Procoagulant tumour microvesicles attach to endothelial cells on biochips under microfluidic flow.

Abdulrahman Algarni [1], John Greenman [1] and Leigh A Madden [1,*]

Department of Biomedical Science, The University of Hull, Hull, HU6 7RX, UK

* Correspondence
Leigh A Madden,
Department of Biomedical Science,
The University of Hull,
Cottingham Road,
Hull,
HU6 7RX, UK
l.a.madden@hull.ac.uk; Tel.: 441482466031

Abstract

Tumour patients are at a high risk of venous thromboembolism (VTE) and the mechanism by which this occurs may involve tumour derived microvesicles (MV). Previously, it has been shown that tumour MV become attached to endothelial cells in static conditions. To investigate whether this process occurs under physiologically relevant flow rates, tumour MV were perfused across a microfluidic device coated with growing human umbilical endothelial cells (HUVECs). Cell lines were screened for their ability to form tumour spheroids and two cell lines, ES-2 and U87 were selected, formed spheroids were transferred to a microfluidic chip and a second endothelial cell biochip was coated with HUVECs and the two chips linked. Media was flowed through the spheroid chip to the endothelial chip and procoagulant activity (PCA) of the tumour media was determined by one-stage prothrombin time assay.
Tumour MV were also quantified by flow cytometry before and after interaction with HUVECs. Confocal images showed HUVECs acquired fluorescence from MV attachment. Labelled MV were proportionally lost from MV rich media with time when flowed over HUVECs and was not observed on a control chip. The loss of MV was accompanied by a proportional reduction in PCA. Flow cytometry, confocal microscopy and live flow imagery captured under pulsatile flow confirmed an associated between tumour MV and HUVECs. Tumour MV attached to endothelial cells under physiological flow rates which may be relevant to the VTE pathways in cancer patients.

Keywords: thrombosis; microparticles; microvesicles; endothelium

Introduction

Microfluidic technology is already showing potential in areas related to medicine and medical diagnostics through the manipulation of biological samples and the use of miniaturized devices [1]. Microfluidic culture systems are able to function, assess, and provide data for nanoenvironments through their ability to mimic *in vivo* biological systems onto closely resembling *in vitro* microfluidic environments [2]. Moreover, microfluidics has the ability to handle microliter volumes in microchannels of 1 μm to 1000 μm and where fluid flow is strictly laminar and concentrations of molecules can be well-controlled. Since the early 1990s, this technology has been used to for biological research methods for specific analyses such as polymerase chain reaction and DNA microarrays and also appears to be an ideal tool for the study of cancer [3]. As such microfluidics has been utilised to study tumour biopsies.

Tumours are known to release subcellular extracellular vesicles (EV) composed of both larger (100-1000 nm) microvesicles (MV) and nano-sized exosomes (<100nm) into the bloodstream [4, 5]. MV are shed from cells via a number of pathways such as apoptosis and membrane remodelling [6, 7]. MV are released into the circulation where they can then be detected in blood samples using a standardised flow cytometry technique [8]. MV were originally thought to be simply inert cellular debris but they have been found to play a number of roles depending on their parent cells and the antigens they retain from the parent cells [9, 10]. Target cells are modulated by MV through their capacity to facilitate cell-to-cell interactions, where proteins and mRNA are transferred to neighbouring cells, raising the expression of protein on the target cell membrane and inducing cell signalling [11, 12]. MV have been implicated in the prothrombotic state associated with cancer and Tissue Factor (TF) -bearing MV (TFMV) in particular are found in cancer patients' plasma and have been suggested as a possible risk factor for occurrence of venous thromboembolism (VTE) [13].

Many tumour cells express TF, especially cancers that originate in the epithelium and TFMV are spontaneously released into the circulation by these tumours [14, 15]. TF is a trans-membrane, 47-KDa-glycoprotein[16] and the key activator for haemostasis, serving as the protein component of tissue thromboplastin [17]. TF also play a vital role in a number of cellular processes including intracellular signalling, cell proliferation, and blood vessel development [18].

TFMV are found in the blood of healthy individuals [19] as well as those with cancer, but levels tend to be higher in cancer patients and this has been recorded in a number of malignancies including breast cancer [20], colorectal cancer [21], and pancreatic cancer [22, 23]. Together, these results suggest the potential of TFMV as a biomarker identifying among cancer patients those who are a high thrombosis risk [24, 25].The procoagulant potential of TFMV mostly dependent on the presence of TF which can drive coagulation[26] and also anionic phospholipid expression, particularly phosphatidylserine (PS). Lacroix and Dignat-George (2012) describe MV that contain both PS and TF as particularly procoagulant [27] and a significant number of prothrombotic conditions have been reported to have elevated MV numbers in plasma [28].

A number of studies have also found links between TFMV and thrombosis using *in vivo* mice models. One such experiment by Thomas et al. [29] involved infusion of MV derived from cancer cells that showed an accumulation at the injury site as well a reduction in tail bleeding time and the time

of arterioles and venules occlusion. The study showed that MV derived from cancer cells and carrying TF and P-selectin glycoprotein ligand 1 (PSGL-1) were active in forming an *in vivo* thrombus [29]. The shedding by a tumour of TF-bearing MV through leaking blood vessels in the tumour mass, tumour-induced upregulation of TF expression in monocytes and endothelial cells, and upregulation of endothelial cell TF expression by chemotherapeutic agents together lead to elevated circulating TF levels [30]. Tumours which are sensitive to chemotherapy would be more likely to cause VTE, given that such tumours are more likely to shed greater numbers of MV via apoptosis [31]. Involvement of TF in tumour progression has also been demonstrated via hematogenous metastasis [32,33]. *In vitro* data has also confirmed a role for TFMV in coagulation and thrombin-generation [34, 35] and they have been shown to promote metastasis through angiogenesis, immune suppression, cancer cell survival, and invasion [13]. All of these processes require the ability to interact with the endothelium.

A microfluidic device has been shown previously to be capable of extracting antigen-specific MV from biologically complex samples, such as serum and conditioned medium from cultured cells. The majority of MV isolated via this method retained their native morphology [36]. Wu et al. developed a microfluidic platform that first filters red blood cells out from blood and then further analyses the remaining vesicles based on their smaller size; this way, over 99% of RBCs can be removed from the initial sample, and the exosomes with desired size were further purified with an efficiency of over 98% [37].Therefore, microfluidic devices are ideal candidates to study translation of biomarkers such as tumour MV to study clinically relevant questions.

The purpose of this present work is to investigate how tumour MV are able to interact with endothelial cells *in vitro* utilising a microfluidic platform. Understanding more fully the mechanisms of endothelial involvement in thrombotic events may help in the development of better therapeutic solutions in cancer management.

Materials and Methods

Cell lines and culture

The ovarian carcinoma cell line ES2 (ATCC, UK) and glioblastoma U87 cell line (ATCC, UK were seeded at 1 x 10^6/ml cells into 25 cm^2 tissue culture flask (Sarstedt, UK) and left to adhere overnight at 37°C in a 5% CO_2 incubator and maintained in McCoy's 5A media or DMEM media respectively, supplemented with 10% (v/v) Foetal Bovine Serum (FBS) and 1% (v/v) Penicillin/Streptomycin (all Lonza, UK). Spheroids were formed using ultra low adherence 96-well plates (ThermoFisher, UK) seeded with 2x10^5 cells and cultivated over 5-7 days prior to use. Primary Human umbilical vein endothelial cells (HUVECs; PromoCell, Heidelberg, Germany) were cultured in complete endothelial cell growth media (ECGM, PromoCell). HUVECs were seeded at 1 x 10^6/ml cells into 25 cm^2 cell+ tissue culture flasks (Sarstedt) and cultured at 37^0C in a 5% CO_2 incubator. HUVECs were utilised at passages 3-6.

Procoagulant activity

The procoagulant potential of cell-free supernatant and cells were measured using the semi-automated Thrombotrack SOLO coagulometer. This machine works by mechanical detection of the clotting endpoint method. Samples (100µl) were placed into a cuvette containing a steel ball and 25mM $CaCl_2$ (100µl) was added; finally, 100µl of control plasma (NormTrol, Helena Biosciences, UK) was added and the time taken for clot formation (prothrombin time, PT) was automatically determined.

CFSE staining protocol

MV released from ES-2 and U87 tumour cells were labelled via 5(6)-carboxyfluorescein diacetate N-hydroxysuccinimidyl ester (CFSE) staining of the parent cells. Harvested cells (1×10^6 cells/ml) from ES-2 and U87 cancer cells were suspended in 1 ml PBS and incubated with CellTrace™CFSE dye (Invitrogen, UK) at 5µM as a final working concentration and incubated for 20 minutes at room temperature or 37 °C in the dark. Stained cells were washed twice with PBS and seeded into a series of 25 cm^2 cell culture flasks in 10 ml of the appropriate medium and incubated for 24 h at 37°C and 5% CO_2. Unlabelled cells were used as negative control.

Microfluidic chips

Two chips were used for HUVECs experiments, either a µ-Slide I Luer (Ibidi, Germany) or a Vena8 endothelial cell biochip (Cellix, Ireland). Slides were treated with UV irradiation for 20 minutes and coated by dispensing approximately 12 µL of type B 2% v/v gelatin (Sigma Aldrich, UK) into the channel. Control chips were also treated in the same way to account for non-specific binding. Then, the biochips were then incubated for 24 hours at 4°C. Cultured HUVECs (2000 cells) were added into each channel and the reservoirs filled with 60µL of media. The biochips were incubated in the CO_2 incubator for 24 hrs at 37°C. Then, labelled/unlabelled MVs were perfused over the HUVECs for 6 hours and PCA, MV quantification and microscopy images were assessed to evaluate MV interaction with HUVECs. Control chips without HUVECs were done in parallel to the experimental setup.

A μ-slide III 3D perfusion was used to hold the spheroids then flow was applied via a syringe pump (4μL/min). A μ-Slide I Luer, which was precoated with HUVECs and was attached to the output of the μ-slide III 3D chip as shown in Figure 1a and b. Samples were then collected via the output of the μ-Slide I Luer into sterile 1.5ml polypropylene tubes.

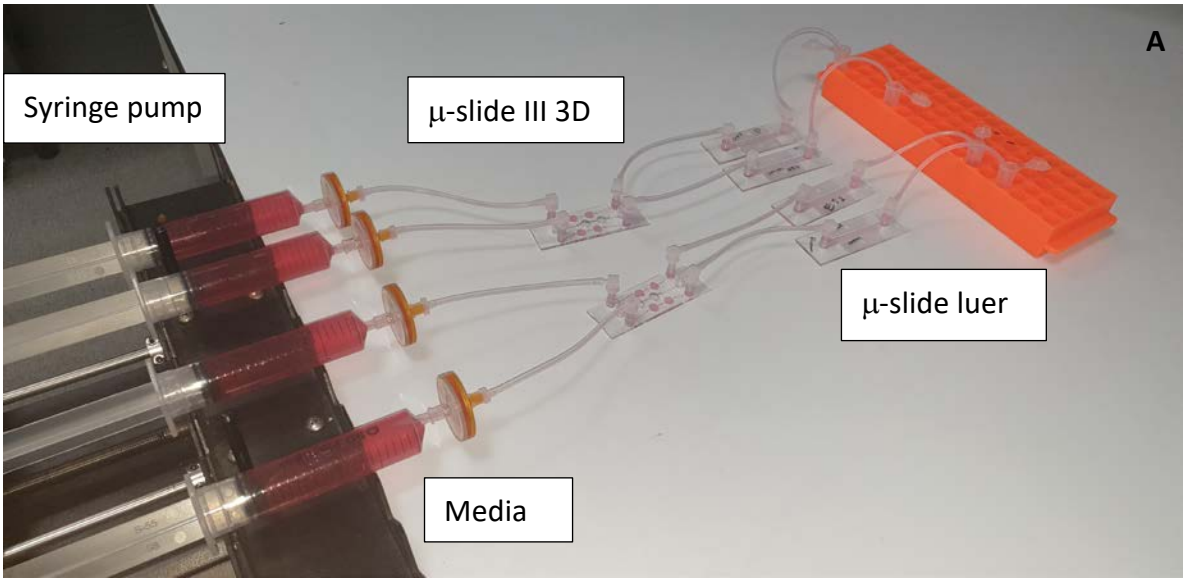

Figure 1a. *Basic experimental setup showing tumour fresh media contained in syringes linked to a multiwell μ-slide III 3D chip containing either ES-2 or U87 spheroids linked through to a μ-Slide I Luer containing HUVECs and finally sample collection tubes. The experiments were carried out in a 37 ℃ incubator.*

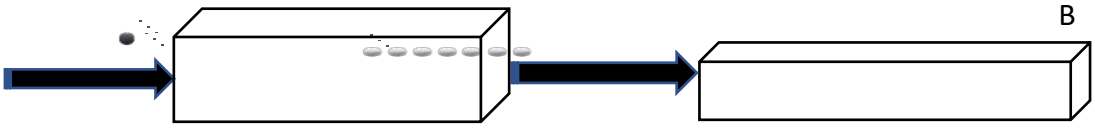

Figure 1b. *Schematic of experimental setup. Media was flowed via either syringe pump (constant flow) or Kima pump (pulsatile flow) through a microfluidic chip containing tumour spheroids. MV are released from the spheroids into the media which is then connected to a second chip coated with HUVECs to study their interaction.*

Ultrafiltration

CFSE labelled-MV cell free media was harvested from ES2 and U87 cells and centrifuged at 300 *g* at 4°C for 4 minutes to remove detached cells. Supernatant (6ml) was collected and filtered through Vivaspin® 6 ml concentrators (Sartorius, UK). MV were recovered from the media concentrate and PCA was assessed. The molecular weight cut off was 100kDa; MVs were presumed not to pass through as filtrate due to their relatively large size in comparison to the cut off value.

Flow cytometry

CFSE Labelled MVs released from ES2 and U87 tumour cells were quantified by flow cytometry before and after being passed through the biochip. Samples (50μl) of labelled (either CFSE or anti-TF: FITC (Bio-rad)) and unlabelled samples were immediately analysed by flow cytometry by adding an equal volume of Accucheck beads (Invitrogen, UK) and 150μl of 0.2μm-filtered sterile

PBS. Unlabelled MV samples were used as negative control. A flow cytometer (BD FACSCalibur) was setup with Megamix SSC beads (Biocytex, France) that are used to define a MV gate according to side-scatter characteristics of the beads (Fig. 2) following the manufacturer's protocol.

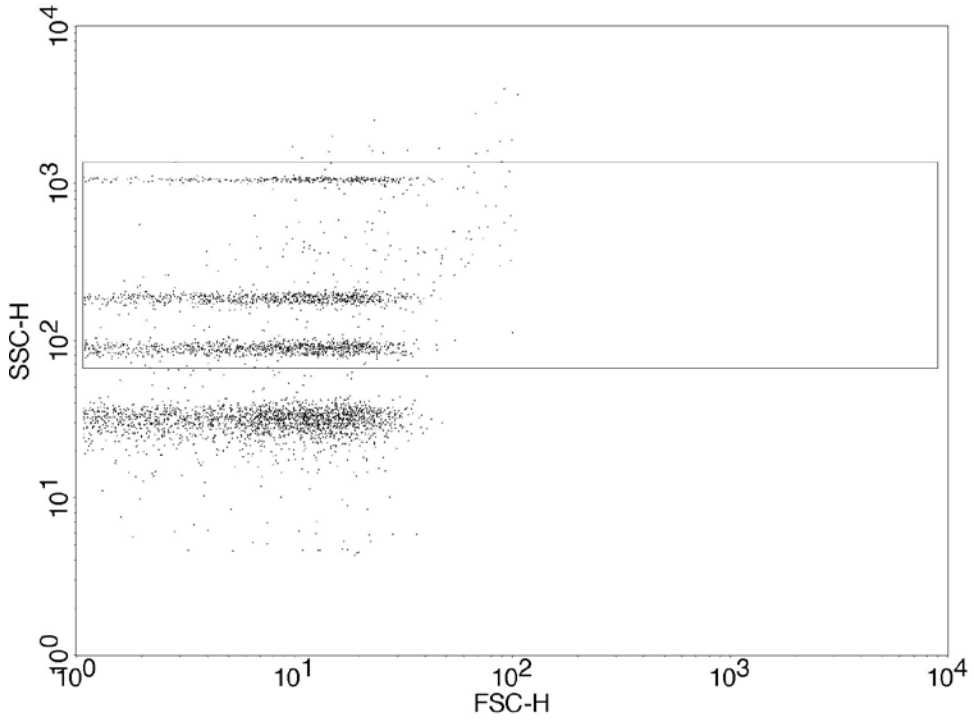

Figure 2. *Defined MV gate based on Megamix SSc beads manufacturer protocol. The box around the 3 differentially sized Megamix SSc beads on side scatter represents the MV gate set at approximately 0.2 to 0.5 μm. The lowest SSc beads are 0.16μm and do not form part of the MV gate.*

Microscopy

The μ-Slide I Luer microfluidics biochip were coated with HUVECs cells and washed twice with PBS to investigate the immobilization of (CFSE)-labelled MVs. Labelled/unlabelled MVs of ES2 and U87 cell free medium were perfused over the HUVECs for 6 hours. Confocal microscopy was performed using a Zeiss LSM710 Laser Scanning Confocal Microscope and images acquired using ZEN software (Zeiss Group, Oberkochen, Germany).

The interaction of MV on HUVECs was further studied using an automated microfluidic platform (VenaFlux and Vena8 Endothelial+ biochips; Cellix, Dublin, Ireland) in order to mimic physiological flow status. The Vena8 chip was coated with HUVECs (same conditions as previously described) and connected to a Kima pump (Cellix) which delivers pulsatile flow with shear stress at 450μl/min for 6 min, followed by 5 min of absence of flow. The flow chamber was then connected to the Mirus Evo Nanopump (Cellix) and the channels were rinsed three times with 25 μl of media prior to each experiment, and MV adhesion was initiated by the addition of CFSE-labelled MV supernatant of (ES-2 and U87) and unlabelled MV as well. Interaction of MV was recorded every second under a shear stress of 1 dyne/cm^2 in phase contrast and the settings were equal in all conditions (exposure time 344 ms, magnification 32×) for 5 min.

Results

Procoagulant activity

PCA of ES-2 and U87 cells and media were assessed via the one stage PT assay. For the same concentration of cells within the assay (3×10^5) the PT was similar between the ES2 (33.0s) and U87 (32.6s). The cell free media harvested at the same time was shown in both cells line to be procoagulant with ES-2 media supporting a PT of 76.9 ±3.4s (n=4) and U87 media was less procoagulant with a PT of 137.1 ±4.3s (n=4). Ultracentrifugation using a Vivaspin (100 kDa MWCO) was shown to remove all associated PCA of the filtrated media confirming that the PCA was MV associated whereas the concentrate diluted with fresh media to the original volume was shown to retain and slightly increase PCA (ES2; 41.4±9.2s, U87; 112.8±13.3s).

TF labelling of MV and interaction with HUVECs

Initially, TF labelling (anti-human TF: FITC) of MV from media of ES-2 and U87 spheroids cultured on a μ-slide 3D chip was used to quantify the interaction with HUVECs under flow and the relationship between TFMV with PCA. Over a time course of 6h TFMV linearly decreased (through 1-6h) from the media collected after perfusion across HUVECs on a μ-slide luer chip when compared to a coated control chip containing no HUVECs (Fig. 3).

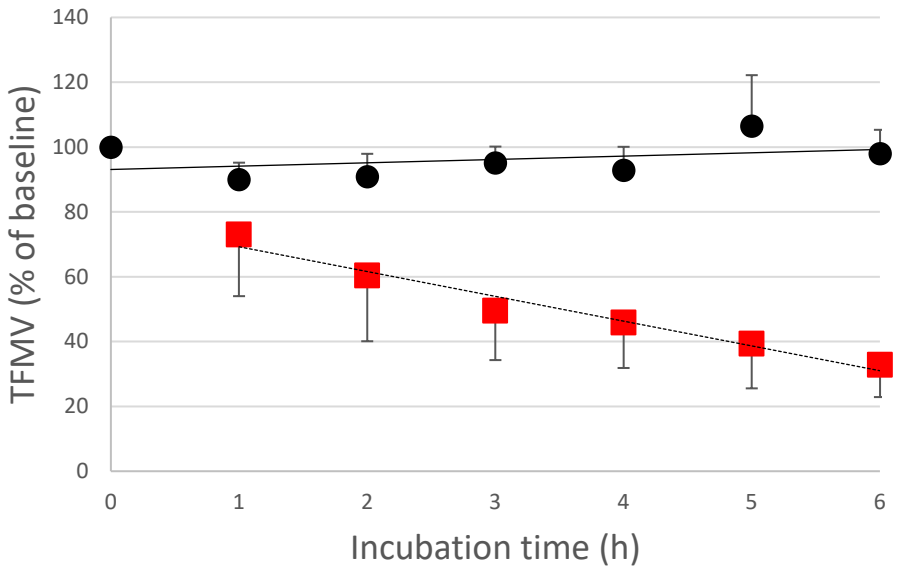

Figure 3. *TFMV of ES-2 and U87 as a percentage of baseline values (n=8) when perfused across a μ-Slide I Luer containing cultured HUVECs (*red square*) or a gelatin control chip (*black circle*) with no HUVECs present (n=4) for 6h. Error bars are SD.*

The loss of TFMV after perfusion across HUVEC coated slides was further investigated through analysis of the PCA associated with the media following perfusion across the 6h time frame. A clear power relationship was observed between TFMV and PCA for both cell lines and the subsequent loss of detected TFMV over time with HUVECs perfusion resulted in a slower PCA (Fig 4). Control samples of MV rich tumour media passed through gelatin coated μ-slide luer chips

showed no change in PCA across the 6h experimental window (ES-2; 224.7±4.8s, U87; 190.2±7.4s) HUVECs were analysed by flow cytometry for TF expression post-perfusion and showed a mean fluorescent ratio (fluorescent intensity $_{TF}$/fluorescent intensity $_{negative\ control}$) increase relative to control HUVECs (ratio of 1) to 2.41±0.13 for HUVECs perfused with ES-2 MV and 2.16±0.26 for HUVECs perused with U87 MV (n=4).

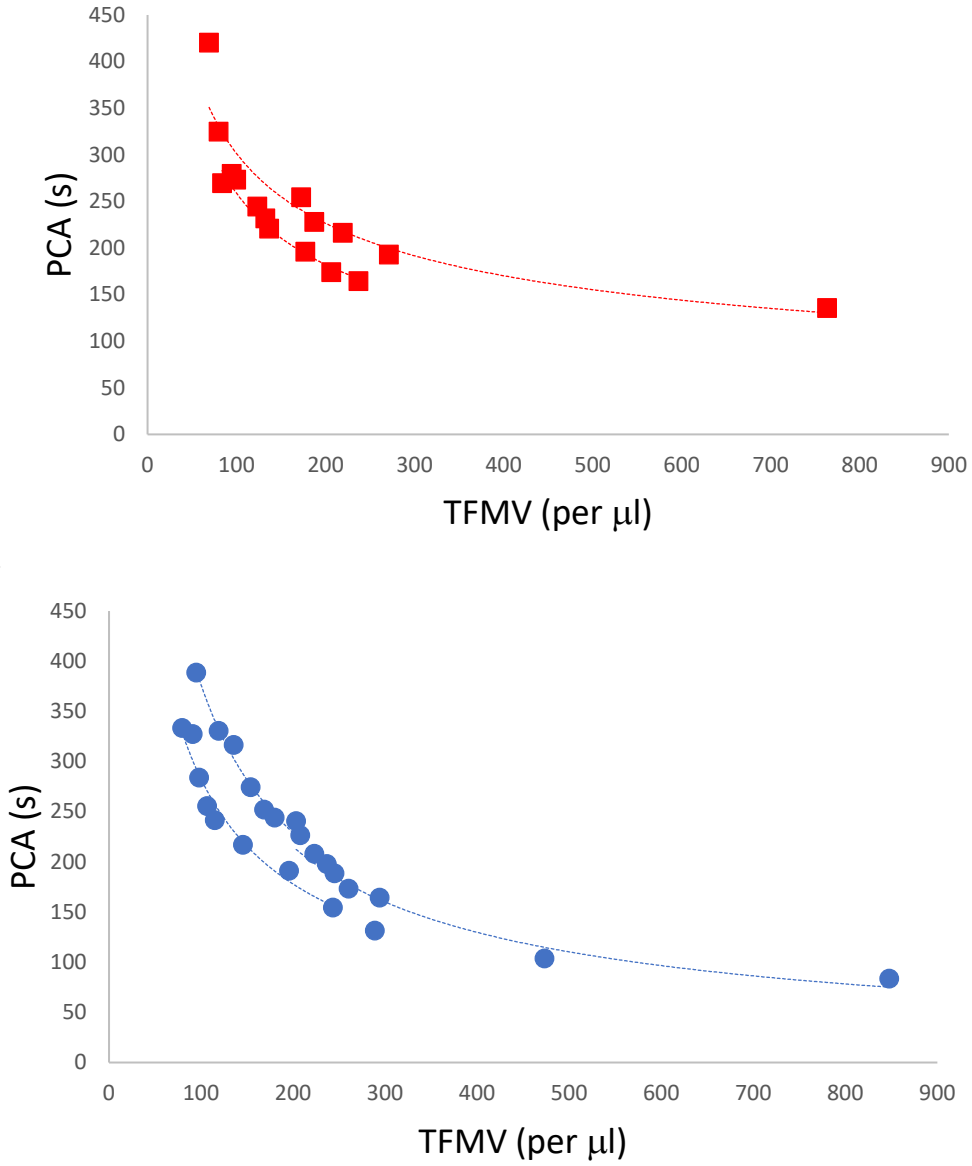

Figure 4. *Relationship between TFMV and PCA of U87(*red square, *n=16) and ES-2 (*blue circle, *n=24) media when perfused over HUVECs for 6h. Lines of best fit are for a power relationship and* R^2 *values range from 0.904-0.985.*

CFSE Labelling and detection of tumour MV

To visualise the observed interaction between MV and HUVECs under flowing conditions, ES2 and U87 (1×10^6 cells/ml) were fluorescently labelled with CFSE and incubated for 24 h. Cell free media from each tumour cell line was then harvested and perfused over HUVECs adhered to a microfluidic chip (μ-Slide I Luer) for 6h (equating to approximately $6.7*10^5$ total MV). PCA and MV quantification was determined before and after being perfused over HUVECs. The MV gate was defined using Megamix SSc beads and CFSE-labelled MV could be clearly identified on a fluorescence plot in comparison to unlabelled MV (Fig. 5). Independent measurements showed a relationship between PCA and the quantity of CFSE labelled MV (Fig. 6).

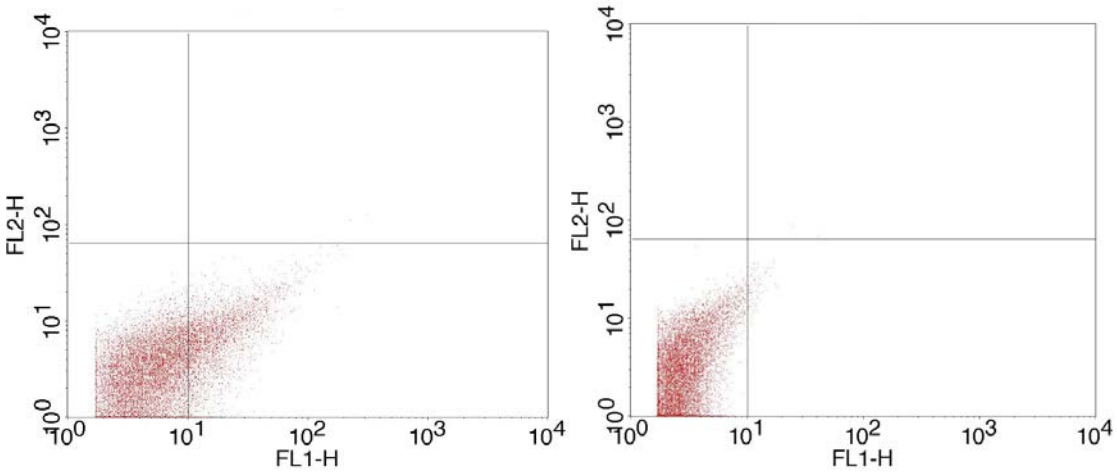

Figure 5. *CFSE fluorescently labelled (left panel) and unlabelled (right) MV populations. The individual MV events in the lower right quadrant correspond to CFSE-labelled MV (increased FL-1 signal) which were then quantified using counting beads.*

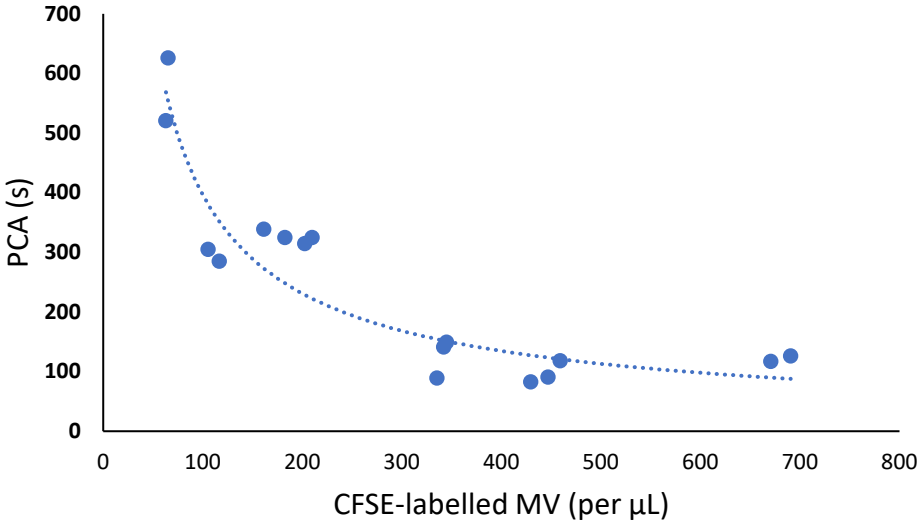

Figure 6. *The correlation between CFSE- labelled MV with PCA of tumour media (n=16, independent measurements)*

HUVECs were detached from the μ-Slide I Luer chip at the end of the 6h period and analysed for PCA and the acquisition of fluorescent properties from the CFSE labelled MV. The PCA of HUVECs ($3x10^5$ per assay) incubated with ES2 tumour media was 149±0.5s and 127±1.2s for HUVECs incubated with U87 tumour media. The PCA of HUVECs with fresh (no TFMV) media was 370±17.5s. The cells were further characterised by flow cytometry and the results showed an increased fluorescence (gained from labelled tumour MV) for HUVECs compared to the control cells (Fig. 7). The mean fluorescent ratio of HUVECs relative to control increased to 10.52±1.77 for HUVECs perfused with ES-2 CFSE labelled MV and 7.53±0.64 for HUVECs perused with U87 MV (n=3).

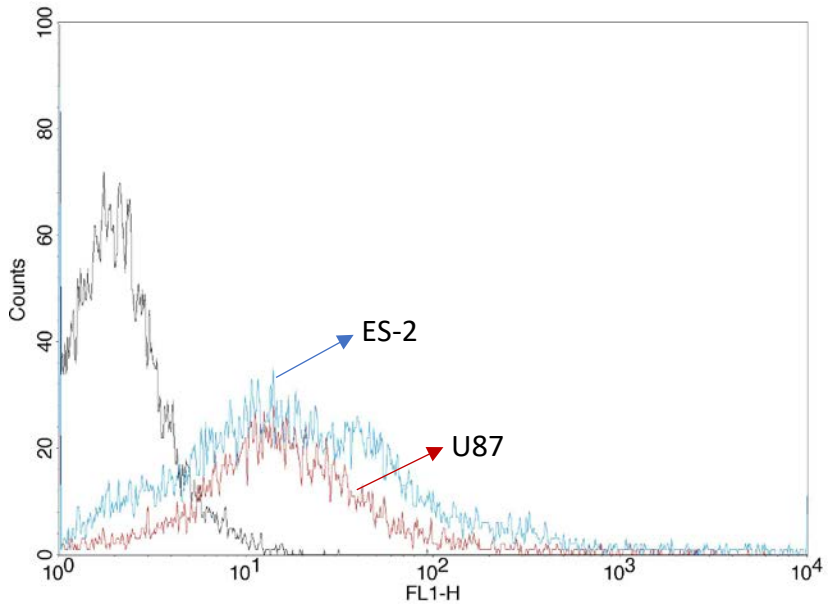

Figure 7. *Representative (n=3) histogram plot of fluorescence of HUVECs perfused with U87 or ES-2 MV rich media for 6h, compared to HUVECs perfused with control media (left peak). MV were labelled with CFSE from the parent cell.*

Confocal microscopy

To further define the MV interaction with HUVECs, CFSE-MV labelled were perfused over HUVECs on a μ-Slide I Luer channel for 6h then washed and analysed by confocal microscopy (Fig. 8). Images obtained showed fluorescence localised at the surface of HUVECs.

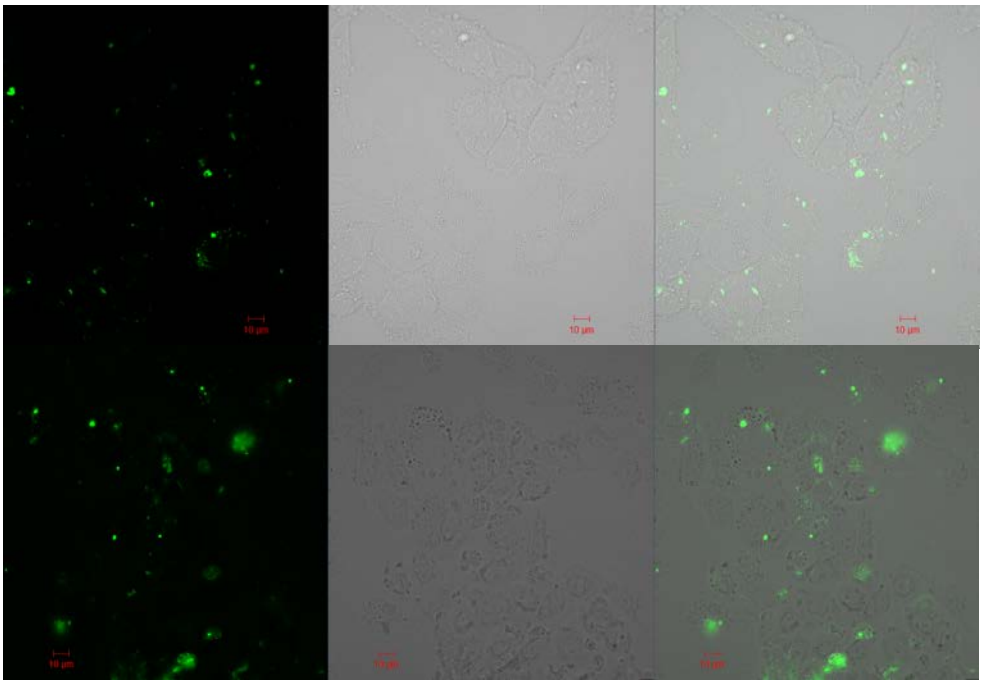

Figure 8. *Confocal microscopy of HUVECs incubated on a μ-Slide I Luer perfused with tumour media (ES-2 top panels, U87 bottom panels) with CFSE labelled MV. The left panels correspond to the fluorescent detection channel, middle panels are brightfield detection channel and the right panels are the combined images.*

Automated image capture under flow

The Cellix system allows for live image capture under physiological flow conditions and was utilised to further confirm that the association of tumour MV with HUVECs, observed by confocal microscopy was not due to any period of static flow between the experiment and analysis. CFSE-MV were constantly passed over a Vena8 microfluidic chip precoated with HUVECs and images captured 'as live' under flowing conditions. MV were again observed to associate with HUVECs (Fig. 9).

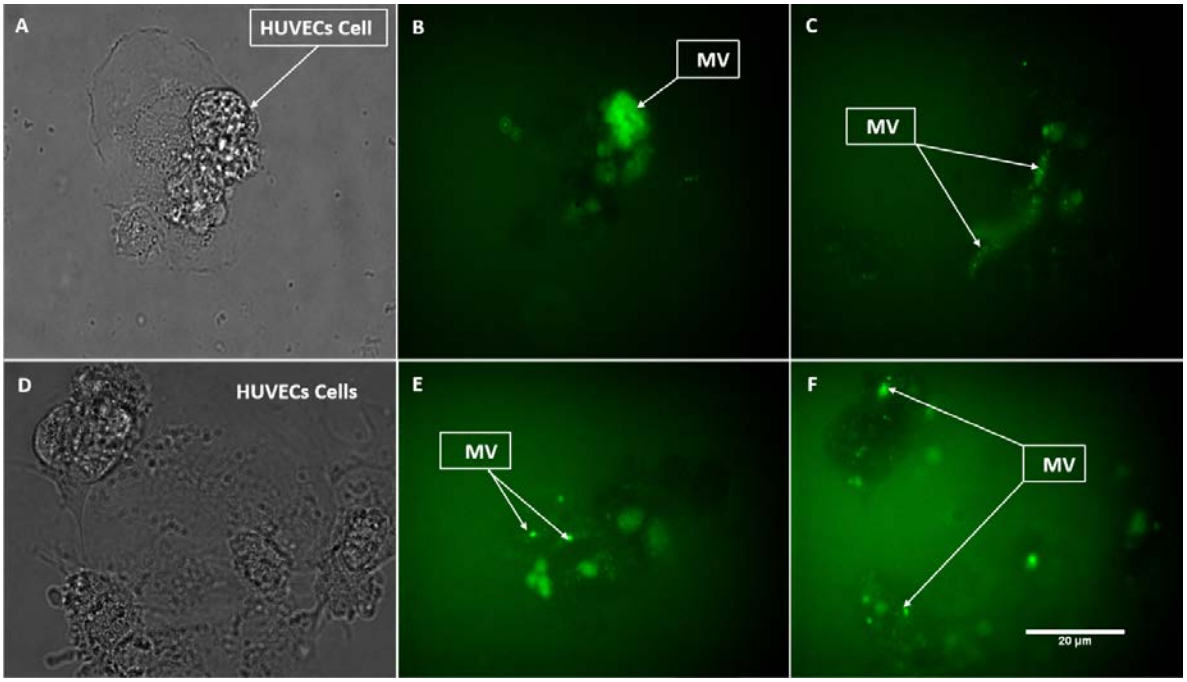

Figure 9. *Still images captured from a live recording showing CFSE labelled MV aggregated on HUVECs under flow. The top panel (A-C), shows labelled ES-2 MV deposition. Image A shows HUVECs cell with unlabelled ES2-MV. Images B and C show two different MV deposition with HUVECs. Image D shows U87 unlabelled-MV with HUVECs. Images E and F represents the labelled U87 MV deposition on HUVECs.*

Discussion

In this study, we demonstrate for the first time that MV formed *in vitro* from tumour spheroids interact with endothelial cells under flow conditions in a dual microfluidic chip assembly. After initially adhering to the HUVEC surfaces in a static condition, TFMV were shown to associate with HUVECs under dynamic flow conditions in a time dependent manner. The correlation of loss of PCA and reduction in detected TFMV when tumour media was passed over HUVECs under flow suggests direct evidence for PCA being determined by TFMV concentration and also that TFMV are lost due to their association with the endothelial cells. Control chips coated with gelatin but without HUVECs showed no loss of MV or PCA through the experiments therefore the observed loss can be attributed to the interaction with HUVECs. We have previously shown that PCA is linked to tumour spent media concentration [23] in a power relationship, as also observed here for MV concentration (Fig. 4). The observation that the concentration of spent tumour media concentration and quantified MV both determine PCA suggests that the MV in tumour media are responsible for the associated PCA. Fluorescent and confocal microscopy (Figs 8 & 9) clearly showed a fluorescence attributed to the presence of CFSE-labelled MV on HUVECs within the microfluidic chip after tumour MV were perfused over the cells. From both TF and CFSE analysis of HUVECs post perfusion the ES-2 MV conferred an increased PCA and showed a greater fluorescence when compared to U87 MV suggesting more MV were associated with HUVECs. ES-2 MV rich media was also found to possess a greater PCA prior to incubation with HUVECs which may be indicative of a greater MV concentration. After removal of MV from the media via

ultrafiltration (Vivaspin, 100kDa) the filtrate no longer supported coagulation. The pore size of these filtration units would allow soluble TF to pass through (47kDa), if present in a monomeric or dimeric form, and so the data suggest that the PCA associated with tumour conditioned media is MV dependent as discussed above.

A possible limitation of the study would be whether the interaction with endothelial cells seen here is ubiquitous and occurs for MV derived from any cell exposed to the circulation. However, the acquisition of a more procoagulant HUVEC phenotype via the acquisition of TF expressing tumour MV as shown here would be more specific to tumour derived MV. Furthermore it has been shown that although flow cytometry is the currently the only standardised method [8] across laboratories for enumeration of MV the method has a size limitation where smaller MV cannot be detected [38]. The described methodology here, using Megamix beads creates an MV size window of detection of 0.2 to 0.5μm (Fig. 2).

The binding of procoagulant tumour MV to endothelial cells could have relevance to the *in vivo* mechanism of VTE formation in cancer patients where TFMV have been proposed to be associated with a high thrombosis risk [24, 25]. If tumour MV are able to bind to endothelial cells within the circulation then this could be a basis for increased procoagulant potential. Future work should focus on the exact mechanism of tumour MV binding to endothelial cells and the response of endothelial cells to the stimuli in terms of activation, apoptosis or altered cell surface marker expression.

It has been proposed that MV endocytosis by endothelial cells occurs through interaction between anionic phospholipids at the MV surface and endothelial cell surface expressed αvβ3 integrin [39]. This process was shown to be inhibited in the presence of annexin V and the internalisation of MV and subsequent protein digestion at the MV surface by trypsin additionally provides evidence of a phospholipid role in binding of MV to endothelial cells [39]. The engulfment and recycling of MV through the Rab family of Golgi-endosomal transport network has also been demonstrated [40]. Furthermore, there is evidence of TF–VIIa–protease-activated receptor (PAR) 2 signalling in thrombin generation (and activation of other trans-membrane G protein-coupled receptors) leading to transcription of prothrombotic genes, signal transduction amplification cascades and also the establishment of tumours [41, 42]. PAR1 can also trans-activate PAR2, which can promote an extra thrombin generation response in the endothelium and tumour environment [43].

When cells are exposed to inflammatory cytokines, leukocytes are more likely to undergo microvesiculation, and are therefore capable of the production of TFMV, which may become associated with developing thrombus via P-selectin glycoprotein ligand 1–P-selectin interactions and also may stabilise the thrombus by fibrin formation induction [44, 45]. Neutrophils can also recruit TFMV [46], and the extracellular traps that they project have been demonstrated *in vitro* to serve as an adherence site for tumour-derived TFMV [47]. This may be a significant process for localising TFMV and concentrating additional TF into the developing thrombus. There is also evidence to suggest that MV are able to transfer their procoagulant potential to other cell types, and in doing so can exacerbate endothelial activation [48] as suggested here. While TF expression can be induced in cultured endothelial cells in response to inflammatory, *in vivo* it is probable that the TF associated

with endothelial cells is derived from TFMV released by monocytes or tumour cells [49, 50]. Moreover, the expression of TF on the endothelium in response to both monocyte-derived MVs and inflammatory mediators accompanies the concomitant translocation of phospholipids such as PS, that could enhance the binding of coagulation factors [51]. The induction of endothelial cell apoptosis is related to MV generation and downregulation of TF pathway inhibitor, thrombomodulin and glycosaminoglycans such as heparan sulphate on the endothelial surface [52]. The resultant impairment of activation of the protein C anticoagulant pathway and reduced antithrombin III activity may be attributed to the disrupted integrity of the endothelium. In addition, activated endothelial cells express cell surface adhesion molecules which increase platelet adhesion and attract monocytes and neutrophils, all of which might further contribute to coagulation initiation or amplification [53, 54].

In summary we report a microfluidic two-chip setup which showed that tumour MV released from spheroids bind to endothelial cell under dynamic, physiologically relevant flow conditions.

Conclusion

Tumour derived procoagulant MV were shown to become associated with endothelial cells under flow conditions within a dual microfluidic setup. Tumour MV were shown be the cause of procoagulant activity *in vitro*.

Acknowledgment

This research was funded through a Northern Border University (Saudi Arabi) PhD scholarship to AA.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Chen, C.; Skog, J.; Hsu, C.-H.; Lessard, R. T.; Balaj, L.; Wurdinger, T.; Carter, B. S.; Breakefield, X. O.; Toner, M.; Irimia, D., Microfluidic isolation and transcriptome analysis of serum microvesicles. *Lab on a Chip* **2010,** *10* (4), 505-511.

2. Choudhury, D.; Ramsay, W. T.; Kiss, R.; Willoughby, N. A.; Paterson, L.; Kar, A. K., A 3D mammalian cell separator biochip. *Lab on a Chip* **2012,** *12* (5), 948-953.

3. Zhang, Z.; Nagrath, S., Microfluidics and cancer: are we there yet? *Biomedical microdevices* **2013,** *15* (4), 595-609.

4. Freyssinet, J. M., Cellular microparticles: what are they bad or good for? *Journal of Thrombosis and Haemostasis* **2003,** *1* (7), 1655-1662.

5. Minciacchi, V. R.; Freeman, M. R.; Di Vizio, D. In *Extracellular vesicles in cancer: exosomes, microvesicles and the emerging role of large oncosomes*, Seminars in cell & developmental biology, Elsevier: 2015; pp 41-51.

6. Aupeix, K.; Hugel, B.; Martin, T.; Bischoff, P.; Lill, H.; Pasquali, J.-L.; Freyssinet, J.-M., The significance of shed membrane particles during programmed cell death in vitro, and in vivo, in HIV-1 infection. *The Journal of clinical investigation* **1997,** *99* (7), 1546-1554.

7. Burnouf, T.; Chou, M.-L.; Goubran, H.; Cognasse, F.; Garraud, O.; Seghatchian, J., An overview of the role of microparticles/microvesicles in blood components: are they clinically beneficial or harmful? *Transfusion and Apheresis Science* **2015,** *53* (2), 137-145.

8. Lacroix, R.; Dubois, C.; Leroyer, A.; Sabatier, F.; Dignat - George, F., Revisited role of microparticles in arterial and venous thrombosis. *Journal of Thrombosis and Haemostasis* **2013,** *11*, 24-35.

9. Nomura, S.; Shimizu, M., Clinical significance of procoagulant microparticles. *Journal of Intensive Care* **2015,** *3* (1), 2.
10. Shai, E.; Varon, D., Development, cell differentiation, angiogenesis—microparticles and their roles in angiogenesis. *Arteriosclerosis, thrombosis, and vascular biology* **2011,** *31* (1), 10-14.
11. Essayagh, S.; Xuereb, J.-M.; Terrisse, A.-D.; Tellier-Cirioni, L.; Pipy, B.; Sié, P., Microparticles from apoptotic monocytes induce transient platelet recruitment and tissue factor expression by cultured human vascular endothelial cells via a redox-sensitive mechanism. *Thrombosis and haemostasis* **2007,** *98* (10), 831-837.
12. Simak, J.; Gelderman, M. P., Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers. *Transfusion medicine reviews* **2006,** *20* (1), 1-26.
13. Hisada, Y.; Alexander, W.; Kasthuri, R.; Voorhees, P.; Mobarrez, F.; Taylor, A.; McNamara, C.; Wallen, H.; Witkowski, M.; Key, N. S., Measurement of microparticle tissue factor activity in clinical samples: A summary of two tissue factor-dependent FXa generation assays. *Thrombosis research* **2016,** *139*, 90-97.
14. Al-Nedawi, K.; Meehan, B.; Micallef, J.; Lhotak, V.; May, L.; Guha, A.; Rak, J., Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nature cell biology* **2008,** *10* (5), 619.
15. Khorana, A. A.; Ahrendt, S. A.; Ryan, C. K.; Francis, C. W.; Hruban, R. H.; Hu, Y. C.; Hostetter, G.; Harvey, J.; Taubman, M. B., Tissue factor expression, angiogenesis, and thrombosis in pancreatic cancer. *Clinical Cancer Research* **2007,** *13* (10), 2870-2875.
16. Han, X.; Guo, B.; Li, Y.; Zhu, B., Tissue factor in tumor microenvironment: a systematic review. *Journal of hematology & oncology* **2014,** *7* (1), 54.
17. Bach, R. R., Tissue factor encryption. *Arteriosclerosis, thrombosis, and vascular biology* **2006,** *26* (3), 456-461.
18. Abdulkadir, S. A.; Carvalhal, G. F.; Kaleem, Z.; Kisiel, W.; Humphrey, P. A.; Catalona, W. J.; Milbrandt, J., Tissue factor expression and angiogenesisin human prostate carcinoma. *Human pathology* **2000,** *31* (4), 443-447.
19. Madden, L. A.; Vince, R. V.; Sandström, M. E.; Taylor, L.; McNaughton, L.; Laden, G., Microparticle-associated vascular adhesion molecule-1 and tissue factor follow a circadian rhythm in healthy human subjects. *Thrombosis and haemostasis* **2008,** *99* (11), 909-915.
20. Trappenburg, M. C.; van Schilfgaarde, M.; Bredewold, E. O.; van Aalderen, M. C.; Spronk, H. M.; ten Cate, H.; Leyte, A.; Terpstra, W. E., Elevated numbers and altered subsets of procoagulant microparticles in breast cancer patients using endocrine therapy. *Thrombosis research* **2011,** *127* (4), 363-369.
21. Hron, G.; Kollars, M.; Weber, H.; Sagaster, V.; Quehenberger, P.; Eichinger, S.; Kyrle, P. A.; Weltermann, A., Tissue factor-positive microparticles: cellular origin and association with coagulation activation in patients with colorectal cancer. *Thrombosis and haemostasis* **2007,** *97* (01), 119-123.
22. Thaler, J.; Ay, C.; Mackman, N.; Metz‐Schimmerl, S.; Stift, J.; Kaider, A.; Müllauer, L.; Gnant, M.; Scheithauer, W.; Pabinger, I., Microparticle‐associated tissue factor activity in patients with pancreatic cancer: correlation with clinicopathological features. *European journal of clinical investigation* **2013,** *43* (3), 277-285.
23. Yates, K. R.; Welsh, J.; Echrish, H. H.; Greenman, J.; Maraveyas, A.; Madden, L. A., Pancreatic cancer cell and microparticle procoagulant surface characterization: involvement of membrane-expressed tissue factor, phosphatidylserine and phosphatidylethanolamine. *Blood coagulation & fibrinolysis* **2011,** *22* (8), 680-687.
24. Dvorak, H.; Quay, S.; Orenstein, N.; Dvorak, A.; Hahn, P.; Bitzer, A.; Carvalho, A., Tumor shedding and coagulation. *Science* **1981,** *212* (4497), 923-924.

25. Falanga, A.; Marchetti, M.; Vignoli, A., Coagulation and cancer: biological and clinical aspects. *Journal of Thrombosis and Haemostasis* **2013,** *11* (2), 223-233.
26. Lacroix, R.; Dignat-George, F., Microparticles as a circulating source of procoagulant and fibrinolytic activities in the circulation. *Thrombosis research* **2012,** *129*, S27-S29.
27. Key, N. S.; Chantrathammachart, P.; Moody, P. W.; Chang, J.-Y., Membrane microparticles in VTE and cancer. *Thrombosis research* **2010,** *125*, S80-S83.
28. Falanga, A.; Marchetti, M.; Russo, L., The mechanisms of cancer-associated thrombosis. *Thrombosis research* **2015,** *135*, S8-S11.
29. Thomas, G. M.; Panicot-Dubois, L.; Lacroix, R.; Dignat-George, F.; Lombardo, D.; Dubois, C., Cancer cell–derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation in vivo. *Journal of Experimental Medicine* **2009,** *206* (9), 1913-1927.
30. Kasthuri, R. S.; Taubman, M. B.; Mackman, N., Role of tissue factor in cancer. *Journal of Clinical Oncology* **2009,** *27* (29), 4834.
31. Boles, J. C.; Williams, J. C.; Hollingsworth, R. M.; Wang, J.-G.; Glover, S. L.; Owens III, A. P.; Barcel, D. A.; Kasthuri, R. S.; Key, N. S.; Mackman, N., Anthracycline treatment of the human monocytic leukemia cell line THP-1 increases phosphatidylserine exposure and tissue factor activity. *Thrombosis research* **2012,** *129* (2), 197-203.
32. Bromberg, M. E.; Konigsberg, W. H.; Madison, J. F.; Pawashe, A.; Garen, A., Tissue factor promotes melanoma metastasis by a pathway independent of blood coagulation. *Proceedings of the National Academy of Sciences* **1995,** *92* (18), 8205-8209.
33. Cole, M.; Bromberg, M., Tissue factor as a novel target for treatment of breast cancer. *The Oncologist* **2013,** *18* (1), 14-18.
34. Diamant, M.; Tushuizen, M. E.; Sturk, A.; Nieuwland, R., Cellular microparticles: new players in the field of vascular disease? *European journal of clinical investigation* **2004,** *34* (6), 392-401.
35. Zwicker, J. I.; Furie, B. C.; Furie, B., Cancer-associated thrombosis. *Critical reviews in oncology/hematology* **2007,** *62* (2), 126-136.
36. Iliescu, F. S.; Poenar, D. P.; Yu, F.; Ni, M.; Chan, K. H.; Cima, I.; Taylor, H. K.; Cima, I.; Iliescu, C., Recent advances in microfluidic methods in cancer liquid biopsy. *Biomicrofluidics* **2019,** *13* (4), 041503.
37. Wu, M.; Ouyang, Y.; Wang, Z.; Zhang, R.; Huang, P.-H.; Chen, C.; Li, H.; Li, P.; Quinn, D.; Dao, M., Isolation of exosomes from whole blood by integrating acoustics and microfluidics. *Proceedings of the National Academy of Sciences* **2017,** *114* (40), 10584-10589.
38. Obeid, S.; Ceroi, A.; Mourey, G.; Saas, P.; Elie-Caille, C.; Boireau, W., Development of a NanoBioAnalytical platform for" on-chip" qualification and quantification of platelet-derived microparticles. *Biosensors and Bioelectronics* **2017,** *93*, 250-259.
39. Terrisse, A.; Puech, N.; Allart, S.; Gourdy, P.; Xuereb, J.; Payrastre, B.; Sie, P., Internalization of microparticles by endothelial cells promotes platelet/endothelial cell interaction under flow. *Journal of Thrombosis and Haemostasis* **2010,** *8* (12), 2810-2819.
40. Collier, M. E.; Mah, P.-M.; Xiao, Y.; Maraveyas, A.; Ettelaie, C., Microparticle-associated tissue factor is recycled by endothelial cells resulting in enhanced surface tissue factor activity. *Thrombosis and haemostasis* **2013,** *110* (11), 966-976.
41. Rickles, F. R.; Patierno, S.; Fernandez, P. M., Tissue factor, thrombin, and cancer. *Chest* **2003,** *124* (3), 58S-68S.

42. Ruf, W.; Disse, J.; CARNEIRO‐LOBO, T. C.; Yokota, N.; Schaffner, F., Tissue factor and cell signalling in cancer progression and thrombosis. *Journal of Thrombosis and Haemostasis* **2011,** *9*, 306-315.
43. McEachron, T. A.; Pawlinski, R.; Richards, K. L.; Church, F. C.; Mackman, N., Protease-activated receptors mediate crosstalk between coagulation and fibrinolysis. *Blood* **2010,** *116* (23), 5037-5044.
44. Østerud, B.; Bjørklid, E. In *Sources of tissue factor*, Seminars in thrombosis and hemostasis, Copyright© 2006 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New …: 2006; pp 011-023.
45. Ruf, W.; Ruggeri, Z. M., Neutrophils release brakes of coagulation. *Nature medicine* **2010,** *16* (8), 851.
46. Martinod, K.; Wagner, D. D., Thrombosis: tangled up in NETs. *Blood, The Journal of the American Society of Hematology* **2014,** *123* (18), 2768-2776.
47. Thomas, G.; Brill, A.; Mezouar, S.; Crescence, L.; Gallant, M.; Dubois, C.; Wagner, D., Tissue factor expressed by circulating cancer cell‐derived microparticles drastically increases the incidence of deep vein thrombosis in mice. *Journal of Thrombosis and Haemostasis* **2015,** *13* (7), 1310-1319.
48. Morel, O.; Toti, F.; Hugel, B. n. d.; Bakouboula, B.; Camoin-Jau, L.; Dignat-George, F. o.; Freyssinet, J.-M., Procoagulant microparticles: disrupting the vascular homeostasis equation? *Arteriosclerosis, thrombosis, and vascular biology* **2006,** *26* (12), 2594-2604.
49. Yu, J.; May, L.; Milsom, C.; Anderson, G. M.; Weitz, J. I.; Luyendyk, J. P.; Broze, G.; Mackman, N.; Rak, J., Contribution of host-derived tissue factor to tumor neovascularization. *Arteriosclerosis, thrombosis, and vascular biology* **2008,** *28* (11), 1975-1981.
50. Osterud, B.; Bjorklid, E., Tissue factor in blood cells and endothelial cells. *Front Biosci (Elite Ed)* **2012,** *4* (1), 289-299.
51. Aird, W. C., The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. *Blood, The Journal of the American Society of Hematology* **2003,** *101* (10), 3765-3777.
52. Pradier, A.; Ettelaie, C., The influence of exogenous tissue factor on the regulators of proliferation and apoptosis in endothelial cells. *Journal of vascular research* **2008,** *45* (1), 19-32.
53. Bombeli, T.; Karsan, A.; Tait, J. F.; Harlan, J. M., Apoptotic vascular endothelial cells become procoagulant. *Blood* **1997,** *89* (7), 2429-2442.
54. Schouten, M.; Wiersinga, W. J.; Levi, M.; Van Der Poll, T., Inflammation, endothelium, and coagulation in sepsis. *Journal of leukocyte biology* **2008,** *83* (3), 536-545.