

## Isolation of Human CD138<sup>+</sup> Microparticles from the Plasma of Patients with Multiple Myeloma<sup>1</sup>

Sabna Rajeev Krishnan<sup>\*</sup>, Frederick Luk<sup>\*</sup>,  
Ross D Brown<sup>†</sup>, Hayley Suen<sup>†</sup>, Yiulam Kwan<sup>‡</sup> and  
Mary Bebawy<sup>\*</sup>

<sup>\*</sup>Graduate School of Health, Discipline of Pharmacy, University of Technology Sydney, NSW 2007, Australia; <sup>†</sup>Institute of Haematology, Royal Prince Alfred Hospital, Camperdown, NSW 2050, Australia; <sup>‡</sup>Department of Haematology, Concord Repatriation General Hospital, Concord, NSW 2139, Australia

### 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- $\mu$ m 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<sup>+</sup>) 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

(0.1–1.0 $\mu$ m) 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

Address all correspondence to: A/Prof Mary Bebawy, Graduate School of Health, Discipline of Pharmacy, University of Technology Sydney, Ultimo, NSW 2007, Australia (PO Box 123). Tel: + 61 2 9514 8349.

E-mail: [mary.bebawy@uts.edu.au](mailto:mary.bebawy@uts.edu.au)

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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<sup>-</sup>) 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<sup>+</sup>/CD45<sup>-</sup> represents the phenotype of mature PCs in bone marrow, and only CD138<sup>+</sup> 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<sup>+</sup> MPs and distinct clinical states. This study describes for the first time the isolation and characterization of CD138<sup>+</sup>/CD41a<sup>-</sup> 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  $-80^{\circ}\text{C}$  for  $>24$  hours and analyzed by surface protein phenotyping.

### Quantitation of Systemic Microparticles

Isolated PC-derived MPs were resuspended in 200  $\mu\text{l}$  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  $\mu\text{l}$  of plasma was calculated using the manufacturer's formula:  $N = [\text{MP number of measured beads}] \times [\text{beads per tube/sample volume added}]$ . 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  $\pm$  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- $\mu\text{m}$  latex beads (R1: the lowest possible limit on FS for BD LSRII) and 1.1- $\mu\text{m}$  beads (R2: represents the upper limit for MPs, Figure 1A).

As platelet-derived MPs comprise the major population systemically, our analysis was confined to CD41a<sup>-</sup> (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<sup>+</sup> CD138<sup>+</sup> (Figure 1B, 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<sup>+</sup> CD138<sup>+</sup> (Figure 1B, 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<sup>+</sup> using fresh sample (Figure 2A) compared with 5.63% for the same sample after freezing (Figure 2B). Consequently, MP isolation was conducted using freshly isolated PFP without freezing.

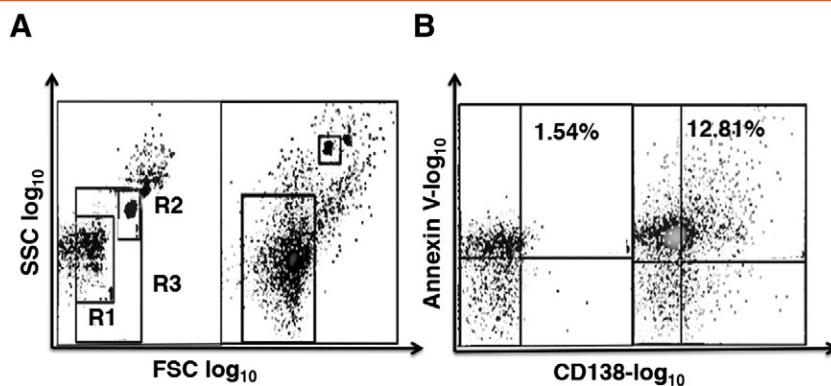
### Total Microparticle Numbers Increase in MM

In considering the cohort data, we observed greater numbers of total MPs in MM patients relative to healthy volunteers (Figure 3A). The absolute number of MPs from MM patients was on average seven-fold greater per  $\mu\text{l}$  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 3B). We observed no significant difference in total MP counts across the different clinical states.

### CD138<sup>+</sup> Microparticle Numbers Increase in MM

In considering the cohort data, we observed greater numbers of CD138<sup>+</sup> MPs in MM patients relative to healthy volunteers (Figure 4A). The absolute number of CD138<sup>+</sup> MPs from MM patients was on average 3.45 fold greater per  $\mu\text{l}$  relative to healthy volunteers ( $U = 632$ ,  $P < .01$ ). Consistent with this, we observed greater numbers of CD138<sup>+</sup> 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<sup>+</sup> 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<sup>-</sup> and may impact MP counts for some patients with PD [22,23]. CD138<sup>+</sup> 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<sup>+</sup> 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 4B). Furthermore, we also identified five patients who were in CR at the time of analysis who had greater CD138<sup>+</sup> MPs counts relative to the rest of the cohort (gray circles, Figure 4B). These same patients were found to clinically relapse a few weeks later, demonstrating the sensitivity and potential for CD138<sup>+</sup> 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  $\mu\text{m}$ ; 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<sup>+</sup> CD138<sup>+</sup>, whereas 12.8% of MPs isolated from MM patient were Annexin V<sup>+</sup> CD138<sup>+</sup> as shown on the right.

### CD138<sup>+</sup> Expression Correlates with Therapeutic Response in Individual Patients

We observed a significant prognostic potential for CD138<sup>+</sup> MPs in predicting “risk of relapse” and therapeutic response in individual patients. We conducted a series of assessments whereby we profiled CD138<sup>+</sup> 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<sup>+</sup> MP count fell in accordance with response to treatment (Figure 5A). 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<sup>+</sup> MPs, whereas we observed no corresponding increase in paraprotein levels observed during this same period (Figure 5B). The patient was then placed on a thalidomide/dexamethasone maintenance regimen. This corre-

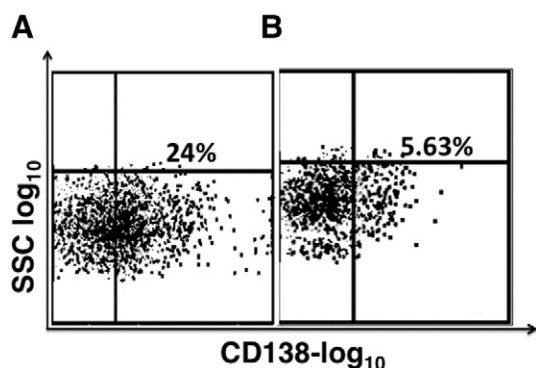
sponded with a drop in both CD138<sup>+</sup> MP counts and IgG levels. The patient underwent autologous stem cell transplant, CD138<sup>+</sup> MP counts increased, and IgG levels stabilized.

### Morphology and Size of the Microparticles

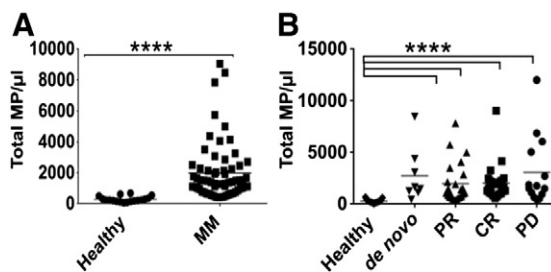
SEM was used to visualize the morphology of MPs across different clinical states. The morphology of MPs was compared with the OPM2 myeloma cell line–derived MPs (Figure 6A, leftmost panel). In *de novo* patients (Figure 6A, second panel from left) and patients in remission (Figure 6A, third panel from 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 6A, rightmost panel). We observed no significant difference in the size across distinct clinical response states with  $0.75 \pm 0.16 \mu\text{m}$ ,  $0.83 \pm 0.53 \mu\text{m}$ , and  $0.87 \pm 0.44 \mu\text{m}$  for the *de novo*, remission, and relapsed cases, respectively (Figure 6B).

### Discussion

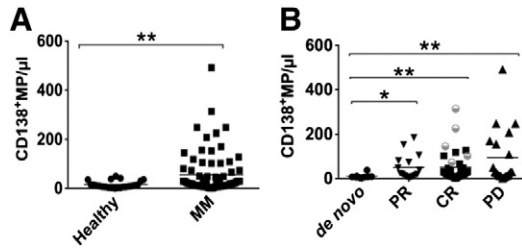
We report on the isolation and detection of non–platelet-derived (CD41a<sup>-</sup>) CD138<sup>+</sup> MPs in the blood of patients with MM. Our data demonstrate that the total MP count is a predictor of the diseased state in MM relative to healthy volunteers and is independent of the



**Figure 2. Sample storage optimization.** PFP was prepared, and the sample was divided in two 200- $\mu\text{l}$  aliquots. One sample was immediately frozen at  $-80^{\circ}\text{C}$ , 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<sup>+</sup> for the fresh MP sample, whereas only 5.63% of MPs were CD138<sup>+</sup> when isolated from PFP post freezing/thawing (B).



**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<sup>+</sup> MP increases in MM.** The CD138<sup>+</sup> MP counts in MM patients and healthy subjects were compared using the manufacturer's formula. (A) CD138<sup>+</sup> MP numbers were significantly greater in the MM cohort relative to the healthy cohort. (B) The absolute numbers of CD138<sup>+</sup> 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<sup>+</sup> MPs in MM patients relative to healthy volunteers. We showed that the CD138<sup>+</sup> 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<sup>+</sup> 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

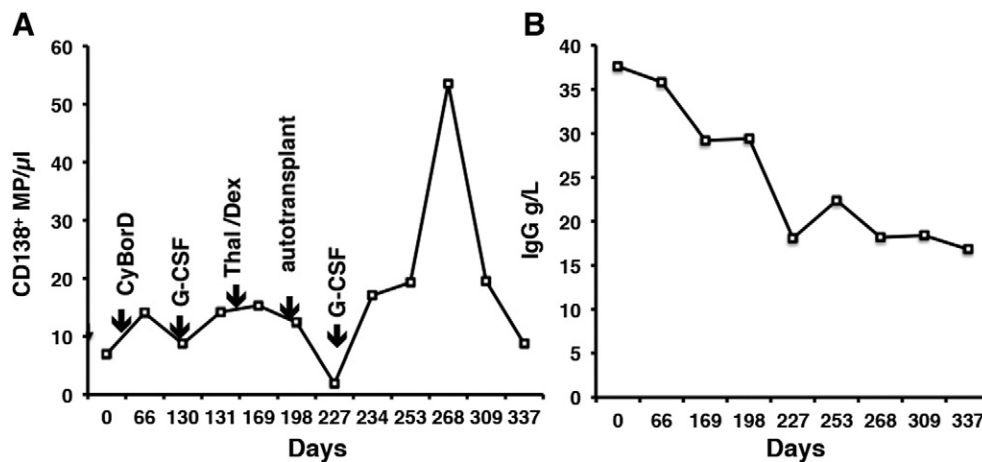
the transition phase from orderly disease state to the progressive, and thus novel systemic markers which can provide for this will aid in improving the clinical management of MM.

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].

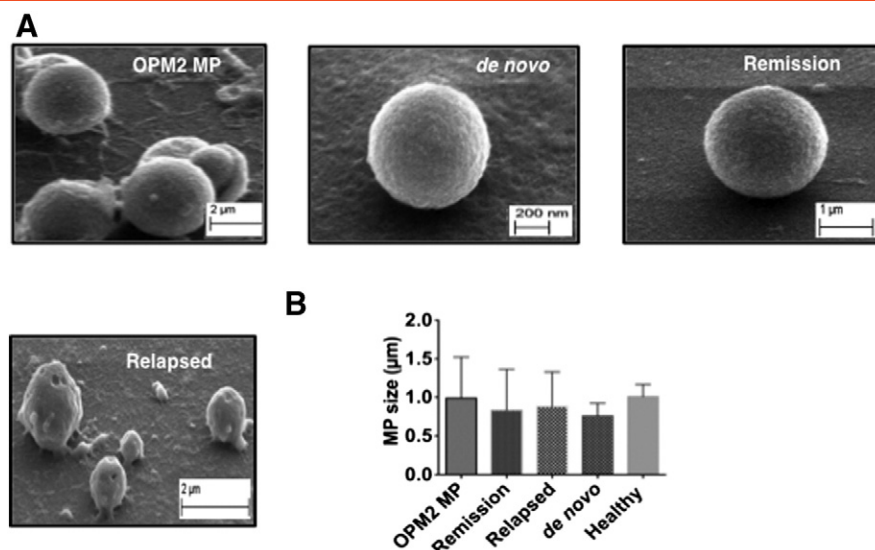
As a stress response, the pliable plasma membrane undergoes lipid bilayer remodeling, which in turn results in systemic shedding of the MPs by most cell types [5,11]. MP levels are elevated in cancer and inflammatory conditions and have a substantial pathophysiological significance [6,11,26]. The surface 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<sup>+</sup> 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<sup>+</sup> MP counts (A) were measured and profiled against IgG levels (B) during the course of clinical interventions. CD138<sup>+</sup> 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<sup>+</sup> MP counts and IgG levels. The patient underwent autologous stem cell transplant, CD138<sup>+</sup>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,  $\times 5.40$  K) were used as a control. (A, second and third from left panel, respectively) MPs isolated from a *de novo* patient sample (magnification,  $\times 24.64$  K) and from a patient in remission (magnification,  $\times 19.40$  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,  $\times 9.18$  K). (B) No significant differences in the size of MPs were observed across the different clinical states. Data are expressed as mean  $\pm$  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<sup>-</sup> PCs in bone marrow has been reported and is identified as immature compared with CD138<sup>+</sup> [31]. According to the literature, CD138<sup>-</sup> PCs were found to be in the S phase and therefore possess higher proliferation potential [31]. We measured greater, albeit insignificant, CD138<sup>+</sup> 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<sup>-</sup>, and may be underestimating MP counts (derived from CD138<sup>-</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> MP count as a response to therapy from baseline and an increase in CD138<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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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