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Circulating microvesicles are less procoagulant and carry different miRNA cargo in myelodysplasia☆

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ABSTRACT

Background and aims: Myelodysplasia (MDS) is characterised by abnormal haematopoiesis and increased risk of bleeding. Microvesicles (MV) play a key role in coagulation and their impact in MDS is unknown.
Methods: Platelet free plasma from 35 red-cell transfusion-dependent MDS patients and 15 controls were analysed. Pro-coagulant function was assessed by the XaCT assay and by thrombin generation (ETP). Total MV were enumerated by nano-tracking analysis. MV subsets were quantified by flow cytometry after staining with specific antibodies for various endovascular cell types. Small RNA was quantitated and sequenced. The MV measurements were correlated with MDS clinical risk scores and level of transfusion dependence.
Results: The pro-coagulant function of MV was significantly lower in MDS. All the MV subtypes, as measured by flow cytometric markers, were also significantly lower. The small RNA and miRNA cargo were significantly higher in MDS. The miRNA profile showed that mir-28 and mir-LETD7 were under expressed whilst mir-584J and mir-4485 were over expressed in MV from MDS.
Conclusions: Circulating MV in MDS show reduced pro-coagulant functional activity, reduced subtypes by flow cytometry and significantly different miRNA content. However, the levels or subtypes of MV did not predict the clinical phenotype or level of transfusion dependence.

1. Introduction

Myelodysplasia (MDS) is a bone marrow dysfunction characterised by disturbed haematopoiesis. Although there are increased numbers of bone marrow precursors in most patients, abnormal haemopoietic maturation leads to fewer red, white blood cells and platelets [1]. Along with clinical features of anaemia, neutropaenia, and thrombocytopaenia, a subset of patients with MDS will experience serious bleeding, and this may not necessarily be dependent on platelet counts [2,3].

Experimental data suggests that both platelet aggregation and phospholipid surface to facilitate thrombus formation are equally important in maintaining normal haemostasis [4]. There is evidence to suggest that high numbers of circulating microvesicles, even in the setting of low platelets, may overcome platelet insufficiency and provide adequate haemostasis. For example, studies show that patients with idiopathic thrombocytopaenic purpura who have very low levels of platelets along with high MV levels do not have clinical bleeding [5]. The ability to generate adequate MV in this instance provides sufficient membrane surface for activated coagulating factors Xa and Va to produce thrombin and generate a fibrin plug in spite of low platelets [6]. Other mechanisms to explain how the insufficiency of platelets is overcome include platelet interaction with leukocyte-derived MV, in models of severe thrombocytopaenia [7]. In a rare clinical context, low levels of circulating MV is associated with bleeding, as observed in Scott syndrome - a disorder with defective vesiculation of platelets [8].

Circulating MV also carry a cargo of small and miRNA which has potential to influence the bone marrow microenvironment as well as the endovascular compartment [9]. Free miRNA in circulation

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complexes with many RNA binding proteins, which regulate its integration in tissue. However, miRNA in MV is unbound and readily available to regulate nuclear transcription and influence cell function [10]. MDS is often characterised by marked increase in apoptosis and resultant vesicular debris/bodies in the marrow generating abnormal levels of MV [11]. We therefore hypothesized that both levels and functional attributes of circulating MV in MDS, as well as their small/miRNA cargo, contribute to the pathophysiology of the disease. Hence the aim of the study was to examine these aspects of MV from MDS compared to normal healthy subjects.

2. Methods

After informed consent, samples were collected from 35 MDS and 15 age similar healthy subjects. Ethics approval was obtained from Hunter New England local health district research ethics committee (06/12/13/5.05).

The healthy controls were voluntary blood donors and these subjects were screened for any significant medical conditions. The control samples were collected in a citrated tube and processed in a manner similar to the MDS cohort.

2.1. Sample preparation

Peripheral blood was collected in 0.109 M tri-sodium citrate and platelet-free plasma was prepared by double centrifugation of whole blood for 15 min at 2500 $\times g$ at room temperature. All samples were processed within 2 h of collection, and aliquots were stored at −80 °C for further batched analysis. Samples were then thawed at 37 °C, 15 min before testing. In the transfusion dependent MDS cohort, blood samples were collected at the nadir of their blood counts i.e. just before the next transfusion.

2.2. Functional coagulation based studies

The factor Xa activation test (XaCT) was performed using the commercially available XaCT test kit (Haematex, Australia) [12]. The measurement of procoagulant activity of MV is based on the ability of vesicles expressing phosphatidylserine to generate Xa. It was performed in duplicate on an automated coagulation analyser (SYSMEX CA-1500; Dade Behring, Newark, DE USA) and results read from a standard curve generated by dilution of a plasma calibrator (expressed in ng/ml).

2.3. Calibrated Automated Thrombogram measurements

Thrombin generation experiments were performed at 37 °C on a fluorometer (Fluoroskan Ascent, Thermo Electron Corporation, Vantaa, Finland) and analysed using the Calibrated Automated Thrombogram (CAT). A sample volume of 80 µl was incubated with 20 µl calibrator or 20 µl specific MP-reagent (Thrombinoscope, Maastricht, The Netherlands) which has been reported to be sensitive to tissue factor bearing MV [13]. Thrombin generation was measured in real time and analysed by the Thrombinoscope software version 3.0029 (Thrombinoscope, Stago Group, Maastricht, The Netherlands) for the endogenous thrombin potential (ETP).

2.4. Nanoparticle tracking analysis (NTA)

The MV enumeration by nanotracking was undertaken on a Nanosight NS500 instrument (Malvern instruments, Malvern, United Kingdom). The scatter and the fluorescence measurements were recorded after incubation with Qdot 625 stain (Life Technologies/Thermo Fisher Scientific, MA, USA) in a dilution of 1:100 with PBS. The capture settings such as camera, focus and gain, were optimized so that particle tracks were clearly visible. If the capture was suboptimal (i.e. event capture rate either < 20 or > 200 tracks) further dilutions were undertaken so that at least 100 completed tracks were recorded [14]. Measurements were taken in triplicate, in scatter and fluorescent mode, for analysis using the Nanosight software (version 2.3 and 3.1); the captured video data was analysed for scatter events (total) and the fluorescent events (total).

2.5. Flow cytometry

Antibodies used included platelet marker CD41a-PE (Clone HIP8, BD Biosciences, CA, USA), red cell marker CD235a-APC (Clone GA-R2, BD Biosciences, CA, USA), endothelial marker CD105-PE (Clone IG2, Beckman Coulter, Marseille Cedex, France), CD14-PC (Clone RMO52, Immunotech, Marseille, France) and tissue factor TF-FITC (Clone VD8, American diagnostics Inc., CT, USA). A 10 µl aliquot of platelet-free plasma in a final volume of 100 µl of PBS (phosphate buffered saline) was taken and incubated (in the dark) with appropriate antibody or isotype control at room temperature for 15 min. For experiments with annexin V-APC (eBioscience, CA, USA), used for marking phosphatidylserine (PS), this incubation was done in a total 50 µl of binding buffer. Then the sample was diluted to 400 µl with filtered PBS or 450 µl of calcium rich buffer respectively. Prior to the analysis, a predetermined number of 10 µm enumeration beads (CountBright beads, Molecular Probes, Life Technologies, Oregon, USA) were added. The flow cytometer was standardized as per the 'ISTH workshop for standardization of flow cytometry for Microparticles' [15]. The gating was performed using Megamix beads (Biocytex, Marseille, France) on a BD FACS Canto instrument (BD Biosciences, San Jose, California, USA) A total of 35 MDS and 11 control samples were analysed, after exclusion of 4 control samples with poor staining. The analysis was undertaken on FACSDiva software.

2.6. Small RNA quantitation

MV were pelleted by centrifuging 1.2 ml of plasma at 21,000 $\times g$ for 60 min at 4 °C. Then small RNA was extracted using the Norgen kit (Norgen Biotek, ON, Canada) for RNA extraction using a slurry based method according to the manufacturer's instructions. The 100 µl of RNA containing eluate was reduced to a final volume of about 7 µl with a speedy-vac. For each sample, 1 µl of the concentrated RNA eluate was used for measurement of small RNA concentration by Agilent Bioanalyzer Small RNA Assay using Bioanalyzer 2100 Expert instrument (Agilent Technologies, Santa Clara, CA).

2.7. miRNA profiling

2.7.1. NGS Library generation and sequencing

Small RNA libraries were constructed with the CleanTag Small RNA Library Preparation Kit (TriLink, Cat# L-3206) according to the manufacturer's protocol. The final purified library was quantified with High Sensitivity DNA Reagents (Agilent Technologies, PO# G2933-85004) and High Sensitivity DNA Chips (Agilent Technologies, PO# 5067-4626). The libraries were pooled, and the 140 bp to 300 bp region was size selected on an 8% TBE gel (Invitrogen by Life Technologies, Ref# EC6215). The size selected library was quantified with High Sensitivity DNA 1000 Screen Tape (Agilent Technologies, PO # 5067-5584), High Sensitivity D1000 reagents (Agilent Technologies, PO# 5067-5585), and the Tailor Mix HT1 qPCR assay (SeqMatic, Cat# TM-505), followed by a NextSeq High Output single-end sequencing run at SR75 using NextSeq 500/550 High Output v2 kit (Cat #FC-404-2005, Illumina, San Diego, CA) according to the manufacturer's instructions.

2.7.2. Bioinformatic analysis

Data consists of single end 76 bp long reads, on 30 samples (10 normal/controls and 20 MDS patients in whom sufficient plasma aliquots were available) for small RNA isolated from MV fraction within the plasma samples. Based on the small RNA species composition,

quality control data, hierarchical clustering based on raw counts, four samples were excluded from the control group due to the higher than 75% proportion of unknown/unmapped reads to identified features. A further six samples from the MDS group were also excluded due to high unknown proportion/unmapped reads. The final cohort for miRNA expression analysis comprised of 6 normal and 14 MDS samples. Data was contig-quantised into regions longer than 20 bp with a maximum gap of 20 bp and at least two reads using Seqmonk v1.41. 85% of contigs had length < 116 bp, and 13.5% of length between 116 bp and 208 bp. Annotation to known features was performed on the significant results.

2.8. Statistical considerations

The statistical analysis was performed using Prism 7 (GraphPad, OK USA). The variables in the data set were evaluated for normality of distribution by a normality test such as the Kolmogorov-Smirnov test to decide whether a nonparametric rank-based analysis or a parametric analysis should be used. The differences between any two groups was assessed by the Mann-Whitney *U* test, and between repeat measures by ANOVA with a p-value < 0.05 considered as statistically significant.

Differential expression analysis was performed in the R statistical environment on contig features using the edgeR and DESeq2 packages, contig counts normalised to library size. Consensus between methods was higher than 70% at the 0.05 FDR level. Significant contig features were then annotated to transcripts from the ENSEMBL GRCh38v90 reference. Two analysis algorithms were used - Bowtie2 and BLAST with parameters for small RNA alignment. Results were considered significant only in those sequences which showed significance across the two algorithms. The significance was set at a p value of 0.05 using adjusted p values for an optimized false discovery rate approach.

3. Results

3.1. Patient characteristics (MDS)

There were 35 MDS subjects, all red cell transfusion-dependent, who were recruited prospectively into the study. The demographic data for the normal and MDS cohorts is shown in Table 1 - the bone marrow characteristics and IPSS risk scores were obtained at diagnosis. The time from diagnosis ranged from < 6 months to 5 years with majority of diagnosis within 2 years (65%, 23/35). The age is recorded at the time of the MV sample collection. The mean age of MDS patients was 78 years (range 56–91) which was significantly higher than the mean age of the normal subjects was 65 years (range 60–73). The full complement of clinical details, including full blood count parameters and WHO bleeding score, is also provided (see Supplementary Table 1).

Table 1
Baseline demographics for normal and MDS. The risk categories for MDS are also detailed. Results presented as median and range for all continuous variables.

	Normal (n = 15)	Range	MDS (n = 35)	Range	p value
Age years	64	60–73	78	56–91	< 0.0001
Gender (M, %)	47		75		0.0049
Hb g/l	127	116–145	84	59–120	< 0.0001
White cell counts 10^9/l	5.4	4.3–8.6	4	1–18	0.1315
Platelets 10^9/l	201	111–313	79	4–434	0.0043
IPSS (n, %) n = 28					
Low			10 (36)		
Intermediate 1			11 (40)		
Intermediate 2			5 (17)		
High			2 (7)		
IPSS-R (n, %)					
Very low			3 (10)		
Low			10 (35)		
Intermediate			9 (43)		
High			4 (15)		
Very high			2 (7)		

3.2. MV in MDS - functional studies

The procoagulant function by ETP and XaCT were both significantly lower in MDS (p < 0.0001 for both) as shown in Fig. 1. The median ETP in MDS was 1094 RFU (relative fluorescence units)/min (IQR of 933–1212) which was nearly half that of the 2190 RFU/min (IQR 1611–2660) measured in normal subjects. Similarly, the median phospholipid measured by XaCT was 438 ng/ml (IQR of 352–525) in MDS, compared to 885 ng/ml (IQR of 659–1035) in normal subjects.

3.3. MV characterisation - nanotracking analysis

In an effort to explain the lower ETP and phospholipid content of the MDS plasma, NTA was used to quantify the absolute number and size distribution of the MV it contained. Surprisingly, the distribution of MV in the size ranges 0–200 nm and 200–400 was similar between MDS and normal subjects as depicted in Fig. 2 (ANOVA one way for repeated measures, p > 0.05 for all comparisons).

In MDS, the median MV levels were noted to be 8.4×10^9/μl (IQR 6–19) in scatter and 4.4×10^9/μl (IQR 3.15–9.4) in fluorescent modes. The scatter mode was undertaken to evaluate all small particles (membrane bound and other particles) whilst the fluorescent mode (using Qdot 625) is specific for biological vesicles.[16]The median measurements for MV in normal subjects was similar - 8.4×10^9/μl (IQR of 1.8–18) in scatter and 7.3×10^9/μl (IQR of 2.6–10) in fluorescent mode. Nanotracking measurements were not significantly different between normal and MDS subjects for both scatter and fluorescent modes (see Fig. 2). There was no significant correlation observed between the MV levels (scatter or fluorescent) by nanotracking analysis and results obtained from functional assays (ETP and XaCT).

3.4. MV subsets in MDS - flow cytometry

Amongst the MV subtype levels tested, CD41, CD105, CD235, TF, annexin V and CD14 expressing MV were significantly lower in MDS compared to normal subjects (see Fig. 3 and Supplementary Table 2). The most common MV subtype in MDS was CD235 red cell MV with a median MV level of 22/μl (IQR of 13–45). On the other hand, CD41 expressing platelet MV were the most abundant MV in normal subjects and showed a median of 255/μl (interquartile range or IQR of 123–834/μl).

3.5. Small RNA cargo in MV from MDS

The quantities of small RNA and miRNA in MV from MDS patients were approximately twice that of the control plasma MV. In MDS, small RNA content was estimated to be 1037 pg/μl (IQR 6161–1921) and miRNA 297 pg/μl (123–581) respectively, which was significantly higher compared to small RNA 458 pg/μl (IQR 175–658) and miRNA content of 129 pg/μl (IQR 23–278) in normal subjects (p = 0.0005 and p = 0.02 respectively; as shown in Fig. 4). The miRNA normalised to mean MV was also compared and the miRNA content continued to be significantly higher in the MDS cohort (p = 0.014).

3.6. MV and correlation with blood counts clinical phenotype, bleeding and transfusion requirements in MDS

Correlation between MV subtypes/functional and blood counts showed that platelet counts correlated positively with platelet MV and inversely with WHO grade 3 or 4 bleeding scores respectively (Supplementary Table 3B). None of the other MV parameters correlated

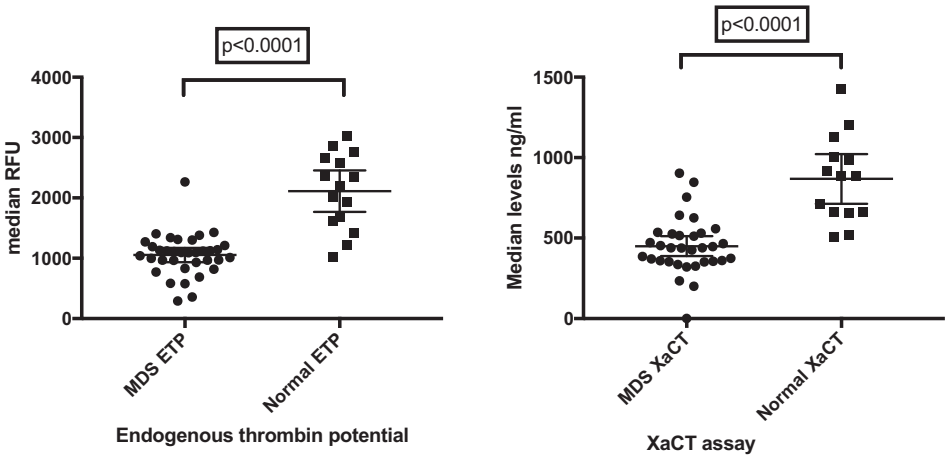

Fig. 1. Comparison of MV parameters by functional evaluation by ETP and XaCT assays between MDS (n = 35) and normal subjects (n = 15). The median ranks and 95% CI are depicted.

with blood counts or WHO grade 3 or 4 bleeding. A similar analysis was also performed in the normal subjects which did not show any significant correlations (Supplementary Table 3A).

The IPSS (international prognostic scoring system) and IPSS-R (in international prognostic scoring system- revised) risk scores were correlated with MV quantitation by flow cytometry, functional, NTA or miRNA analysis in MDS (see Supplementary Table 4). The IPSS-R score negatively correlated with CD235 red cell MV. It was also observed that PEAK thrombin, a functional MV parameter, correlated with IPSS-R and IPSS ($p = 0.048$ and $p = 0.039$ respectively). The XaCT test, which measures phospholipid MV contribution to coagulation, inversely correlated with IPPS score. The prognostic scores, the cytogenetic risk and severity of the WHO bleeding scores did not correlate with any other MV subsets, small RNA levels or MV levels measured by NTA analysis.

A subgroup analysis comparing MDS patients requiring red cell transfusions every 4 weeks or less (n = 5) compared with those requiring a red cell transfusion beyond 4 weeks (n = 4) was undertaken. These patients were selected as they all had platelets > 50 × 10^9/l and were not platelet transfusion dependent. No significant differences between the MV levels in these two groups of MDS by flow cytometry, functional, NTA analysis or small RNA cargo were observed.

3.7. miRNA expression analysis

The miRNA analysis showed a total of two mirs were under expressed whilst two others were over expressed. The differential expression specifically showed that mir-28 and mir-LET7D were under expressed whilst the mir-548J and mir-4485 were overexpressed in the MDS cohort compared to the normal cohort. The relation of these mirs to MDS and platelet activity was not known at the time of the analysis.

4. Discussion

MDS is a clonal bone marrow disorder associated with abnormal blood counts, transfusion dependence and risk of progression to leukemia. It is characterised by morphologically abnormal precursors in the bone marrow and increased apoptosis due to ineffective haemopoiesis. The evaluation of circulating MV in MDS has previously not been undertaken.

4.1. MV in MDS by functional coagulation, flow cytometry and NTA analysis

The bleeding diathesis of MDS is not completely explained by thrombocytopaenia. Our results show significantly lower procoagulant MV function by both ETP and XaCT tests, and lower CD41 MV (platelet derived) by flow cytometry compared to normal even when adjusted for platelet count. This is consistent with both a reduced production of large MV and reduced MV function. This explains a further part of the bleeding in MDS and presumably reflects the abnormal megakaryopoiesis that is often observed in MDS. This deficiency in 'procoagulant MV' in MDS is likely to be contributing to higher bleeding risk apart from the thrombocytopaenia alone.

However, in our small series, the variable nature of bleeding in our patients with MDS is not explained by the variation in CD41 MV as shown by the similarity of results in the 2 groups, one with increased blood transfusion requirements, and one without. As anticipated, platelet counts correlated with platelet MV and inversely with WHO grade

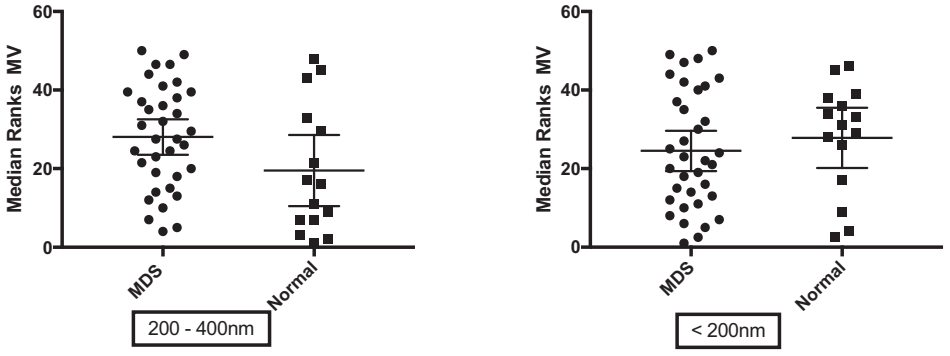

Fig. 2. Comparison of MDS (n = 35) and normal MV (n = 15) levels for 200–400 nm and < 200 nm using NTA in fluorescent mode. The median ranks and 95% CI are depicted.

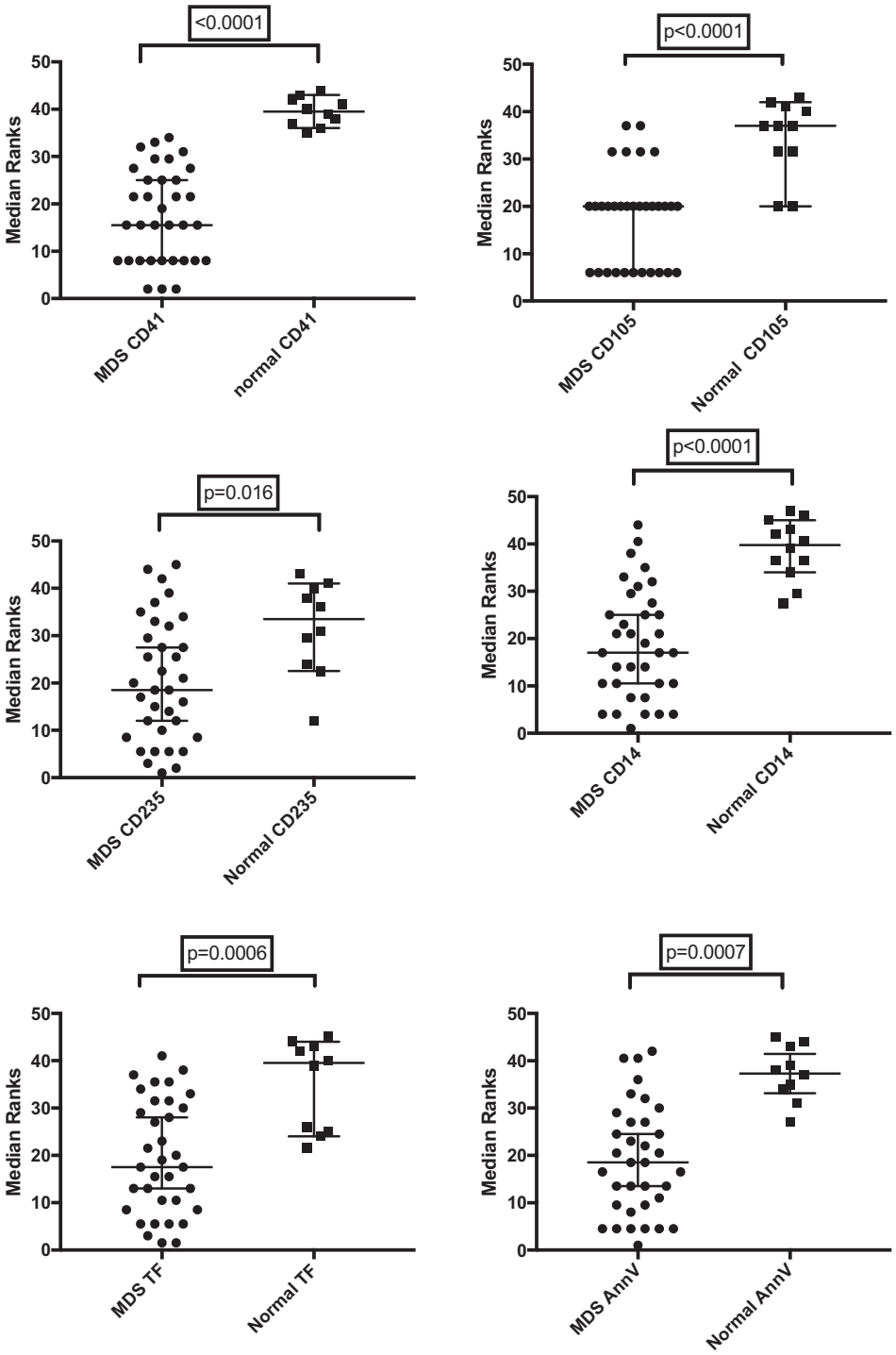

Fig. 3. Comparison of MV parameters by flow cytometry between MDS (n = 35) and normal subjects (n = 11). The median ranks and 95% CI are depicted.

3 or 4 bleeding scores respectively indicating that low platelet counts were associated with more clinically significant bleeding. In other studies, in non-MDS patients, the procoagulant function and flow cytometry results have been shown to correlate [17,18]. It is likely that the MV detected by flow is only the larger 'procoagulant functional' MV.

In MDS where there is significant apoptosis and abnormal megakaryocytes, it might have been expected that MV would be normal to increased. NTA measuring small MV shows normal numbers as predicted, but flow shows a marked reduction in large MV particularly platelet derived. This aspect will need further investigation with an increase in subject numbers to confirm these results and determine the origins of the smaller MV.

It could also be postulated that the MV levels/function in circulation do not completely reflect the MV generation in the bone marrow (BM). This study did not examine any BM samples. This may be technically difficult given the way BM is collected.

4.2. MV and correlation with MDS risk scores and frequency of red cell transfusions

The clinical risk scores (IPSS and IPSS-R) were correlated with the

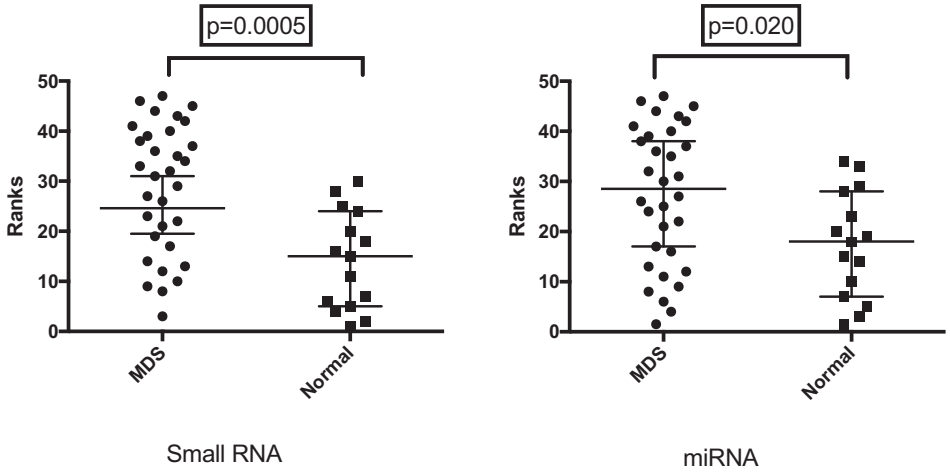

Fig. 4. Comparison of total small and miRNA isolated from MV between MDS (n = 35) and normal subjects (n = 15). The median ranks and 95% CI are depicted, normalised to small and miRNA obtained per 1 μl of MV pellet from 1.2 ml of plasma.

MV numbers and function. These clinical risk scores - IPSS and IPSS-R, are calculated on a combination of cytopaenias, bone marrow blast percentage and cytogenetic abnormalities. The IPSS-R, a more recent prognostic score, has cytopaenias defined in greater detail than IPSS [19]. The IPSS-R correlated negatively with CD235 MV as detected by flow cytometry indicating higher risk score was associated with lower red cell MV (Supplementary Table 4). In this study, there was a correlation between IPSS-R/IPSS and functional assessment of MV (PEAK thrombin and XaCT tests). However, the clinical significance of these observations is uncertain. A small subgroup analysis did not show any obvious differences in those who had a higher frequency of red cell transfusions. This finding needs to be evaluated in a larger cohort.

4.3. miRNA in MV

The total small RNA and miRNA content in MV was significantly higher in MDS patients compared to normal. In fact, the small RNA and miRNA contained were approximately twice that of the control plasma MV. These continued to be significantly higher in MDS even after normalisation to MV by NTA.

This study subsequently focussed on analysis of miRNA within MV as they have been shown to have an important role in transcription regulation and potentially transferrable between cells of the endovascular system even at distant sites [11,20]. We found that differential expression of several species of miRNA was different between the normal and MDS cohorts.

Specifically, mir-28 and mir-LET7D were under expressed. The mir-28 controls cell proliferation and post transcriptional gene silencing. It is reported to target the regulation of the thrombopoietin receptor, being overexpressed in patients with high platelets (in a subset of myeloproliferative disorders) [21]. Elevated mir-28 has also been reported to be been significantly associated with thrombosis/pulmonary embolism [22]. Under expression of mir-28 could therefore contribute to the thrombocytopaenia and/or bleeding by influencing platelet production/function and endothelial cell proliferation.

Interestingly mir-LET7D has been found in isolates of exosomal RNA in certain tissues and secretions (saliva and breast milk) though its functional annotation is not clear [23]. The detection of this mir-LET7D provides surrogate evidence that the miRNA isolated from MV in this study are indeed the species specific to MV and/or exosomes.

It was also observed that mir-548J and mir-4485 were over-expressed. Interestingly, mir-548J is associated with breast cancer cell invasion and metastasis [24]. Its role in myelodysplasia is not clear and needs evaluation in a larger cohort to study association with disease risk, progression and clinical behaviour. The role of mir-4485 has been reported to be under expressed in patients with chronic hepatitis and is reported as a marker for progression of liver disease; its role in MDS is not understood [25].

The small sample size and lack of a validation cohort for the RNA analysis are the main limitations of this study. In addition, the age of the normal cohort was lower than the MDS cohort and this was unavoidable given the few 'normal healthy' subjects above the age of 65 years who could be recruited into the study. This could have influenced MV results, however, previous studies have shown that older age cohorts (> 60 years) have a relatively uniform range of MV [26]. This study did not include any marrow samples for MV measurement to evaluate the differences between plasma and bone marrow MV but this would be a technically challenging exercise to undertake.

5. Conclusions

This analysis of MV in MDS shows significant differences in levels, function and small RNA content as compared to healthy normal controls. The lower levels of MV subtypes by flow cytometry and lower procoagulant function of MV in MDS appear to be a hallmark of this disease process. The clinical IPSS-R risk score correlated with the functional ETP parameter, however, quantitative or functional differences in MV did not correlate with level of transfusion dependence. The under and over expression of expression of miRNA in circulating MV in MDS is hypothesis generating and should be further explored.

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AKE, LL, MS and GI conceived the project; GI provided access to samples and in interpretation of data; MS provided access to coagulation testing and flow cytometry; AKE carried out the project and data analysis; AKE, LL, MS and GI were responsible for data interpretation and manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcmd.2018.11.001.

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