

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

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*A thesis submitted in the fulfilment of the requirements of  
the Degree of Doctor of Philosophy*

**Discipline of Pharmacy  
Graduate School of Health**



**| U | T | S |**

**UNIVERSITY OF TECHNOLOGY SYDNEY**

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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 - 4<sup>th</sup> 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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# Table of Contents

Title Page .....	i
Certificate of Original Authorship .....	ii
Dedication .....	iii
Acknowledgements .....	iv
Table of Contents .....	v
Lists of Figures and Tables .....	xi
List of Abbreviations.....	xv
Publications and Provisional Patent.....	xix
Conference Presentations .....	xvi
Graduate certificates (LEAP modules) .....	xviii
Awards and Scholarships .....	xix
Abstract .....	xx

## Chapter 1

1. Multiple myeloma and persistence of drug resistance in the age of novel drugs (Review).....	1
---	---

## Chapter 2

2. Systemic signatures in multiple myeloma and their role in clinical management.	3
2.1 Abstract .....	4
2.1 Introduction .....	5
2.2 Multidrug resistance in multiple myeloma .....	8
ABC transporters and drug resistance in multiple myeloma .....	9
2.3 CD138 and its significance in myeloma pathophysiology.....	13
2.4 Current systemic markers used clinically in the management of MM.....	17
Stage, tumor burden and disease progression: .....	17
$\beta$ -2-microglobulin ( $\beta_2$ M).....	18
Paraprotein monoclonal or M protein .....	19
Free light chains .....	19

Acute phase proteins as a measure of tumor burden .....	19
Aberrant cellular respiration: .....	20
Defective bone physiology:.....	20
2.5 Membrane vesiculation and microparticle formation .....	23
2.6 Significance of extracellular vesicles.....	25
2.7 Microparticle physiology in cancer.....	29
a) Tumor survival.....	30
b) Immune evasion.....	31
c) Extracellular matrix degradation.....	33
d) Vascularisation, invasion and migration leading to metastasis ....	34
e) Microparticle cargo .....	36
f) Extracellular vesicles in myeloma.....	39
2.8 Extracellular vesicles as a prognostic in myeloma .....	42
2.9 Conclusion.....	45
References .....	49

**Aims and Hypothesis..... 63**

**Chapter 3**

**3.The development of validated protocols and workflows for the isolation, quantitation and phenotyping of microparticles from myeloma patients. .... 65**

3.1 Abstract .....	66
3.2 Introduction.....	67
3.3 Materials and Methods.....	69
3.3.1 Antibodies and other reagents.....	69
3.3.2 Pre-analytical protocols.....	70
3.3.3 Study design and sample collection .....	70
3.3.4 Storage and freeze-thaw .....	70
3.3.5. MP isolation from cell lines and patient samples.....	71
3.3.6 MP sample preparation for Scanning electron microscopy .....	72
Preparation of PEI solution: .....	72
Coating Coverslips with PEI:.....	72
3.3.7 Validation of MP cargo .....	73

Preparation of MP and cell lysates:.....	73
Protein quantitation:.....	73
Electrophoresis and Western blotting.....	73
3.4 Analytical methods.....	74
3.4.1 Flow cytometry.....	74
Instrument calibration.....	74
Comparative assessment of MP resolution and gating parameters..	74
Immunolabeling workflow.....	75
Titration of antibodies.....	75
3.4.2 Optimization of immunolabeling workflow.....	76
1) Immunolabeling MP pellet (no wash step):.....	76
2) Immunolabeling with a wash step in between.....	77
3) Direct immunolabeling of the platelet-free plasma.....	
Sequential and simultaneous immunolabeling.....	77
3.5.Data acquisition.....	77
3.5.1Platelet-derived MP exclusion and surface antigen detection.....	78
3.5.2Enumeration of MPs.....	79
3.5.3 Statistical analysis.....	79
3.6 Results and Discussion.....	80
3.6.1 Pre-analytical assessment.....	80
3.6.2 Storage and freeze-thaw.....	80
3.6.3 Validation of MP cargo by Western blot.....	82
3.7 Analytical assessment.....	85
3.7.1 Flow cytometry.....	85
3.7.2 Instrument calibration, comparative assessment of MP resolution and gating parameters.....	85
3.7.3 Immunolabelling workflow.....	87
A) Titration of antibodies.....	87
B) Comparative analysis of direct immunolabeling of PFP vs MP pellet with respect to antigen detection..	89
1. Adaptability of the workflow to a clinical setting:.....	93

2. Recovery of MPs after ultracentrifugation: .....	93
C) Sequential vs simultaneous antigen immunolabelling .....	93
3.8.Data acquisition.....	96
Platelet MP exclusion and surface antigen detection and enumeration .....	96
3.9 General Discussion.....	98
References .....	101

## Chapter 4

<b>4.Isolation of human CD138<sup>+</sup> microparticles from the plasma of patients with multiple myeloma.</b> .....	104
---	-----

## Chapter 5

<b>5.P-glycoprotein expression in microparticles is indicative of disease progression and treatment unresponsiveness in myeloma.</b> .....	106
5.1 Abstract .....	107
5.2 Introduction .....	108
5.3 Materials and Methods .....	111
5.3.1 Reagents & Antibodies .....	111
5.3.2 Study design and patients.....	111
5.3.3 Isolation and flow cytometric detection of MPs. ....	112
5.3.4 Surface phenotyping of systemic MPs and quantitation. ....	112
5.3.5 Statistical analysis .....	112
5.4 Results .....	113
5.4.1P-gp <sup>+</sup> MP numbers are elevated in <i>de-novo</i> and progressive disease MM patients.....	114
5.4.2 CD138 <sup>+</sup> MPs do not express significant levels of P-gp on their surface. ....	117
5.4.3 CASE 1: 58-year-old female patient with aggressive disease .....	120



5.4.4 CASE 2: 66-year-old female patient in progressive disease..	123
5.4.5 CASE 3: 63-year-old male patient in stable condition .....	124
5.4.6 CASE 4: 71-year-old male patient in partial remission. ...	127
5.4.7 CASE 5: 62-year-old male patient in remission – long-term survivor.....	128
5.4.8 Annexin <sup>+</sup> MP represents a more aggressive state in MM..	131
5.4.9 Annexin V <sup>+</sup> and CD138 do not co-express in progressive disease. ....	134
5.5 Discussion .....	137
Acknowledgements .....	143
References .....	144
Supplementary figures .....	148

## **Chapter 6**

<b>6. A Novel approach for Individualized Risk-stratification in multiple myeloma</b> .....	152
References .....	170
<b>7. Appendices</b> .....	173
7.1 Executed non-disclosure assignments.....	173
7.2 Conference Proceedings Articles .....	180
7.3 Human Research Ethics Committee approval documents .....	183
7.4 Consent for Reproducing the Content from Published articles.....	198

# List of Figures and Tables

## Chapter 2

Figure 2.1 P-gp mediated drug efflux in MM .....	11
Figure 2.2 Role of CD138 in MM pathology .....	16
Figure 2.3 Current systemic signatures of MM pathology .....	22
Figure 2.4 Schematic representation of membrane vesiculation .....	27
Figure 2.5 Extracellular vesicles in cancer .....	38
Figure 2.6 Microparticle subtypes in MM .....	45
Figure 2.7 Study design.....	64

## Chapter 3

Figure 3.1 Sample storage optimization.....	81
Figure 3.2 Validation of MP cargo constituents by Western blot.....	83
Figure 3.3 Comparative assessment of MP resolution and establishment of the MP gating parameters .....	86
Figure 3.4 Determination of antibody concentration for optimal MP staining.....	88
Figure 3.5 Immunolabeling workflow .....	90
Figure 3.6 Immunolabeling optimization.....	92
Figure 3.7 Sequential Vs simultaneous immunolabeling approaches.....	95
Figure 3.8 Detection of distinct MP populations from the platelet free plasma of MM patients .....	98

## Chapter 5

Figure 5.1 P-gp <sup>+</sup> MP increases in MM.....	115
Figure 5.2 CD138 <sup>+</sup> MPs do not significantly express P-gp. ....	118
Figure 5.3 P-gp <sup>+</sup> MPs in a 58-year-old patient with aggressive disease during the course of treatment (patient 1).....	121
Figure 5.4 Elevated levels of ‘dual positive’ MPs in patient 1 with aggressive disease. ....	123

Figure 5.5 66-year-old female patient in progressive disease (patient 2) and 63-year-old male patient (patient 3) in stable condition .....	127
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).....	131
Figure 5.7 Annexin+ MP represents a more aggressive state in MM.....	134
Figure 5.8 Annexin V+ and CD138 do not co-express in progressive disease.....	136

Supp.fig.5.1 Gating strategy to define parameters for +/- staining in patient 1 (aggressive disease).....	149
Supp.fig.5.2 Isotype-matched control to define parameters for +/- staining in patient (PD) and 3 (stable) .....	150
Supp.fig.5.3 Isotype-matched control to define parameters for +/- staining in patient 4 (PR) and 5 (remission) .....	151
Supp.fig.5.4 Isotype-matched control to define parameters for +/- staining patient 1 in partial remission and ‘dual positive’ population in May 2015 .....	152

Table 5.1 MP subtypes across different clinical states .....	137
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## **Chapter 6**

Figure 6.1 Graphical representations of prognosis and risk-stratification in MM based on MP phenotype. ....	166
---	-----

## 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	<i>MAF</i> 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
<i>mir</i>	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 membrane domain
TNF- $\alpha$	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<sup>+</sup> microparticles from the plasma of patients with multiple myeloma patients. Neoplasia

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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

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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 & 21<sup>st</sup> 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 10<sup>th</sup>, 2016**

3MT Thesis 2013, 2014 and 2015, Graduate School of Health.

## **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.

Rajeev Krishnan S, Luk F, Brown RD, Kwan YL, Bebawy M. ***“Microparticles as novel prognostic markers in multiple myeloma”***

American Association of Cancer Research - Annual meeting 2015-April 18-22,2015. Philadelphia, PA

## **Conference Attendance**

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

Workshops

Uniquet 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.

**Graduate certificates from ATN e Grad school ( LEAP and MORE modules)**

Certificate in Research Commercialization- Australian Technology Network- e-Grad School LEAP (Learning Employment Aptitude program) University of Queensland, Australia.

Certificate in Leadership and Communication- Australian Technology Network- e-Grad School LEAP (Learning Employment Aptitude program) Curtin University, WA, Australia.

Certificate in Critical and Creative thinking- Australian Technology Network –e-Grad School MORE (Modules On-line for Research Education), RMIT University, VIC, Australia.

Certificate in Global Sustainability - Australian Technology Network- e-Grad School LEAP (Learning Employment Aptitude program), QUT, Australia.

## **Awards and Scholarships**

Recipient of Australian Postgraduate Award (APA)-1 Jan-2012- 30-June 2014 – Faculty bestowed APA to the value of \$23,728 AUD (indexed annually) based on the progress made during Masters by Research and subsequent upgrade to Doctoral candidate.

Awarded Vice Chancellor's Postgraduate Research Student Conference Fund, Round 1, 2013, University of Technology, Sydney to the value of \$1,200 AUD.

Awarded Vice Chancellor's Postgraduate Research Student Conference Fund, Round 3, 2014, University of Technology, Sydney to the value of \$1,200 AUD.

## **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- $\mu$ m 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™ beads.

We observed significantly greater levels of total MP and CD138<sup>+</sup> 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<sup>-</sup> CD34<sup>+</sup> P-gp<sup>+</sup>) 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.