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

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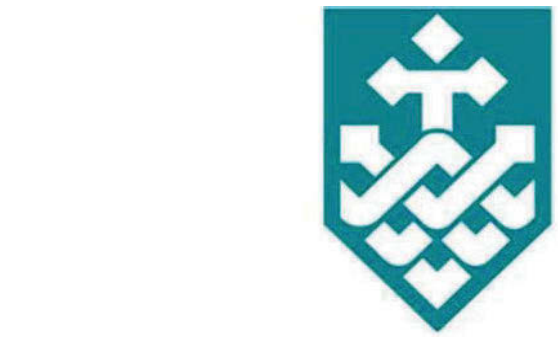

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CERTIFICATE OF ORIGINAL AUTHORSHIP

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.

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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

Rajeev Krishnan, S., Brown RD., Suen H., Kwan YL and Bebawy M P-glycoprotein expression in microparticles is indicative of disease progression and treatment unresponsiveness in myeloma-under embargo

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**

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 -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.

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 Doctoaral candidate.

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

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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.