello-Wilks
Chairperson
UTS Biosafety Committee

RESEARCH STUDY
CHARACTERIZATION OF MYELOMA DERIVED MICROPARTICLES FOR A NOVEL DIAGNOSTIC TO PREDICT TREATMENT OUTCOME

PARTICIPANT CONSENT FORM FOR THE HEALTHY VOLUNTEERS

I, ……[name] of

……[address]

have read and understood the Information for Participants for the above named research

study and have discussed the study with …… .

- I have been made aware of the procedures involved in the study, including any known or expected inconvenience, risk, discomfort or potential side effect and of their implications as far as they are currently known by the researchers.
- I understand that my participation as a healthy volunteer in this study is only to help the researchers to compare between people with blood cancer and normal age and gender matched healthy individuals.
- I freely choose to participate in this study and understand that I can withdraw at any time.
- I also understand that the research study is strictly confidential.
- I hereby agree to participate in this research study.

Name (Please Print): .....

Signature:..... **Date:** .....

.....
Name of Person who conducted informed consent discussion (Please Print):

Signature:..... **Date:** .....
of Person who conducted informed consent discussion

RESEARCH STUDY

"Characterization of Myeloma Derived Microparticles for the Development of a Novel Diagnostic to Predict Treatment outcome".

(School of Pharmacy, Graduate School of Health, The University of Technology Sydney in collaboration with
The Sydney Medical School, The university of Sydney, The Institute of Haematology, Royal Prince Alfred Hospital and Department of Haematology, Concord Repatriation General Hospital)

INFORMATION FOR PARTICIPANTS

Introduction

You are invited to take part in a research study which aims to develop a new blood test that may be useful in predicting the best treatment outcome for patients with Multiple Myeloma- a common type of blood cancer. You are being asked to take part because you have been diagnosed with Multiple Myeloma and are being treated at Royal Prince Alfred or Concord Hospitals.

The study is being conducted by A/Prof Mary Bebawy (School of Pharmacy, Graduate School of Health, The University of Technology, Sydney in collaboration with Prof. Georges Grau, University of Sydney. The clinical collaborators involved in the study include Dr. Yiu Lam Kwan (Staff Specialist, Haematology, Concord General Repatriation Hospital) , Dr. Ross Brown (Principal Hospital Scientist, Royal Prince Alfred Hospital, Sydney) along with Prof. Douglas Joshua, (Institute of Haematology, Royal Prince Alfred Hospital, Area Head Haematology, SSWAHS)

Procedures

If you agree to participate in this study, you will be asked to give an extra 4ml blood sample for research purposes when you come to the clinic for your routine assessment each time over 6 months.This does not involve any major risks. We will also collect information about your age, gender and the treatment you are receiving from your medical records. This information will be stored in the research team's secured database in a coded form for correlation purpose. The details will be individually de-identified form.

Risks

Only a very small amount (up to 4ml) of blood is required in each visit over 6 months for the study. Extra inconvenience and discomfort will be minimal. Collection of blood may involve some minor discomfort, bruising and on rare occasions local infection. Trained pathology collection staff will take your blood sample. You will not receive the results of testing.

It is important that participants in this study are not pregnant and do not become pregnant during the course of the study. If at any time you feel you may have become pregnant, it is important to let the researchers know immediately.

Benefits

While we intend that this research study furthers medical knowledge and may improve management of Multiple Myeloma in the future, it may not be of direct benefit to you.

Compensation

Every reasonable precaution will be taken to ensure your safety during the course of the study. In the event that you suffer any injury as a result of participating in this research project, hospital care and treatment will be provided at no extra cost to you.

Costs

Participation in this study will not cost you anything, nor will you be paid

Confidentiality

If you consent to take part in this study, your hospital medical records may be inspected by the researchers, by regulatory authorities or by the Concord Hospital Human Research Ethics Committee. By signing the attached consent form, you are giving permission for this to be done. All details obtained by those named will remain confidential. A report of this study may be submitted for publication, but individual participants will not be identifiable in such a report.

Withdrawal from the study

Participation in this study is entirely voluntary. You are in no way obliged to participate and - if you do participate - you can withdraw at any time. Whatever your decision, please be assured that it will not affect your medical treatment or your relationship with medical staff.

Further Information

When you have read this information, clinical collaborators of the study Dr. Kwan (Concord), Prof Joshua and Dr. Brown (RPAH) will discuss it with you further and answer any questions you may have. You can also contact Dr. Kwan on 9767 5154 or Professor Joshua on 9515 8038 with any concerns, questions you may have**.** If you would like to know more at any stage, please feel free to contact A/ Prof. Mary Bebawy, Graduate school of Health, School of Pharmacy, University of Technology, PO Box 123, Ultimo, Sydney, PH: 9514 8305. This information sheet is for you to keep.

This study has been approved by the Sydney Local Health District (SLHD) Human Research Ethics Committee - CRGH. If you have any concerns or complaints about the conduct of the research study, you may contact the Executive Officer of the Ethics Committee, on (02) 9767 5622.

The conduct of this study at Royal Prince Alfred Hospital has been authorized by SLHD. Any person with concerns or complaints about the conduct of this study may contact the Research Governance Officer Lesley Townsend on T: (02) 9515 6766 or E: lesley.townsend@email.cs.nsw.gov.au.

RESEARCH STUDY
CHARACTERIZATION OF MYELOMA DERIVED MICROPARTICLES FOR A NOVEL DIAGNOSTIC TO PREDICT TREATMENT OUTCOME

PARTICIPANT CONSENT FORM FOR THE HEALTHY VOLUNTEERS

I, ……[name] of

……[address]

have read and understood the Information for Participants for the above named research

study and have discussed the study with …… .

- I have been made aware of the procedures involved in the study, including any known or expected inconvenience, risk, discomfort or potential side effect and of their implications as far as they are currently known by the researchers.
- I understand that my participation as a healthy volunteer in this study is only to help the researchers to compare between people with blood cancer and normal age and gender matched healthy individuals.
- I freely choose to participate in this study and understand that I can withdraw at any time.
- I also understand that the research study is strictly confidential.
- I hereby agree to participate in this research study.

Name (Please Print): .....

Signature:..... **Date:** .....

.....
Name of Person who conducted informed consent discussion (Please Print):

Signature:..... **Date:** .....
of Person who conducted informed consent discussion.

RESEARCH STUDY

"Characterization of Myeloma Derived Microparticles for the Development of a Novel Diagnostic to Predict Treatment outcome".

(School of Pharmacy, Graduate School of Health, The University of Technology Sydney
in collaboration with
The Sydney Medical School, The University of Sydney, The Institute of Haematology, Royal Prince Alfred Hospital and Department of Haematology, Concord Repatriation General Hospital)

INFORMATION FOR HEALTHY VOLUNTEERS

Introduction

You are invited to take part in a research study which aims to develop a new blood test that may be useful in predicting therapeutic outcome for patients with blood cancer. You are being asked to take part because you are a healthy individual who can provide a control for the study group. People often volunteer to take part in medical research because they have a medical condition and the research may offer a chance of improving their condition. Your role in this study is different. You are healthy and as such you need to carefully consider your participation.

Patients with Multiple Myeloma (a blood cancer) who are attending Royal Prince Alfred and Concord Hospitals will also be sought as potential participants in the study group. The study is being conducted by A/Prof Mary Bebawy (School of Pharmacy, Graduate School of Health, The University of Technology, Sydney in collaboration with Prof. Georges Grau, Sydney Medical School, University of Sydney. The clinical collaborators involved in the study include Dr. Yiu Lam Kwan (Staff Specialist, Haematology, Concord General Repatriation Hospital) and Dr. Ross Brown (Principal Hospital Scientist, Royal Prince Alfred Hospital, Sydney) along with Prof Douglas Joshua (Institute of Haematology, Royal Prince Alfred Hospital, Area Head Haematology, SSWAHS)

Procedures

If you agree to participate in this study, you will be asked to provide a sample of 4ml blood for research purposes. This does not involve any major risk. You are being asked to take part because you are a healthy individual. You are not taking any cytotoxic drug currently and it is understood that you have not undergone any treatment for any type of cancer in the past 5 years. Your results will be used for comparison purposes.

Risks

Only a very small amount (up to 4ml) of blood is required for the study. Collection of blood may involve some minor discomfort, bruising and on rare occasions local infection. Trained pathology collection staff will take your blood sample. You will not receive the results of testing.

It is important that participants in this study are not pregnant. If at any time you feel you may have become pregnant, it is important to let the researchers know immediately.

Benefits

While we intend that this research study furthers medical knowledge and may improve treatment of blood cancers in the future, it will not be of direct benefit to you.

Compensation

Every reasonable precaution will be taken to ensure your safety during the course of the study. In the event that you suffer any injury as a result of participating in this research project, hospital care and treatment will be provided at no extra cost to you.

Costs

Participation in this study will not cost you anything, nor will you be paid

Confidentiality

If you consent to take part in this study, all details obtained by those named will remain confidential. A report of this study may be submitted for publication, but individual participants will not be identifiable in such a report.

Withdrawal from the study

Participation in this study is entirely voluntary. You are in no way obliged to participate and - if you do participate - you can withdraw at any time. Whatever your decision, please be assured that it will not affect your medical treatment or your relationship with medical staff.

Further Information

When you have read this information, clinical collaborators of the study Dr. Kwan (Concord), Prof. Joshua and Dr. Brown (RPAH) will discuss it with you further and answer any questions you may have. You can also contact Dr. Kwan on (02) 9767 5154 or Professor Joshua on (02) 9515 8038 with any concerns, questions you may have If you would like to know more at any stage, please feel free to contact A/ Prof. Mary Bebawy, Graduate school of Health, School of Pharmacy, University of Technology, PO Box 123, Ultimo, Sydney, PH: 9514 8305. This information sheet is for you to keep.

This study has been approved by the Sydney Local Health District (SLHD) Human Research Ethics Committee - CRGH. If you have any concerns or complaints about the conduct of the research study, you may contact the Executive Officer of the Ethics Committee, on (02) 9767 5622.

The conduct of this study at Royal Prince Alfred Hospital has been authorized by SLHD. Any person with concerns or complaints about the conduct of this study may contact the Research Governance Officer Lesley Townsend on T: (02) 9515 6766 or E: lesley.townsend@email.cs.nsw.gov.au

We need your help as a healthy volunteer for the research project

"Characterization of Myeloma derived microparticles for a novel cancer diagnostic"

HREC Reference Number: CH62/6/2011-150
HREC/11/CRGH/223

The research team needs age and gender matched healthy volunteers to compare the research findings amongst Myeloma patients to healthy subjects.

You can help us if you

- Are aged above 18 years and healthy
- Have not undergone treatment for any type of cancer in the past 5 years.
- Are not on any cytotoxic drugs currently

Please note that pregnant women are specifically excluded.

Participation involves

- Attending the blood collection centre at Concord Haematology, Concord Repatriation General Hospital, Concord.
- Going through the consent process with clinician (5-10 min)
- Provide 4 ml blood for analysis (venepuncture will be carried out by trained pathology staff).
  You will not be given the results of the tests. Results will be used for comparison purposes in the research.

  For more information on participation please contact Dr. Yiu Lam Kwan at email: Yiu.Kwan@sswahs.nsw.gov.au.

  - This study has been granted ethics approval by Concord Hospital Human Research Ethics Committee.
  - All participant details will be strictly confidential.
  - Participation is entirely voluntary.

We need your help as a healthy volunteer for the research project

"Characterization of Myeloma derived microparticles for a novel cancer diagnostic"

HREC Reference Number: CH62/6/2011-150
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You can help us if you

- Are aged above 18 years and healthy
- Have not undergone treatment for any type of cancer in the past 5 years.
- Are not on any cytotoxic drugs currently

Please note that pregnant women are specifically excluded.

Participation involves

- Attending the blood collection centre at Institute of Haematology, RPAH, Camperdown.
- Going through the consent process with clinician (5-10 min)
- Provide 4 ml blood for analysis (venepuncture will be carried out by trained pathology staff).
  You will not be given the results of the tests. Results will be used for comparison purposes in the research.

  For more information on participation please contact Prof. Douglas Joshua at email: douglas.joshua@sswahs.nsw.gov.au or Dr. Ross Brown at email : ross.brown@email.cs.nsw.gov.au

  - This study has been granted ethics approval by Concord Hospital Human Research Ethics Committee.
  - All participant details will be strictly confidential.
  - Participation is entirely voluntary.

ADDRESS FOR ALL CORRESPONDENCE
RESEARCH DEVELOPMENT OFFICE
ROYAL PRINCE ALFRED HOSPITAL
CAMPERDOWN NSW 2050

TELEPHONE: (02) 9515 6766
FACSIMILE: (02) 9515 7176
EMAIL: lesley.townsend@email.cs.nsw.gov.au
REFERENCE: X12-0019

NSW GOVERNMENT Health
Sydney
Local Health District

21 February 2012

A/Professor M Bebawy
Graduate School of Health
School of Pharmacy
University of Technology
PO Box 123
BROADWAY NSW 2007

Dear Professor Bebawy,

Re: Protocol No X12-0019 - "Characterization of myeloma derived microparticles for the development of a novel diagnostic to predict treatment outcome"

HREC/11/CRGH/223 **SSA/12/RPAH/10**

Thank you for submitting a Site Specific Assessment Form for this study. I am pleased to inform you that authorisation has been granted for it to be undertaken at the Haematology Unit, Royal Prince Alfred Hospital.

The approved information and consent documents for use at this site are:

- Information for Health Volunteers (RPAH Version 4, 1 January 2012)
- Participant Consent Form for Healthy Volunteers (Version 2, 21 December 2011)
- Information for Participants (RPAH Version 3, 1 January 2012)
- Participant Consent Form (Version 1, 21 December 2011)

The following conditions apply to this research study. These are additional to those conditions imposed by the human research ethics committee (HREC) that granted ethical approval:

1. A copy of the annual report and any other reports to the approving HREC, accompanied by a copy of the HREC's acknowledgement letter, should be provided to me for review.

D.A. Spandidos
International Journal of Oncology - Spandidos Publications <ijo@spandidos-publications.com>
Tue 26/07, 5:23 PMSabna Rajeev Krishnan

Spandidos Publications Ltd
International Journal of Oncology
July 26, 2016
Dear Dr Sabna Rajeev Krishnan

Int J Oncol. 2016 Jul;49(1):33-50.
Title: Multiple myeloma and persistence of drug resistance in the age of novel drugs (Review).

Thank you for your inquiry.
Permission is hereby granted for reproduction of the above for the purpose outlined in your message.

Yours sincerely,
The Secretariat
for the Accounts Dept.

Professor D.A. Spandidos
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E-mail: contact@spandidos-publications.com
Website: www.spandidos-publications.com

From: Sabna Rajeev Krishnan [mailto:Sabna.RajeevKrishnan@student.uts.edu.au]

Sent: Monday, July 25, 2016 4:04 PM
To: contact@spandidos-publications.com
Subject: Permission to reuse my review article in my Thesis

Dear Sir/Madame,

I would like to use my review article published in International Journal of Oncology "Multiple myeloma and persistence of drug resistance in the age of novel drugs" (Review) DOI: 10.3892/ijo.2016.3516 in my Thesis. Would you please advise if there is any thing I need to do to obtain permission from Spandidos publications?

Sabna Rajeev Krishnan
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