Chemotherapy treatment of multiple myeloma patients increases circulating levels of endothelial microvesicles

Jessica Hall, M. Adeola Adesanya, Eric Gardiner, Haz Sayala, Leigh Madden, Anthony Maraveyas

Dear Editor,

One of the principal complications unexpectedly reported in many clinical studies of multiple myeloma (MM) patients treated with immunomodulatory drugs (IMiD) based regimens is the development of thrombosis. However, the increased risk of venous thromboembolism (VTE) associated with IMiD is only observed as a synergistic effect when thalidomide (Thal) and/or lenalidomide (Len) are given in combination with other drugs, and not as a single-agent therapy for which VTE incidence remains below 5% in both newly diagnosed and relapsed/refractory patients. Specifically, Thal or Len with concurrent dexamethasone (Dex) has been shown to increase the risk of VTE (1). Moreover, an extremely high rate of thrombosis was initially observed in a trial comparing Dex alone versus Dex plus Len in newly diagnosed patients; 9 out of the first 12 patients (75%) enrolled in the Len/Dex arm experienced thromboembolic events (including one ischemic stroke) in the absence of thromboprophylaxis, while no events were observed in the control arm (n=9) (2). Routine thomboprophylaxis is now recommended by the International Myeloma Working Group, European Society for Medical Oncology and American Society of Clinical Oncology for patients with MM who are receiving IMiD-based combination regimens, and as with other areas of thromboprophylaxis, a risk stratified approach is appropriate (3). The exact mechanisms by which chemotherapeutic agents increase VTE risk are predominantly undefined, both in vitro and in vivo. Possible mechanisms involve inducing release of cytokines and subsequently the expression/activity of procoagulant molecules, such as tissue factor (TF), or by reducing the production of endogenous anticoagulant proteins such as protein C and protein S during chemotherapy-induced cell

damage and associated tumour lysis, causing direct damage to the vascular endothelium (4), which may be accompanied by microvesicle (MV) release.

In this study we investigated MV populations through chemotherapy in 15 newly diagnosed MM patients (mean age 69.2 (± 10.6) years) whom received cyclophosphamide, Thal, and Dex (CTD; 21 day cycle; n=4), an attenuated dose of CTD (CTDa; 28 day cycle; n=6), or an attenuated dose of Len, cyclophosphamide, and Dex (RCDa; 28 day cycle; n=1), and relapsed patients whom received Len/Dex (28 day cycle; n=4). Additionally, antibiotics and antiemetic prophylaxis were given to patients during treatment according to local protocols. Since all MM patients received combination therapy with IMiD, they were consequently prescribed concurrent LMWH anticoagulation, specifically a prophylatic dose of dalteparin (typically 5000 U/day). Thus, no symptomatic DVT or pulmonary embolism events were observed in any of the MM patients in this study. However, 2 out of the 15 MM patients (13%) suffered cardiovascular events during chemotherapy; these were newly diagnosed patients receiving either CTD or CTDa and endured fatal stokes. *Both patients were male, a 47 year old with no prior history of thrombotic risk factors, and an 80 year old with history (CABG surgery, TIA, hypertension and angina). Baseline values of TF+MV in these cases were 177 and 348 respectively (the mean for the group was 215 per μL of platelet poor plasma [PPP]).*

Blood samples were taken through chemotherapy (baseline = T1 (n=15), end of 1st cycle = T2 (n=10), end of 2nd cycle = T3 (n=10) and end of chemotherapy = T4 (n=5)) and assessed for endothelial microvesicles (EMV) populations using a FACS Calibur flow cytometer, validated by the ISTH working group on enumeration of MV. A statistical comparison of each of the MV populations measured in this study over time (T1-T4) was performed. Specifically, a marginal model (with no random effects) was fitted to the $\log_e$ transformed data for quantified MV using the SPSS MIXED procedure. An unstructured correlation matrix with no pattern assumed for the variances and covariances of the variable values within a patient at each time point was used. Significance between model-estimated marginal means (T1-T4) for each variable was calculated using paired

Student's t tests. P values < 0.05 were considered to be statistically significant. All statistical analyses were performed with IBM SPSS (v. 20.0).

Baseline populations of MV are shown in Figure 1a. The median (IQR) level of CD105+ EMV at T1 was 563.0 [321.0–744.0]/μl PPP and was unchanged at T2 (605.0 [339.5-1766.3]/μl PPP), but increased at T3 by 2.4-fold (1375.0 [361.3–1794.0]/μl PPP), and remained elevated at T4 (1190.0 [763.5–2184.5]/μl PPP) as shown in Fig 1b. The estimated marginal mean of circulating CD105+ EMV was significantly elevated at T4 in comparison to T1 (P < 0.0005) and T2 (P = 0.001).

The median (IQR) level of CD106+ EMV (391.0 [243.0–631.0]/μl PPP) measured at T1 was shown to sequentially increase, through T2, (599.5 [314.8–1413.0]/μl PPP), T3 (1058.5 [298.8–1514.0]/μl PPP) and T4 (1491.0 [994.5–1588.0]/μl PPP), shown in Fig 1c. CD106+ EMV were significantly elevated at T4 in comparison with T1, T2 and T3 (P = 0.001, P = 0.004, P = 0.031, respectively).

There was a clear trend of increasing levels of circulating CD54+ EMV from T1 (242.0 [179.0–387.0]/μl PPP) to T2, (400.5 [240.0–601.8]/μl PPP) T3 (465.5 [283.8–960.8]/μl PPP), and T4 (680.0 [560.5–764.0]/μl PPP, shown in Fig 1d. CD54+ EMV were significantly elevated at T4 in comparison T1 and T2 (P 0.0005 and P = 0.011, respectively). CD144+ EMV was unchanged through T1 (130.0 [73.0–216.0]/μl PPP; T1) T2 and T3 (145.0 [50.5–422.5]/μl PPP; T2; 128.5 [76.3–287.0]/μl PPP; T3), but increased significantly (p=0.017) at T4 (305.0 [162.0–400.5]/μl PPP), Fig. 1e. CD138+ plasma cell-derived MV numbers were significantly elevated at T4 (P = 0.004) in comparison to T1.

Strong statistical correlations were found to exist between all markers of EMV (Pearson's correlation coefficient r = 0.66 to 0.94, P < 0.0005) indicating that the detected MV were of homogenous/endothelial origin. Correlations between MV of differing origins are shown in Table 1. Changes in individual markers showed some variation but the overall trend was for increased EMV through chemotherapy (all markers significantly correlated) and no change in TF+MV,

although post chemotherapy samples were scarce (n=5) and the statistical test used accounted for missing data.

Elevated plasma levels of EMV reflect endothelial cell injury and are now considered a biomarker of vascular dysfunction (5). Furthermore, previous studies have demonstrated that MV from various cellular origins may also induce endothelial dysfunction (6). In particular, high levels of EMV have been observed in cardiovascular diseases, including acute coronary syndromes (7). While, in VTE, marked activation of the endothelium, platelets, and leukocytes has been reported and shown to involve the generation of EMVand formation of EMV-monocyte conjugates and platelet-leukocyte conjugates (8). In addition, endothelial cell damage has been described as a mechanism for the increased incidence of VTE observed when anti-angiogenic drugs were combined with chemotherapy agents such as cisplatin or gemcitabine (9). EMV shed into the peripheral circulation of MM patients that received chemotherapy with IMiD, either Thal or Len, may be a consequence of endothelial cell injury and/or activation and subsequently may be involved in thrombogenicity associated with anti-cancer agents in vivo.

In this study, 4 newly diagnosed MM patients received pulsed high-dose Dex (40 mg; days 1-4 and 12-15 of a 21 day cycle of CTD) and 4 relapsed MM patients received weekly high-dose Dex (40 mg; days 1, 8, 15, and 22 of a 28 day cycle of Len/Dex). Kerachian et al. demonstrated that treating endothelial cells in vitro with high-dose Dex significantly elevates CD106 and CD54 mRNA expression levels (10). In addition, studies have reported that Dex modulates the expression of endothelial haemostatic elements; specifically inducing vWF and TF (10), while down-regulating thrombomodulin (10). Furthermore, EMV derived from TNF-α-stimulated endothelial cells have been shown to bind to monocytes in vitro, involving the interaction between CD54 on EMV and β2 intergrin on monocytes, which induces TF-dependent PCA in monocytes (11). In our study, TF+MV showed no significant change in expression upon chemotherapy administration (Fig. 1f) and this is supported by similar studies that show a general absence of TF expression in plasma cells in MM, in contrast to solid malignancies (12).

In this study, the number of circulating plasma cell-derived MV or more specifically, tumour cell-derived MV in MM patients were elevated 6-8 weeks after chemotherapy with IMiD, either Thal or Len, relative to baseline, suggesting that the tumour cells have undergone apoptosis in the presence of cytotoxic agents as the number of tumour cell-derived MV correlated with the number of PS+ MV (Table 1). In addition, previous studies have shown that human acute monocytic leukaemia THP-1 cells exposed to daunorubicin *in vitro* release higher numbers of MV than untreated tumour cells (13).

Despite the relatively small size of the study population, the data presented in this pilot study suggests that the host response to treatment may contribute to the relatively increased thrombogenicity observed in MM patients who received chemotherapy in combination with IMiD, either Thal or Len. This is a markedly different procoagulant profile than solid malignancies. However, further studies are justified to evaluate the impact of circulating MV in chemotherapy-associated VTE in cancer patients. In this study, we have demonstrated changes in MV populations occur throughout chemotherapy on MM patients, and suggest that these differences are due to the influence of IMiD-based chemotherapy. The finding of elevated EMV following administration of IMiD-based chemotherapy lends support to previous studies in the literature that suggest further clinical evaluation for the role of EMV as biomarkers of potential or ongoing thrombosis.

Acknowledgement

JW was funded by Yorkshire Cancer Research (Hul001PhD).

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Rajkumar SV, Blood E, Vesole D, Fonseca R, Greipp PR. Phase III clinical trial of thalidomide plus dexamethasone compared with dexamethasone alone in newly diagnosed multiple myeloma: A clinical trial coordinated by the eastern cooperative oncology group. J Clin Oncol. 2006;24(3):431-6.
2. Zonder JA, Barlogie B, Durie BG, McCoy J, Crowley J, Hussein MA. Thrombotic complications in patients with newly diagnosed multiple myeloma

treated with lenalidomide and dexamethasone: benefit of aspirin prophylaxis. Blood. 2006;108(1):403; author reply 4.
3. Lyman GH, Khorana AA, Kuderer NM, Lee AY, Arcelus JI, Balaban EP, et al. Venous thromboembolism prophylaxis and treatment in patients with cancer: American Society of Clinical Oncology clinical practice guideline update. J Clin Oncol. 2013;31(17):2189-204.
4. Falanga A, Marchetti M. Anticancer treatment and thrombosis. Thromb Res. 2012;129(3):353-9.
5. Burger D, Touyz RM. Cellular biomarkers of endothelial health: microparticles, endothelial progenitor cells, and circulating endothelial cells. J Am Soc Hypertens. 2012;6(2):85-99.
6. Brodsky SV, Zhang F, Nasjletti A, Goligorsky MS. Endothelium-derived microparticles impair endothelial function in vitro. Am J Physiol Heart Circ Physiol. 2004;286(5):H1910-5.
7. Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, et al. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. Circulation. 2000;101(8):841-3.
8. Chirinos, J. A., et al. (2005). "Elevation of endothelial microparticles, platelets, and leukocyte activation in patients with venous thromboembolism." J Am Coll Cardiol 45(9): 1467-1471.
9. Kuenen, B. C., et al. (2002). "Dose-finding and pharmacokinetic study of cisplatin, gemcitabine, and SU5416 in patients with solid tumors." J Clin Oncol 20(6): 1657-1667.
10. Kerachian MA, Cournoyer D, Harvey EJ, Chow TY, Neagoe PE, Sirois MG, et al. Effect of high-dose dexamethasone on endothelial haemostatic gene expression and neutrophil adhesion. J Steroid Biochem Mol Biol. 2009;116(3-5):127-33.
11. Sabatier F, Roux V, Anfosso F, Camoin L, Sampol J, Dignat-George F. Interaction of endothelial microparticles with monocytic cells in vitro induces tissue factor-dependent procoagulant activity. Blood. 2002;99(11):3962-70.
12. Cesarman-Maus G, Braggio E, Maldonado H, Fonseca R. Absence of tissue factor expression by neoplastic plasma cells in multiple myeloma. Leukemia. 2012;26(7):1671-4.
13. Boles JC, Williams JC, Hollingsworth RM, Wang JG, Glover SL, Owens AP, 3rd, et al. Anthracycline treatment of the human monocytic leukemia cell line THP-1 increases phosphatidylserine exposure and tissue factor activity. Thromb Res. 2012;129(2):197-203.

Table 1

Statistical correlations between MV of differing cell origin.

Figure 1

Quantified MV numbers at (A) entry to the study and (B-F) baseline through chemotherapy of specific markers (B) CD105, (C) CD106, (D) CD54, (E) CD144 (F) and (F) Tissue factor. The number of samples available were as follows T1; n=15, T2; n=10, T3; n=10 and T4; n=5. Platelet free citrated plasma was isolated

by 2-step centrifugation and 25mL of sample was incubated with 5mL of specific antibody for 30 minutes prior to addition of counting beads (25mL) and filtered PBS (150mL). A MV gate was setup based on Megamix SSC beads (Biocytex) using ISTH protocol. BD FACSCaliburs were used running CellQuest Pro software.

Authors

Jessica Hall

M. Adeola Adesanya

Hull York Medical School, University of York and University of Hull, Hull, UK

Eric Gardiner

Department of Psychological Health and Wellbeing, University of Hull, Hull, UK

Leigh Madden

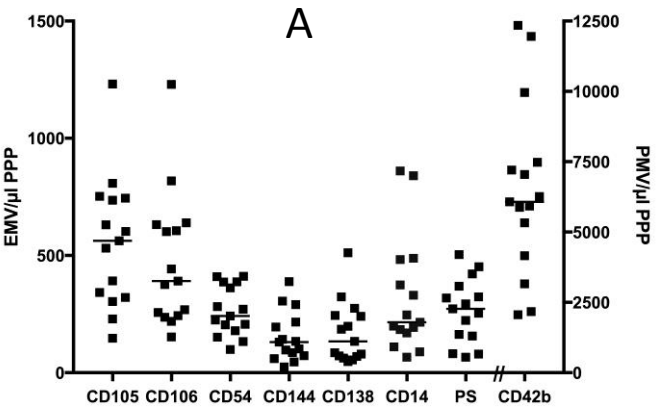
School of Biological, Biomedical and Environmental Sciences, University of Hull, Hull, UK. Corresponding author

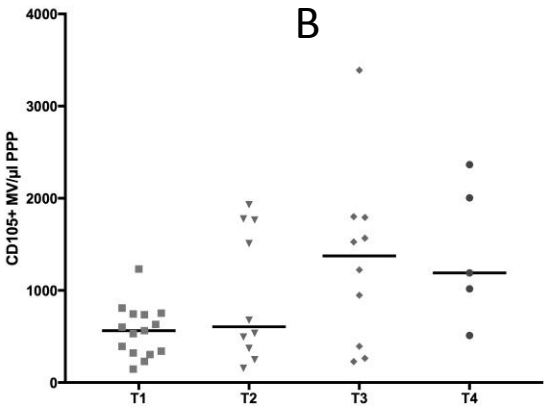
E-mail address l.a.madden@hull.ac.uk

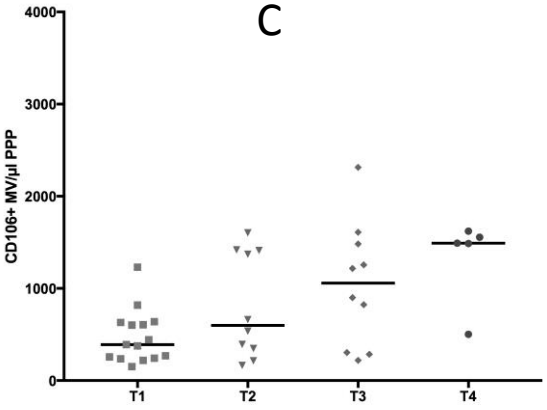
Haz Sayala

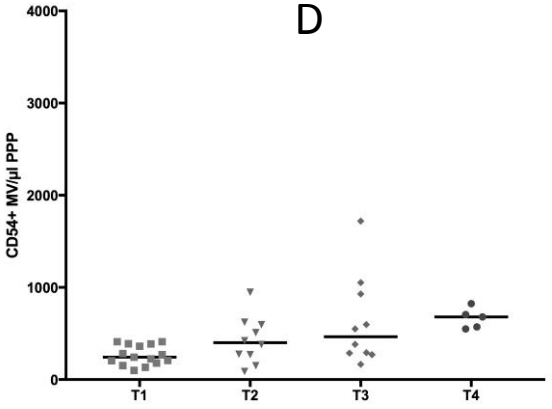
Anthony Maraveyas

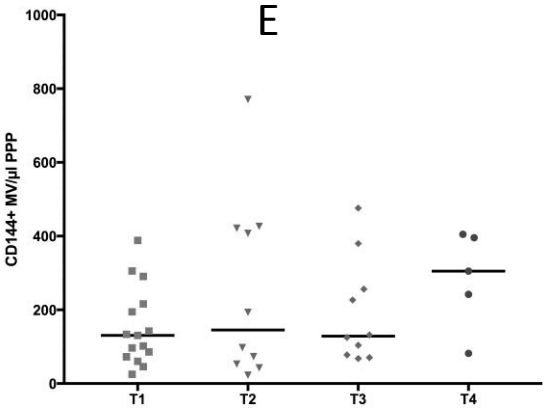
Queen's Centre for Oncology and Haematology, Castle Hill Hospital, Cottingham, UK

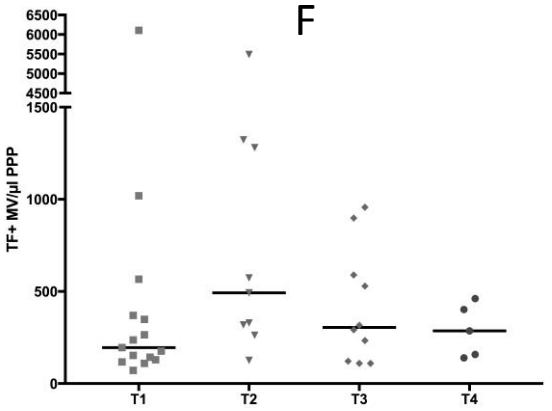

A
EMV/µl PPP
PMV/µl PPP
1500
1000
500
0
12500
10000
7500
5000
2500
0
CD105
CD106
CD54
CD144
CD138
CD14
PS
CD42b


B
CD105+ MV/µl PPP
4000
3000
2000
1000
0
T1
T2
T3
T4


C
CD106+ MV/µl PPP
4000
3000
2000
1000
0
T1
T2
T3
T4


D
CD54+ MV/µl PPP
4000
3000
2000
1000
0
T1
T2
T3
T4


E
CD144+ MV/µl PPP
1000
800
600
400
200
0
T1
T2
T3
T4


F
TF+ MV/µl PPP
6500
6000
5500
5000
4500
1500
1000
500
0
T1
T2
T3
T4

Correlations between subtypes of MV	Pearson's correlation coefficient (*r*)	P value (two-tailed)
PMV and monocyte-derived MV CD42b and CD14	0.31	P = 0.053
PMV and plasma cell-derived MV CD42b and CD138	0.26	P = 0.11
PMV and PS+ MV CD42b and annexin V	0.63	P < 0.0005
PMV and TF+ MV CD42b and CD142	-0.25	P = 0.11
Monocyte-derived MV and plasma cell-derived MV CD14 and CD138	0.63	P < 0.0005
Monocyte-derived MV and PS+ MV CD14 and annexin V	0.54	P < 0.0005
Monocyte-derived MV and TF+ MV CD14 and CD142	0.03	P = 0.85
Plasma cell-derived MV and PS+ MV CD138 and annexin V	0.51	P = 0.001
Plasma cell-derived MV and TF+ MV CD138 and CD142	0.34	P = 0.03
PS+ MV and TF+ MV Annexin V and CD142	-0.05	P = 0.75
EMV and PMV CD105 and CD42b	0.39	P = 0.01
EMV and monocyte-derived MV CD105 and CD14	0.85	P < 0.0005
EMV and plasma cell-derived MV CD105 and CD138	0.54	P < 0.0005
EMV and PS+ MV CD105 and annexin V	0.53	P < 0.0005
EMV and TF+ MV CD105 and CD142	0.11	P = 0.49

Table 1