

**CIRCULATING TUMOUR CELLS SHED LARGE EXTRACELLULAR
VESICLES IN MICROFLUIDIC CAPILLARY MODELS TO
INDUCE SYSTEMIC INFLAMMATION**

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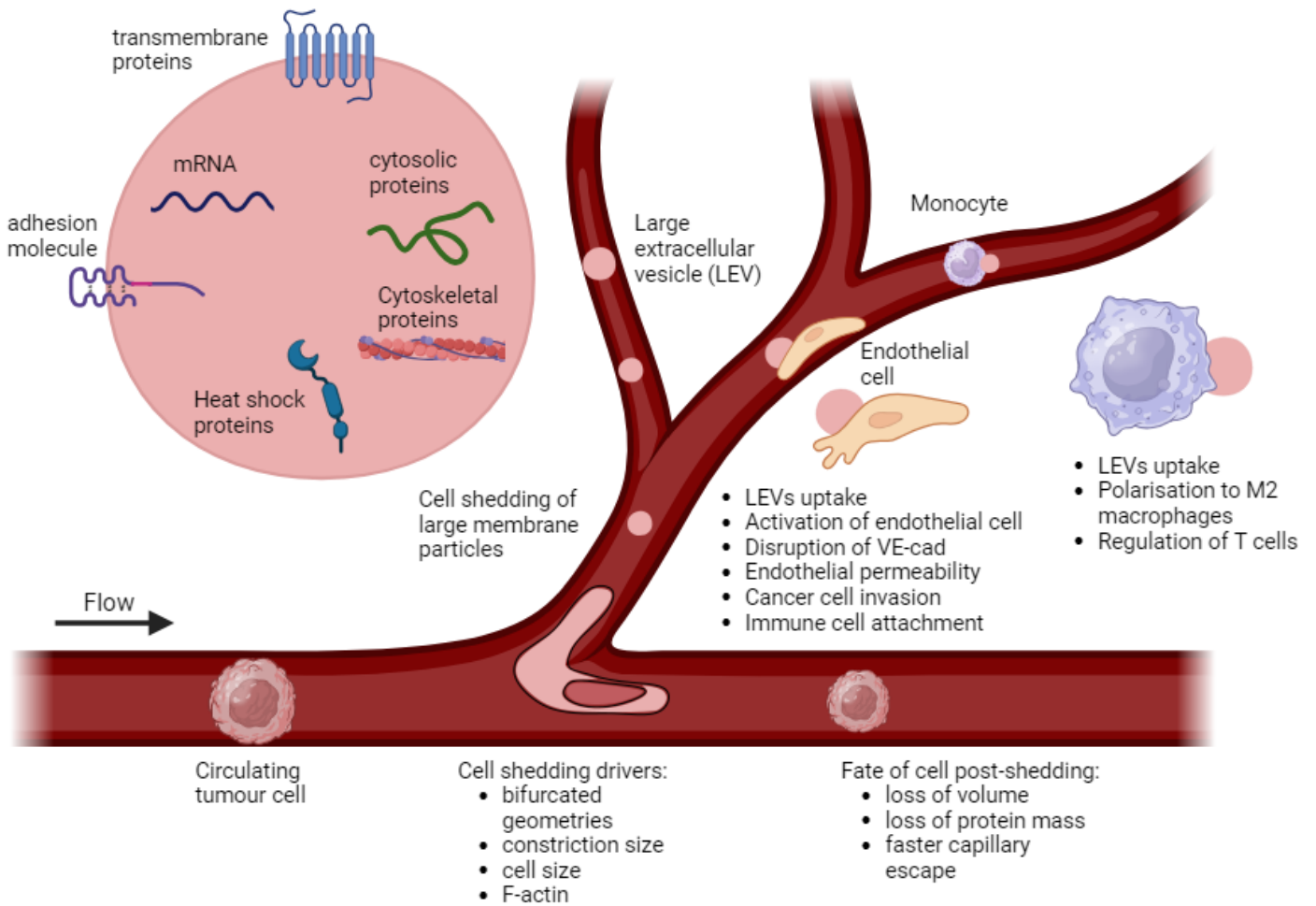
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Graphical Abstract



Abstract

Tumour cells are often released in the circulation and disseminate through capillary beds to seed eventually metastasis, the major cause of cancer-related deaths. Capillary beds or the microcirculation comprise numerous interconnected non-bifurcated and bifurcated capillaries of narrow sizes and diverse geometries entrapping circulating tumour cells (CTCs), yet the direct impact of capillary bifurcations on CTCs transit remains largely underexplored. In this work, microfluidic technology was utilised to fabricate these geometries, mimicking the conditions met in the microcirculation by CTCs. During CTCs transit, these shed large cytoplasmic microparticles predominately in capillary bifurcations, later verified as large extracellular vesicles (LEVs). Their biogenesis was dependent on multiple parameters, including bifurcations, capillary size, shear, cell size and F-actin. Cell shedding led to reduction of tumour cell volume and protein quantity and enabled faster escape from capillary bifurcations. Successful separation of LEVs from co-transiting tumour cells within the microfluidic devices permitted their downstream investigation. LEVs contained a vast array of proteins, whose role was linked to several pathways that suggested cell reprogramming. Independent co-cultures of LEVs with endothelial cells, monocytes or M1 macrophages revealed internalisation of LEVs by all tested cell types. In respect to endothelial cells, LEVs disrupted endothelial cells' intercellular junctions, enhancing the permeability against invading cancer cells. Moreover, LEVs promoted the activation of endothelial cells by an increase in expression and production of relevant markers, which in turn led to monocyte stimulation and their attachment. In respect to monocytes, exposure to LEVs led to differentiation of both monocytes and M1 macrophages to pro-metastatic M2 macrophages, validated via typical M2 macrophage markers and enabled enhanced co-interactions with the

endothelium and modulation of immune T cells. Altogether, these findings suggest that capillary bifurcations are a favourable location of shedding of LEVs, whose protein diversity enables activation of multiple cell types to promote metastasis.

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Statement of Originality

Hereby I declare that the research work in this thesis is my own and all other contributions are acknowledges and referenced.

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What cancer research truly means to me

These past 4 years has been an immensely full and fulfilling journey both at a scientific and a personal level. It has been accompanied by many challenges and obstacles, which allowed me to find my chances and motivation to become better and evolve mentally and skill wise in order to overcome them. I wholeheartedly believe that there is always a lesson to learn, whether an outcome or an interaction is positive or negative (or somewhere in between) and that is always the way to go forward or fail better or succeed more broadly. Experience, learn, be critical and find the right ingredient and the right combination. This very concept can be taught in life, should be taught by our societies and when embraced, can be integrated in research. It's all about the mental attitude and fortitude that an individual shapes. And even before this scientific journey (PhD), my past has shown me a path. A path that I chose to walk and a path that became my hope and that slowly turned to my determination and conviction. To beat cancer. No matter how tough that sounds, this is the reason I work so hard, why I've made several choices in my personal life and the why I pursue continuous evolution from my own self and mind.

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To beat cancer and protect as many people as I can from this disease.

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Abbreviations

CTCs: Circulating tumour cell

EVs: Extracellular vesicles

LEVs: Large extracellular vesicles

LOs: Large oncosomes

LMPs: Large microparticles

DMEM: Dulbecco's modified eagle medium

FBS: Fetal bovine serum

SCLC: Small cell lung carcinoma

CDX: Cell line-derived xenograft

RPMI: Roswell park memorial institute

CAFs: Cancer-associated fibroblasts

PBS: Phosphate buffered saline

IPA: Isopropanol

PDMS: Polydimethylsiloxane

BSA: Bovine serum albumin

MISEV: Minimal information for studies of extracellular vesicles

Il-6: Interleukin 6

VE-cad: Vascular endothelial cadherin

VCAM: Vascular cell adhesion molecule

ICAM: Intercellular cell adhesion molecule

NO: Nitric oxide

TNF- α : Tumour necrosis factor α

HUVECs: Human umbilical vein endothelial cells

HEPES: 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid

CM: Conditioned media

EIPA: Ethyl isopropyl amiloride

PCR: Polymerase chain reaction

VEGF: Vascular endothelial growth factor

CD206: Cluster of differentiation 206

FN: Fibronectin

CXCL10: C-X-C motif chemokine ligand 10

RPLO: Ribosomal protein large P

Arg-1: Arginase 1

BCL2: B-cell lymphoma 2

Vim: Vimentin

CD44: Cluster of differentiation 44

ALDH-1: Aldehyde dehydrogenase

RIPA: Radioimmunoprecipitation assay

EMT: Epithelial-to-Mesenchymal transition

ROCK: Rho-associated protein kinase

NK: Natural killer

LDH: Lactate dehydrogenase

ITGB-1: Integrin beta-1

EpCAM: Epithelial cell adhesion molecule

MHC II: Major histocompatibility complex II

VWF: Von Willebrand factor

TRAIL: TNF-related apoptosis inducing ligand

CD163: Cluster of differentiation 163

TAM: Tumour-associated macrophages

UF: Ultra-filtered

DTT: Dithiothreitol

IAA: Iodacetamide

CHAPTER 1

LITERATURE REVIEW

1.1 Cancer

1.1.1 Cancer incidence

Cancer is one of the leading causes of death worldwide, representing one of two most frequent causes of death, with cardiovascular diseases (1). Worldwide cancer statistics have depicted the increasing rates of both cancer incidence and cancer mortality. Worldwide data comparison between 2010 and 2019, reveal that the number of new cancer cases had increased from 18.7 to 23.6 million, whereas cancer deaths increased from 8.2 to 10.6 million (2). Dissection of these numbers by cancer types, reveals that 8/36 cancers involved in the study are the most commonly diagnosed and lethal cancers, occurring in the lung, breast, stomach, colorectum, prostate, liver, uterus and pancreas, representing altogether 56.2% and 62% of the total cancer cases and total cancer deaths, respectively, worldwide in 2020 (3,4) (**Table 1-1**) (4). It is noteworthy that there are numerous types of cancer, with multiple classification criteria i.e. organ inflicted by cancer or cell of origin within a particular organ or staging or mutational status (5), yet the aforementioned organs represent the most commonly inflicted locations. For instance, it is argued that based on molecular classification criteria, among 1000 tumours from colorectal cancer patients, there were 32 subtypes identified, which is a perfect paradigm of the extreme complexity that cancers present (6).

Table 1-1: Most commonly diagnosed and lethal cancers per cancer site (4)

CANCER SITE	NUMBER OF NEW CASES (%)	NUMBER OF NEW DEATHS (%)
BREAST	11.7	6.9
LUNG	11.4	18
PROSTATE	7.3	3.8
COLORECTUM	9.8	9.2
STOMACH	5.6	7.7
LIVER	4.7	8.3
UTERUS	3.1	3.4
PANCREAS	2.6	4.7

In general, cancers are driven majorly by genetic and epigenetic factors, as reviewed extensively elsewhere (7–9). In respect to the commonest cancer types, as cited above, the majority of cancer cases appear to a great extent due to lifestyle choices that drive epigenetic changes at a cellular level or promote inflammation that is often a precursor of cancer, although some cancers like breast or prostate also present high risk contribution from inheritable factors (10,11). Namely, alcohol consumption, smoking, sustenance, exposure to chemicals/heavy metals, environmental pollutants, sun radiation, infections, obesity and excessive stress are all corroborated and supported by clinical data as major risk factors that drive development and progression of cancer (10). Despite efforts to campaign against these risk factors, the numbers of cancer cases grow annually.

Revisiting carefully at the data that highlight how cancer incidence and mortality grow every year, there are several parameters contributing. Firstly, the global number of population increases annually, increasing thus the chances that more people will get cancer (4). Secondly, advances in medicine and technology have increased sharply the life expectancy of an individual, which correlates to an increase in cancer incidence and mortality, since cancer is largely an age-dependent disease (12,13), whereby the human body accumulates damage and inflammatory conditions that favour mutations and cancer. Last but not least, these continually growing numbers are also ascribed to the inability to diagnose cancer early on an initial stage and treat effectively patients (14).

1.1.2 Cancer prognosis

Prognosis of cancer varies majorly by cancer type and stage of cancer. Primarily, cancers such as those in lung, liver, pancreas and stomach present much lower 5-year survival rates compared to thyroid, bladder and kidney cancers among others (3). However, despite these distinct values, the increase in 5-year survival rates per cancer type has been very narrow for most cancers. In some cancers such as prostate cancer better therapeutic alternatives exist, but the majority of cancers have seen a minor increase. Moreover, the 5-year survival rates change dynamically by cancer stage (3). Cancer stages are classified as Stage 0 (carcinoma in situ) Stage 1 (localised), Stage 2 (Regional), Stage 3 (Disseminated) and Stage 4 (Metastatic) (5,15). In most metastatic cancers, the 5-year survival rates do not surpass 20% 5-year survival rates, establishing a clear and sound correlation between cancer metastasis and mortality (3). In fact, because medicine has advanced at a stage that allows improved therapeutic options

and altogether a more effective therapeutic or surgical management of localised tumours, patients live longer, yet often end up developing metastases. While research on therapeutic options is growing incessantly, late diagnosis and failure to prevent metastases, which present an interdependent relationship, remain the two major bottlenecks that drive high percentages of mortality (14,16).

Cancer diagnosis efficacy is usually dictated by screening and effectiveness of screening tools (17). One major hindrance at detecting cancers very early is the lack of appropriate screening criteria. Some types of cancer such as breast, ovarian and prostate cancers (inheritable at higher percentages than other cancers) are somewhat easier to screen, due to family history and location of organ. On the other hand, organs such as pancreas, lungs, liver and the stomach for example (18–20), require usually a highly invasive approach to screen an individual for cancer (with no symptoms for example), while other methods lead potentially to high false positives (21). Moreover, these cancers biogenesis is highly correlated to lifestyle factors, which further complicates the introduction of a screening tool in the general population (10). The lack of other biomarkers, e.g. obtained from the blood, saliva or urine to identify disease at a very early stage (where cancer is likely localised) do not exist for the majority of cancers, yet there is a spark of research currently towards that direction (22).

Inevitably, the longer the delay for cancer diagnosis to occur, the higher the chances are that cancer cells will spread, disseminate and metastasise to one or more organs. As will be expanded in following sections, once cancer cells begin metastasising, the therapeutic window becomes very limited. Cancer cells become more diverse genetically, more aggressive, can infiltrate in multiple organs, can cause inflammation to endothelial cells lining blood vessels or immune cells, affect the coagulation and the neurological system (23,24), requiring thus tremendous efforts to fight off all at the same time in order to prevent and treat metastasis. This evidence highlights the argument that cancer metastasis is not one but many diseases at the same time (25,26). So far, the advance of immunotherapies and targeted therapies (sometimes in combination) along with other first-line treatment options have enabled new strategies to combat metastatic cancer and prolong patient lives, though these benefits are far away from eliminating the disease and prevent cancer metastasis mortality at large patient percentages. Metastasis remains a very complicated state of cancer, covering a broad range of steps and interactions in the whole human body and despite the increased

understanding of this process, there are still numerous mechanisms that still need to be unveiled.

1.2 Cancer metastasis

1.2.1 Initial steps of cancer metastasis

Metastasis is defined as the dissemination of cancer cells from one organ to another. Starting with cancer cell growth, survival and proliferation in the primary tumour, genetic and epigenetic mutations as well as the numerous contributions from biomechanical forces and intracellular interactions within the tumour microenvironment, start shaping the process of metastasis (**Fig. 1-1**) (23). All the above factors support the initial steps of migration of single cancer cells or clusters (23,27). Clusters are sub-classified as homotypic clusters, formed by aggregates of cancer cells or heterotypic clusters, formed by aggregation of cancer cells with fibroblasts, immune cells or platelets (27). Briefly, cancer cells break through the barriers of the vasculature and enter in the circulation by undergoing a process termed intravasation, which is driven by multiple factors, including hypoxia, mechanical pressure, influence of the tumour microenvironment. Then, they begin their dissemination to other organs by transiting through the bloodstream that eventually paves the path and access to other organs (23,28). It is noteworthy that other routes such as neural tracking or vasculogenic mimicry drive dissemination and serve as alternate routes of dissemination (29,30), albeit are not perceived as the dominant mechanisms and there is still contention in the community regarding their existence.

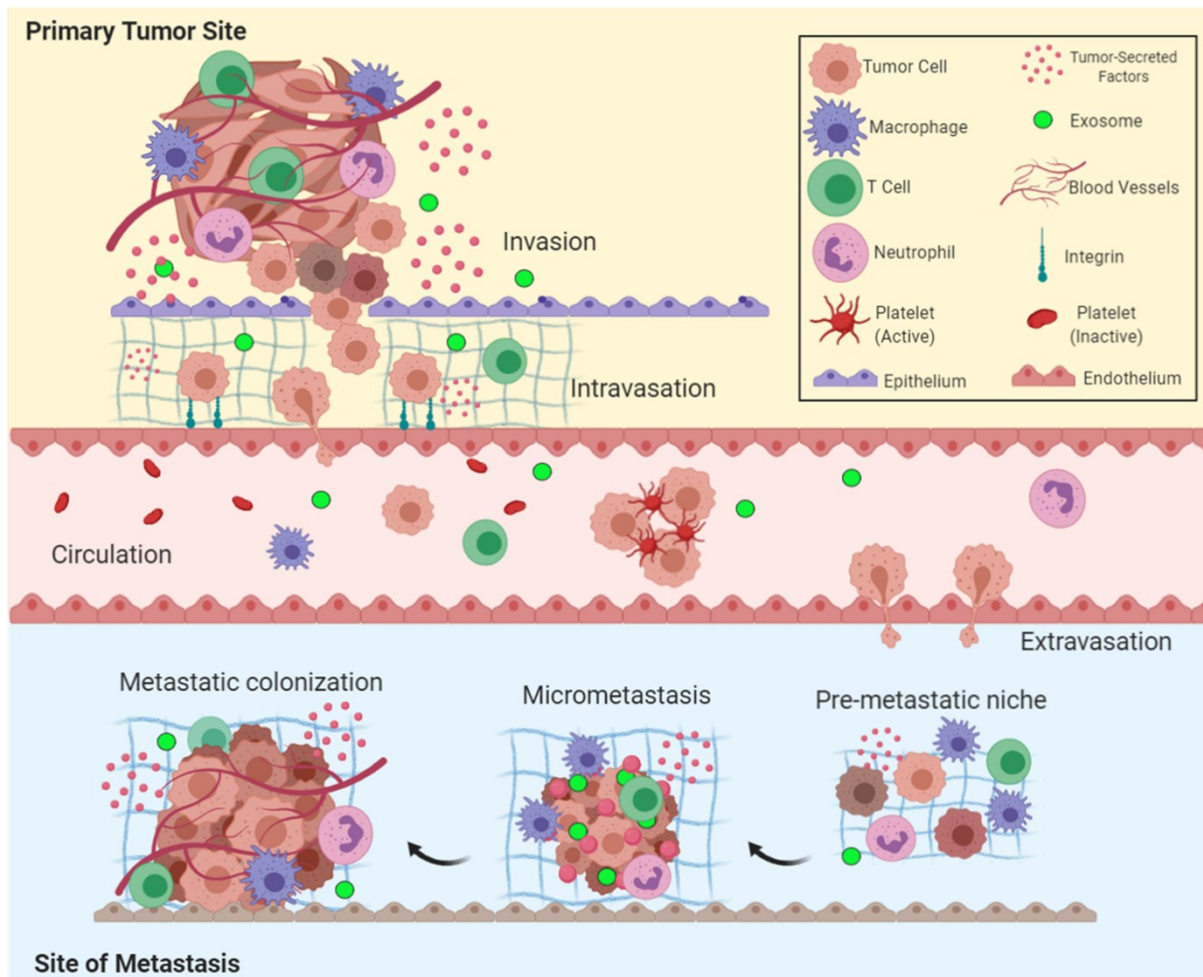


Figure 1-1: The metastatic journey of cancer cells from the primary tumour to macrometastases (23)

1.2.2 Circulating tumour cell fate in the microcirculation

Once away from the primary tumour, tumour cells that transit the blood of patients are defined as circulating tumour cells (CTCs) (28). CTCs need to survive and extravasate to escape the vasculature and by cooperating with the pre-metastatic niche, they develop micro- and macrometastasis (**Fig. 1-1**) (23). Growing evidence suggests that the ability of CTCs to metastasise successfully is affected during their journey in the microcirculation (31,32) (**Fig. 1-2**) (31). An interconnected network of small vessels i.e. arterioles and capillaries (3-8 μm) that present bifurcating and narrow constrictions as well as favourable low blood velocities are characterised as the microcirculation or microvasculature or capillary bed (33-35). These features render this location a unique microenvironment in the blood of patients that affects CTCs fate (28,33,34). In particular, CTCs get trapped quickly and remain in the environment of microcirculation for much longer timeframes, compared to their transit time in arteries and

veins (36). Follain et. al. have demonstrated in zebrafish models how the low flow profiles of capillaries favour the entrapment and deceleration of CTCs, which provides sufficient time to arrest and adhere to the endothelium and eventual extravasation (34). In a previous commentary, it was discussed briefly how important biomechanical forces and intercellular interactions are, dictating CTCs fate in the microcirculation (31), which will be elaborated subsequently.

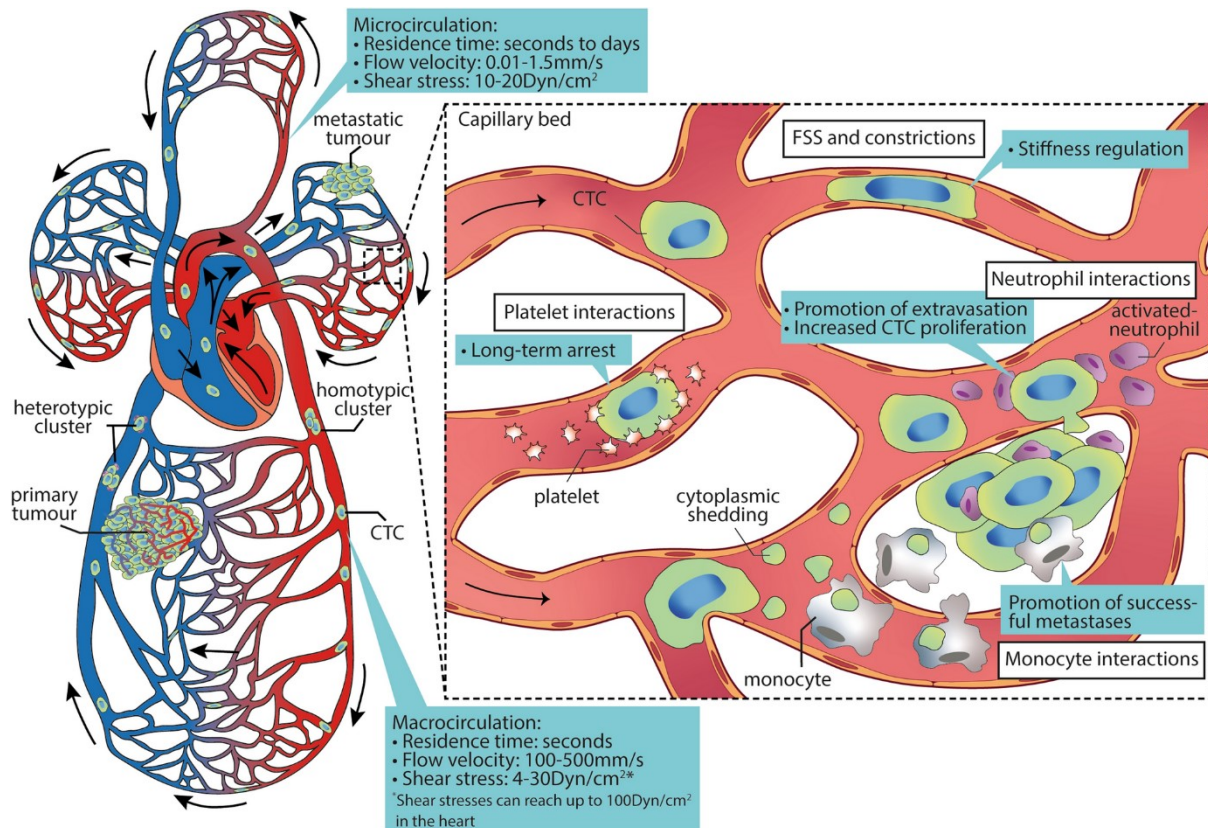


Figure 1-2: Biomechanical forces and intercellular interactions on CTCs (31)

The major biomechanical forces applied to CTCs during their transit in capillaries, affecting their survival, are fluid flow (shear stress) and constriction forces (31,32,35,37). It was shown previously in vitro that while the majority of CTCs die off post shear exposure, a subset of CTCs acquires features that allow them to survive (38). One such strategy is the acquisition of Epithelial-to-Mesenchymal transition (EMT) upon exposure to shear, whereby cancer cells convert to more motile phenotypes (38–40). In one particular study, CTCs were exposed to high shear stress and the CTCs that underwent EMT (verified by gained gene expression of mesenchymal and loss of epithelial markers), were recirculated at the same high shear stress, revealing an increase in their survival compared to those that had not undergone EMT. The

authors demonstrated that the EMT phenotype was accompanied by upregulation of anti-apoptotic genes and downregulation of pro-apoptotic genes. Mechanistically, they have demonstrated that the EMT response by CTCs is driven by activation of Jun N-Terminal Kinase signalling, which regulates mechanical stimuli and was found to be higher expressed after CTCs treatment with 20 dyne/cm² compared to untreated cells (38). In a different study, activation of the Rho-associated protein kinase (ROCK) pathway by fluid shear stress enhanced the ability of CTCs to survive hematogenous dissemination. Injected cancer cells in mice showed enhanced intravascular survival, compared to injected cancer cells that were pre-treated with a ROCK inhibitor, blebbistatin (41). Another recent study identified ATOH8, a protein acting as a mechanosensory unit, to be highly expressed upon exposure of CTCs to laminar shear stress. These pro-survival effects seem to have been conferred upon CTCs due to enhanced metabolic activity induced by elevated expression of ATOH8, which ultimately supported intravascular survival (42). A different protective concept of CTCs under shear exposure highlighted the shear-induced nuclear expansion of multiple cancer cell lines between 20 dyne/cm² and 0 dyne/cm², which was verified by estimating nuclear volume and histone acetylation levels via molecular assays. When shear-induced histone acetylation that led to nuclear expansion was inhibited, suspended CTCs died off at significantly higher rates, suggesting that the downstream effects of shear protect at least a fraction of CTCs that expand their nuclei (43). While more studies are focused on the effect of fluid shear on CTCs fate, the influence of constriction forces on cell survival during CTCs transit in capillaries is less explored. Nath et al. used a single linear (non-bifurcated) microfluidic channel (7 µm) to mimic capillary constrictions and observed that about 50% of cells that transited remained viable (44). On the other hand, Au et al. have shown that the majority of clusters of cancer cells were able to transit 7 µm single linear microfluidic channels, without damage or cell death (45). This suggests that clusters may possess an enhanced protection capacity, yet direct comparison to single cell transit and the transit behaviour of clusters in capillary bifurcations or more complicated geometries remain contentious. Kurma et al. review several microcirculation mimicking microfluidic models that have been exploited to investigate cancer cell stiffness, deformation, cytoskeleton, transit time among many others, during encounter of capillaries (46), however CTCs singlets and clusters' viability during and post-capillary transit remains very little explored.

While the biomechanical forces of the microcirculation clearly affect CTCs fate, the residence time of CTCs in the microcirculation can be from minutes to days (36), which provides sufficient time for interactions with the various immune cells or platelets in the bloodstream. Among the immune cells in the human circulation, neutrophils are the most abundant one (47). Spiegel et al. studied how neutrophils in mice that bore primary tumours affected CTCs. They showed that injected metastatic D2A1 cells in the blood of healthy mice were cleared at high rates (about 70%) from a natural killer (NK) cell response, whereas in mice that bore primary tumours, only 20% of injected cancer cells were cleared. This protective mechanism was due to neutrophils that were mobilised by primary tumours, since depletion of neutrophils curbed this protective effect. These data suggest that primary tumours had an effect on neutrophils, potentially polarising them to an activated phenotype, that enabled them to act in favour of CTCs survival (48). Indeed, several studies have highlighted how neutrophils may be polarised to a N2 activated phenotype that is considered pro-tumorigenic and pro-metastatic (49,50). This can be achieved either by physical contact with cancer cells or from secreted factors such as TGF β or exosomes (49,51,52). Szczerba et al. investigated for instance heterotypic clusters of cancer cells and neutrophils, formed in the primary tumour, that indoctrinated neutrophils to a phenotype presenting genetic attributes of a polarised pro-tumorigenic N2 state and further promoted proliferation and metastatic seeding (49). A similar concept to neutrophil activation has been shown for platelets, whose activation by cancer cell factors led to a reduced NK cell-induced clearance of CTCs (53,54). Moreover, activated platelets possess the ability to adhere to CTCs and protect them by downregulating NK cell activity, transferring immune antigens to cancer cells to evade immune surveillance (53,55) or even induce EMT (56), whose role in flow-induced cell death has been expanded earlier. In another study, adhered platelets around CTCs surface area were shown to protect CTCs from shear stress compared to CTCs alone, reducing the levels of cancer cell released lactate dehydrogenase (LDH) (an enzyme indicative of membrane damage) from 31% to 8% (57). Altogether, increasing knowledge suggests that residence and transit of CTCs in capillary beds allows for enhanced intercellular and biomechanical interactions that can affect their survival. CTCs survival is a critical success factor during metastasis as it increases the availability of CTCs candidates for downstream steps, including arrest, endothelium adhesion and extravasation, crucial for metastasis (58).

Briefly, the vascular endothelium (expanded later) is a protective barrier between organ tissues and the blood stream. The endothelium is lined up by endothelial cells that are tightly connected, preventing the entry of CTCs (59). However, the endothelium is often disrupted when cancer cells break away from the primary tumour and similarly, it is disrupted when CTCs try to invade another organ tissue (58,59). Nonetheless, the endothelium plays a key role in CTCs responses during their transit in blood vessels. Follain et. al have demonstrated in vivo in zebrafish models that it was locations with low velocity profiles that promoted arrest of CTCs (34). By injecting cells smaller than the measured vessels in the monitored zebrafish vessel areas, they observed that many cells arrested in areas with low velocity profiles, despite lack of cell to vessel constriction. Then, they identified that 51% of injected cells that were arrested, were present in vessels larger than 10 μm , suggesting that smaller vessels and narrower constrictions though important may not be the sole contributors for CTCs arrest. The authors have further shown that stable adhesion to the endothelium was achieved due to adhesion molecules, such as integrins, whose inhibition disrupted long-term adhesion (assessed at 3hr). They consolidated these findings by tuning the blood flow velocities to lower or higher levels, establishing a correlation between lower flow profiles with CTCs arrest, endothelium adhesion and extravasation, whose increase led to higher metastases formation (34). Osmani et al. have delved deeper in the adhesion molecules of cancer cells that permitted stable arrest and adhesion to the endothelium under shear forces, identifying Integrin beta-1 (ITGB1) as a critical mediator of this process (60). On the other hand, Offeddu et. al used their microfluidic platforms, mimicking the microvasculature to study CTCs arrest. While their observations agreed with previous data in respect to low velocity profile-induced CTCs arrest, they show that arrest efficiency is determined by physical trapping, especially at capillary bifurcations (61). In agreement to this idea, Kienast et al. have shown convincing data from mice studies, whereby injected human cancer cell lines were tracked in real time, revealing significant deceleration at vascular bifurcation geometries, compared to non-bifurcated ones, suggesting the importance of bifurcation points in physical entrapment (33). Prior work established that the ROCK/RhoA pathway gets activated during shear exposure (41), while Huang et. al. further demonstrated that CTCs passing through 3D printed microstrains imposing constriction forces on them led to upregulation of the RhoA protein, validated by western blot (62). Moreover, they showed that this activation resulted in a morphological change of CTCs (elongated $\rightarrow$ spherical) that promoted stable arrest and

eventually metastases formation. Using cytochalasin, an agent that inhibits F-actin polymerisation, they proved that this morphological switch was dependent on F-actin, since Cytochalasin-treated cells failed to change their morphology and thus arrest and metastasise successfully (62). Overall, while conflicting data emerge regarding the role of fluid velocities and capillary contributions in CTCs arrest and entrapment, it is most likely that CTCs hijack both features of the microcirculation to metastasise more successfully.

Intercellular interactions with CTCs are another important mediator of CTCs arrest and endothelium adhesion. Chen et al. suggested a strong correlation between systemic inflammation and metastases formation, brought together by neutrophils. Chemically stimulated neutrophils via LPS (inflamed phenotype) were co-injected with melanoma cells in a 3D perfusable microvascular network, which led to greater heterotypic aggregation and arrest in bifurcation points, compared to the interactions between non-stimulated neutrophils and melanoma cells. Stimulated neutrophils further disrupted the endothelium integrity through secreting interleukins and altogether led to significantly higher extravasation events of melanoma cells compared to non-stimulated (63). Spiegel et al. have showcased too that neutrophils that were indoctrinated by the presence of primary tumours in mice, modulated positively CTCs arrest and extravasation (48). In another study, Spicer et al. demonstrated via intravital microscopy in the lung microvasculature that inflamed neutrophils (attached to the endothelium) acted as adhesion bridges to incoming CTCs. The authors recorded more neutrophil-cancer cell adhesion events, when neutrophils were inflamed compared to either non-inflamed neutrophils or neutrophil-depleted animals (64). Similar to stimulated neutrophils, stimulated or activated platelets can interact with cancer cells, form intravascular aggregates and increase the arrest and adhesion of CTCs to the endothelium, steps which correlate to enhanced metastases formation (65,66). The use of aspirin had successfully reduced platelet activation, reducing thus the heterotypic aggregation, which in turn abrogated CTCs adhesion and metastases formation (67). Other drugs such as Ticagrelor have been shown to reduce platelet activation, by downregulating P-selectin protein expression, a protein which contributes to enhanced heterotypic interactions with breast cancer cells. The authors have further showcased that administration of Ticagrelor reduced significantly the colony units in lungs of mice, which could be partially attributed to disruption of platelet adherence on CTCs (68,69). Altogether, these data highlight that the

microcirculation represents an environment with a repertoire of functionalities, that enables both biomechanical and intercellular interactions with CTCs. Both mechanical features - particularly the blood flow- of the microcirculation and interactions with other cells found in the blood can promote metastasis by regulating crucial steps such as CTCs arrest, endothelium adhesion and extravasation, rendering the microcirculation a favourable location to assist CTCs in metastasis. However, collectively, the direct role of capillaries, especially of capillary bifurcations on CTCs transit and fate remains largely underrepresented.

1.2.3 Capillary bifurcations are important during CTCs journey towards metastasis

CTCs survival, arrest, endothelium adhesion and extravasation are most often the focus of studying intravascular dissemination during metastasis, both in in vivo and in vitro models. However, even in vivo, these phenomena are recorded hours post-injection of cancer cells, when CTCs are already arrested, adhered or even extravasated (34). Thus, the direct interactions of CTCs with the biomechanical features of the microcirculation i.e. capillary geometries and bifurcations may be missed. For instance, Kienast et al. tracked down via live imaging injected cancer cells in mice brain's vasculature and quantified the diameters of cells and small vessels they initially encountered (33). By comparing instances where cells were smaller than vessels diameters to instances where cells were equally-sized to vessels diameters, they observed that transit velocities of cancer cells were lower in the latter case (33). Additionally, they quantified lessened transit velocities of injected cancer cells at capillary bifurcations, as opposed to non-bifurcated capillaries (33). Furthermore, due to a live imaging approach, the authors were able to verify the slow movement of CTCs, which in some cases took more than a single day to change position and location in the capillary bed (33); observations that would not have been possible without real time imaging. These data prime this short timeframe as crucial to study in more depth CTCs fate, when they encounter and transit in the microcirculation. In another study, Yamauchi et al. visualised trafficking of injected cancer cells in the skin vessels in mice, observing their entrapment and deformation in capillary bifurcations. With their live imaging approach, they tracked cancer cells' positions relative to various capillary diameters and quantified that 80% (16/20) of cells present in capillaries ($>8\text{ }\mu\text{m}$ in diameter) migrated, whereas 84% (21/25) of cells present in capillaries ($<8\text{ }\mu\text{m}$ in diameter) did not migrate, suggesting the importance of narrower capillary sizes in

trapping CTCs (70). In another study, Au et al. quantified in vitro tumour cell velocities in different capillary sizes, using microfluidic models of capillaries, demonstrating that the higher the cell diameter in respect to vessel diameter, the lower the cell's velocity (45). These findings are in agreement with previous in vivo findings, presented by Kienast et al. (33), as described above. Au et al. further demonstrated that homotypic cancer cell clusters, larger than single cells, would stay trapped for hours and move very slowly within capillary bifurcations of zebrafish animals (45), findings which agree with the aforementioned studies. Furthermore, real-time imaging of cluster transit in straight capillary models revealed that clusters reorganised into single-file chains, a phenomenon which they postulated that was partially influenced by the adhesion forces within cells in the cluster (45). Cumulatively, these data lend credence to the importance of real-time imaging approaches to record cellular responses and understand better the phenomena that affect CTCs trafficking in the microcirculation.

By performing intravital imaging (live imaging of animals through microscopy) on the lungs of live mice, Headley et al. observed that injected melanoma cells got trapped in their lung microvessels and shed large nuclei-free large microparticles (LMPs) from the membrane of CTCs that were $>1\ \mu\text{m}$ in diameter -also termed as cytoplasts by the authors- while most cells remained viable (**Figure 1-3**) (71). Similar nuclei-free LMPs have been identified in other in vivo studies either in mice or in zebrafish models (62,70,72). However, the apparent lack of investigation of their biogenesis mechanisms and nature across the literature suggests that they may have been incorrectly characterised and considered (by visual observations only) as apoptotic fragments and thus ditched (62,70,72). Instead, Headley et al. proved that these LMPs they observed were not a product of apoptosis, since the treatment of cells with an apoptosis inhibitor did not reduce or abolish LMPs generation. What is intriguing in Headley's et al. observations, is the calculation of a lower shedding frequency present in lung slices from mice (static conditions), suggesting that various parameters of the microcirculation such as shear stress and capillary geometries may drive cellular shedding of LMPs (71). While this phenomenon and the nature of these shed LMPs in general remain immensely underrepresented, this shedding process may bring together phenomena that have been described in patients previously, yet they remain unconnected.

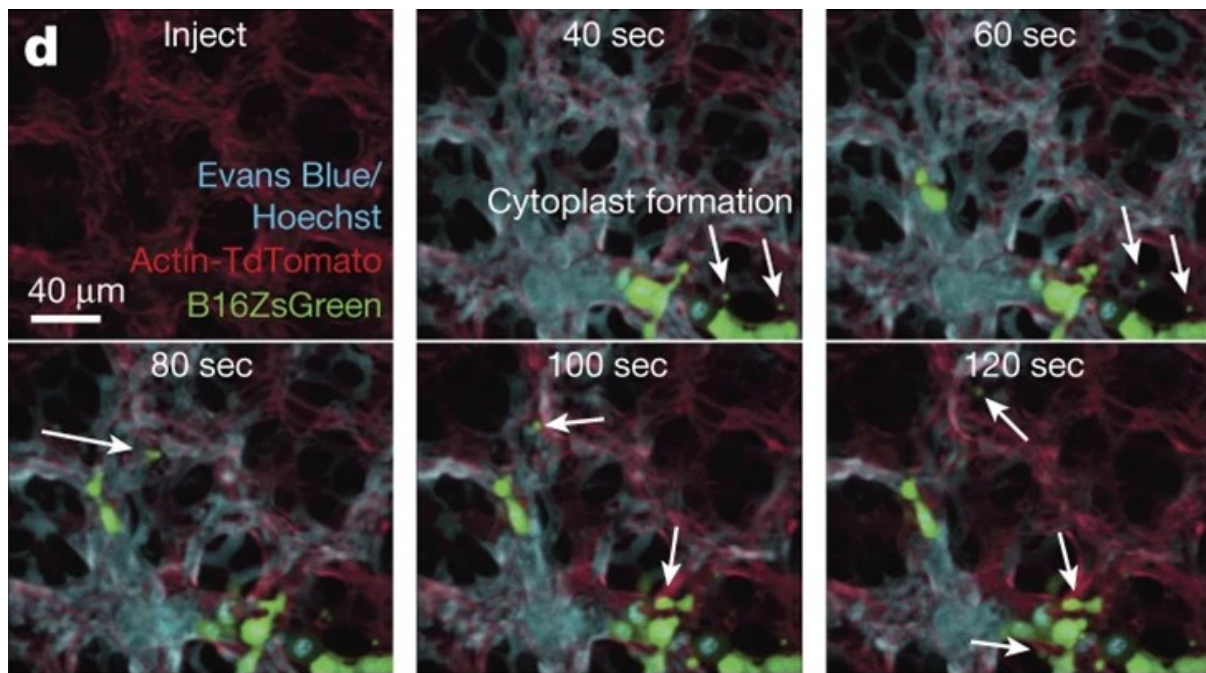


Figure 1-3: Circulating tumour cells shed part of their membrane when encountering capillary beds in the lung microcirculation in vivo. (71)

On the one hand, two independent studies have used CTCs from patients, isolated from different vascular locations to compare their size (73,74). For instance, the cell surface area of CTCs isolated pre-capillary transit (hepatic veins) from patients with hepatocellular carcinoma was larger ($100.58 \pm 73.54 \mu\text{m}^2$ vs $49.69 \pm 29.09 \mu\text{m}^2$), compared to the cell surface area of CTCs isolated from the periphery (post-capillary) (74), suggesting that capillary beds or the microcirculation affects CTCs fate in unexplored ways. On the other hand, two independent groups have identified similar large nuclei-free microparticles in the blood of patients with prostate, metastatic breast, metastatic colorectal and non-small cell lung cancers that were co-isolated with CTCs (75–77), termed as tumour-derived extracellular vesicles (EVs) and large EVs (LEVs) by the authors. Both Nanou et al. and Gerdtsen et al. showed that these microparticle counts were significantly higher compared to CTC counts (76,77). For instance, in patients with castration-resistant prostate cancer ($n=190$), the average microparticle count was at least ten-fold higher compared to CTC counts (100 vs 10 counts), while some patients presented about 5000 microparticle counts per 7.5 ml of blood draw. It is noteworthy that the 97.8% of patients (186 out of 190) presented higher microparticle counts compared to CTCs (76). It should be noted that there is no direct proof that these large particles isolated from patients are the same as the ones observed by Headley

et al. (71). Nonetheless, their similarities suggest that at least a fraction of these are likely originating from CTCs in the circulation. Interestingly, while the presence of large particles in the blood of patients (75) and the CTCs size reduction post capillary transit (73,74) appear as two distinct phenomena, the recent real-time observations of cellular shedding from Headley et al. (71), may link these disconnected phenomena. While Headley et al. have ascribed cellular shedding to shear stress (71), the microenvironment of the microcirculation, consisting of an interconnected network of numerous and diverse capillary geometries of distinct angles, constriction widths, symmetry configurations and multiple bifurcations among others (33), presents many features that may affect cellular shedding. Thus, it is imperative that the features of the microcirculation are studied and dissected in depth to understand better not only the biogenesis mechanisms of LMPs but also unexplored haemodynamic and transit phenomena of both single and clustered cancer cells.

To conclude, it appears that definitions and terminologies for these large microparticles vary. The terms large microparticles (also cytoplasts), tumour-derived EVs and LEVs have been used by Headley et al., Nanou et al. and Gerdtsson et al. respectively (71,76,77). It remains unknown to which extent these present similarities and differences and to delve deeper into the nature of these identified large particles, it is prerequisite to introduce the broad field of extracellular vesicles.

1.3 Extracellular vesicles

EVs are defined as particles secreted by a cell, enclosed in a lipid bilayer, lacking nuclei and unable to replicate (78). They contain biological material, including almost all the major biological constituents of a cell, such as nucleic acids (RNA/DNA), proteins and lipids (78). Extensive research has delineated several aspects of their roles and functionalities in healthy and diseased conditions, including cancer and they can be secreted by all types of cells (79,80). However, the diversity in which they are found and the numerous classification criteria that exist, merge or overlap to enable distinction between exosomes or microvesicles for example, puzzle further the field (78,81). For instance, when a new type of particle does not meet all current criteria for a particular type of EV, then a new type of EV has been discovered. This heterogeneity has on the one hand expanded the field of EVs immensely, but has also contributed to confusion and miscommunication among the research community

(81). The International Society for Extracellular Vesicles has proposed a statement in respect to the minimal information required to characterise, analyse, classify and study the various different subtypes of extracellular vesicles (78). This allows and necessitates the classification of an observed particle to facilitate the accurate communication between researchers that may have observed similar particles yet are unaware of their nature. Given the focus of part of this thesis is the type of large particles, the general categories of EVs that will be covered below are sub-classified as exosomes, microvesicles and large vesicles, however a more extensive list reviewed and brought together by others can be seen in **Table 1-2** (71,82–85). It should be noted that size and overlap exists among different types of EVs, additional classification criteria such as molecular markers, more specific mechanisms of biogenesis and downstream functionalities are important part of the discussion.

Table 1-2: Types of EVs, size, biogenesis mechanism and downstream functionalities (71,82–85)

EV TYPE	SIZE	BIOGENESIS
Exosomes ⁸²⁻⁸⁵	30-120 nm ^{83,85} / 40-150 nm ⁸⁴	Multivesicular bodies ⁸²⁻⁸⁵
Small ectosomes ⁸³	30-150 nm ⁸³	Outward budding of the plasma membrane ⁸³
Microvesicles ⁸²⁻⁸⁵	150-1000 nm ^{83,84} / 50-1000 nm ⁸⁵ / 100-1000 nm ⁸²	Plasma membrane shedding ⁸²⁻⁸⁵
Apoptotic bodies ⁸²⁻⁸⁴	1000-5000 nm ^{83,84} / 800-5000 nm ⁸²	Apoptosis ⁸²⁻⁸⁴
Migrasomes ^{82,83}	500-3000 nm ^{82,83}	Retraction fiber ^{82,83}
Large oncosomes ⁸²⁻⁸⁵	1-10 μ m ⁸²⁻⁸⁵	Plasma membrane shedding ⁸²⁻⁸⁵
Exophers ⁸³	3.5-4 μ m ⁸³	Unknown ⁸³
Large microparticles ⁷¹	0.5-25 μ m ⁷¹	Shear & capillary derived ⁷¹

Matrix vesicles ⁸⁴	40-1000 nm ⁸⁴	Matrix binding and release ⁸⁴
Autophagic EVs ⁸⁴	40-1000 nm ⁸⁴	Autophagosome ⁸⁴
Stressed EV ⁸⁴	40-1000 nm ⁸⁴	Plasma membrane shedding ⁸⁴

1.3.1 Types of extracellular vesicles

The dominant criteria used to distinguish between these types of vesicles are frequently the biogenesis mechanism and the size, though discordances still exist. For instance, the size of exosomes has been reported from many to be from 30-120 nm (85), whereas other reviews highlight a size range of 40-150 nm, but it is generally accepted that usually exosomes are not larger than 150 nm (84). Microvesicles on the other hand are usually considered as larger than either 50- or 100 nm up to 1000 nm. Along with their size difference, exosomes are secreted by multivesicular bodies that are formed inside the cell, whereas microvesicles are released from the surface of cell membrane, during blebbing (86). The biogenesis and size range of the other EVs that are smaller than 1 µm are expanded in **Table 1-2** (71,82–85).

In respect to large vesicles, accepted consistently as larger than 1 µm in diameter, the four major types that are reported are apoptotic fragments (usually 1-5 µm large), large extracellular vesicles (LEVs), large oncosomes (LOs) and large microparticles (LMPs) (71,76,77,84,86,87). Throughout research studies, some of these terms, especially revolving around the former three, are used interchangeably, yet it is unclear whether they are the same or not. Focusing on the last three mentioned types of large vesicles, the majority of the research literature reports typically their size range from 1-10 µm (71,76), though values outside this range have been reported (71,77). However, the field remains very vaguely described and disagreements still exist. For clarity, the terms that will be used will be the ones that the authors of the studies employed, yet these need to be cautiously described given the incomplete validation based on the position statement from the International Society for Extracellular vesicles (78).

For instance, Gerdtsson et al. enumerated LEVs from the blood of castration resistant prostate cancer patients and characterised LEVs size, ranging from 6.3-20.4 µm, which deviates from

the usual range of 1-10 μm , reported for LOs. It needs to be mentioned that the authors omitted nuclei-negative, Cytokeratin-positive objects, due to their inability to verify the positivity of LEVs for a membrane structure, for smaller objects than 6.3 μm (77). Headley et al. measured the size of LMPs that were generated post melanoma cells injection in lung microvessels during live imaging, identifying a size range between 0.5-25 μm with a mean of 5 μm , while the majority of the observed LMPs was below 10 μm (71). Several other studies report LEVs or LOs isolation from cultured cancer cell lines, by incubation in serum-free media, which triggers shedding and release of large vesicles or LOs in the media (88,89). For instance, Johnson et al. isolated EVs from a medulloblastoma cell line that were split in small, medium and large vesicles, with the latter being between 0.935-5.7 μm (90). What becomes obvious from all these studies is that while there appears to be an overlap in size, there is also a large gap in the size ranges. From a first glance, this difference may be attributed to in vitro vs in vivo vs patient data. For example, human breast cancer CTCs are significantly larger than most commercial cell lines (34). A second argument can be made regarding the biogenesis of these LEVs, reported under various names. For instance, the LMPs observed by Headley et al. are generated during melanoma cell transit in lung microvessels, exposed to capillary forces and shear stress (71). On the other hand, as explained above, LEVs isolated from a medulloblastoma cell line were obtained by serum-depletion under static conditions and lack of any additional mechanical pressure (90). Identification of LEVs directly from blood draws from patients (75,77) unfortunately does not reveal any information regarding location origin (generation in primary tumour or during circulation) or biogenesis mechanism.

It is at this point that the field of large particles becomes rather blurry. First of all, it is debatable whether these three types (LEVs, LOs and LMPs) -as described in the previous paragraph- are similar or not. Starting off with the biogenesis mechanism, many studies that isolate LEVs from cancer cell lines via either serum depletion or inhibition of certain proteins, distinguish generated LEVs from LOs, while other studies employ the same strategy and name the collected particles as LOs, suggesting that they are the same as LEVs (87–89,91). Despite lack of complete understanding, LOs pose a fundamental difference, which lies in the enhanced quantities of DNA and the presence of a nucleus that have been identified previously (91), yet not all studies studying LOs have identified them positive for nuclei. Moreover, in these studies, LEVs or LOs appear to be generated during plasma blebbing

(87,89), as shown in relevant images. This reasoning and level of acceptance from the research community seems to classify LOs as a sub-type of LEVs. Interestingly, LOs have been identified both in human biopsy samples (localised tumours) and in the circulation of mice (87). In a follow-up study, the same group identified Cytokeratin-positive LOs, obtained and isolated from the plasma of patients with prostate cancer (88). It remains to be explored whether LOs were directly shed in circulation from transiting CTCs (as Headley et al. observed for LMPs)(71) or whether they entered the bloodstream post their generation in the primary tumour. It is thus highly possible that the LEVs or LMPs of higher than 1 μm identified in the blood of patients represent a mix of LOs and shear-generated LMPs, which warrants future research. Nonetheless, it is crucial that researchers that observe and study these particles stick to the guidelines of EVs classification criteria, many of which require protein markers validation (78), that will improve communication among the research community in a united effort to elucidate the complex field of LEVs.

1.3.2 EV Biologics

1.3.2.1 Proteins

LEVs or LOs similar to exosomes and microvesicles, carry molecular cargo, including proteins (78,92). The presence of distinct types of proteins within LEVs has been previously demonstrated, including cytosolic (enclosed within LEVs) and membrane-associated (93). For instance, Ciardiello have identified the presence of adhesion molecules on LOs originating from prostate cancer cell lines, such as integrin α -V and matrix metalloproteinases, verified via multiple techniques including western blotting, immunofluorescence and flow cytometry (89). Minciacchi et al. studied the proteome of large oncosomes, obtained from prostate cancer cell lines, which revealed the presence of cytosolic proteins such as heat shock proteins and cytokeratin-18 (filamentous). Interestingly, they also compared the proteomic profiles between large oncosomes and exosomes, discovering that about 25% of all 407 proteins found, were differentially expressed, suggesting crucial differences between exosomes and LOs. Moreover, the authors showed that LOs were specifically enriched in metabolic enzymes (88). This is an interesting point, given that Headley et al. have visualised the presence of mitochondria via live imaging and flow cytometry (for quantitative analysis), suggesting that mitochondrial proteins involved in metabolic processes are present both in LOs and shear-derived LMPs (71). It remains to be tested how many similarities and how many

differences exist between these two. Johnson et al. demonstrated via fluorescence imaging that other cytoskeletal proteins than Cytokeratins such as F-actin are also present in LEVs (90). Gerdts et al. performed imaging mass cytometry, employing an antibody panel in combination with imaging to verify proteins identified in prostate cancer patient-isolated LEVs. While LEVs were negative for nucleus (verified by immunofluorescence staining using a nuclear stain), nuclear proteins were identified; albeit at low levels. Originating from the surface of cells, LEVs were enriched naturally for membrane proteins (77). Interestingly, while LEVs retained higher levels of epithelial proteins, they also expressed vimentin, a marker for mesenchymal cells, which are found more commonly in circulation, suggesting that LEVs may in fact be generated during CTCs transit. As expected, LEVs expressed consistently PSA protein, which is used as a traditional screening marker to diagnose prostate adenocarcinoma (77). In another study focusing on glioma cells, LEVs expressed tetraspanins, such as CD63, albeit at lower levels compared to their expression in smaller vesicles, whereas epidermal growth factor receptor (EGFR) was enriched in larger vesicles (94). While several studies have enquired the protein content of LEVs, these still represent a small fraction of the proteins known to exist in cells or other EVs and they are restricted to LOs that have been generated due to plasma blebbing. However, there is almost no information on the protein content of large particles, generated during CTCs transit in the microcirculation.

1.3.2.2 Nucleic acids

Nucleic acids represent another major molecule in cells and EVs alike (95). Notably, EVs of all sizes by definition do not contain nucleus (78). However, there are a few reports that attribute the presence of nucleus to LOs (91), which sparks a debate whether they can be considered or not LEVs. Nonetheless, all EVs, from exosomes to microvesicles to LEVs present a variety of RNA and DNA molecules (95). Chen et al. have performed an extensive study on the RNA content of exosomes by comparing it with their cancer cell of origin, from samples that were obtained from patients. The first difference they observed was that exosomes encompassed RNA molecules with a larger number of nucleotides. They further identified about 50% variance between the transcriptomic profiles of EVs compared to their cells of origin. Looking at enrichment analysis, they identified 8000 RNA molecules that were differentially expressed between EVs and cells, with exosomes being enriched predominately for effector and regulatory molecules (96). Focusing on LEVs, Di Vizio et al. used propidium iodide to stain DNA

and RNA from LEVs isolated from the conditioned media of a prostate cancer cell line (LNCaP). The isolated shed vesicles were positive for RNA but not DNA, according to the results obtained from agarose electrophoresis, with or without treatment of DNase or RNase (87). In contrast, Vagner et al. identified about a 7-fold increase of DNA in LEVs over small EVs, isolated from prostate cancer and glioblastoma cell lines. To recapitulate their findings in patients, the authors isolated LEVs and small EVs from the blood of patients with prostate cancer, extrapolating significantly higher amounts of double stranded DNA in LEVs compared to small EVs (91). Ji et al. studied more in depth the RNA molecules found within EVs, secreted by a colon cancer cell line (97). By centrifuging cell supernatant at 10000 x g for 30 min, they isolated what they termed as shed microvesicles (97), though it remains unclear whether this represented a mix of LEVs and microvesicles, despite reports on pure LEVs populations isolation after centrifugation at 10000 x g for 30 min (89). By performing RNA sequencing, the authors identified multiple RNA types, including miRNA, rRNA, tRNA, snRNA, snoRNA, srpRNA and mRNA degraded fragments. Furthermore, they showed that out of 254 miRNAs that were highly expressed, only 20 miRNAs represented about 91% of the total reads (97). In another study, Morello et al. interrogated a tumorigenic KRAS-transformed RWPE-2 cell line, which generated at least 2-fold higher LEV shedding events per cell, compared to the non-tumorigenic one. Applying a miRNA array, they found out that different miRNA molecules were represented in the cell line compared to isolated EVs. Using a small fraction of discovered miRNAs, they validated molecularly with real-time PCR that many miRNA molecules are differentially expressed between LEVs and their cells of origin (98). Bertolini et al. that showed the presence of mRNA transcripts within LOs, when examining secreted LOs from glioblastoma neurospheres. 4 mRNA transcripts were identified and enriched in LOs that were isolated from glioblastoma neurospheres with high expression of V1G1, compared to ones isolated from low V1G1 expression (99). Altogether, LEVs contain various RNA molecules and our understanding will only increase by integrating more genomic and transcriptomic approaches routinely in LEV and LOs investigation. Last but not least, it becomes increasingly clear that similar to protein differences, LEVs and smaller EVs, such as exosomes differ also in their RNA content, both qualitatively and quantitatively.

1.3.2.3 Lipids

A less commonly studied type of biological molecule that can be found in cells and EVs are lipids (100). Llorente et al. examined the lipid content of exosomes via molecular lipidomics that were secreted by a prostate cancer cell line (PC3), determining distinct lipid profiles between exosomes and prostate cancer cells. Among the 23 lipid classes that were used as a basis for comparison, the majority of those tested were upregulated in exosomes, while enrichment of certain classes in cells was also observed. Moreover, analyses of lipid species revealed about 300 different molecular lipid species present in exosomes, many of which had distinct expression profiles from their cell of origin, including glycosphingolipids and cholesterol (101). Brzozowski et al. used a similar approach to compare shed EVs from either non-tumorigenic or tumorigenic or metastatic prostate cells. From a total of 187 lipid species quantified, the lipid profiles of shed EVs among these three cell types were significantly distinct. EVs that were purified from tumorigenic or metastatic prostate cell lines revealed a higher composition of glycerophospholipids, sterol lipids and sphingolipids, whereas non-tumorigenic cells secreted EVs that comprised of fatty lipids and glycerolipids. The authors conclude that differences in the lipid profile between non-tumorigenic and metastatic cell lines may present a basis for the use of LEVs and their lipid content as predictive biomarkers (102). Another type of EVs, microvesicles has been shown by Gabrielli et al. to incorporate higher lipid concentrations of N-arachidonylethanolamine compared to exosomes and in general, the overall load of EVs (mix of exosomes and microvesicles) was about 2900 times higher compared to the respective cell of origin (103). The authors further validated via fluorescence microscopy and immunostaining that microvesicles were positive for lipids(103). In another study by Haraszti et al., both the proteome and lipidome of exosomes and microvesicles were examined for various cell lines, including glioblastoma, hepatocellular carcinoma and human bone marrow-derived mesenchymal stem cells. One major finding was the differences both in the total lipid and protein composition between exosomes and microvesicles. The other finding that the authors demonstrated was that both the proteome and lipid content of exosomes released either by tumorigenic or non-tumorigenic cell lines was distinct, further suggesting that EVs can be used to diagnose malignancies (104). While lipidomic analyses exists in the literature for exosomes and microvesicles, there are minimal or potentially no studies in LEVs of greater than 1 μm in diameter. However, given the fact

that LEVs are significantly larger than exosomes or microvesicles and originate from the cell membrane, it is highly likely that they will contain significant amounts of lipid concentrations and a broad list of lipid classes.

To recap, the studies that have so far demonstrated the wide repertoire of LEVs composition in respect to proteins and nucleic acids, emphasise the importance of understanding and interrogating more extensively these, in multiple cancer cell lines and patient samples. In addition, this section's literature review emphasises that molecular cargo differences exist between a. distinct types of EVs, b. EVs and cells and c. EVs obtained from tumorigenic and non-tumorigenic cell lines. A better understanding of the molecular content of LEVs is crucial to enable their co-utilisation in the clinical setting but will also allow the research community to delve deeper in LEVs contributions to downstream functionalities during cancer progression. Nonetheless, while a few studies have profiled LOs and LEVs (77,88), shear-derived LMPs remain largely underexplored. It is currently unknown which biomolecules they contain, at what quantities, how they can affect cancer metastasis and whether they can be considered EVs in the first place.

1.3.3 LEVs use in liquid biopsies

Another important aspect of studying the content of LEVs is their utilisation potential in liquid biopsy strategies, similar to exosomes use (105). Pezziccoli et al. grouped collectively studies that support the notion that LEVs can be effectively used for diagnosis/prognosis, as predictive biomarkers and for monitoring therapy response and therapy resistance (100). In the last decade, the massive expansion of techniques to isolate and study smaller vesicles such as exosomes from the blood of patients has bloomed their potential use in early diagnosis and prognosis (106). LEVs on the other hand are less studied, yet they are about 10-100 times larger (diameter-wise) compared to exosomes, suggesting that they may encapsulate significantly higher amount of proteins and nucleic acids as well as present more diversity, a notion bolstered by Minciacchi et al. that found protein differences between exosomes and LOs (88). Additionally, Vagner et al. demonstrated that by comparing equal amounts of LEVs and small EVs, the large fraction of DNA mass was recovered in LEVs (91). Another advantage of utilising LEVs is the multitude of techniques that exist that facilitate LEVs purification and recovery. Pezzicoli et al. review these and highlight how ultracentrifugation is particularly very useful by separating LEVs from exosomes for example

(100). For instance, Minciacchi et al. have separated LEVs from nano-sized EVs i.e. exosomes by applying different centrifugation speeds. Supernatant was obtained from pelleted cells and debris and centrifuged at 10000 x g to retrieve LEVs population, documented in numerous studies as a standard centrifugation speed, whereas the supernatant was further centrifuged at 100000 x g to retrieve nano-sized EVs (88). While these data highlight an easy method of purification and separation from cells and between distinct types of EVs, particularly LEVs, these studies are restricted to conditioned media from cultured cancer cells; yet the situation in blood isolated from patients is more convoluted.

Nanou et al. have characterised digitally stored images from archives, identifying LEVs co-collected with CTCs from blood draws. Pooling images from patient data, whose blood was analysed with the Cell Search system that operates based on recognition of epithelial cell adhesion molecule (EpCAM+), Cytokeratin+ (CK8, CK18, CK19) while negative for CD45 (immune cell marker), they identified LEVs at higher counts than collected CTCs in all cancer types (76). A limitation of correctly identifying LEVs of cancer cell origin was their higher mean count in healthy donors (0-20 range) (76), which in theory should be 0, reported previously from the same group (76,107). This is one of the reasons why understanding and comparing the proteome or the molecular content of cancer-originating LEVs holistically compared to other LEVs secreted from immune cells or other cells in the bloodstream is vital to distinguish these and facilitate the identification and collection only of cancer-secreted LEVs. In a similar approach, Gerdtsen et al. also analysed previously acquired images of CTCs collected from a clinical trial from patients with castration-resistant prostate cancer. CTCs were originally scanned at 10x in slides and these images were reanalysed using a trained algorithm to identify and enumerate LEVs. The authors have also identified an increased frequency of LEV counts compared to CTCs and positivity for cytokeratin (77).

The higher frequency of LEV counts could be partially attributed potentially to their smaller size and the lack of nucleus compared to CTCs, while this is not demonstrated yet. The nucleus is evidenced as the stiffest organelle in a cell and is often reviewed as a limiting factor in the successful navigation of cells in narrow spaces (108,109), which could explain partially the frequent entrapment of at least a fraction of CTCs. CTCs are often entrapped in small capillaries (110) and thus the ability of LEVs to escape these may be the reason of their higher counts. Furthermore, LEVs positivity for cytokeratin seems to correlate with independent

studies that have identified elevated plasma levels of Cytokeratin 18 in patients with prostate cancer compared to healthy donors (88), suggesting that LEVs can be used clinically. Especially at early stages of cancer, where the number of invading cells in the bloodstream is low, the expected CTC number tends to be low (111). However, if LEVs are generated in circulation, for which there is strong evidence, their numbers may be sufficient for diagnosis, even in early stages, given their generally higher counts compared to CTCs (76,77). Moreover, Nanou et al. identified a cut-off value of 105 LEVs, whereby patients exhibited three-fold higher median overall survival, when LEVs number was lower (76). This number is not large enough to obtain, when the same groups showed that thousands of LEVs can be collected from the blood of patients, suggesting their high potential to clinical utility. Overall, the higher LEV counts in the blood of patients, the presence of proteins on LEVs and their differences from CTCs and other smaller EVs, suggest cumulatively that LEVs should be used as complementary biological material from patients to improve diagnosis, prognosis, patient monitoring and overall amount of extracted information. However, to successfully lead LEVs towards that direction, a deep examination of their proteome is required.

1.3.4 Role of EVs in cancer metastasis

The role of EVs in cancer metastasis is highly diverse (**Figure 1-4**) (112). More specifically, smaller EVs, such as exosomes and microvesicles in cancer progression and metastasis are more extensively studied (113,114), yet there is a lot of ground still to be covered. In general, a great number of studies document the significant role of exosomes in cancer, including intercellular communication, immune cell regulation, tumour microenvironment remodelling and reprogramming, tumour angiogenesis, tumour cell migration and invasion, EMT induction, drug resistance mechanisms, apoptosis suppression and immune T cell evasion among others (80,113,114). Moreover, exosomes have been identified in the blood of patients (115,116) and there is evidence backing the concept of exosomes causing thrombosis and clotting, dysfunctions to the endothelium via vascular remodelling and formation of a pre-metastatic niche and an inflammatory milieu that can facilitate metastasis by CTCs (117–119). Extensive research on tumour-secreted microvesicles, reviewed by Zhu et al. and supported by significant volume of research efforts reveal that cumulatively tumour-secreted microvesicles activities to promote cancer progression converge to a large extent with those of exosomes (114).

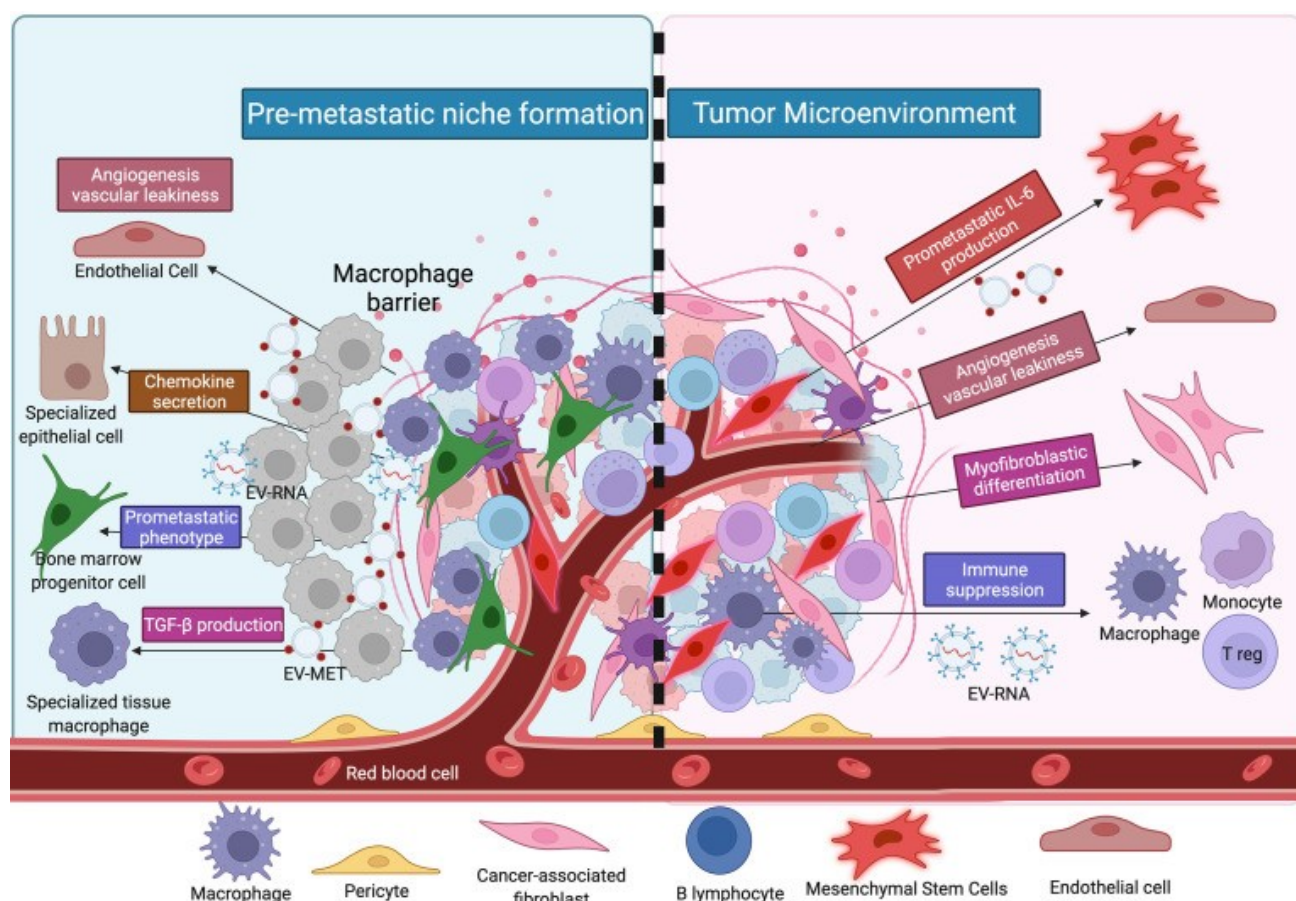


Figure 1-4: Role of EVs in cancer metastasis. (112)

On the other hand, the role of LEVs is less explored, yet numerous studies attest to their various roles. Of course, the range of LEVs-based activities may be different due to their differences in biological cargo or biogenesis mechanisms. For instance, LOs that are generated in the primary tumour can induce fibroblast reprogramming (120). On the other hand, shear-derived LMPs are generated directly in circulation and their activities may be restricted to interactions in the blood compartment i.e. with immune cells that transit (71). It remains to be seen whether LEVs have the ability to attach to the endothelium or extravasate through it. Tersely, LEVs and LOs generated under static conditions have been described to: a) interact with other cell types, including fibroblasts, immune cells, endothelial cells and platelets b) mediate transfer of biological material, c) increase proliferation and survival ability d) disrupt endothelial barrier integrity e) regulate immune cells f) contribute to the formation of a pre-metastatic niche and g) confer drug resistance and potentially other bountiful mechanisms that are yet to be explored, as the field of LEVs is more broadly unfolded (71,89,98,100,120,121).

More specifically, Minciacchi et al. demonstrated that LOs isolated from prostate cancer cell lines were internalised upon co-culture from normal prostate fibroblasts, inducing their differentiation. Reprogrammed fibroblasts released Interleukin-6 and matrix metalloproteinase-9 and also had an upregulated expression of α -smooth muscle actin, a marker of fibroblast activation. LOs have thus stimulated altogether a reprogrammed fibroblast phenotype that exhibited a pro-vascularised phenotype and stimulated tumour growth in mice (120). Bertolini et al. studied the influence of LOs, obtained from glioblastoma neurospheres, on a glioblastoma cell line, demonstrating their uptake and delivery of molecular cargo, which recipient cells retained up to 90 days, as validated molecularly by PCR. LO-induced cargo delivery improved several aspects of cultured cells, including proliferation, survival, ability to form neurospheres and their motility. To further prove the importance of these findings, the authors isolated LOs from the serum of glioblastoma patients and co-cultured these with recipient cells, as described previously, demonstrating again their successful uptake and similarities with the LOs that were isolated from the glioblastoma neurospheres (99). In another study, Minciacchi et al., following their results on LOs proteomics tied to a metabolic-specific role, they investigated how LOs affect cancer cell metabolism. By co-culturing LOs with their cells of origin, DU145 (prostate cancer cell line), they validated the upregulation of a metabolic enzyme, GOT1, claiming thus that LOs transferred protein material. Another interesting finding, emerging from that study is the increased metabolic activity of cells post-exposure to LOs, compared to both untreated and exosome-treated cells, which increased the percentage of cells that were found in the proliferative S phase (88). This constitutes a powerful suggestion that LOs and LEVs, whose biological content is different than exosomes, are in fact capable of inducing different changes to cells, with which they interact. Spurling et al. identified a suspended vesicular population in their cultured melanoma cells, which retained cytoplasmic staining, similarly as described for LEVs. Upon their co-culture with a brain endothelial cell line in vitro, the authors extrapolated a reduced endothelial barrier resistance in the co-culture compared to untreated cells, reflective of the disrupted endothelium barrier. They further confirmed the disruption of endothelial integrity by visualising the differences observed in endothelial junction, by staining VE-cadherin, which revealed lack of staining intensity and morphological disorganisation. While the authors did not calculate the size of these vesicular structures, it is apparent from recorded images that they match the size of LEVs (121). It needs to be noted

that the authors refer to these as suspended cells, obtained from an adherent melanoma cell line. However, the retainment of cytoplasmic stain eliminates the possibility of these being apoptotic suspended cells. What was intriguing was the fact that they observed some of these vesicular structures to be positive for nuclear stain, yet there was no clear morphology of a nuclei structure (121). In their published images, the nuclei stain appears to resemble fragments of DNA, which is supported by Vagner et al. that quantified DNA on LOs (91). In a study led by Ciardiello et al., the authors isolated LOs from a more aggressive sub-line of prostate cancer DU145 cell line, resulted in increased adhesion of parental DU145 cells in vitronectin, an ECM molecule after co-culture for 60min, compared to LOs that were isolated from the parental cell line. Using similar experimental conditions, the authors showed that DU145 cells developed the ability to invade through Matrigel at higher percentages when co-cultured with LOs from the more aggressive subline, compared to DU145 control cells or cells treated with LOs isolated from control cells (89). These findings insinuate that LEVs contributions and downstream functionalities change dynamically and reflect at least to some extent their cell of origin. Last but not least, Headley et al. tested the impact of shear-derived LMPs in early metastasis. Upon their generation, Headley et al. quantified the percentages of various immune populations that ingested these i.e. cellular uptake. They further showed that macrophages that had ingested LMPs presented a distinct phenotype. To evaluate the involvement of this immune cell-loading phenomenon on metastasis, they compared wild type animals with animals, whose C-C chemokine receptor type 2 (CCR2) was knocked out, compromising thus monocyte recruitment. This led to a series of responses by reducing total metastasis and the number of macrophages that were positive for large microparticles ingestion in CCR2-compromised animals, while concurrently increased the numbers of immunostimulatory dendritic cells positive for large microparticles ingestion and cytotoxic CD8⁺ T cells (71). Altogether, while the number of studies that focus exclusively on the functionalities of LEVs (diameter >1 μ m) is small, the wide range of biological cargo and their interactions with cancer, immune, fibroblast and endothelial cells, highlight their importance in metastasis. However, the majority of these studies, with the exception of Headley et al., use LOs or LEVs that were isolated from the conditioned media of cultured cancer cells in static conditions. There is currently very little understanding on what capabilities shear-derived LMPs possess and how they can influence the pre-metastatic niche or cancer metastasis. Ultimately, the presence of shear-derived LMPs or LEVs in the blood raises the

possibility that they can interact and influence multiple cell types, such as endothelial cells that line blood vessels and immune cells, though these remain largely underexplored.

1.4 Role of vascular endothelium in cancer

The vascular endothelium represents an organ of endocrine origin with a multitude of roles. It consists of endothelial cells that line all the blood vessels of a human body and the lymphatic system. It is a vital organ that controls and regulates blood flow, platelet aggregation, immune responses, angiogenesis and inflammation, all of which are crucial for homeostasis (122). Various blood cells, including red blood cells, platelets, immune cells of both lymphoid (i.e. T cells) and myeloid origin (i.e. monocytes) transit through the vessels, arteries, veins and capillaries, as a transport route that enables them to perform their functionalities (123). Blood vessels i.e. capillaries are supporting life, by enabling the transport of oxygen as well as other nutrients from the blood to the organ tissues that require these to survive and be sustained (122). However, such a complicated system will be eventually under attack from various endogenous or exogenous factors that evidently lead to endothelium imbalance, instability and disruption, presenting thus phenotypes that are considered activated, dysfunctional and pro-inflammatory (124–127).

1.4.1 Markers of activated endothelial phenotypes

Endothelium activation and subsequent acquisition of pro-inflammatory features can be either the start of a disease/condition or the consequence of one (125). For instance, endothelium activation can lead to the development of atherosclerosis, blood clots, vascular remodelling and stroke among others (125). On the other hand, endothelium activation can be caused by diabetes, cancer, hypertension, smoking, blood clots and infectious diseases among others (128). More specifically, there are numerous mechanistic factors that can drive endothelium activation. For instance, Theofilis et al. review several such mechanisms by highlighting how bacterial endotoxins, pro-inflammatory stimuli i.e. interleukins and reactive oxygen species among others stimulate endothelial activation and dysfunction (128). Moreover, immune cells or platelets at an activated state themselves i.e. in autoimmune diseases, have been shown to correlate positively to endothelial activation. For instance, activated platelets secrete chemokines, coagulation factors, tumour-necrosis factor, inflammatory cytokines, all of which lead to upregulation of endothelial activation markers

(129). Furthermore, stimulated neutrophils i.e. during sepsis have a similar effect perpetuating the activation and dysfunction of the endothelium (130). Overall, endothelium activation is measured, assessed and validated by many different metrics and factors, which are important to delve deeper into (131).

Endothelium activation and dysfunction drives a series of changes in the individual cells that line the vascular endothelium (126,127,131). As elaborated above, stimuli such as pro-inflammatory factors can induce changes in endothelial cells. Primarily, expression of proteins such as selectins, intercellular- and vascular cell adhesion molecules (ICAM/VCAM) and major histocompatibility complex II (MHC II) are upregulated in the surface of endothelial cells, compared to non-activated or healthy endothelial cells (131–133). Another important mediator of healthy and functional state of endothelial cells are the intracellular levels of nitric oxide, whose increased intracellular levels have been correlated to an activated endothelial phenotype (134). Another activation marker of endothelial cells is the presence and secretion of several pro-inflammatory or coagulation-inducing molecules either in the plasma (patients) or in the cultured media (cell culture) from activated endothelial cells. Such molecules constitute, von Willebrand factor (VWF), P-selectin, platelet-activating factor, or many cytokines and chemokines such as interleukins or granulocyte colony stimulating factor among many others, reviewed extensively (131,135). Last but not least, another critical marker of activated endothelium is the loss of intercellular junctions (59). Tight and adherens junctions are present in the surface of endothelial cells and play a pivotal role in keeping the cells together and firmly attached (136). However, upon stimulation with TNF- α , a pro-inflammatory stimulation agent, endothelial cells start losing their junctions, leading to detachment from their neighbouring cells, leading to disruption of the endothelial barrier integrity (59,137,138). This variety in molecular changes leads cumulatively to an activated and pro-inflammatory phenotype of endothelial cells, whose continuous activities further perpetuate inflammation.

Activated endothelial cells are able to recruit immune cells i.e. leukocytes/monocytes or platelets. Via molecules such as VCAM-1, they enable physical interactions and attachment of leukocytes and monocytes for example, that would not occur in healthy endothelial cells (123,139). Through their secretion of VWF and platelet-activating factor, activated endothelial cells can stimulate platelet aggregation and activation that eventually leads to the

formation of blood clots and thrombosis (131,140). Moreover, by secreting granulocyte colony stimulation factor, activated endothelial cells drive generation of larger concentrations of white blood cells, leading to an increased immune response that often can sustain inflammation and tissue damage (123,135,141). Loss of intercellular junctions can often lead to a disrupted and disorganised lining of blood vessels, which facilitates leakage, permeability and extravasation of other cell types or factors (59). Moreover, activated endothelial cells secrete a wide variety of interleukins, many of which are associated with polarisation/differentiation of monocytes to macrophage populations, either pro-inflammatory M1 or anti-inflammatory M2 (135,142,143). At this state, it needs to be clarified that endothelium activation is also a natural defence mechanism upon tissue injury. For example, upon bleeding, the endothelium of a human becomes activated, recruiting and stimulating platelet production to clot the wound (140). However, in diseases or conditions that are sustained for long periods of time, i.e. cancer, many of these roles and functionalities of the activated and pro-inflammatory endothelial phenotypes are hijacked to support cancer progression and metastasis as a principal accomplice (144).

1.4.2 Activated endothelial phenotypes in cancer

The endothelium is involved in many different steps in cancer metastasis, including cancer cell intravasation, arrest, adhesion and extravasation (33,34,59,62,144,145). The purpose of this section does not serve to expand on each rather to emphasise a compelling correlation between these metastatic steps and a proinflammatory, pro-coagulant endothelium phenotype. Chen et al. utilised an in vitro self-organised microvasculature model, demonstrating that either melanoma cells or melanoma cells co-perfused with LPS-stimulated neutrophils (activated state) increased ICAM-1 expression on the surface of endothelial cells, which rendered endothelial cells activated, as established previously. Interestingly, treatment of endothelial cells with an anti-ICAM-1 antibody resulted in reduced number of heterotypic interactions between LPS-stimulated neutrophils and melanoma cells, compared to non-activated endothelial cells. Moreover, activation of endothelial cells was responsible for recruitment and attachment of LPMs-stimulated neutrophils, highlighting the various roles of activated endothelial cells (63). Ludwig et al. observed via intravital microscopy enhanced melanoma cell interactions with endothelial cells in skin vessels (postcapillary venules) in wild type mice compared to P-selectin negative mice. They further showed that the enhanced

interactions of melanoma and endothelial cells in wild type mice were not assisted by activated platelets (that also express P-selectin), suggesting that it was activated endothelial cells that favoured melanoma cell interactions (146). Klemke et al. have shown that activated endothelial cells, expressing higher expression of VCAM-1, facilitated transendothelial migration of human melanoma cell lines, compared to non-activated endothelial cells, suggesting that activated endothelial cells can support intravasation and extravasation (147). Läubli et al. found out that colorectal cancer cells in combination with platelets activated endothelial cells. This activation led to enhanced recruitment and interactions with monocytes that promoted formation of metastatic deposits (148). Dhimi et al. have demonstrated that breast cancer cells induced secretion of coagulation factors from endothelial cells, matching their activated status, which in turn enhanced cancer cell adhesion to the endothelium and subsequent extravasation (149). Overall, these studies highlight a correlation between cancer-induced activated endothelium and several steps of cancer metastases, yet it remains debatable which molecular mechanism cancer cells utilise to drive endothelial cell activation.

Riera-Domingo et al. demonstrated that conditioned media (CM) from cancer cells, which contained secreted factors, converted quiescent endothelial cells (high TNF-related apoptosis inducing ligand-TRAIL- expression) to activated (low TRAIL expression), facilitating the establishment of metastatic formation. This phenotypic conversion was accompanied by an increase in expression of both adhesion molecules such as ICAM-1 and VCAM-1 and secreted chemokines i.e. CCL2. This led conversely to an increase in cancer cell adhesion and immune cell infiltration in the lung of mice. These phenomena enhanced cancer cell burden, as expected, yet was reversed by pre-treatment of mice with a combination of ICAM-1 and E-selectin blocking antibodies, both of which were upregulated in activated endothelial cells (150). Mejía-Rangel also studied the effects of cancer cell CM on activation of human umbilical vein endothelial cells (HUVECs). By collecting CM from a luminal B subtype of breast cancer, they demonstrated that CM-treated HUVECs exhibited an enhanced ability to interact with attaching monocytes (151). Monocyte recruitment and adhesion plays a major role during cancer metastasis and is largely mediated by upregulated adhesion molecules on the surface of endothelial cells, such as VCAM-1, ICAM-1 and E-selectin, all of which were validated via western blotting to be expressed on HUVECs treated with breast cancer CM or TNF- α (151),

which functions as a pro-inflammatory stimuli. By performing transcriptome analysis on HUVECs that were treated with CM, they identified upregulated expression of adhesive molecules and factors that can be secreted from activated endothelial cells, relating to inflammation. According to their bioinformatic analysis, the transcriptomic profile matched a pro-adhesive and pro-inflammatory phenotype (151). It is noteworthy that many of these factors, such as interleukins, promote monocyte polarisation to macrophages, further triggering inflammation (135,142). In respect to extravasation, Tomita et al. highlight the fact that vascular remodelling and permeability, both of which enhance tumour cell extravasation can be initiated by two dominant mechanisms. On the one hand, physical interactions of arrested and adhered tumour cells with endothelial cells can indoctrinate the vascular integrity and either transmigrate paracellularly (in-between adjacent endothelial cells) or transcellularly (through a cell) or by inducing vascular remodelling (59). Follain et al. have extensively detailed the visual differences between vascular remodelling and paracellular migration, whereby in the former scenario endothelial cells spread around the tumour cell surrounding it and slowly enclose it (34). Tomita et al. further document how vascular permeability is promoted, for example, by downregulation or disruption of tight and intercellular junctions, formed between adjacent endothelial cells (59), a disruption often induced by secreted factors from cancer cells (152–155). For instance, Dwyer et al. demonstrated that interleukin 8, secreted by glioblastoma cells was responsible for inducing junction instability in endothelial cells (153). While it is evident that cancer cells secrete and release various factors in their cultured media or in the blood of patients, the collective list of factors is not only limited to cytokines, interleukins or chemokines, but can include exosomes, microvesicles and LEVs.

1.4.3 Interactions of endothelial cells with EVs

Various studies attest the secretion of multiple cytokines, chemokines, growth factors and in general pro-inflammatory molecules by cancer cells (156). As established previously, these factors are identified as potent activators of endothelial cells. However, in the aforementioned studies, endothelial cells were stimulated with CM from cancer cells, which is known to contain not only individual molecules i.e. cytokines but also EVs, including exosomes, microvesicles and LEVs (88,156). Indeed, Ghorbanian et al. showcased that exosomes, in particular, isolated from an epithelial ovarian cancer cell line stimulated

endothelial activation by increasing intracellular and secreted levels of VEGF, proliferation and migration, findings that are consistent with an activated phenotype of endothelial cells (157). Adesanya et al. isolated microvesicles from a head and neck cell squamous carcinoma cell line and upon incubation with human umbilical vein endothelial cells (HUVECs), they initially determined a faster coagulation response, induced by HUVECs. Furthermore, they stained the surface of HUVECs for tissue factor, identifying an increased expression, when cocultured with cancer microvesicles which coupled together support the acquisition of a pro-coagulant phenotype, which aligns with an inflammatory role of endothelial cells (158). In fact, there is a large body of work identifying smaller EVs such as exosomes and microvesicles in driving changes to endothelial cells (159–166), yet there are almost no reports on the impact of LEVs. Spurling et al. document via multiple series of confocal Z-stack imaging that large vesicular bodies, collected from melanoma cell culture and separated from other components, were ingested by endothelial cells. Since the authors refer to these bodies as suspended cells throughout their literature, it is unclear whether they can be classified as LEVs or LOs, yet based on the descriptions of LEVs features throughout their findings, they appear to be so (121). This study highlights the uptake of LEVs by endothelial cells under static conditions and the disruption of the endothelial integrity (121), suggesting that LEVs have the ability to interact and affect endothelial cells. However, excluding the study from Spurling et al. (121) that utilised LEVs generated under static conditions, the current literature is devoid completely of any related studies on the interactions and influence of shear-derived LMPs on activation of endothelial cells and subsequent acquisition of proinflammatory phenotypic traits.

1.5 Role of immune cells in cancer

The immune system is an extremely intricate network that coordinates different organs, cells and proteins to act together against foreign organisms or endogenous threats to an organism. Paradigms of foreign pathogens that cause disease are bacteria, viruses, fungi, parasites, protozoa and toxins among others. The immune system's protective activity is not solely restricted to invading pathogens or microorganisms, rather it fights off endogenous threats that arise in an organism, i.e. cancer development. The immune reaction is broadly categorised into innate and adaptive immunity, with the former being present from the birth

of an organism, whereas the latter continuously develops and evolves through the exposure to pathogens or other threats (167,168). One of the main types of immune response is the cellular one, described extensively and elaborately by Chaplin et al. (168). The main blood cells are of lymphoid origin i.e. T or NK cells and of myeloid origin i.e. monocytes, neutrophils, red blood cells among others. In response to infections or pathogens, the fraction of cells that attack and defend the organism are termed leukocytes or white blood cells. These are comprised by T lymphocytes, neutrophils, monocytes, eosinophils, basophils and natural killer cells. Neutrophils and T lymphocytes are the most prevalent leukocytes, constituting 40-60% and 20-40% of the leukocyte population at a given time, while monocytes represent about 2-8% of overall leukocytes in the blood (167). While the less prevalent among the three, monocytes respond to infections and other harmful factors by differentiating to macrophages, whose activation patterns, markers and downstream functionalities are crucial.

1.5.1 Activated phenotypes of monocytes

Similar to the role of the activated endothelium, monocytes activation can be part of the pathogenesis mechanisms of very serious diseases, including multiple sclerosis, tumour metastasis and liver fibrosis, as highlighted by Karlmark et al (169). On the other hand, monocyte activation can be one of many consequences or symptoms of diseases or inflammatory conditions, including cancer progression and metastasis, autoimmune diseases i.e. rheumatoid arthritis, inflammatory bowel diseases, cardiovascular diseases and blood disorders among many others (170–172). Various mechanisms exist capable of activating and recruiting monocytes to sites of injury, infection and inflammation (170,172). Proinflammatory signals, adhesion molecules in the endothelium, soluble factors from stimulated platelets, secreted chemokines and cytokines are some of the factors that inform monocytes that a particular location in the human body is under attack or that it has sustained injury (123,139,170,173,174). Given their ability to traffic relentlessly in the bloodstream, monocytes are able to recognise instantly the location that requires their recruitment. It is often suggested that monocytes will reach a site of injury i.e. in tissue and will infiltrate and differentiate in a more specialised immune cell type, macrophages, that would be relevant for the defence, healing or repair mechanism (167,170,172,175,176). Monocytes can differentiate to numerous subtypes of macrophages, all of which express distinct surface

proteins, secrete different types of molecules and even perform different functionalities (175,177,178). While some macrophage types are more well-established and accepted across the research community, there is also significant division in the community in respect to certain macrophage subtypes (179).

The main differentiated macrophage populations that emerge from monocytes are the pro-inflammatory M1 macrophages or the anti-inflammatory M2 macrophages (175,176,180,181). Differentiation of monocytes to M1 macrophages is usually stimulated by interferon gamma or lipopolysaccharides (LPS) among other factors, whereas differentiation to M2 macrophages by stimulation with interleukin-4 or interleukin-10 among others (180,182). In general, briefly, M1 macrophages express in their surface and secrete proinflammatory molecules i.e. TNF- α , are positive for T cell antigens (MHC II) to boost immunity and produce ROS as a cell- or pathogen-dependent killing mechanism (172,176,177,182). Altogether, these activities cooperate to defend against pathogens, for instance. Naturally, post-tissue injury, a healing process follows. This time, M2 macrophages emerge, consistently shown to produce and secrete anti-inflammatory cytokines, such as IL-10 and TGF- β , to halt inflammation and shift the balance towards wound healing (172,176,177,182). M2 macrophages express many other cell surface markers, such as cluster differentiation (CD206) or CD163 (180,183), which are involved in processes such as tissue repair and remodelling as well as immunomodulation (184). Another trait that indicates activation to M2 phenotypes is the release of pro-fibrotic molecules, such as Galactin-3 or fibronectin among many others that promote the formation of a dense ECM-rich fibrotic stroma (185–187). Another major trait of M2 phenotype is the secretion of pro-angiogenic factors, with many studies verifying that M2 macrophages are potent in inducing angiogenesis and capillary tube formation, restoring therefore blood flow in damaged areas of tissue (188). Last but not least, M2 macrophages have been shown in several instances to reverse T cell immunity and promote an immunosuppressive environment that counters off inflammation (189–191). It should be noted that in contrast to M1 macrophages, M2 macrophages are more complicated, given the fact that M2 macrophages do not present consistently similar levels of protein expressions, activation markers or functionalities, which led to their division and subsequent sub-classification (183,192). For instance, some subtypes of M2 macrophages are capable of inducing angiogenesis and tissue remodelling, while fail to suppress T cells, while

other M2 subtypes can perform all three mentioned activities (177,178,192,193). Nonetheless, M2 macrophages serve as a mode of immune cell activation that normally suppresses inflammation to allow sufficient time for downstream activities, including the remodelling and wound healing of damaged or injured tissue. However, in diseases such as cancer, which is a chronic disease with persisted inflammation, M2 macrophage healing mechanisms are hijacked to promote cancer progression and metastasis (194,195).

1.5.2 M2 macrophages in cancer

M2 macrophages contribute to cancer progression in various ways (195–197). For instance, M2 macrophages can secrete anti-inflammatory cytokines that suppresses inflammation and tumour death (178,194,197–199). Moreover there are multiple mechanisms through which M2 macrophages can modulate negatively immune responses, especially by suppressing T cells activation and response (190,200–203). In addition, M2 macrophages can reprogram cancer-associated fibroblasts and increased ECM production to lead to a fibrotic environment, which blunts T cells activity (186,204). Suarez-Lopez et al. have identified the role of MK2 in activation of M2 macrophages, whose impairment led to reduced tumorigenesis and angiogenesis (validated by endothelial markers presence *in vivo*) and reduced ability of capillary tube formation *in vitro* (205). In another study, Wei et al. investigated the role of M2 macrophages in cancer cell survival upon exposure to chemotherapy drugs, such as 5-fluorouracil. The authors demonstrated that non-stimulated (M0) macrophages did not confer any tumour-promoting effects, whereas secreted factors from M2 macrophages not only conferred resistance to cancer cell death by the drug tested, but also reversed the drug's effect on diminishing the colony forming ability of colon cancer cells (206). The role of M2 macrophages against chemotherapeutic drugs has also been validated by Genin et al. (180). Dannenmann et al. characterised molecular markers from tumour-associated macrophages that were isolated from primary tissue from clear cell renal carcinoma patients, matching their molecular profile to that of macrophages with an M2 signature. After demonstrating that this M2-like transcriptional signature correlated to reduced survival and tumour progression in patients, they further showed that cancer cells were able to convert M1 to M2 macrophages. To investigate deeper the effect of tumour-associated macrophages (TAMs), which matches an M2-like signature, on T cells, they compared cytokine production levels from CD4⁺ T cells, isolated from peripheral blood,

compared to co-cultured CD4⁺ cells with tumour-isolated TAMs. TAMs increased significantly anti-inflammatory cytokines such as transforming growth factor beta, interleukin-4 and interleukin-10, while reduced the secretion of proinflammatory cytokines such as interferon gamma, tumour necrosis factor alpha and interleukin-2; though only the latter reached statistical significance (207). More specifically, during cancer metastasis, M2 macrophages have been found in association with CTCs, that were isolated as heterotypic clusters from the blood of prostate cancer patients, as shown by Osmulski et al. (208). The authors demonstrated that M2 macrophages conferred a distinct mechanical phenotype on CTCs, enabling them to survive shear stress and increase their adhesive properties. This phenotype was distinct from CTCs profiled either alone or with M1 macrophages, that were also isolated as heterotypic clusters (208). Borriello et al. made an interesting comparison in respect to cancer cell behaviour in the microvasculature of mice lungs, observed and recorded via intravital microscopy. The authors compared spontaneously metastasising tumour cells (shed from primary tumour) against intravenously injected tumour cells, observing through their live imaging approach that metastasising tumour cells were entrapped for longer, extravasated quicker and presented higher dormancy and stem cell markers. One of the critical dormancy markers was the presence of nuclear receptor subfamily 2 group F member 1 (NR2F1), whose levels were upregulated in disseminated tumour cells rather than the primary tissue, yet the authors suggest that the short half time circulation is not enough to reflect this increase, rather that this increase occurs in locations near to intravasation sites. Indeed, the authors verified the increase of NR2F1 in a location, termed as tumour microenvironment of metastasis doorways, rather than farther from these structures. Upon NR2F1 knockdown, it was shown that the ability of cells to grow had improved, which matches the concept of dormancy loss, so that cancer cells can start proliferating and grow in a metastatic lesion. Interestingly, the authors quantified the levels of NR2F1 in tumour cells in proximity to macrophages in primary tumours. Lastly, in macrophage-depleted mice, tumour cells lost their ability to reside for long periods of time in the lung microvasculature, extravasate or survive for long post-extravasation (209). Altogether, while cancer cells can activate and induce differentiation of monocytes to M2 macrophages persistently, whose tumour-promoting effects are well-documented, it remains debated in the literature which are the major mechanisms that cancer cells utilise to achieve that.

1.5.3 EVs influence differentiation to macrophages

Similarly to what was described in the previous section, where interactions of EVs with the endothelium were revisited, primary tumours and cancer cells secrete a variety of factors that have the potential to influence and drive differentiation of monocytes or M1 macrophages to pro-metastatic M2 macrophages (156,210,211). This can occur via individual or a combination of cytokines i.e. interleukins or through EVs (156,212,213). For instance, Jiang et al. purified isolated conditioned media from cultured bladder cancer cell lines and upon co-culture with bone marrow-derived macrophages, they validated the macrophage conversion to an M2 phenotype (increase of M2 marker, CD206 -both in protein and gene levels) rather than an M1 phenotype (reduction of M1 marker, inducible nitric oxide synthase gene). The authors further demonstrated that exosomes could be isolated and purified from the conditioned media and that macrophages were able to ingest them. Exosomes effect on macrophages presented a very similar molecular signature as the treatment with conditioned media. Moreover, the authors used an exosome inhibitor that interfered with generation of exosomes from cells, demonstrating that it inhibited the induction of M2 macrophages. These findings, coupled together, suggest strongly that from the total components of the conditioned media, exosomes are a strong and dominant contributor of the M2 phenotype induction, rather than other factors in the conditioned media. Lastly, they presented in vivo evidence that the exosome inhibitor shifted downwards both the tumour volume and the percentage of M2 macrophages, while the percentages of CD4+ and CD8+ T cells went up, clearly indicating the significant exosome-mediated role on driving the induction of an immunosuppressive M2 phenotype (214). While a significant amount of work has been done on examining the role of cancer exosomes on polarisation to M2 macrophages (213,215–219), only two studies, at the best of my knowledge, focus on the investigation between the interactions of shear-derived LMPs with immune cells (71,220). Back in 2016, Headley et al. demonstrated the uptake of shed LMPs in vivo by various immune cells of myeloid origin. These uptake events occurred in the pre-metastatic lungs of mice, in the microcirculation, including neutrophils, monocytes, macrophages and dendritic cells. Notably, the authors also reported a mobility and trend towards extravasation for both conventional monocytes and macrophages that were positive for large microparticles ingestion. While this behaviour was not correlated directly to metastases formation, the authors demonstrated that metastatic

growth was dependent on monocyte recruitment, which increased the number of microparticles-positive monocyte and correlated with a higher number of lung metastases. Interestingly, knockout of monocytes, revealed a shift in microparticle uptake, with more dendritic cells (immunostimulatory) ingesting particles, while macrophages were less frequent. This led to an increase of cytotoxic T cells, suggesting that monocytes-ingested microparticles confer some type of immunosuppression. However, this immunosuppressive mechanism does not appear to be direct, in the sense that microparticle-ingested monocytes suppress immunity, rather it appears to be a competition between monocytes and dendritic cells, where monocytes are able to ingest more particles and thus shift the balance towards an immunosuppressive environment, as the authors suggest (71). In a similar setting, Hyenne et al. demonstrated that exosomes, isolated from melanoma cell lines could be ingested in zebrafish models by macrophages but not by neutrophils (221). In comparison to Headley, where neutrophils ingested LMPs (71), it remains unclear whether neutrophils in zebrafish models behave differently, as opposed to mice neutrophils. However, both studies provide compelling evidence that macrophages are capable of ingesting EVs of various sizes under flow conditions, bolstering the notion that cellular uptake of EVs can occur during their transit among other blood cells. Nonetheless, the interactions between shear-derived LMPs and monocytes or M1 macrophages, their uptake mechanisms, the conferred molecular changes and downstream effects are largely underexplored.

1.6 Methodology approaches

The metastatic journey of CTCs and their clusters in the bloodstream involves many steps, such as survival, immune evasion, arrest, adhesion to endothelium and extravasation (28,33,34,60,222–225). These phenomena are currently the most explored so far among others, yet still not fully understood. In particular, biomechanical factors and intercellular interactions play a pivotal role in determining CTCs fate towards metastases formation (31,32). However, the immediate interactions of CTCs when they encounter capillary beds or the microcirculation remains largely underexplored. One of the reasons is that many studies focus on investigating arrest and extravasation and thus do not image directly CTCs transit, rather evaluate what occurs hours later post-injection (34,62). However, Headley et al. and others have demonstrated the importance of tracking down CTCs transit upon injection in

vivo (33,70,71). Nonetheless, the microcirculation represents a vast network, comprising of interconnected capillaries, with the presence of bifurcated or non-bifurcated capillaries, narrow constrictions or small arterioles (non-constricted) and a broad diversity of diameters and geometries (33,34,226,227). Thus, to increase understanding of the impact of individual features of the microcirculation on CTCs, it is crucial to utilise technologies that allow the dissection and investigation of single parameters. However, the current in vitro models are not completely representative in respect to the diversity of capillary geometries, inclusion of bifurcations, small sizes and implementation of flow. This can be overcome by utilising the microfluidic technology.

1.6.1 Introduction to microfluidic technology

Microfluidics is defined as a technology that enables manipulation of small volumes of fluid, by involving channels of small sizes, ranging from ten to hundreds of micrometers. Convery et al. provide a historical journey to the original concept of microfluidics, birthed and advanced slowly by the need to create reliable systems for microelectronics (228). However, the revolution of the microfluidic technology as a routine in today's labs, emerged slowly from the 90's, when Manz et al. demonstrated that a microfluidic device could operate as a complete system for sampling, sample transport, sample preparation (i.e. chemical reactions) and chemicals detection (229). The advances in micromanufacturing techniques, such as replica moulding, rapid prototyping and others, have boomed the field of microfluidic platforms presenting diverse functionalities, including droplet microfluidics, paper analytical devices, open microfluidics, organ-on-a-chip (228), which are routinely used in biomedical research, expanded in following paragraphs.

Fluids' behaviour on the microscale differs from the behaviour of fluids in the macroscale. For example, factors such as surface tension, surface-to-volume ratio, energy dissipation, electrokinetic effects, viscosity, shear, laminar flow and fluid resistance become more dominant at small scales (230,231). The flow properties within microscale devices are described by dimensionless numbers, such as the Reynolds number (Equation 1), whose values differ between micro- and macroscale flow physics.

$Re = \rho * u * D_h / \mu$, where ρ is fluid density, u is fluid velocity, μ is fluid viscosity and D_h is the hydraulic diameter. (Equation 1)

Reynolds number is used to distinguish between laminar and turbulent flow profiles. Laminar flow allows the control of particle velocity across the flow stream in respect to time ($Re < 2300$), whereas turbulent flow dictates chaotic motion ($Re > 2300$) (231). Another important property of microscale flow, particularly in biological systems, where cell movement can be investigated, is the fluid viscosity, defined as the rate of shear force to velocity gradients (231). The importance of fluid shear, which is defined as the frictional fluid forces acting on the surface of cells for example, is important in microfluidic applications, enabling the mimicking of fluid shear values found in arteries, veins or capillaries (231). Altogether, control over these and other physical parameters enables the utility of microfluidic technology in biomedical applications.

1.6.2 Microfluidic technology on cancer research

The advances in microfluidic engineering techniques and continuous evolution to address biological questions, has expanded and diversified their use in biomedical research. Particularly, microfluidic technology is used in liquid biopsy approaches, precision medicine, cell culture systems, drug screening tools, mimetic models of metastasis among many others (232–238). More specifically, Belotti et al. review extensively how microfluidic technology is utilised to improve patient diagnosis by identifying tumour material from early on (233). For example, Mishra et al. devised a microfluidic chip that leverages size-based inertial separation to separate CTCs from other blood cells and platelets from the blood of patients by negative selection, without the need for accounting for CTCs antigen markers (239). Alternatively, antigen-dependent microfluidic devices are also utilised significantly, reviewed elsewhere (238). More specifically, microposts within microchannels were coated with antibodies that recognise CTCs, enabling their capture as the blood is filtered through the device (240). Regmi et al. describe in detail the various label-free and label-based microfluidic approaches used to isolate CTCs (237). Additionally, a large body of work utilised microfluidic chips to isolate CTC clusters, exosomes and circulating-free DNA, which represent other blood markers relevant to cancer disease (233,239,241–243). Sun et al. provide an extensive review on the utility of microfluidics to drug screening efforts (236). Eduati et al. engineered a microfluidic platform that enables combinatorial drug screening on solid biopsies, overcoming the hurdle of material availability, by enabling parallel testing of 56 different conditions per biopsy (244). Khot et al. fabricated a 3D microfluidic chip for cancer spheroid culture, probing the effects

of chemotherapeutic drugs on cytotoxicity (245). Overall, microfluidic technology can be readily used as a diagnostic and screening tool, with potential to be implemented in clinical settings for early diagnosis but also personalised medicine (246,247).

Another area that microfluidic technology is used massively is to address more fundamental cancer research questions, namely cancer metastasis, the influence of tumour microenvironment, angiogenesis, cancer phenotypes among many others (235,237,238,247). Truong et al. developed a 3D microfluidic co-culture platform, successfully mimicking the interactions between breast cancer cells and cancer-associated fibroblasts (CAFs), demonstrating that CFAs influence breast cancer cell invasion (248). Agliari et al. engineered microfluidic models encompassing microchannels that allowed them to visualise splenocyte migration towards cancer cells that constantly secreted chemical factors (249). Wang et al. fabricated microfluidic channels filled with ECM proteins and investigated the ability of human lung cancer cells to form invadopodia, degrade the ECM and invade through (250). Kim et al. co-cultured human umbilical vein endothelial cells with glioblastoma cells within a microfluidic device and observed faster growth and more tortuous branching by HUVECs, after exposure to factors secreted from glioblastoma cells (251). Zervantonakis et al. modelled the interface between tumour cells and vascular cells, correlating the chemical factors secreted by tumour cells and macrophages to activation of endothelial cells which consequently led to enhanced intravasation of tumour cells (252). Altogether, microfluidic technology is utilised in many areas of cancer research, recapitulating the importance of the tumour microenvironment and processes such as intravasation and extravasation. However, there is another important aspect of cancer metastasis i.e. CTCs transit in blood vessels, which is crucial at determining the success of metastasis (31,32,34). CTCs are exposed to intercellular interactions and biomechanical forces such as shear stress and constriction forces (31,32,34,35,227). Chen et al. created a microfluidic 3D perfusable microvascular network (formed by HUVECs) to investigate cancer cell extravasation (63). The authors observed via live imaging the entrapment and extravasation of injected tumour cells. In fact, their model enabled the incorporation of healthy or inflamed neutrophils to simulate the more complex interactions that occur in the blood between tumour cells and blood cells to promote tumour cell extravasation (63). Regmi et al. used a microfluidic system coupled to a peristaltic pump to stimulate continuous exposure of tumour cells to shear stress (253). The

authors identified that high shear stress values up to 60 dyne/cm² reduced significantly tumour cell viability, which was also dependent on circulation time (253). Nath et al. used microfluidic technology to mimic in vivo capillary constrictions by engineering a single straight channel, constricted at 7 µm diameter (44). After HELA cells transited the constricted microchannel, the authors examined cell viability, quantifying that 50% of cells had died (44). Cognart et al. engineered microfluidic models mimicking unconfined geometries to evaluate the effects of circulation only and confined geometries (6 µm) to evaluate the effects of constriction on cell transit parameters, morphological and molecular changes (254). The authors further evaluated genes relevant to epithelial and mesenchymal phenotypes, demonstrating that unconfined circulation-mimicking conditions conferred changes in EMT transcriptional programs (254). Au et al. engineered microfluidic models of straight channels of 5-, 7- and 10 µm widths to explore and compare the transit dynamics between breast cancer cell clusters and their singlets (45). This live imaging approach enabled the visualisation of cell rearrangement within clusters to transit successfully through narrow constrictions (45). Overall, these studies highlight how the microfluidic technology can be successfully utilised in addressing the effects that the complex environment of the circulation and capillary beds can impose on CTCs.

1.6.3 Choosing microfluidic technology to mimic capillary bifurcations

Delving deeper in the suitability of microfluidic technology, the use of polydimethylsiloxane (PDMS) from the late 20th century, rendered PDMS-based microfluidic chips very popular, due to their quick and cheap fabrication (228). Furthermore, PDMS can bond to glass i.e. glass slides, rendering it a portable technology and facilitating its incorporation into imaging systems (228,237). Having said that, PDMS is optically clear, a property which allows the real-time study of biological processes through microscopy (255). For example, Au et al. applied a live imaging approach, visualising the transit dynamics of both clustered and single cancer cells in straight constriction channels (45). PDMS permeability to gases (255), facilitates further its use for multi-hour live imaging, in microscopes that are equipped with gas chambers and CO₂ feed to ensure viable cultures for hours or days. Additionally, the progress and improvements on photolithography and soft lithography techniques have enabled the formation of channel geometries with submicron features that resemble capillaries found in

vivo (44,45). Despite the in vivo complexity of blood vessels and particularly capillaries i.e. subsequent capillary bifurcations of various sizes (33,227), microfluidic technology allows the engineering and replication of channels with controlled physical parameters to reduce the in vivo complexity and investigate single factors impact on CTCs transit. As described earlier, microfluidic chips with narrow constrictions have been previously employed to investigate cell deformability, transit time, viability and mechanical phenotypes (44,45,256), however microfluidic models that mimic capillary bifurcations are at best largely underexplored.

Despite their multiple advantages, the use of microfluidic technology to mimic capillaries also present limitations. Engineering of microchannels with soft lithography, although enables formation of small channels i.e. 5 μm , leads to the formation of channels with rectangular cross sections, which are not representative of in vivo circular microvessels (255). Other techniques such as the wire casting technique enable the engineering of channels with circular cross sections but not at sizes smaller usually than 10 μm . Another limitation is that PDMS is a stiffer material, reported at 360 kPa to 3Mpa (255), as opposed to blood vessels, whose stiffness can be as low as a few kPa. Despite these limitations, microfluidic-based capillary bifurcations remain a useful in vitro tool to dissect the in vivo capillary bed complexity into single factors and investigate their impact on CTCs phenomena.

Currently, the journey of CTCs in the circulation is mostly explored in respect to arrest, endothelium adhesion and extravasation. However, CTCs navigate through a series of complicated blood microenvironment networks, comprising bifurcated or non-bifurcated capillaries, various constriction sizes and a diversity of geometries tuned by angle and symmetry. Many current microfluidic chips or in vitro models only recapitulate the simplest capillary geometry such as straight channels, lacking bifurcated geometries (44,45,254). Other work highlighted the use of microfluidic chips, encompassing a series of parallel and vertical (equally spaced) micropillars to create bifurcated pathways, but the design was not physiological to in vivo bifurcated capillaries (257). Wang et al. have engineered bifurcated geometries with large angles between daughter channels, but they investigated neutrophils transit phenomena and did not implement fluid flow, instead operated the devices using chemoattractant gradients (258). Other work utilises self-organised HUVEC-based microvascular networks to mimic smaller vessels, but the larger diameters do not represent accurately the typically smallest blood capillaries (5-10 μm). Altogether, there are not enough

studies across the literature that investigate the impact that capillary bifurcations can impose on CTCs and current in vitro models do not recapitulate accurately the small sizes and diverse geometries of capillary bifurcations. Thus, in this project, the microfluidic technology was used to fabricate and engineer different geometries found in the microcirculation, including bifurcated, non-bifurcated, non-constricted, different sizes of daughter channels in bifurcations as well as distinct symmetric and angular parameters. Altogether, the microfluidic technology was used effectively as an approach to study the fate of CTCs and their clusters in respect to cellular shedding and other unexplored phenomena during transit in microcirculation-mimicking geometries.

Subsequently, prior observations on cellular responses during their transit in capillary bifurcations revealed the shedding of LMPs from the cell membrane. Understanding of the nature of these LMPs, what they are, how they can be utilised and what their impact on metastasis is, required some initial guarantees. To approach the problem of studying a population of unexplored observed particles in the most accurate way, primarily their purity should be maximal, which was achieved by employing traditional purification techniques for other reported large particles. Then, large numbers of LMPs needed to be generated, given requirements of certain molecular techniques for adequate material i.e. PCR, which was secured by the high-throughput nature of microfluidic devices, designed previously. Last but not least, retrieval of this LMP population from the collected fluid post-purification would be required to utilise this population for downstream analysis. Information on the application of previous techniques on purification of EVs (88,100), indeed indicated the application of a certain range of centrifugation speeds that allowed pelleting of LMPs and thus the use of molecular techniques i.e. immunofluorescence to study their proteins. Altogether, the resolution of all these three steps rendered it possible to investigate a pure population of sufficient number of LMPs not only molecularly but also in respect to their interactions with other cell types.

1.7 Hypothesis & Aims

Summarising the literature review, there are numerous aspects of CTCs transit in the bloodstream in respect to shedding of LMPs and the nature or role of LMPs that remain largely underexplored:

- Complete repertoire of biogenesis mechanisms of LMPs during transit of CTCs in capillary beds
- Cells' consequences post shedding
- Behaviour of clusters in respect to shedding or dissociation in capillary bifurcations
- Active ways to manipulate shedding
- LMPs purification methods
- Nature of LMPs as LEVs?
- LEVs biological material
- Implication to cancer metastasis pathways
- Interactions with other cell types such as endothelial cells and monocytes
- The ability of shed LEVs to interact and induce phenotypic changes on above cell types
- Functionalities of LEV-activated cell types?

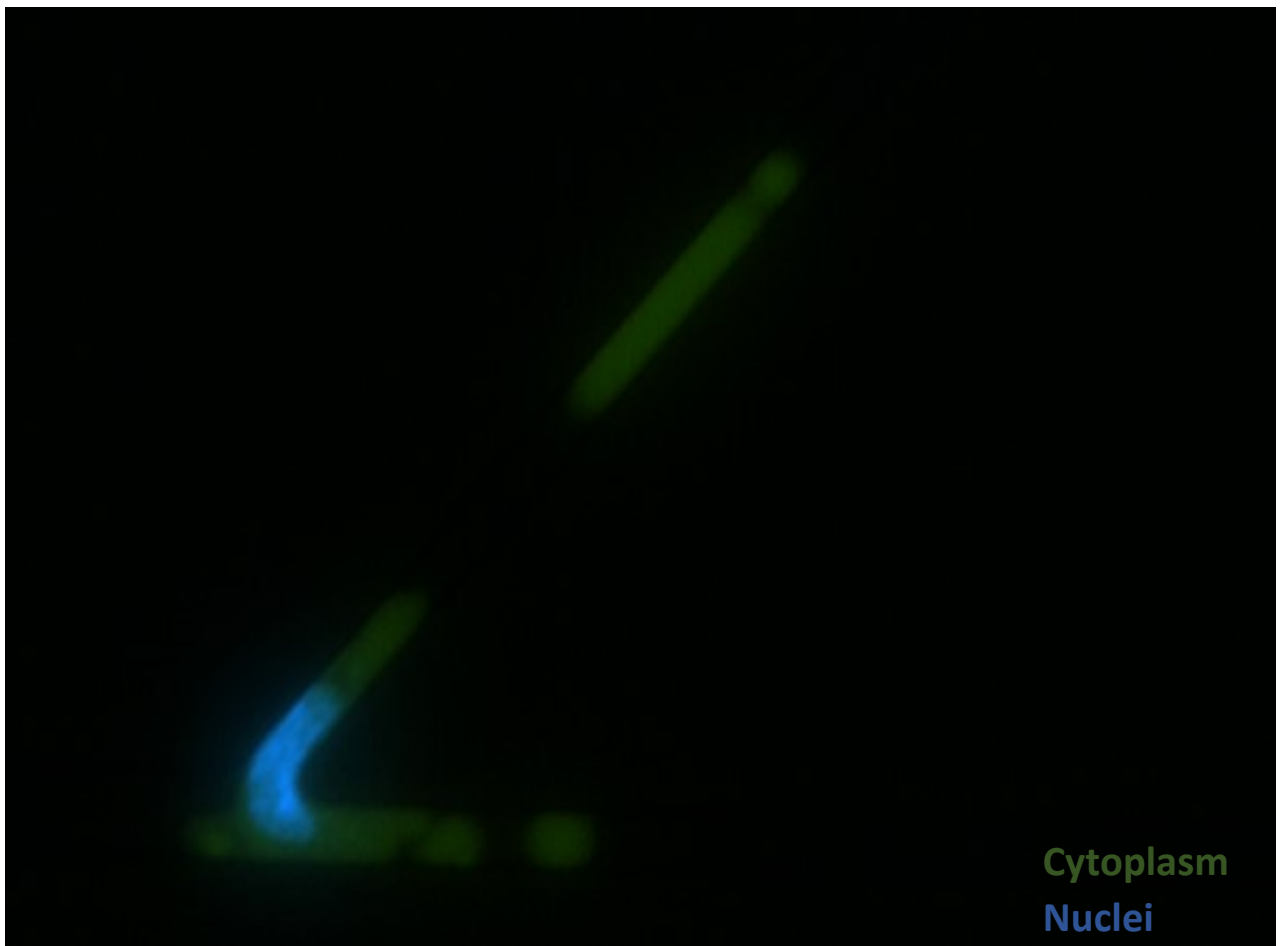
Thus, I hypothesise that CTCs transiting in the microcirculation shed LEVs, which are rich in proteins, enabling them to influence metastasis by inducing activated endothelial and pro-metastatic M2 macrophage phenotypes that can prolong systemic inflammation in cancer patients.

The experimental findings obtained from this thesis are split and allocated in five chapters, where in each, a particular theme is examined and investigated. In **Chapter 2**, the factors that drive cellular shedding of LMPs during transit in microfluidic devices of various capillary geometries as well as the consequences of cell shedding are elucidated. In **Chapter 3**, microfluidic devices are utilised to study for the first time the transit and haemodynamic phenomena that occur during transit of clustered cancer cells upon encounter of capillary bifurcations. Following up on the shedding of LMPs, **Chapter 4** focuses on the purification, characterisation of the biological content and the nature of these shear- and capillary bifurcation-derived LMPs, which were successfully verified as LEVs. As of right now, there is no evidence whatsoever in respect to the interactions between shear-derived LEVs and endothelial cells. Thus, in **Chapter 5**, I delve deeper in these interactions i.e. cellular internalisation, verify molecular changes towards the activation of endothelial cells and study the downstream effects of activated endothelial cells. Last but not least, **Chapter 6** revolves around the interactions of shed LEVs with two major immune cell populations that influence cancer, monocytes and M1 macrophages. In this Chapter, the mechanistic interactions and

phenotypic changes of monocytes and M1 macrophages to pro-metastatic M2 macrophages are validated upon exposure to shed LEVs, as well as downstream effects. Altogether, this research thesis provides evidence about potential CTCs transit phenomena in the bloodstream that may affect metastasis.

CHAPTER 2

Circulating tumour cells shed large microparticles during their transit in capillary geometries



2.1 INTRODUCTION

Circulating tumour cells (CTCs) and their clusters disseminate from the primary tumour to metastasise to distant organs (23,34,222). During dissemination in the bloodstream, CTCs encounter capillary beds (henceforth referred to also as microcirculation or microvasculature), including small arterioles and capillary networks, whose narrow constrictions, bifurcated geometries and favourable flow profiles facilitate their entrapment (31,33,34). Live imaging approaches have previously documented the importance of capillary bifurcations particularly in slowing down CTCs in vivo (33,110). Evidently, CTCs remain in the microvasculature from minutes to days (33,259), from where eventually they extravasate (34,62,227), while their transit in arteries and veins is rapider (222). Significant amount of research findings has implicated both biomechanical and intercellular interactions as important mediators of CTCs survival, arrest, endothelium adhesion and extravasation (31–34,41,60,62,71,225,260). However, the direct impact of the microcirculation and especially the diversity of capillary geometries on CTCs behaviour remains immensely underexplored.

Previously, in a study involving cancer patients, CTCs were isolated from metastatic cancer patients from two distinct locations, namely from hepatic veins (before microcirculation transit) and from peripheral veins (after microcirculation transit) (73). When the authors compared the size of CTCs that were isolated from these distinct compartments, by calculating their surface area, they identified a significant reduction in the cell surface area of CTCs that were collected from peripheral veins, findings verified by an independent research group (73,74). According to the bloodstream haemodynamic routes, CTCs that were isolated from peripheral veins would have transited at least once from two independent capillary networks before being collected, namely the lung and the finger capillaries (74). In another study, Nanou et al. have used a software algorithm to analyse digitally stored images of previously enumerated and collected CTCs, identifying co-isolated objects that had retained staining for cytoplasm but were negative for nuclei (76). These objects were identified as particles, termed by the authors as tumour-derived extracellular vesicles (EVs) and were larger than 1 μm in diameter (75,76). These particles had been identified in four distinct cancer types, including colorectal carcinoma, metastatic breast cancer, castration-resistant prostate cancer and non-small cell lung carcinoma. In fact, they were found in significantly higher counts compared to CTCs, suggesting that a relationship between CTCs transit and the

origin of tumour-derived EVs may exist (76). Similar large particles termed as large EVs (LEVs) or large oncosomes (LOs) have been isolated from cancer patients by others (77,91). While these two distinct phenomena of CTCs surface area reduction and the presence of large particles in the blood of patients may appear initially unconnected, there are clues in the literature that suggest otherwise.

Almost twenty years ago, imaging of injected CTCs *in vivo* and real-time observations of their transit in the skin microvessels of mice, revealed the entrapment of CTCs in capillary bifurcations. What the authors further recorded close to the entrapped cells, was cytoplasmic-positive material that lacked nuclei (70). About twelve years later, Headley et al. injected melanoma cells in mice and through intravital microscopy observed on real time their arrival in lung microvessels and the shedding of cytoplasmic-positive particles from the membrane of cells that lacked nuclei, which the authors termed generally as large microparticles (LMPs) and later in the study as cytoplasts (71). Similar large particles have been observed in a zebrafish model, post CTCs transit in capillary networks, though the authors considered these simply as cellular apoptotic fragments (62). It is noteworthy to mention that Headley et al. used apoptosis inhibitors to treat cells and in their live imaging approach observed nonetheless the generation of LMPs at levels similar to untreated cells, validating the notion that LMPs are not a product of apoptosis (71). Interestingly, Headley et al. identified a 4-fold increase of cell shedding frequency under flow in the lung microvessels compared to static conditions, examined in lung slices, suggesting a shear dependency (71), and thus, the terminology shear-derived LMPs will be used henceforth to refer to these particles from this particular study. Altogether, these studied cumulatively bolster the notion that CTCs shed nuclei-free LMPs in the human microcirculation, which could affect the size of CTCs post-shedding and partially explain the presence of large particles in the blood of cancer patients. However, the microcirculation is an extremely intricate microenvironment, which comprises bifurcated and non-bifurcated geometries, narrow constrictions of various sizes, non-constricted small vessels and diversity of angles and symmetry among other features (33,34,71,227). Despite the few findings attained in the literature in respect to this cellular shedding phenomenon, the complete repertoire of LMPs biogenesis mechanisms and the fate of cells post-shedding remain largely ambiguous.

In this work, the application of microfluidic tools was utilised to mimic capillary geometries, which were fabricated using photo- and soft lithography. These engineered microfluidic devices were fabricated to examine the transit behaviour and shedding patterns of both single and clustered cells in distinct capillary geometries. Primarily, I demonstrated that the combination of shear and constricted bifurcations are important mediators of LMPs biogenesis, generated both by single and clustered cells. Then, I showed that physical properties of the cell such as size act synergistically with capillary bifurcations to modulate shedding frequencies. The release of LMPs also led to a reduction in cell volume, facilitating CTCs transit, by reducing the residence time of cells in capillary bifurcations post-shedding. Last but not least, the role of cytoskeletal proteins, namely F-actin was also evaluated, demonstrating that its inhibition led not only to reduced shedding but also to enhanced lysis during cell transit. Altogether, I hypothesise that both the mechanical features of capillary beds and biophysical cell properties synergise to promote LMPs biogenesis from CTCs and influence their transit.

2.2 MATERIALS & METHODS

2.2.1 Cell culture & reagents

All cell lines and reagents were obtained from ATCC and Sigma Aldrich, respectively, unless purchase is reported from different vendors. MDA-MB-231 (breast cancer), OVSAHO (ovarian cancer), LNCaP (prostate cancer), Me290 (melanoma), PDAC (pancreatic) and B16F10 (melanoma) cell lines were grown in StableCell™-Dulbecco's Modified Eagles Medium (DMEM) media, supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin and incubated at 37°C/5% CO₂. All cell types were sub-cultured every 2-3 days as adherent cell lines, as will be expanded later. MDA-MB-231 breast cancer cells were used throughout the project (in all upcoming Chapters) and their culture process will not be repeated in detail.

Moreover, a CTC-derived cell line was obtained from small cell lung cancer (SCLC) patients following informed consent and appropriate protocols that were ethically approved. For the formation of a CTC-derived cell line, CTCs were purified and separated from the blood of SCLC patients via RosetteSep™ and were implanted subcutaneously in mice, which enabled the development of cell line-derived xenograft (CDX) tumours in mice. The formed tumours were

isolated from mice and dissociated in single cells, which created the CDX19 CTC-derived cell line (261). CDX19 cell line was grown in Roswell Park Memorial Institute (RPMI-1640) media, supplemented with 20% (v/v) FBS, Human Insulin solution (0.005 mg/mL), Human Transferrin (0.01 mg/mL), β -estradiol (10 nM) and Hydrocortisone (10 nM), all of which represent the final working concentrations. CDX19 cell line was incubated at 37°C/5% CO₂ and passaged every 3 days, as an adherent cell line.

Primary cancer associated fibroblasts (CAFs) cell lines derived and developed from patients with triple negative breast cancer were provided by Breast Cancer Now Tissue Bank. CAF cell lines were grown in DMEM:F12 media, further supplemented with 1% (v/v) penicillin/streptomycin, 2.5 µg/ml final concentration of amphotericin-B and 10% (v/v) heat-inactivated FBS.

2.2.2 Single cell and cluster suspensions preparation

Single cells were harvested from 90% confluent 48-well plates (Corning, UK) or 25 cm² tissue culture flasks (Corning, UK), by firstly washing with PBS briefly and adding trypsin at a concentration of 0.25% (v/v) for 5 min at 37°C/5% CO₂. Serum-containing media was then added to flasks or wells to inactivate trypsin and the dissociated and harvested cells were centrifuged at 180 x g for 5 min to pellet the cells. The pellet was either resuspended with fresh media and transferred to new flasks or where relevant, was resuspended with a staining solution for downstream experiments. The staining solution was formulated at 1 ml final volume, diluting Calcein-AM (ThermoFischer Scientific, UK) at a final working concentration of 5 µM and Hoechst 33342 at a final working concentration of 16.2 µM in media. Cells were stained for 15 min at 37°C/5% CO₂.

In addition to single cells, experiments for cell clusters, aggregates of single cells, were also performed. Single cells were initially harvested as above and equal suspensions of 10000 counted cells (100 µl of 100000 cells/ml) were added to pre-coated round 96-well plates (Corning,UK) for 1 day at 37°C/5% CO₂. To form cell aggregates, round 96-well plates were pre-treated with 1% Pluronic F-127 (Thermo Fischer Scientific, UK) at room temperature for 1 hr to prevent cell attachment. Where relevant, the formed large aggregates were stained with staining solution, as formulated above. To prevent spheroids disruption, the staining solution was added directly to the wells, without removing any well-filling media. To retain

the original dilutions of the staining agents at their final working concentration, the staining dyes were prepared initially at twice the final working concentrations and equal volume of staining solution was added to equal volume of the filled well. Lastly, the spheroids were resuspended multiple times gently to disrupt them in smaller clusters and the cluster suspension was used for downstream experiments.

2.2.3 Cell treatment

Cells were treated with various compounds for downstream analysis. Single cell and/or clustered populations were treated with a. Cytochalasin D (Merck Life Science, UK) termed as Cyto D from now on, a F-actin polymerisation inhibitor (262,263) or b. Colchicine (Merck Life Science, UK), termed as Colch from now on, a F-actin polymerisation promoter (264). 10 μ M, and 50 μ M were the final working concentrations respectively. Treated single or clustered cells were used in relevant experiments under either static or microfluidic transit experiments, as will be described subsequently. Validation of the compounds' activity was performed through fluorescent imaging of F-actin (described below).

2.2.4 Immunofluorescence

10000 counted cells (100 μ l of 100000 cells/ml) were counted via a haemocytometer (Fischer Scientific, UK) and were added in separate wells of a 96-well plate, according to relevant experimental conditions for 1 day to attach and grow. Cells were then treated or not with above compounds (Cyto D, Colch) as described and after 3 hours incubation, media was removed. Cells were fixed with 4% formaldehyde solution (diluted in PBS) for 12 min at room temperature. Cells were then washed 3 times for 3 min per wash with phosphate buffer saline (PBS) and further incubated at room temperature for 5 min with a Triton X solution of 0.1% (diluted in PBS). Then, cells were washed again 3 times for 3 min per wash with PBS and finally incubated with Phalloidin-iFluor 647 antibody (Abcam, UK/ab176759), after 1000 times dilution (from the vial) with PBS, for 1 hr at room temperature. Hoechst 33342 was added for 5 min at room temperature and 3-4 multi-fluorescent images were obtained using the Nikon microscope at 20x magnification per condition, as described followingly.

2.2.5 Fluorescent microscopy

Generally, for acquisition of fluorescent or brightfield images of cells, a Nikon Eclipse Ti2 multi-fluorescent inverted microscope with Okolab incubated stage (Nikon, USA) was used.

Well plates were loaded onto the stage of the microscope and were imaged to obtain fluorescent and/or brightfield images, using the respective filters and excitation/emission values, relevant to the dyes used. Images were obtained at 4x, 10x, 20x or 40x magnification, according to the experiment's needs. The Nikon Eclipse microscope was an integral microscopy tool, used consistently throughout the project and its use won't be described in detail in upcoming Chapters, unless a particular analyses or technique is employed.

2.2.6 Designs of capillary geometries

To mimic the bifurcation geometries that are found in vivo, Murray's law was employed. Murray's law states that the parental vessel is connected mathematically to the two daughter vessels. In particular, the cubed diameter of the parent vessel is equal to the sum of the cubed diameters of each daughter vessel (265) (**Fig. 2-2A**) (**Equation 2** see below). To mimic the physiological sizes of circular capillaries, usually smaller than 10 μm , the following, **Equation 1**, was used to calculate from the equivalent diameter of circular capillaries, the rectangular dimensions of engineered microfluidic based capillary geometries. The values of d_e (**Equation 1**) were set in a range of 5-10 μm to mimic circular capillaries. The following equation was used to calculate the dimensions of microfluidic rectangular channels, as elaborated in **Table 2-1**.

$d_e = 1.3 \cdot (a \cdot b)^{0.625} / (a+b)^{0.25}$, (**Equation 1**), where a, b are the dimensions of the rectangular geometries, with a being the width of a vessel (depicted as d_0 for parent vessel and d_1 or d_2 for daughter vessels) and b the height (7 μm). To match Murray's law, the following thus needs to be true: $d_{e0}^3 = d_{e1}^3 + d_{e2}^3$ (**Equation 2**).

For each particular bifurcation geometry, the width values of the microfluidic-based vessels were calculated (**Table 2-1**), so that **Equation 2** remains true. It should be noted that in **Table 2-1**, the values of d_0 , d_1 and d_2 , represent the values a_0 , a_1 and a_2 in **Equation 1** (d_0 for parent vessel and d_1 or d_2 for daughter vessels). Moreover, the symmetry and the angle of the two daughter channels were varied to examine several features of capillary beds. In addition, a single non-bifurcated microfluidic device (of capillary dimensions of 7 μm) and a non-constricted microfluidic device (of dimensions of 20 μm) were additionally fabricated.

Table 2-1 Microfluidic devices' design parameters.

Name	d_0 (μm)	d_1 (μm)	d_2 (μm)	Angle (θ°)	Symmetry
EWS	10	6.3	6.3	87°	YES
EWA	10	6.3	6.3	87°	NO
UWS	10	5.5	7	87°	YES
UWA	10	5.5	7	87°	NO
ENS	10	6.3	6.3	43.5°	YES
ENA	10	6.3	6.3	43.5°	NO
UNS	10	5.5	7	43.5°	YES
UNA	10	5.5	7	43.5°	NO
ENA_5/5	7.9	5	5	43.5°	NO
ENA_7/7	11.3	7	7	43.5°	NO
ENA_9/9	14.7	9	9	43.5°	NO
UNA_5/7	9.6	5	7	43.5°	NO
UNA_5/9	11.4	5	9	43.5°	NO
Non-bifurcated	7	N/A	N/A	N/A	N/A
Non-constricted	50	20	20	87°	YES

All geometries were designed using Autocad.

2.2.7 Microfluidic devices fabrication & preparation

The fabrication of all microfluidic devices was performed using the techniques of standard photolithography and soft lithography. Succinctly, silicon wafers (MSE Supplies, Germany) were cleaned first with 99% (v/v) IPA and 100% (v/v) acetone and then dried with a nitrogen gun. To ensure complete removal of humidity, wafers were added for 5 min at 200°C on a hot plate. Then, wafers were treated with oxygen plasma (Harrick plasma cleaner, UK) at 0.5 Tor O_2 at 30 W for 1 min. The wafers were directly transferred in the spin coater (Elveflow, France), where 2 ml of a GM1050 SU-8 negative photoresist (Gersteltec, Switzerland) were poured on top of the wafer. The wafer was spun first at 1050 rpm for 10 s and then at 950

rpm for 45 s to define a single height of 7 μm for capillary microfluidic devices (EWS, EWA, UWS, UWA, ENS, ENA, UNS, UNA, ENA_5/5, ENA_7/7, ENA_9/9, UNA_5/7 and UNA_5/9, non-bifurcated- Table 2-1). For the non-constricted microfluidic devices (Table 2-1), the single height of SU-8 thickness was set at 20 μm by using a GM1070 SU-8 negative photoresist and spin coating at 1600 rpm for 10 s and 3000 rpm for 45 s. When the process of spin coating was finalised, wafers were heated independently first for 2 min at 65 °C and then for 20 min at 95 °C. After cooling down at room temperature for about 10 min, wafers were loaded independently in a UV mask aligner (KLOE, France). The autocad designs of all relevant geometries were sent to a company (Microlitho), which created the chromium glass masks with the relevant designs.. The masks were then loaded on top of the wafer and UV exposure followed for 30s at 100% power. Then, wafers were heated for 2 min at 65 °C and then for 20 min at 95 °C and then developed in 5 ml filled plates with SU-8 developer solution (Kayaku Advanced Materials, USA) for 5 min to remove non-polymerised (non-UV exposed) SU-8. The wafers were heated for a last time for 10 min at 95 °C and then the height of the features was measured.. These measurements were obtained using an optical surface profilometer (FILMETRICS, USA), with an accepting range of $\pm 5\%$ deviation.

The fabricated wafer (called also as wafer master) was placed in a petri dish, taped around its edges that did not interfere with the features and a mix (10:1 w/w ratio) of Sylgard™ 184 Polydimethylsiloxane prepolymer with its crosslinker (Dow Corning) was poured onto the petri dish to cover the wafer master. The petri dish was placed under vacuum to be degassed for about 30 min and then transferred to an oven to polymerise at 65 °C overnight. The polymerised PDMS wafer masters were sliced out of the petri dish and both the inlets and outlets of the microfluidic devices were punched using biopsy punches of 4- and 2 mm (IHC WORLD, USA), respectively. Glass slides were cleaned with IPA, dried and treated with plasma along with the cut PDMS microfluidic devices at 0.5 mm Tor at 30 W for 2 min. Then, PDMS devices were bonded at glass slides, pressed gently and heated for 10 min at 95 °C to ensure stable attachment.

For downstream experiments, microfluidic devices that encompassed formed channels (**Fig. 2-1A-C**) were first flushed 1x with 70% v/v ethanol, as a step to clean and make wet the channels. Then, devices were flushed 1x with PBS to clean ethanol and finally flushed again with 3% (v/v) Bovine Serum Albumin (BSA) solution to refrain cells from attaching to the

surface of the channels. These washing steps were performed by connecting a 10 ml polypropylene syringe with a 22-gauge needle (Needlez, UK) and then to a one-inch tubing of outer diameter of 2 mm (Qosina, UK), whose end was inserted in the outlet of the microfluidic devices. Pressure was applied by pulling the plunger. Microfluidic devices were prepared in the same manner to generate LMPs throughout the upcoming Chapters, which will be expanded then. On the other hand, for cell transit experiments, a similar setup was used, but instead 80 cm long tubing was connected to the syringe setup (**Appendix 3**). This time, the tubing/syringe was prefilled with PBS by pulling the plunger. After this, the plunger was removed and the syringe acted as a reservoir. The free end of the tubing was inserted in the outlet of the microfluidic devices. Pressure was applied by lowering the height difference of the syringe in respect to the microfluidic device.

2.2.8 Microfluidic devices transit experiments

To perform live imaging experiments, 5 0 μ l of either single cell suspensions (0.2×10^6 cells/ml) or clusters (0.2×10^6 cells/ml) were prepared, as described previously, and were added in microfluidic device inlets. The outlets of the microfluidic devices were connected as previously to the tubing/syringe setup and the microfluidic device was loaded on the stage of the Nikon microscope. To mimic the physiological range of human capillary velocities, the syringe was lowered at 30 cm height difference in respect to the microfluidic device, which represents human capillary blood pressures (45,266) (**Appendix 3**). The pressure differential that was created, enabled cell transit from the inlet of the devices to the outlet. Multifluorescent images were obtained every 300 ms at 10x or 20x magnification, compiled together at the end as video series. Thus, real time cellular responses during cell transit under flow in the microfluidic devices were recorded and analysed, as subsequently will be elaborated on. To mimic static conditions, cells were added in the inlet of the microfluidic devices, but no pressure was applied, for the same time that the cell transit experiment lasted. In relevant experiments, single or clustered cells were pre-treated as previously described.

2.2.9 Microfluidic devices transit video analysis

Analysis of single cells' or clusters' transit behaviours was performed manually, by monitoring and following their transit, via the recorded video series. Shortly, the first analytical metric

was the direction that cells transited through when encountering a capillary bifurcation. The number of cells that transited through the d_2 channel in the bifurcation was divided by the overall number of cells transiting in either daughter channel (d_1 or d_2), that was defined as transit direction d_2 frequency.

Observations of cell shedding were validated by tracking the detachment of LMPs from the cell membrane- that retained the cytoplasmic fluorescent dye, but that were negative for Hoechst (nuclear) signal. Shedding frequency for single cells was extrapolated by dividing the number of shedding cells to the overall number of cells that transited. For clusters, shedding frequency was calculated by dividing the number of clusters that shed at least once to the overall number of clusters that transited. Shedding frequencies were recalculated for clusters, by splitting the datasets for clusters that consisted only of doublets or more than 3 cells. Additionally, the number of shed LMPs per single cell or per cluster was also noted. The nuclear content frequency was calculated based on number of cells or LMPs that were positive for Hoechst. Similarly, the frequency of shed LMPs that transited in a different or the same direction in respect to their shedding cells was calculated by dividing them with the overall number of cell shedding events. Lastly, the shedding time was defined as the time it took for a cell to shed, once it has been trapped in the capillary bifurcation. The time difference was calculated by the time frames presented through the software in the recorded video series.

The diameters of the cell's cytoplasm or nucleus were extrapolated using the diameter measurement option from the software. Diameters were quantified before cells encountered capillary bifurcations, where they were constricted only in the z-axis. According to previous methodology employed by Au et al. (45), the spherical cell volume values were obtained using the quantified cell diameter readings, either for cytoplasm or nuclei. Volume-shedding frequency data were plotted with average volume per experiment added in x axis and shedding frequency per experiment in the y axis.

Observations of cell lysis in live imaging experiments was verified by complete loss of the cytoplasmic signal, while nuclear signal was retained. Lysis frequency was calculated by dividing the number of lysed cells to the overall number of cells that were recorded to transit. Lysis frequency was also calculated for shedding cells only, by dividing the number of post-shedding lysed cells to the overall number of post-shedding cells.

Cells' residence time within capillary bifurcations was defined as the time difference between frames of cells encountering a capillary bifurcation till their exit. For shedding cells, the time difference from the event of shedding till the cell's exit was also estimated from the respective time frames.

The extrapolation of cell volume within the capillary bifurcation was calculated by employing a similar methodology previously published, by Au et al. (45). In the capillary bifurcation, cells were deformed and assumed to be considered as three cylinders with three semi-circular caps. The surface area and volume of the cap were calculated directly, according to equations used for surface area and volumes of half circles (**Equation 3 & Equation 4**). To calculate the non-cap volume of cells, a series of calculations were performed. By measuring the total surface area of cells via the Nikon microscope software, the no-cap surface area of cells was estimated by subtracting the cap surface area from the total surface area (**Equation 5**). Then, the no-cap volume of cells was calculated by multiplying the height of the devices to the no-cap surface area of cells (**Equation 6**). Finally, the overall volume of a cell trapped in capillary bifurcation was obtained by adding the no-cap and cap volume values (**Equation 7**). Volume losses were calculated as the percentage difference between cell volume before and after shedding. Individual cell volumes with their respective cell volume losses were plotted in X-Y plot. The same set of values was also plotted as a histogram. Similarly, individual cell volumes before and after shedding were plotted in X-Y graph.

$$A_{\text{cap total}} = A_{\text{cap}}(d_0) + A_{\text{cap}}(d_1) + A_{\text{cap}}(d_2), \text{ where } A_{\text{cap}} = (\pi/2) * (d/2)^2 \quad (\text{Equation 3})$$

$$V_{\text{cap total}} = V_{\text{cap}}(d_0) + V_{\text{cap}}(d_1) + V_{\text{cap}}(d_2), \text{ where } V_{\text{cap}} = (4/6) * \pi * (d/2)^3 \quad (\text{Equation 4})$$

$$A_{\text{no cap total}} = A_{\text{total}} - A_{\text{cap total}} \quad (\text{Equation 5})$$

$$V_{\text{no cap total}} = A_{\text{no cap total}} * \text{Height} \quad (\text{Equation 6})$$

$$V_{\text{total}} = V_{\text{no cap total}} + V_{\text{cap total}} \quad (\text{Equation 7})$$

2.2.10 Viability assay

100 µl of single cell suspensions (100000 cells/ml) were grown for a day in separate wells of a 96-well plate at 37°C/5% CO₂. Cells were treated with Cyto D or Colch for either 3 or 24 hr to evaluate short and long exposure on cell viability. Control cells' viability at 0 hr was also examined. Once the media was removed from wells of both treated and untreated cells,

staining solution for live/dead staining was added in each well. Staining solution was prepared by diluting 1:1000 times Calcein-AM, Hoechst 33342 and Propidium iodide (Thermofischer Scientific, UK) at final working concentrations of 5 μ M, 16.2 μ M and 1 μ g/ml, respectively, in 1 ml PBS. The staining solution was incubated with cells for 15 min at 37°C/5% CO₂. Across each condition, 4-5 multifuorescent images were recorded at 20x magnification, using the Nikon microscope. To enumerate alive or dead cells, alive cells were defined as Calcein⁺, Hoechst⁺ but Propidium Iodide⁻, whereas dead cells were Calcein⁻, Hoechst⁺ and Propidium Iodide⁺. Viability was quantified by dividing the number of alive cells to the overall number of Hoechst-positive cells. Images were analysed in the Nikon microscope software and all conditions were tested in triplicates. The same process was repeated for assessing and quantifying cluster viability. Viability of clusters was quantified by dividing the number of alive cells to the overall number of Hoechst-positive cells within all imaged clusters.

2.2.11 Post transit cell analysis

Cells or LMPs were also analysed post-transit from the microfluidic devices. The contents of the devices were either collected directly from the outlet of the devices or emptied from the syringe/tubing setup directly to wells of a 96-well plate for downstream analysis. The diameters of LMPs were quantified using the diameter measurement option of the Nikon microscope's software. Viability of collected cells, either as single or clusters, was evaluated with the staining solution for live/dead assay, as described above. To quantify LMPs : cell ratio, collected post transit either for single or clusters, several multifuorescent images were taken using the Nikon microscope. Both cell and LMP numbers were counted and the ratios were extrapolated, independently for single or cluster-based experiments.

2.2.12 Sample preparation for scanning electron microscopy (SEM)

Shed LMPs and cells post microfluidic device transit were collected and centrifuged at 180 x g for 5 min to pellet cells and retain LMPs in the supernatant (more in detail in Chapter 4). LMPs or control cells were cultured for a day in square glass slides of 10 x 10 mm. Media was removed and glass slides were fixed with 4% formaldehyde for 15 min at room temperature to crosslink proteins. A subsequent step of fixation followed by chemical treatment with 1% (v/v) Osmium tetroxide (Agar Scientific, UK) to crosslink lipids. The fixed specimen was

dehydrated in a series of ethanol washings, starting from 30% to 100% (v/v) ethanol, with an increment step of 10% for 5 min per step. After dehydration, the specimen was dried chemically, using Hexamethyldisilazane (Merck, UK) for 3 mins, preferably used for cell preparations. The fully dried specimen was attached to a sticky carbon tape that was already attached to a metal stub, used for loading the sample. To further increase the conductivity of the sample and improve imaging resolution, silver-containing glue was added on top of the specimen. Finally, to improve the image resolution even further, the specimen was coated with 15 μm Nickel using a sample sputter coater Q150TS equipment (Quorum technologies, UK), a conductive material to improve electron scattering.

The fully prepared sample that was attached on the metal stub, was loaded in the stage holder of the Zeiss Auriga 40 Cross beam scanning electron microscope and vacuum was applied to seal the vacuum chamber. Utilising secondary electron detection, 5 kV acceleration voltage was applied to image the sample. The scanning speed was adjusted to improve resolution of the image and sample was scanned in a range from 1000x to 8000x magnification and images were generated.

2.2.13 Graphs & Statistical analyses

All experiments were run independently and repeated at least three times for each experimental condition. Data were reported as mean $\pm$ standard deviation (SD) and graphs were plotted and statistically analysed by using the GraphPad Prism (version 9.0) software. Statistical analysis between two experimental groups was done by applying the two-tailed student's t test. For statistical comparison of at least three experimental groups, one-way analysis of variance (Bonferonni or Turkey's multiple comparisons) was applied with a 95% confidence interval (CI). Non-significant statistical differences were denoted as ns and presented P values ≥ 0.05 . On the other hand, statistical significance was achieved if P values were lower, with various levels of significance described as a. significant (0.01 to 0.05, single asterisk*), b. very significant (0.001 to 0.01, double asterisk **), and c. extremely significant (0.0001 to 0.001, triple asterisk *** or < 0.0001 , quadruple asterisk ****). Illustrations were prepared using BioRender software. Graphs & statistical analyses was performed in the same way, as described, throughout all Chapters and will not be detailed in the upcoming chapters.

2.3 RESULTS

2.3.1 Development of microcirculation-mimicking microfluidic devices

To mimic the diversity of microvessel geometries commonly found in the microvasculature in vivo i.e. capillaries or small arterioles (33), different PDMS-based variants of these were engineered via photolithography (**Fig. 2-1A-C & Fig. 2-2A-C**). These microfluidic variants included constricted (synonymous for capillary) non-bifurcated channels (7 μm width x 7 μm height cross sections) (**Fig. 2-2C**), constricted bifurcated channels (see **Table 2-1** for all capillary bifurcation models) (**Fig. 2-2A**) and non-constricted bifurcated models (20 μm width x 20 μm height cross sections) (**Fig. 2-2B**). The different bifurcated models were formed by tuning several parameters between the daughter channels across each distinct bifurcated variant, including channel width (d), angle between the two daughter channels (θ) and symmetric/asymmetric geometry (with symmetric geometry defined as splitting the bifurcation in two equal halves), as can be seen in **Fig. 2-2A** and **Table 2-1**. The channel widths of the bifurcated models were designed based on Murray's law, which dictates physiologically branching of human vessels (265,267). These microfluidic variants allowed the systematic exploration of the influence of multiple microvessel geometries on cell transit.

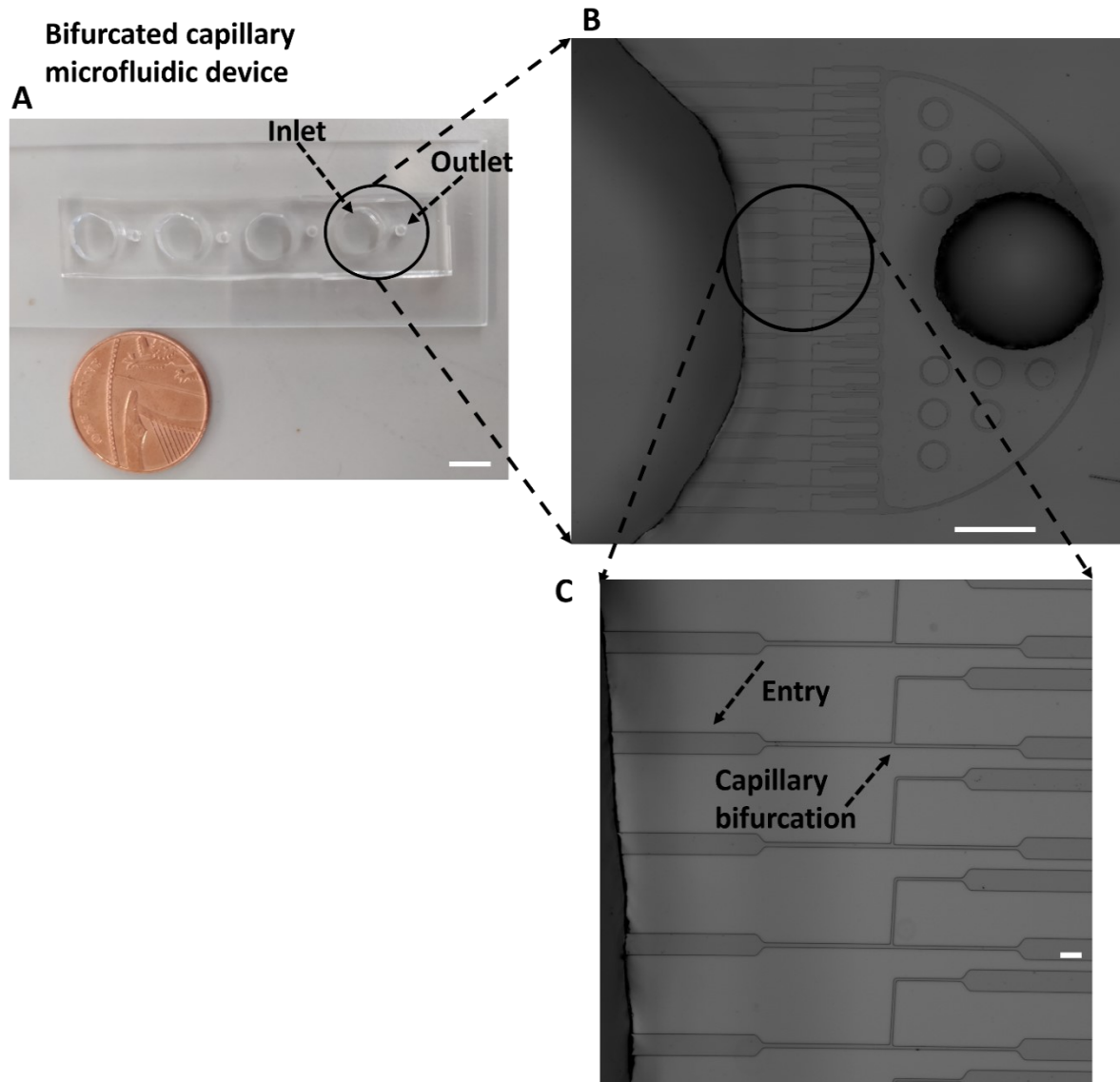


Figure 2-1: Example of a microfluidic device

(A) Photo of a microfluidic device, consisting of four independent pairs of inlets and outlets. Scale bar: 6 mm. (B) 4x brightfield image of a series of capillary bifurcation channels. Scale bar: 1 mm. (C) 20x brightfield image of capillary bifurcation channels. Scale bar: 50 μm .

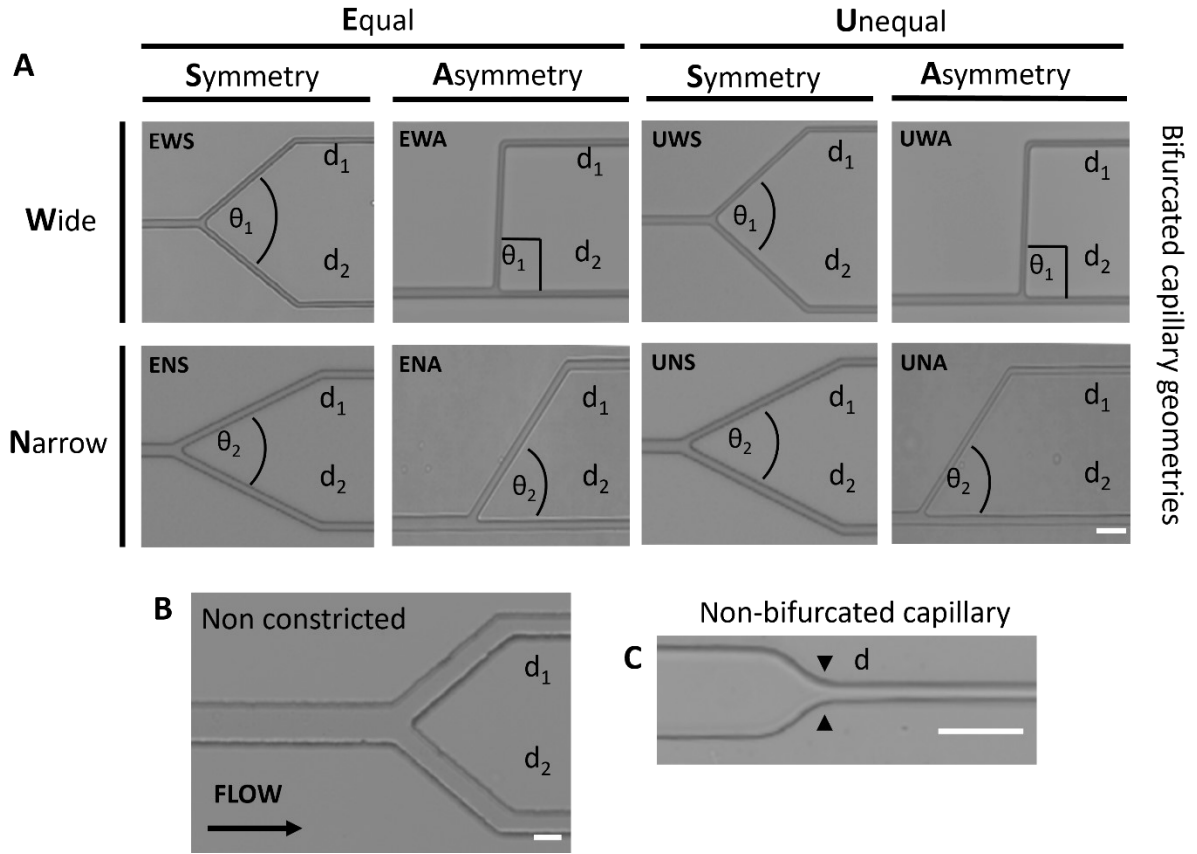


Figure 2-2: Microfluidic capillary-scale bifurcations.

(A) Brightfield images of microfluidic capillary bifurcations at 20x (**Table 2-1**). E: Equal, U: Unequal, W: Wide, N: Narrow, S: Symmetry, A: Asymmetry. Scale bar (applies in all images): 20 μm . (B) Brightfield image of a non-constricted geometry at 20x. Scale bar: 20 μm . (C) Brightfield image of a non-bifurcated capillary geometry at 20x. Scale bar: 50 μm . d represent diameters and θ represent angles between daughter channels (**Table 2.1**).

2.3.2 Cancer cells transit preferably in bifurcation daughter channels with a larger width

Single MDA-MB 231 breast cancer cells were added in all the constricted bifurcated microfluidic devices to examine primarily the influence of these geometries on cell transit direction. The mean transit percentages of cells that transited through d_2 channel were $46.27 \pm 7.71\%$ (EWS), $52.97 \pm 6.7\%$ (EWA), $46 \pm 8.01\%$ (ENS), $51.43 \pm 3.09\%$ (ENA), $81.4 \pm 7.3\%$ (UWS), $86.1 \pm 6.92\%$ (UWA), $77.47 \pm 10.21\%$ (UNS) and $80.53 \pm 3.75\%$ (UNA), as seen in **Fig. 2-3A**. Grouping these datasets in different combinations revealed that only one parameter contributed to transit direction preferences, which was whether the daughter channels of a

bifurcation had the same width or not. Cells transited preferably through d_2 rather in d_1 in unequal capillary bifurcations, with d_2 presenting the larger channel width in unequal bifurcations, compared to their equal counterparts (unequal: $81.4 \pm 7.1\%$ vs equal: $49.1 \pm 6.5\%$, $P < 0.0001$), as seen in **Fig. 2-3B**.

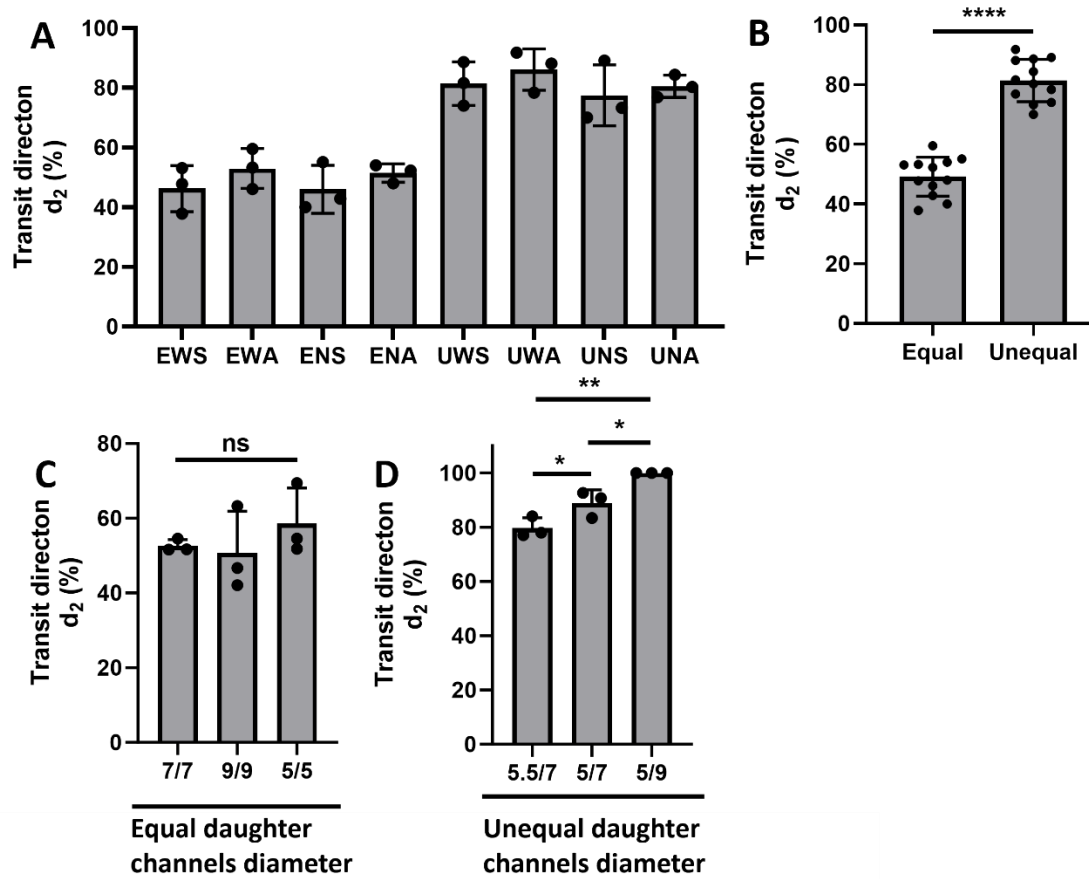


Figure 2-3: Cells prefer transit through channels with larger sizes.

(A) Quantification of percentage of MDA-MB 231 cells that transited in d_2 daughter channel across all 8 microfluidic devices ($n=3$ for all). (B) Replotting of above data in respect to equal or unequal geometries. 3 biological replicates per type of geometry, subclassified together as equal or unequal. (C) Quantification of percentage of MDA-MB 231 cells that transited in d_2 daughter channel across 3 equal capillary bifurcated geometries (ENA_5/5, ENA_7/7 and ENA_9/9) (**Table 2-1**). 3 biological replicated per type of geometry. (D) Quantification of percentage of MDA-MB 231 cells that transited in d_2 daughter channel across 3 unequal capillary bifurcated geometries (UNA, UNA_5/5 and UNA_5/9). 3 biological replicated per type of geometry.

To investigate solely the influence of channel width on cell transit behaviour, another set of bifurcated capillary geometries was used, by keeping unchanged both the angle (θ_2) and the

asymmetry. The mean transit percentages of cells through d₂ channel, were 52.63 ± 1.66% (ENA_7/7), 50.7 ± 11.17% (ENA_9/9), 58.61 ± 9.47% (ENA_5/5), 79.67 ± 3.78% (UNA_5.5/7), 88.87 ± 4.89% (UNA_5/7), 100 ± 0% (UNA_5/9). As expected from above findings, there was a clear difference again in cell transit direction between equal and unequal bifurcations (**Fig. 2-3C-D**). More specifically, the differences of channel (constriction) width across the equal bifurcated capillary geometries did not promote cell transit through d₂ (no statistical significance for all combinations) (**Fig. 2-3C**), with all mean values being close to 50%. On the other hand, increasing the difference of width in the two daughter channels of a capillary bifurcation had a significant difference (5/7 vs 5/9: P<0.05, 5.5/7 vs 5/9: P<0.005 and 5.5/7 vs 5/7: P<0.05), as observed by the increasing percentages in **Fig. 2-3D**.

2.3.3 All tested cell types shed large cytoplasmic microparticles at bifurcations

The transit of MDA-MB 231 breast cancer cells across capillary bifurcations revealed the shedding of large particles from the membrane of the cell (**Fig. 2-4A-B & Video 1-Appendix 11**), which appeared to be initially cytoplasmic fragments that retained the stain from their cell of origin. To corroborate the generality of these observations, multiple cell types were tracked down, including CTCs-grown cell lines (cell line-derived xenograft line), multiple cancer cell lines and non-cancerous cancer-associated fibroblasts, all of which shed these large particles (**Fig. 2-7A**) to determine whether these fragments originated from the cytoplasm alone, all tested cells were stained both for their membrane and nucleus and real-time imaging of their transit revealed the generation of large nuclei-free particles (**Fig. 2-4B-D & Fig. 2-5A**), in line with Headley's findings in vivo for injected melanoma cells (71). Tracking their transit revealed that MDA-MB 231 cells initially deformed to squeeze through and reach the bifurcation, where they remained trapped, released the particles within seconds (**Fig. 2-4B & Fig. 2-5B**) and continued their transit. The vast majority of cells that shed escaped from a different daughter channel, in contrast to the direction in which the shed particles transited (**Fig. 2-5C**). Measuring the size of collected particles, size calculations from fluorescent images revealed their large diameter, ranging from 1.17-11.32 µm with a mean of 4.9 ± 2 µm (**Fig. 2-5D**). While these large particles appeared highly spherical in the low magnification fluorescent images (**Fig. 2-4C**), analysis with a scanning electron microscope, which depicts a 4500-

zoomed particle, revealed that particles retained to an extent their sphericity but not as exosomes (**Fig. 2-4D, Appendix 5**). Interestingly, the SEM images of large particles resemble large EVs, isolated from the blood of prostate cancer patients by Nanou et al, where their surface appeared rougher and less spherical (75). Comparing the membrane of these large particles with whole cell membranes (**Appendix 4**), they appeared similar, given their generation from the membrane of cells. The live capture of LMPs biogenesis during cancer cell transit in capillary bifurcations under shear conditions highlights the important role of the microcirculation on cell shedding and suggests that the terminology of shed LMPs should be adjusted to shear- and capillary-derived LMPs. For simplification, the term LMPs or shear-derived LMPs will be used henceforth.

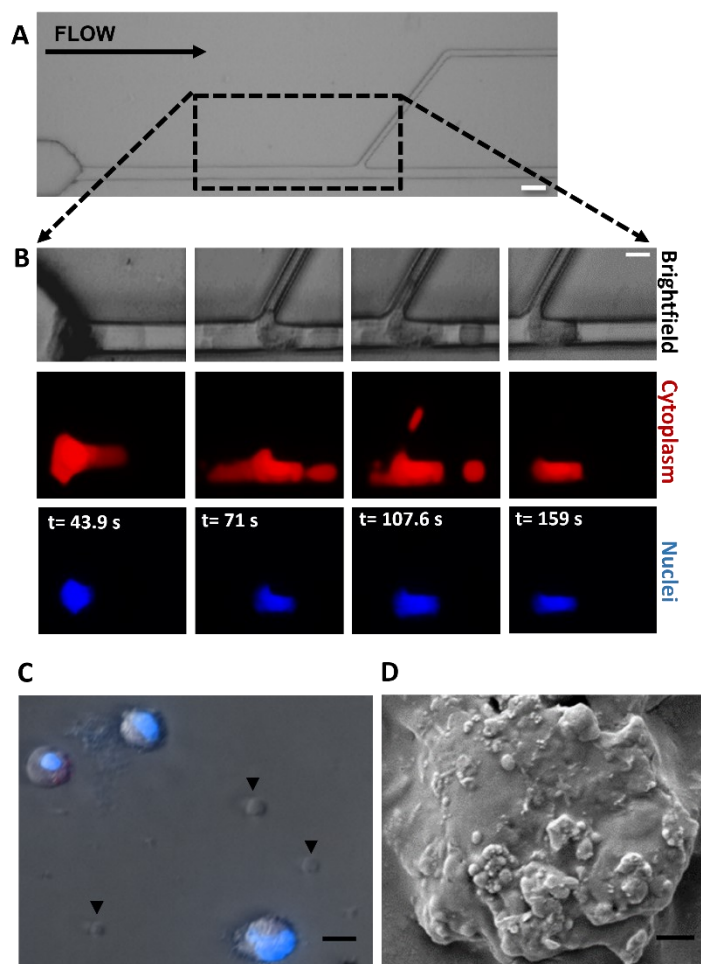


Figure 2-4: Tumour cells shed LMPs at capillary bifurcations.

(A) Brightfield image of a microfluidic capillary bifurcated geometry (UNA_59) at 20x. Scale bar: 10 μm. (B) Snapshots of timelapse series of a MDA-MB 231 breast cancer cell shedding 2 LMPs at capillary bifurcations of (A) at 20x. Cell cytoplasm is depicted red and cell nuclei blue. Scale bar: 10 μm. (C)

Fluorescent image of nucleated MDA-MB 231 cells and nuclei-free LMPs at 20x. Scale bar: 10 μm . (D) Scanning electron microscopy image of a single LMP at 3000x. Scale bar: 2 μm .

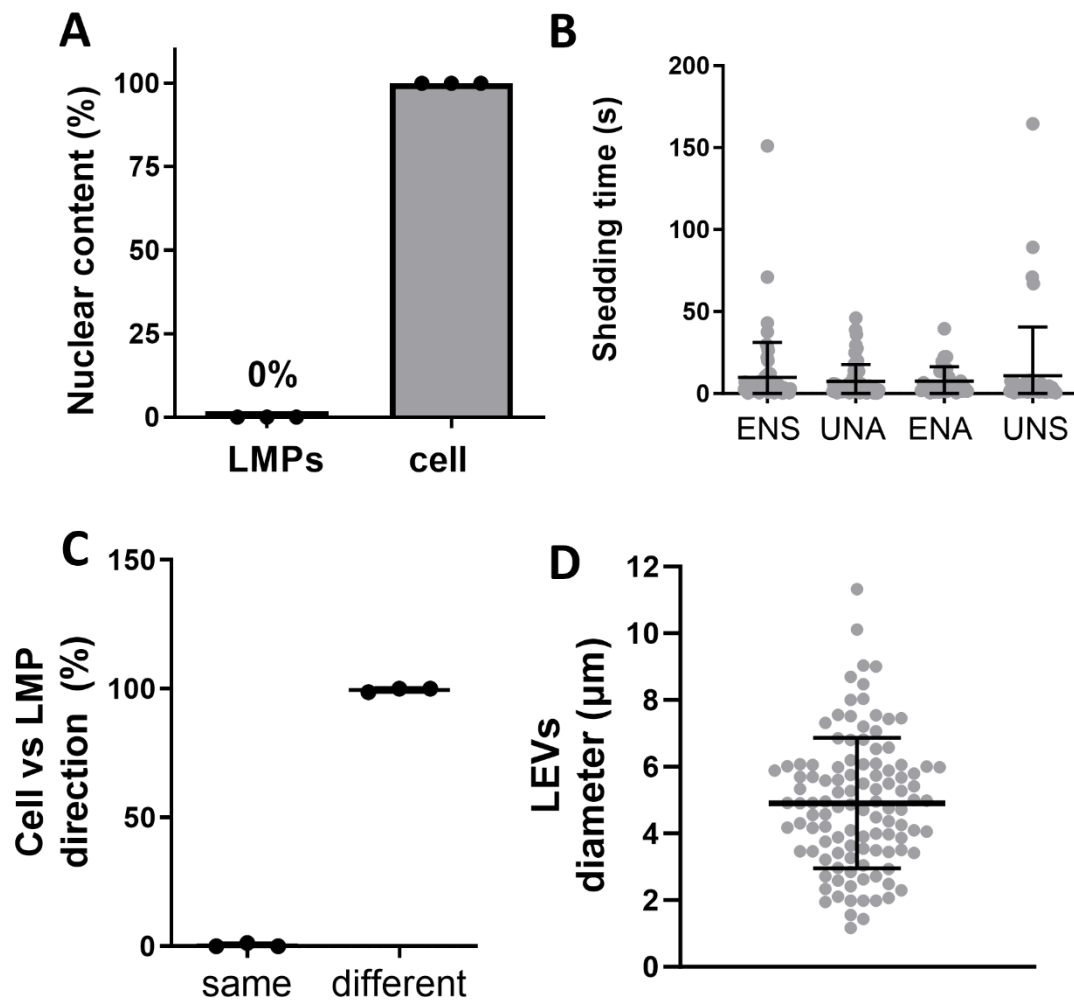


Figure 2-5: Features of shed LEVs.

(A) Quantification of percentage of MDA-MB 231 cells or LMPs that are positive for nuclei (n=3 biological replicates). (B) Shedding time of MDA-MB 231 cells that transited across 4 distinct capillary bifurcated geometries. Each data point represents a single cell that shed (n=66 for ENS, n=61 for UNA, n=31 for ENA, n=46 for UNS). (C) Percentage of MDA-MB 231 cells that shed and transited either in the same or a different daughter channel with their shed LMPs (n=3 biological replicates). (D) Diameter of shed LMPs (μm). Each data point represents a single LMP (n=111).

2.3.4 Bifurcations influence the phenomenon of cell shedding

To examine the influence of various microvessels in cell shedding, shedding frequencies and the LMP : cell ratio were estimated across transit of cancer cells in microfluidic devices. Transit

of MDA-MB 231 cells in constricted bifurcated devices enabled higher mean shedding frequencies of transiting cells, compared to constricted non-bifurcated models (bifurcated vs linear: $28.5 \pm 19.4\%$ vs $3.7 \pm 5\%$, $P < 0.05$), as seen in **Fig. 2-6A**. To investigate further which features of bifurcated geometries promoted cell shedding, the data was categorised and replotted, according to three features, i. angle, ii. equal or unequal daughter channel widths and iii. symmetry (**Fig. 2-6B-D**). Shedding frequency was not influenced neither from daughter channel width equalness (equal vs unequal: $30 \pm 20.31\%$ vs $27.08 \pm 19.23\%$, ns) nor from symmetry (symmetry vs asymmetry: $30.04 \pm 20.33\%$ vs $27.04 \pm 19.20\%$, ns), whereas differences in angle presented a profound difference in the means of cell shedding frequency (wide vs narrow: $15.7 \pm 15.6\%$ vs $41.3 \pm 13.7\%$, $P < 0.0005$), as calculated in **Fig. 2-6B-D**. Several other cell lines were used to measure shedding frequency, all of which presented consistently means of shedding frequency that were higher than 40% (**Fig. 2-7A**).

I next wondered whether other features of constricted geometries affected shedding frequency i.e. constriction width. In the alternative series of bifurcated capillary geometries tested, angle and symmetry were kept constant. Indeed, by initially comparing equal bifurcations (same daughter channel width), it was found out that lowering the channel width of the constriction, had a massive increase in the mean of cell shedding frequency (**ENA_9/9 vs ENA_7/7**: $6.1 \pm 3.5\%$ vs $47.9 \pm 7\%$, $P < 0.0005$; **ENA_9/9 vs ENA_5/5**: $6.1 \pm 3.5\%$ vs $70.1 \pm 6.2\%$, $P < 0.0001$; **ENA_7/7 vs ENA_5/5**: $47.9 \pm 7\%$ vs $70.1 \pm 6.2\%$, $P < 0.005$), as quantified in **Fig. 2-7B**. Additionally, increasing the width difference of two daughter channels in unequal bifurcations had a minor effect (**Fig. 2-7C**) on enhancing cell shedding, albeit significant (59 vs 57: $52.2 \pm 2.7\%$ vs $46.1 \pm 2.5\%$, $P < 0.05$).

Given the inability to image on real time transit of cancer cells in non-constricted bifurcated devices, I collected and enumerated the LMPs : cell ratio post-transit. Cells that transited through non-constricted devices presented about 2-fold significant increase compared to static conditions (shear vs static: 0.09 ± 0.02 vs 0.05 ± 0 , $P < 0.05$), as quantified in **Fig. 2-7D**, suggesting the contribution of shear in cell shedding. On the other hand, when the LMP : cell ratio was calculated for cells that had transited bifurcated capillary geometries, there was about 9-fold higher shedding frequency, compared to static ones (0.45 ± 0.04 vs 0.05 ± 0 , $P < 0.0001$), as quantified in **Fig. 2-7D**, suggesting that while shear can induce shedding, it is

the combination of shear and capillary bifurcations that promotes cell shedding greatly, rendering capillary bifurcations a favourable location. Interestingly, the large variance of shedding frequency percentages across bifurcated models suggests that additional factors may be at play.

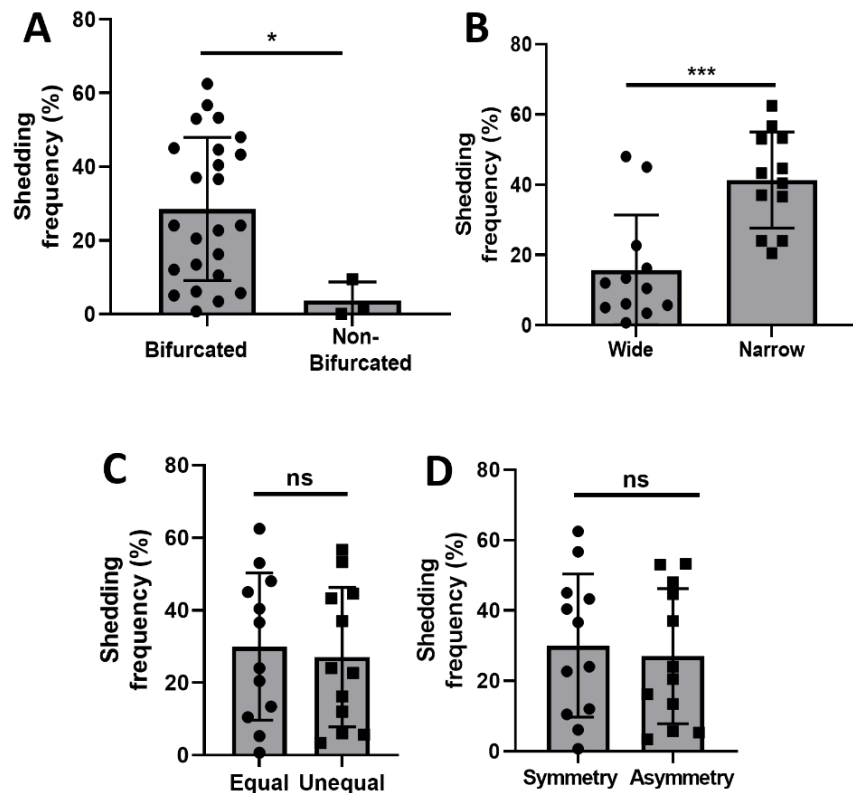


Figure 2-6: Factors that promote cell shedding.

(A) Percentage of MDA-MB 231 cells that shed at least one LMP across bifurcated or non-bifurcated capillary geometries. 8 distinct types of capillary bifurcation geometries were classified together as bifurcated (n=24, 3 biological replicates per each of 8 distinct geometries and n=3 biological replicates for non-bifurcated geometries). (B-D) Replotting of above data for bifurcated capillaries in respect to angle (B), equal diameters of daughter channels (C) or symmetry (D) (n=12 for all).

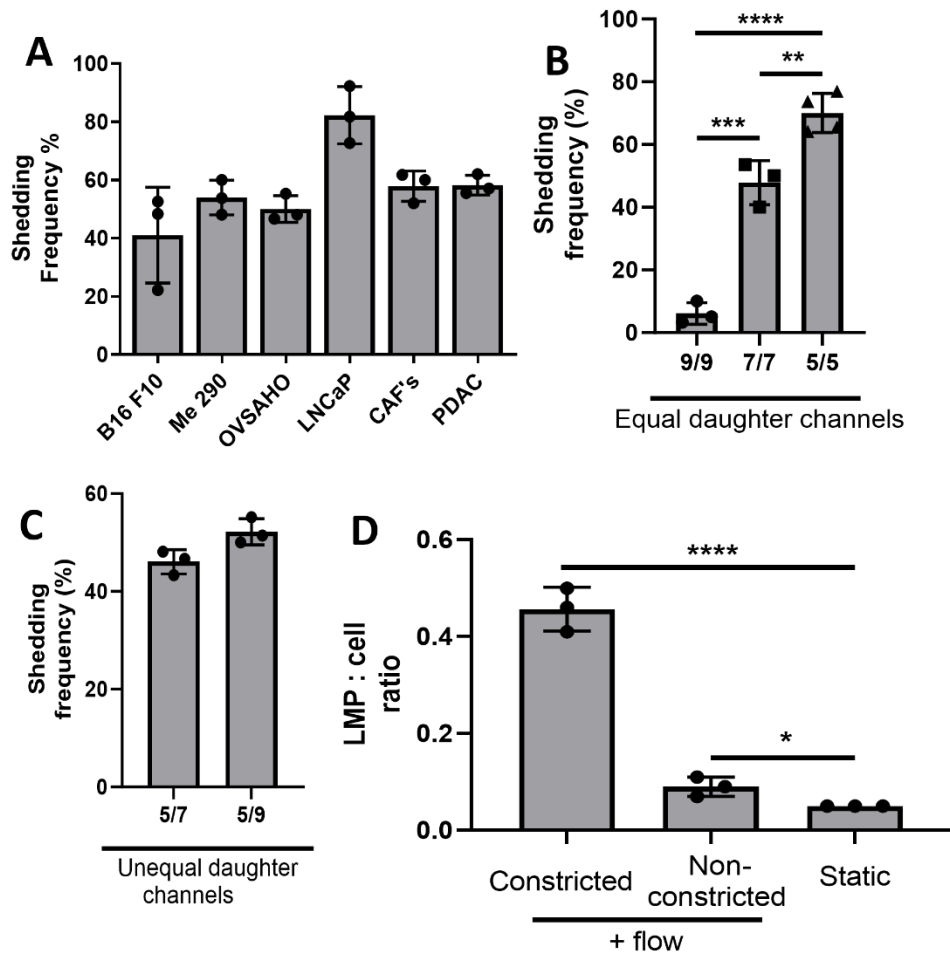


Figure 2-7: Factors that promote cell shedding.

(A) Percentage of various cell lines that shed at least one LMP (n=3 biological replicates per cell line). (B) Percentage of MDA-MB 231 cells that shed at least one LMP across 3 equal bifurcated capillaries (ENA_9/9, ENA_7/7 and ENA_5/5) (n=3 biological replicates per type of geometry). (C) Percentage of MDA-MB 231 cells that shed at least one LMP across 2 unequal bifurcated capillaries (UNA_5/7 and UNA_5/9) (n=3 biological replicates per type of geometry). (D) Extrapolation of the ratio of collected LMPs and MDA-MB 231 cells in 3 conditions: post-transit from capillary bifurcations, post transit from non-constricted geometries and static (n=3 biological replicates per type of geometry).

Homotypic cancer cell clusters transit in capillary vessels of cancer patients, similar to singlets (110). As expected, MDA-MB-231 breast cancer cell clusters were able to shed multiple or single LMPs in capillary bifurcations (**Fig. 2-8A**) at significantly higher shedding frequencies compared to singlets ($83.8 \pm 5.9\%$ vs $52.2 \pm 2.7\%$, $P < 0.0005$), as compared in **Fig. 2-8B**. Similar to single cells, shear had an enhanced impact on cluster shedding compared to static conditions by itself ($9.5 \pm 1\%$ vs $0.9 \pm 0.5\%$, $P < 0.0005$) (**Fig. 2-8C**), highlighting overall the

cooperation between shear and capillary bifurcations. As predicted, clusters also shed more LMPs than singlets (2.2 ± 1.2 vs 1.11 ± 0.4 , $P < 0.0001$) (**Fig. 2-8D**), with at least 70% of clusters shedding more than one LMP (**Fig. 2-8E**). As expected, the larger a cluster was, the higher the probabilities of at least one shedding event occurring. In fact, all but one cluster (36/37) - composed of at least 3 cells- shed, whereas 68.7% (119/173) doublets shed (**Fig. 2-8F**). Finally, by calculating the percentage of clusters where all or some cells shed, no significance occurred ($36.7 \pm 3.8\%$ vs $44.4 \pm 3.6\%$, ns)(**Fig. 2-8G**).

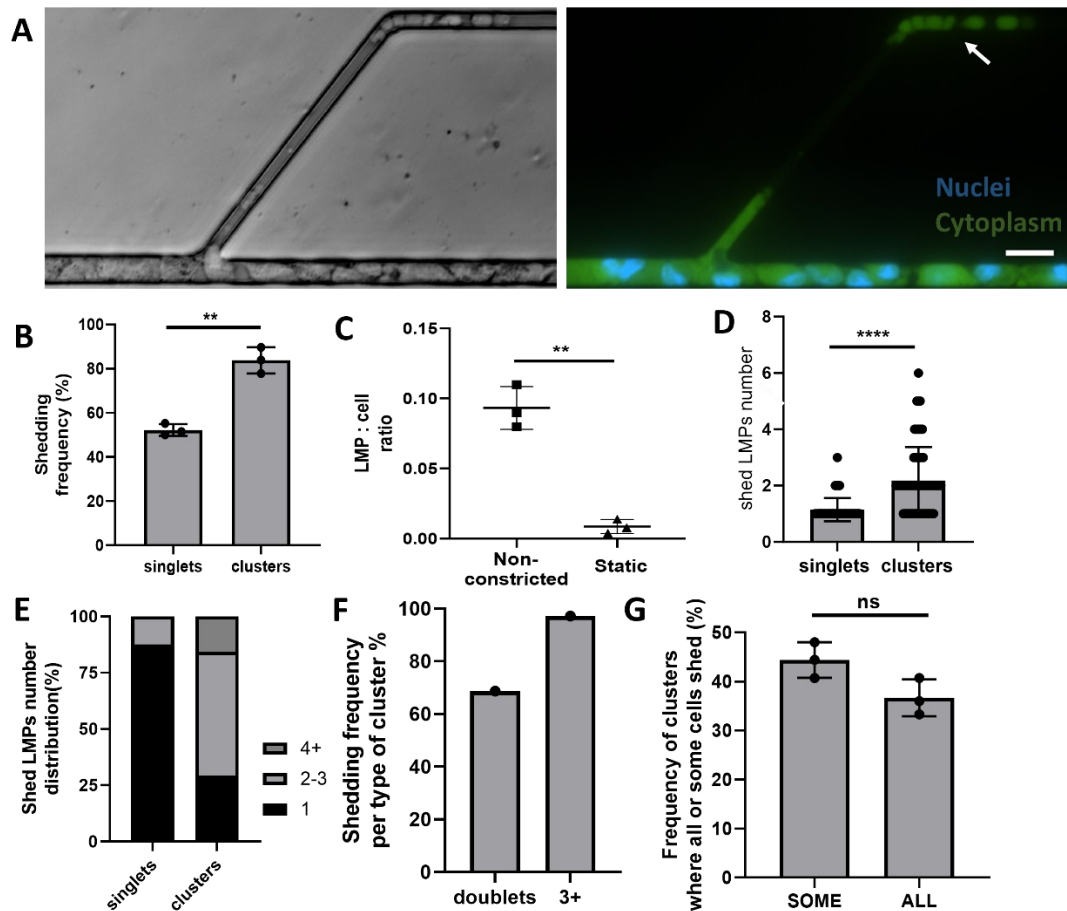


Figure 2-8: Clusters of tumour cells shed LMPs.

(A) Multifluorescent image of an 8-cell cluster shedding multiple LMPs in a capillary bifurcation. Cytoplasm was stained green and nuclei blue. Arrow depicts multiple shed LMPs. Scale bar: 20 μ m. (B) Percentage of single or clustered MDA-MB 231 cells that shed at least one LMP in capillary bifurcations (n=3 biological replicates). (C) Extrapolation of the ratio of collected LMPs and clusters in 2 conditions: post transit from non-constricted geometries and static (n=3 biological replicates). (D) Number of LMPs shed by single (n=48) or clustered cells (n=66), obtained from (B). Each data plot represents the number of shed LMPs per either single or cluster. (E) Distribution of numbers of shed LMPs by single

(n=48) or clustered cells (n=66). (F) Percentage of doublets or clusters of at least 3 cells that shed at least one LMP in capillary bifurcations. (G) Percentage of clusters where all cells or some cells within a cluster shed LMPs in capillary bifurcations (n=3 biological replicates).

2.3.5 CTC shedding of LMPs is a size dependent phenomenon

I noticed quite frequently that cells that appeared larger, ended up shedding LMPs, suggesting that cell size could be an inherent physical property that regulated cell shedding. I tracked MDA-MB-231 breast cancer cells transit of non-shedding cells and of cells that eventually shed (**Fig. 2-9A-B**) and retrospectively quantified their cytoplasmic volume before their entrapment in the capillary bifurcation, which enabled the identification of a major difference in cytoplasmic cell volume. I demonstrated that the mean volume of cells that eventually shed was significantly higher ($2421 \pm 677.6 \mu\text{m}^3$ vs $1269 \pm 398.6 \mu\text{m}^3$, $P < 0.0001$), and this pattern was consistent across all bifurcated capillary geometries (**Fig. 2-9C**), as presented for each of 8 tested bifurcation geometries: **EWS** ($2420 \pm 811.5 \mu\text{m}^3$ vs $1108 \pm 300.2 \mu\text{m}^3$, $P < 0.0001$), **UWA** ($2648 \pm 621.9 \mu\text{m}^3$ vs $1430 \pm 503.1 \mu\text{m}^3$, $P < 0.0001$), **EWA** ($2641 \pm 878.7 \mu\text{m}^3$ vs $1104 \pm 392.1 \mu\text{m}^3$, $P < 0.0001$), **UWS** ($2370 \pm 459.5 \mu\text{m}^3$ vs $1406 \pm 352.4 \mu\text{m}^3$, $P < 0.0001$), **ENS** ($2045 \pm 364.3 \mu\text{m}^3$ vs $1259 \pm 259.9 \mu\text{m}^3$, $P < 0.0001$), **ENA** ($2628 \pm 776.6 \mu\text{m}^3$ vs $1354 \pm 434.3 \mu\text{m}^3$, $P < 0.0001$), **UNS** ($2565 \pm 659 \mu\text{m}^3$ vs $1408 \pm 388.2 \mu\text{m}^3$, $P < 0.0001$), **UNA** ($2340 \pm 601.5 \mu\text{m}^3$ vs $1365 \pm 336.9 \mu\text{m}^3$, $P < 0.0001$) (**Fig. 2-9D-E**). Difference in mean volumes was quantified also in non-bifurcated capillary geometries ($3949 \pm 860.8 \mu\text{m}^3$ vs $2022 \pm 668.4 \mu\text{m}^3$, $P < 0.0001$) (**Fig. 2-10A**). Additionally, two melanoma cell lines tested (**Fig. 2-10B-C**), exhibited a similar volumetric difference when tracked down in capillary bifurcations geometries (B16F10: $2607 \pm 810.9 \mu\text{m}^3$ vs $1378 \pm 314.8 \mu\text{m}^3$, $P < 0.0001$; Me290: $1804 \pm 366.5 \mu\text{m}^3$ vs $970.1 \pm 226.3 \mu\text{m}^3$, $P < 0.0001$). Lastly, when the nuclear volume was measured (**Fig. 2-10D**), following the same methodology, there was also a noticeable difference between non-shedding cells and cells that eventually shed ($696.6 \pm 151.9 \mu\text{m}^3$ vs $418.8 \pm 94.3 \mu\text{m}^3$, $P < 0.0001$).

To investigate further whether individual features of bifurcated geometries affected cell size-dependent shedding, I replotted the datasets from the 8 distinct constricted bifurcated devices in respect to each individual design parameter. By comparing the mean volumes of cells that shed only (**Fig. 2-10E**), there was no statistically significant increase or decrease in mean cell volume for any particular design parameter (**wide**: $2538 \pm 748.6 \mu\text{m}^3$, **narrow**: 2384

$\pm 650.1 \mu\text{m}^3$, **equal**: $2390 \pm 728.1 \mu\text{m}^3$, **unequal**: $2456 \pm 617.1 \mu\text{m}^3$, **symmetry**: $2316 \pm 593.7 \mu\text{m}^3$, **asymmetry**: $2523 \pm 737.7 \mu\text{m}^3$; ns in all multiple comparisons).

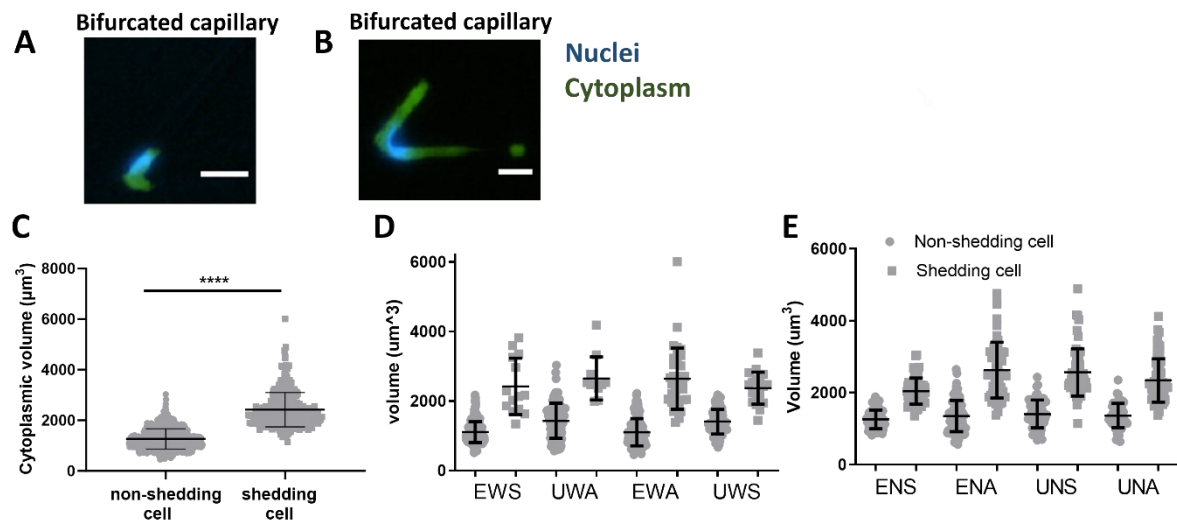


Figure 2-9: Cell size promotes shedding.

(A-B) Multifluorescent images of a MDA-MB 231 cell not shedding (A) or shedding (B) at 20x while transiting in capillary bifurcations. Cytoplasm was stained green and nuclei blue. Scale bar: 20 μm . (C-H) Each data point represents a single cell. (C) Cytoplasmic volume (μm^3) of non-shedding (n=852) and shedding MDA-MB 231 cells (n=323) during transit in 8 different bifurcated capillary geometries. All data were pooled together. (D) Cytoplasmic volume (μm^3) of non-shedding (circle) and shedding MDA-MB 231 cells (square) during transit in 4 wide bifurcated capillary geometries. (E) Cytoplasmic volume (μm^3) of non-shedding (circle) and shedding MDA-MB 231 cells (square) during transit in 4 narrow bifurcated capillary geometries.

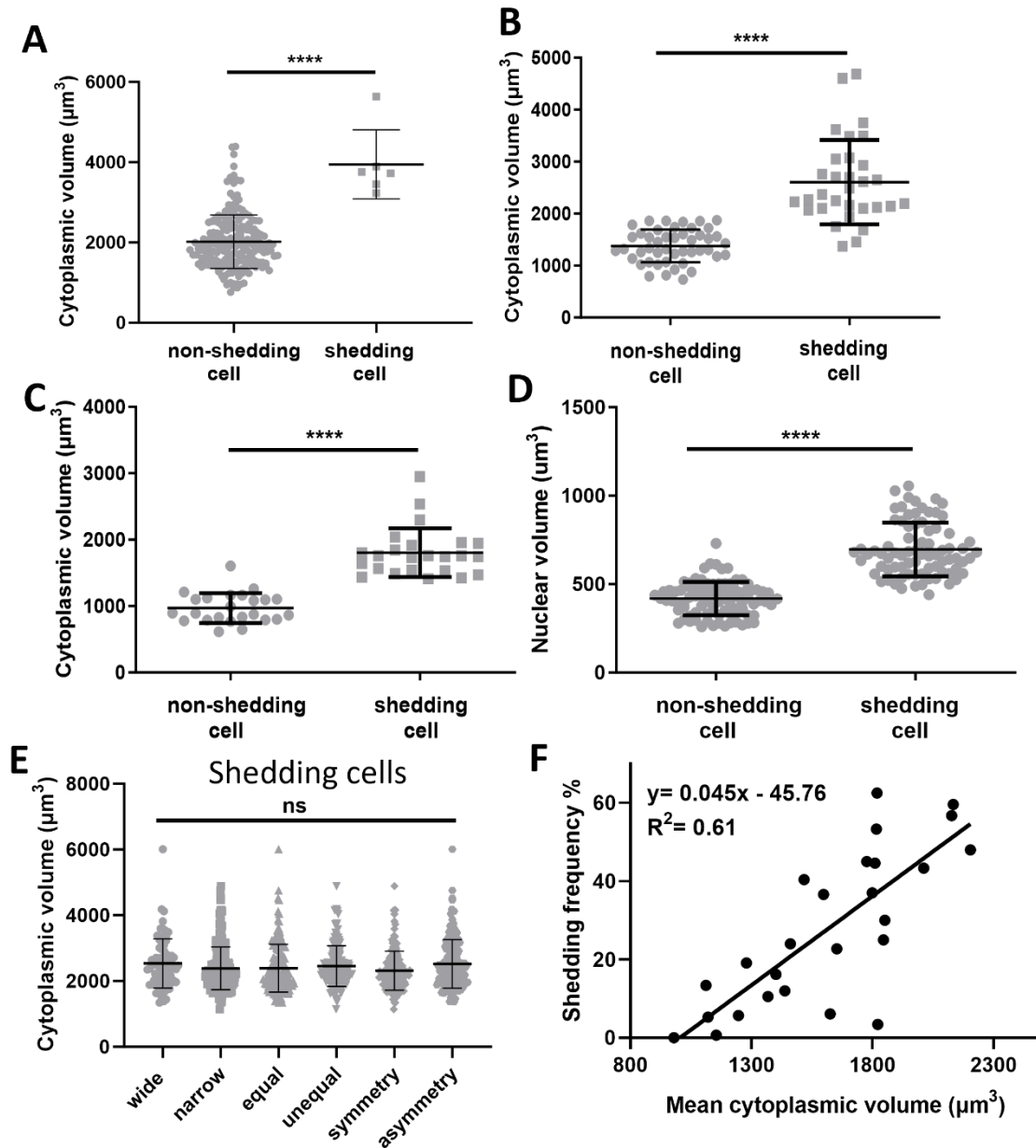


Figure 2 - 10: Cell size promotes shedding.

(A) Cytoplasmic volume (μm^3) of non-shedding (n=198) and shedding MDA-MB 231 cells (n=6) during transit in non-bifurcated capillaries. (B) Cytoplasmic volume (μm^3) of non-shedding (n=46) and shedding B16F10 melanoma cells (n=31) during transit in bifurcated capillaries. (C) Cytoplasmic volume (μm^3) of non-shedding (n=24) and shedding Me290 melanoma cells (n=24) during transit in bifurcated capillaries. (D) Nuclear volume (μm^3) of non-shedding (n=87) and shedding MDA-MB 231 cells (n=78) during transit in bifurcated capillaries. (E) Cytoplasmic volume (μm^3) of shedding MDA-MB 231 cells during transit in various bifurcated capillary geometries, as classified per design parameter (n=79 for wide, n=244 for narrow, n=170 for equal, n=153 for unequal, n=159 for symmetry, n=164 for asymmetry). (F) Graph plot of mean cytoplasmic volume (μm^3) in x axis vs shedding frequency in y

axis. Each data point represents a single experiment. Data from all 8 bifurcated capillary geometries were brought together (n=24).

As previous findings highlighted, cell shedding frequencies presented large variances (**Fig. 2-6A**), so given the cell size contribution to shedding, I wondered whether cell shedding frequencies were affected by the distribution of cell volumes across each experiment. By plotting shedding frequencies (Y axis) vs mean volume (X-axis) -for each single experiment- a positive correlation was identified ($R^2 = 0.61$) (**Fig. 2-10F**), suggesting that shedding frequency is at least partially a function of cell size.

2.3.6 Cell shedding of LMPs affects cell volume and transit

The large size of shed LMPs prompted me to investigate whether shedding affected cells, namely their cell volume. As observed in **Fig. 2-11A**, shedding appears to reduce the surface area of a cell. Thus, cytoplasmic volumes before and after cell shedding, during cell entrapment in capillary bifurcations were calculated. Cell volume was reduced significantly after shedding in all 4 tested constricted bifurcated geometries (**UNA**: $2467 \pm 663.8 \mu\text{m}^3$ vs $1991 \pm 508.6 \mu\text{m}^3$, $P < 0.0001$; **UNS**: $2569 \pm 906.8 \mu\text{m}^3$ vs $2034 \pm 638.1 \mu\text{m}^3$, $P < 0.0005$; **ENA**: $2686 \pm 833.6 \mu\text{m}^3$ vs $2100 \pm 626.4 \mu\text{m}^3$, $P < 0.0001$; **ENS**: $2195 \pm 481.8 \mu\text{m}^3$ vs $1701 \pm 360.4 \mu\text{m}^3$, $P < 0.0001$) (**Fig. 2-11B-E**). The percentage of volume losses was calculated (**Fig. 2-11F**) and statistical significant difference was extrapolated between unequal and equal capillary bifurcation geometries (**unequal**: $18.9 \pm 9.9\%$ vs **equal**: $22.8 \pm 9.1\%$, $P < 0.005$) (**Fig. 2-11G**). To test this idea further, capillary bifurcated geometries, only whose channel width was altered, were employed this time. In **Fig. 2-11H**, there is a clear reduction in the means of volume losses, as the width difference between two daughter channels increases, yet statistical significance was reached only between each unequal geometry to the equal one (**ENA_7/7**: $22.3 \pm 11.9\%$ vs **UNA_5/7**: $15.6 \pm 8.4\%$, $P < 0.005$; **ENA_7/7**: $22.3 \pm 11.9\%$ vs **UNA_5/9**: $13 \pm 6.5\%$, $P < 0.0001$; **UNA_5/7**: $15.6 \pm 8.4\%$ vs **UNA_5/9**: $13 \pm 6.5\%$, ns). Interestingly, while cells' volume was reduced post-shedding significantly, their volume was still significantly larger than non-shedding cells, as shown for all tested 4 microfluidic devices in **Fig. 2-12A-D**.

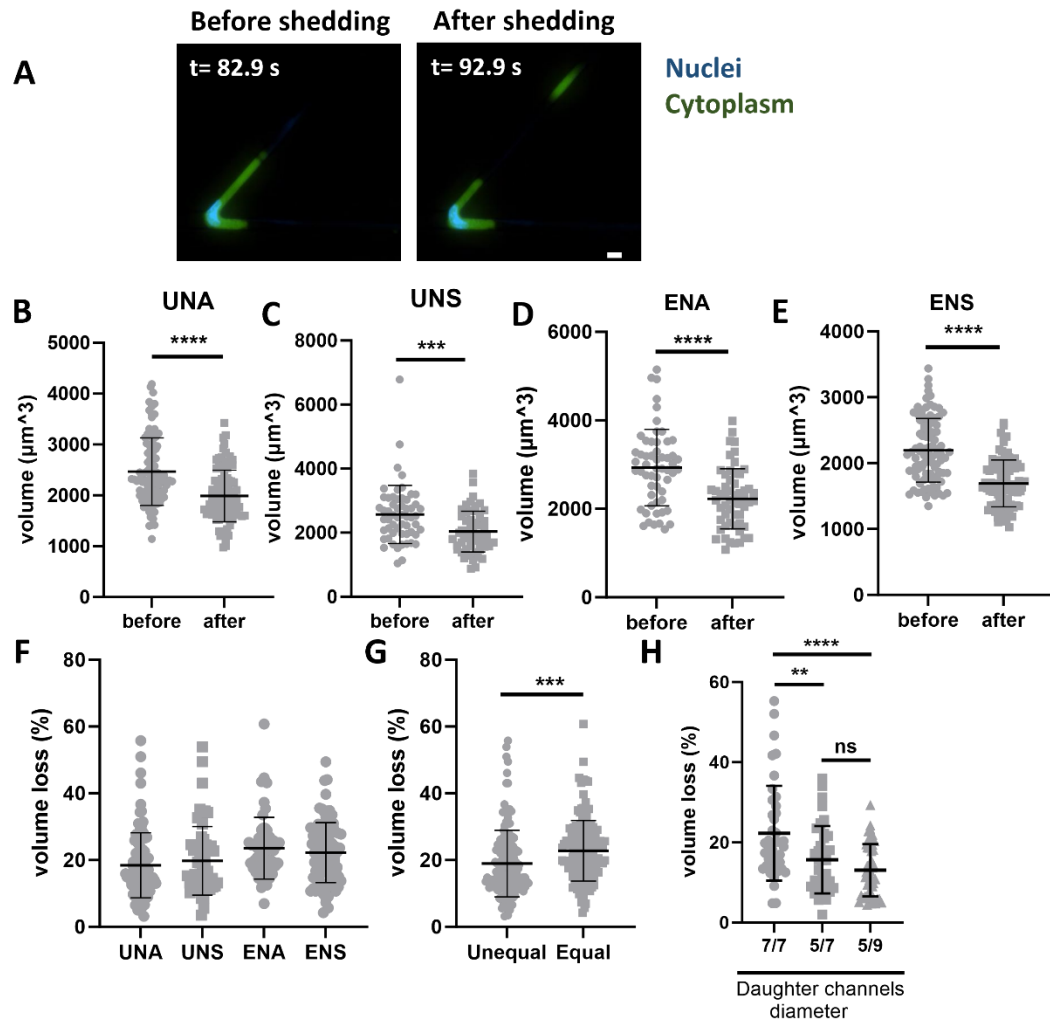


Figure 2-11: Cell shedding of LMPs leads to cell volume reduction.

(A) Multifluorescent images of the area of a MDA-MB 231 cell before and after shedding while trapped in a capillary bifurcation. Cytoplasm is stained green and nuclei blue. Scale bar: 10 μm . (B-H) Each data point represents a single cell. (B-E) Cytoplasmic volume (μm^3) of MDA-MB 231 cells post transit across 4 distinct bifurcated capillary geometries, before and after shedding (n=97 for UNA, n=57 for UNS, n=56 for ENA, n=92 for ENS). (F) Calculation of volume loss post shedding for above data (n=97 for UNA, n=57 for UNS, n=56 for ENA, n=92 for ENS). (G) Replotting of data from (F) in respect to equal (n=154) vs unequal (n=148) capillary bifurcations. (H) Calculation of volume loss post shedding of MDA-MB 231 cells that shed in 3 distinct capillary bifurcations (ENA_7/7-n=42, UNA_5/7-n=39 and UNA_5/9-n=44).

This volume reduction prompted me to investigate further what its impact on cell fate is. Therefore, by tracking down the residence time of transited MDA-MB 231 breast cancer cells in capillary bifurcations, as expected, I initially quantified a large difference in mean residence

times between non-shedding cells and cells that eventually shed (10.1 ± 4 s vs 27.5 ± 1.5 s, $P < 0.005$) (**Fig. 2-12E**). However, when the residence time of shedding cells was noted only post-shedding, there was no statistical difference between non-shedding and shedding cells (27.5 ± 1.6 s, 13.8 ± 2.4 s, ns) (**Fig. 2-12E**), suggesting that shedding promotes faster transit through capillary bifurcations.

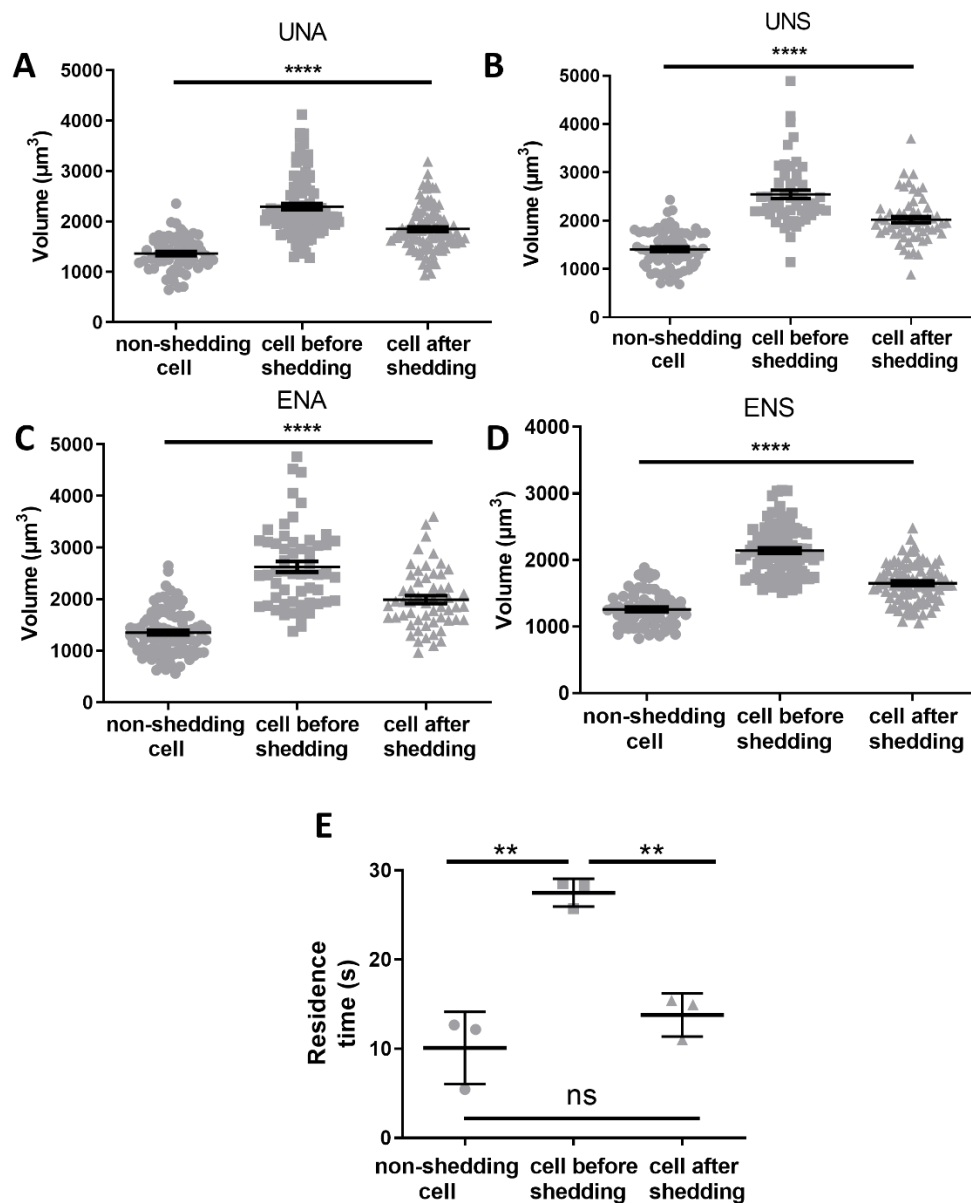


Figure 2-12: Cell shedding of LMPs leads to cell volume reduction, enabling faster transit.

(A-D) Cytoplasmic volume (μm^3) of non-shedding (n=62 for UNA, n=71 for UNS, n=95 for ENA, n=68 for ENS) or shedding (n=97 for UNA, n=57 for UNS, n=56 for ENA, n=92 for ENS) (before and after) MDA-MB 231 cells post transit across 4 distinct bifurcated capillary geometries. Each data point

represents a single cell. (E) Residence time of non-shedding or shedding (before and after shedding) MDA-MB 231 cells during transit in ENA capillary bifurcation (n=3 biological replicates).

Finally, by plotting the individual volume of cells that eventually shed with their respective volume loss, presented very weak correlations, both for unequal ($R^2=0.1$) and equal ($R^2=0.06$) bifurcations (**Fig. 2-13A&C**). On the other hand, by plotting the volumes of individual cells before and after shedding, there was a strong linear correlation, both for unequal ($R^2=0.73$) and equal ($R^2=0.84$) capillary bifurcations (**Fig. 2-13B&D**), suggesting that cells lose volume at similar levels. To validate this notion, a histogram depicting the volume loss distribution per counts was plotted, highlighting that there is a pattern of increased counts of volume loss percentages between 14 and 28% (**Fig. 2-13E**).

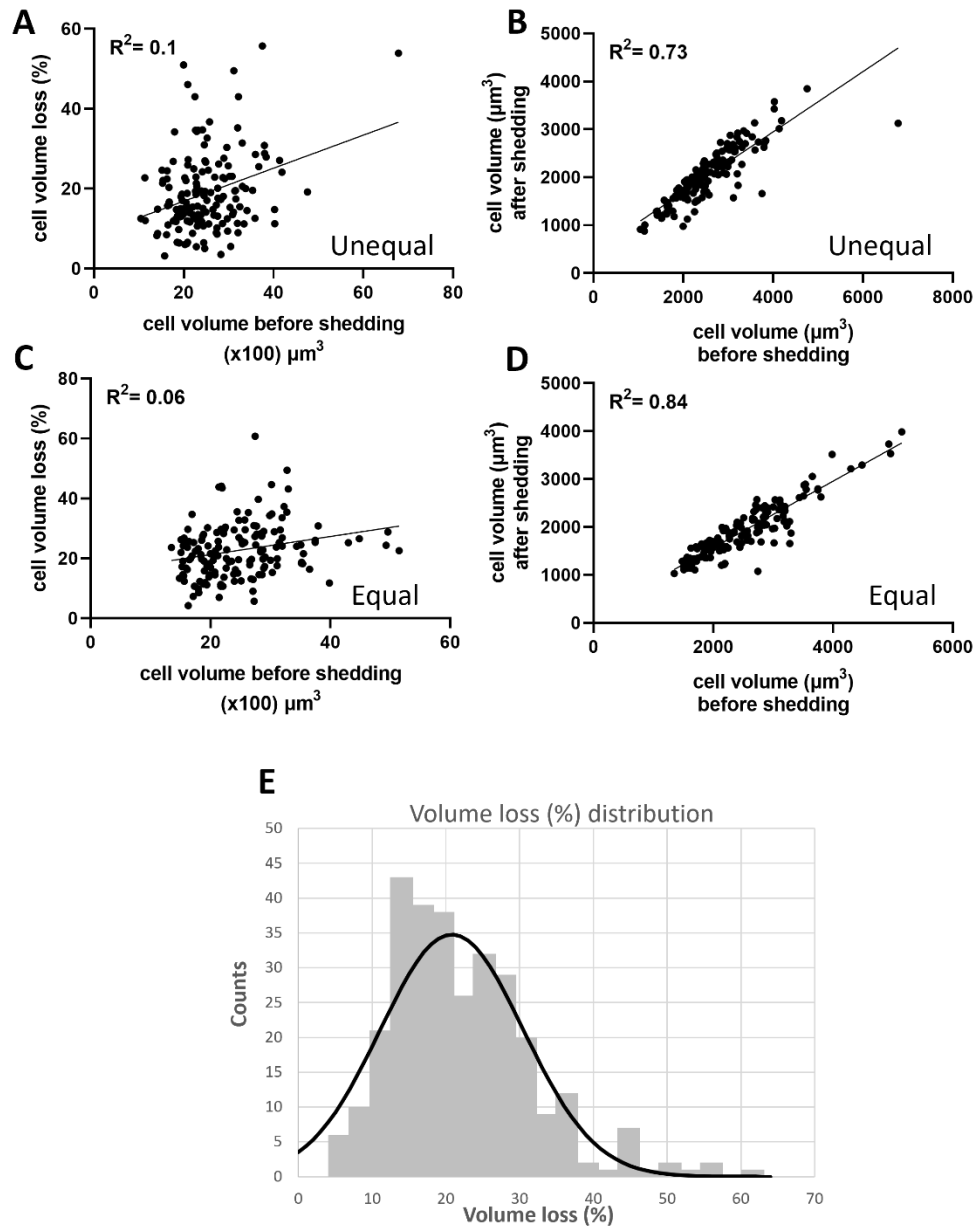


Figure 2-13: Cell volume relationships pre- and post-shedding

(A-D) Each data point represents a single cell. (A) Graph plot of cytoplasmic volume before shedding (x axis) with volume loss (y axis) in unequal capillary geometries (n=154). (B) Graph plot of cytoplasmic volume before shedding (x axis) with cytoplasmic volume after shedding (y axis) in unequal capillary geometries (n=154). (C) Graph plot of cytoplasmic volume before shedding (x axis) with volume loss (y axis) in equal capillary geometries (n=148). (D) Graph plot of cytoplasmic volume before shedding (x axis) with cell volume after shedding (y axis) in unequal capillary geometries (n=148). (E) Histogram of volume loss distribution (n=302).

2.3.7 Interfering with cellular F-actin promotes cell shedding and lysis

After characterising features of capillaries i.e. bifurcation angles and physical properties of cells i.e. size as important mediators of cell shedding, I sought out to investigate whether interfering with the cell's cytoskeleton, which confers structural integrity to cell membranes (268) could affect cell shedding. To test this, I employed F-actin polymerisation-modulating agents such as Cytochalasin D (Cyto-D) and Colchicine (Colch) to inhibit and promote F-actin polymerisation, respectively (262,264). As expected, Cyto-D inhibited F-actin polymerisation, as can be seen by lack of intense F-actin antibody staining around the cells' nuclei, whereas Colchicine promoted its polymerisation, observed by enhanced F-actin staining, compared to untreated cells (**Fig. 2-14A**). The viability of cells treated with these compounds remained unaltered compared to controls (consistently higher than a mean value of 85%), both at 3hr and 24hr, as quantified from live/dead staining assay (**Fig. 2-14B-C**).

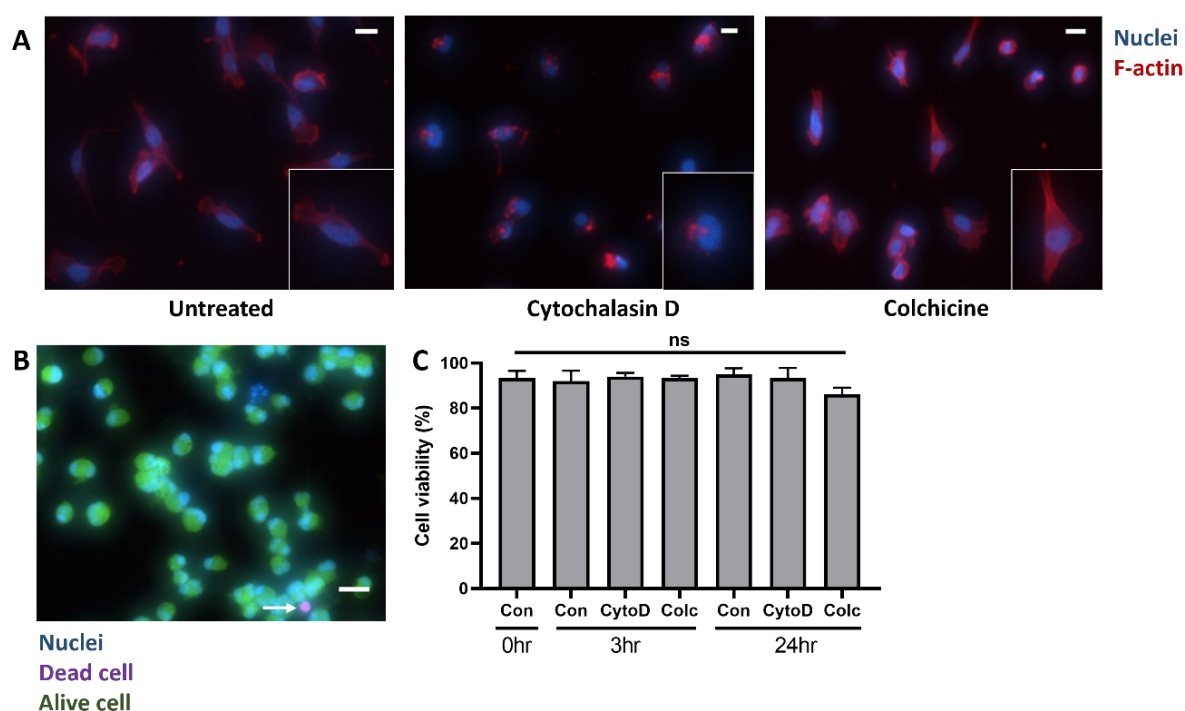


Figure 2-14: Cell cytoskeleton modulation by F-actin interfering compounds.

(A) Multifluorescent images of untreated, Cytochalasin D treated or Colchicine treated MDA-MB 231 cells at 20x. F-actin cytoskeleton was stained red and nuclei blue. Scale bar: 20 μ m. (B) A characteristic multifluorescent image of live/dead assay for MDA-MB 231 cells treated with Cyto D in 3hr. Nuclei was stained blue, dead cells as red (shown as purple) and alive cells as green. Arrow indicates a dead

cell. Scale bar: 50 μ m. (C) Quantification of MDA-MB 231 cell viability of either untreated, Cyto D treated or Colchicine treated MDAMB 231 cells at 3 or 24hr (n=3 biological replicates for all).

Modulating F-actin polymerisation significantly affected cellular shedding in capillary bifurcations, by increasing or decreasing the LMPs : cell ratio, respectively, compared to untreated (Cyto-D: 1.34 ± 0.45 vs untreated: 0.46 ± 0.04 , $P < 0.05$; Colchicine: 0.11 ± 0.06 vs untreated: 0.46 ± 0.04 , $P < 0.005$), as can be seen in **Fig. 2-15A**. Additionally, through live imaging, I compared on real-time the shedding frequency of Cyto-D treated cells in capillary bifurcation geometries, which exhibited a statistically significant difference compared to untreated cells ($82.5 \pm 4.4\%$ vs $38.2 \pm 18.7\%$, $P < 0.05$) (**Fig. 2-15B**). To validate the impact of cytochalasin on cells' transit in other microvessels, I calculated an increase in LMPs : cell ratio in non-bifurcated capillary geometries (Cyto-D: 0.23 ± 0.04 vs untreated: 0.08 ± 0.01 , $P < 0.005$), and a 10-fold increase in non-constricted geometries (Cyto-D: 0.92 ± 0.13 vs untreated: 0.09 ± 0.02 , $P < 0.0001$) (**Fig. 2-15C-D**). To exclude the possibility that the increase in cell shedding occurred due to the direct effects of Cytochalasin, the very low increase, albeit significant, of LMPs : cell ratio (Cyto-D: 0.07 ± 0 vs untreated: 0.05 ± 0 , $P < 0.005$) (**Fig. 2-15E**), suggests that it's the combination of shear and capillary bifurcations that cooperate with Cyto-D treated cells to promote greatly shedding. Colchicine had the opposite effect by reducing significantly the LMP : cell ratio both in non-constricted and static conditions (**Fig. 2-15D-E**).

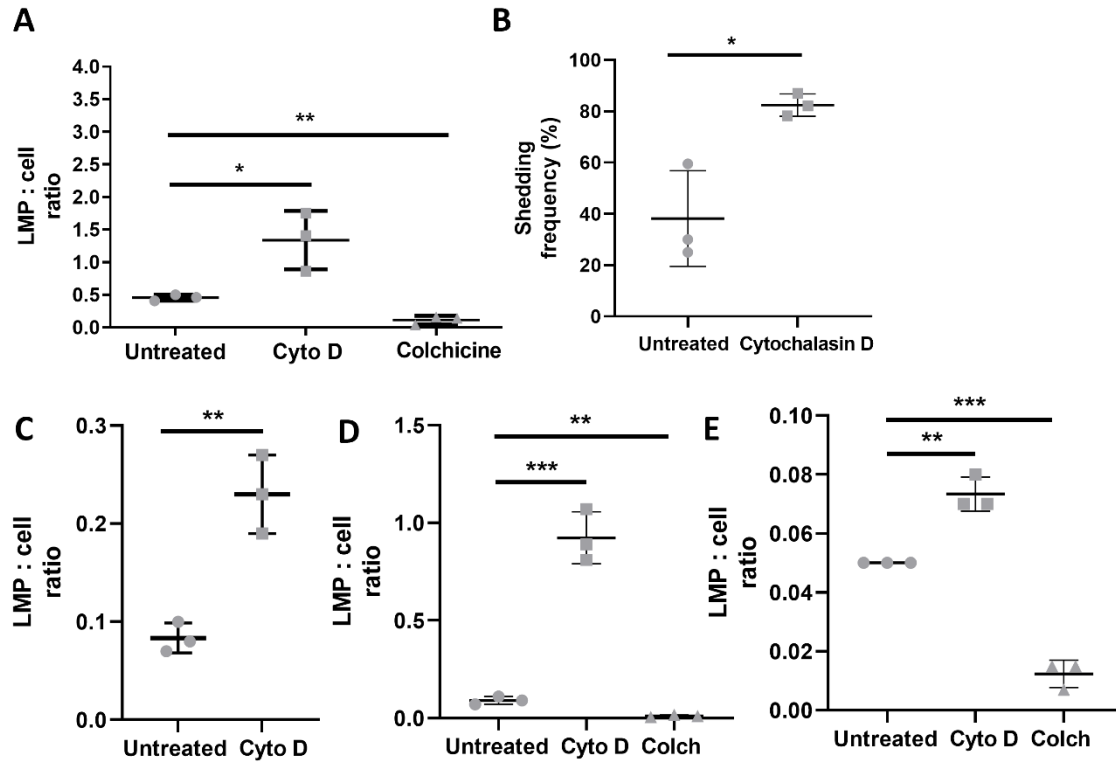


Figure 2-15: F-actin modulation compounds affect cell shedding.

(A) Extrapolation of the ratio of collected LMPs and MDA-MB 231 cells in 3 conditions post-transit from capillary bifurcations: untreated, Cytochalasin-D treated or Colchicine treated. (B) Percentage of untreated or Cytochalasin-D treated MDA-MB 231 cells that shed during transit in capillary bifurcations (live imaging). (C) Extrapolation of the ratio of collected LMPs and MDA-MB 231 cells in 2 conditions post-transit from non-bifurcated capillaries: untreated or Cytochalasin-D treated. (D) Extrapolation of the ratio of collected LMPs and MDA-MB 231 cells in 3 conditions post-transit from non-constricted geometries: untreated, Cytochalasin-D treated or Colchicine treated. (E) Extrapolation of the ratio of collected LMPs and MDA-MB 231 cells in 3 conditions from static conditions (no flow): untreated, Cytochalasin-D treated or Colchicine treated. (A-E) $n=3$ biological replicates for all.

On the other hand, treatment with Cytochalasin only slightly increased (not statistically significant) the shedding frequency of clusters compared to untreated ($89.2 \pm 9\%$ vs $83.8 \pm 5.9\%$, ns) (**Fig. 2-16A**) or against single cells that were treated with Cytochalasin ($89.2 \pm 9\%$ vs $82.5 \pm 4.4\%$, ns) (**Fig. 2-16B**), during their transit in capillary bifurcations. Though there was an increase of the LMP : cell ratio in non-constricted devices post Cytochalasin treatment for clusters (Cyto-D: 0.15 ± 0.04 vs untreated: 0.09 ± 0.01 , ns), there was still no statistical

significance (**Fig. 2-16C**). Cytochalasin also had no direct effect on clusters shedding in static conditions (**Fig. 2-16D**). A striking difference was noticed when shedding frequencies between singlet and cluster data were brought together in non-constricted geometries (**Fig. 2-17A**). While single cells faced a major increase in the LMPs : cell ratio, upon Cyto-D exposure in non-constricted geometries (**Fig. 2-15D**), clusters resisted that change, by retaining a low ratio (**Fig. 2-16C & Fig.2-17A**). By staining clusters for F-actin, the staining of the cytoskeleton between cells was strongly expressed (**Fig. 2-17B**), in contrast to single cells that had markedly lost their F-actin staining around their nuclei (**Fig. 2-14A**). While initially it was extrapolated that cyto-D treated clusters released more LMPs per cluster, this difference was most likely attributed to the larger number of cells within clusters in the cyto-D treated cohort (**Fig. 2-17C-D**).

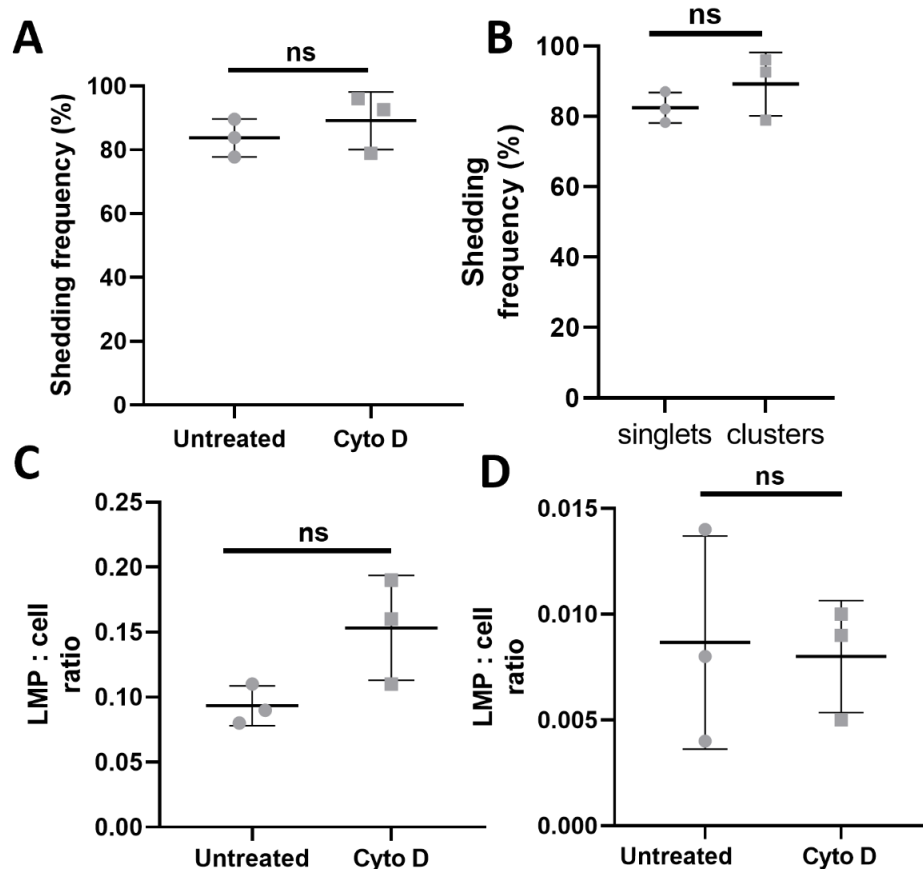


Figure 2-16: F-actin modulation does not affect cluster-dependent shedding.

(A) Percentage of untreated or Cytochalasin D treated MDA-MB 231 clusters that shed during transit in capillary bifurcations. (B) Percentage of MDA-MB 231 clusters or MDA-MB 231 singlets that were treated with Cytochalasin that shed during transit in capillary bifurcations. (C) Extrapolation of the

ratio of collected LMPs and MDA-MB 231 cells within clusters in 2 conditions post-transit from non-constricted geometries: untreated and Cytochalasin-D treated. (D) Extrapolation of the ratio of collected LMPs and MDA-MB 231 cells within clusters in 2 conditions in static conditions: untreated and Cytochalasin-D treated.

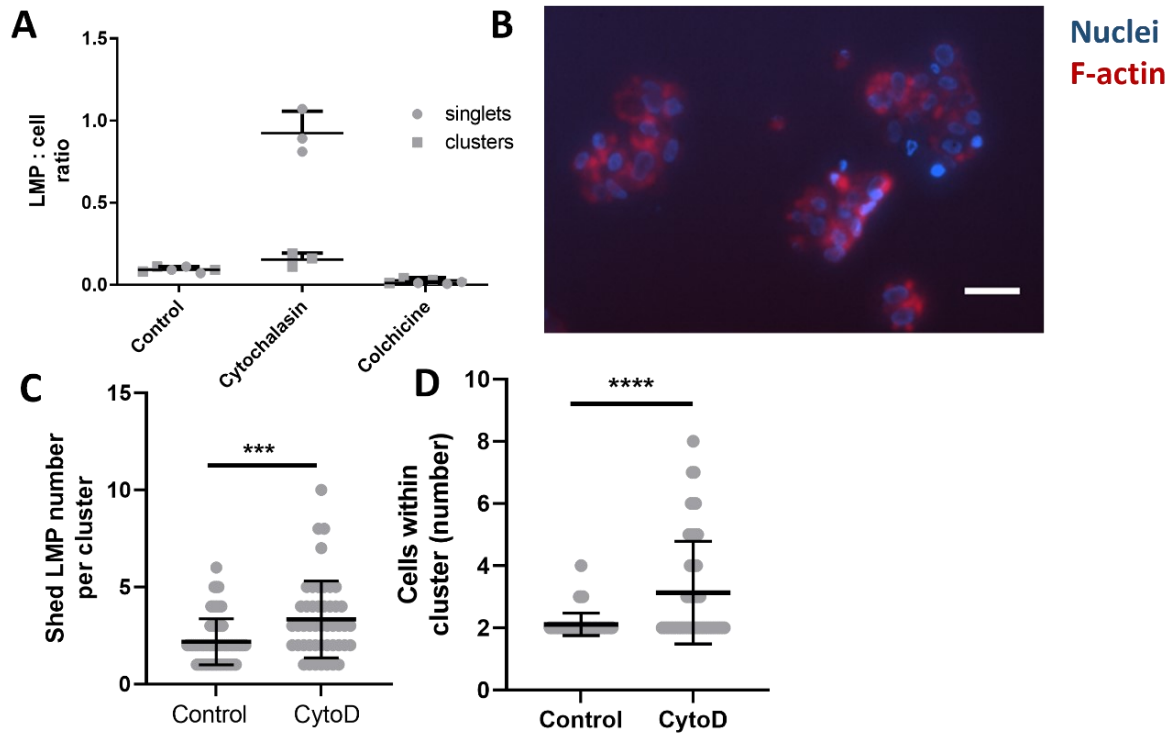


Figure 2-17: F-actin modulation does not affect cluster-dependent shedding.

(A) Compiled data between single and clustered MDA-MB 231 cells, that were enumerated along with collected LMPs post-transit in non-constricted geometries. (B) Multifluorescent image of MDA-MB 231 clusters that were treated with Cytochalasin and then stained for F-actin (red) and nuclei (blue). Scale bar: 50 μ m. (C) Number of shed LMPs per untreated (n=66) or Cytochalasin D treated (n=48) cluster of MDA-MB 231 cells. Each data point represents an individual cluster. (D) Number of cells per untreated or Cytochalasin D treated cluster of MDA-MB 231 cells. Each data point represents an individual cluster. (A-E) n=3 biological replicates for all.

During real time cell transit experiments in capillary bifurcations, I noticed an increased number of cell lysis events, especially when cells were treated with Cyto-D. Cell lysis during cell transit in the microfluidic devices was defined as complete loss of the cytoplasmic stain (**Fig. 2-18A**). By quantifying the cell lysis events in capillary bifurcations, there was a profound difference in Cyto-D treated cells compared to untreated ($41.5 \pm 7.9\%$ vs $3.5 \pm 1.4\%$, $P < 0.005$) (**Fig. 2-18B**). In the Cyto-D treated cohort, the majority of cell lysis events were observed for

shedding cells ($86.4 \pm 4\%$ vs $13.6 \pm 4\%$, $P < 0.0001$) (**Fig. 2-18C**). Given this observation, I replotted the lysis datasets only for cells post shedding, still identifying a large increase in Cyto D-treated cells, compared to untreated ($46.4 \pm 11.1\%$ vs $13.9 \pm 7.4\%$, $P < 0.05$) (**Fig. 2-18D**).

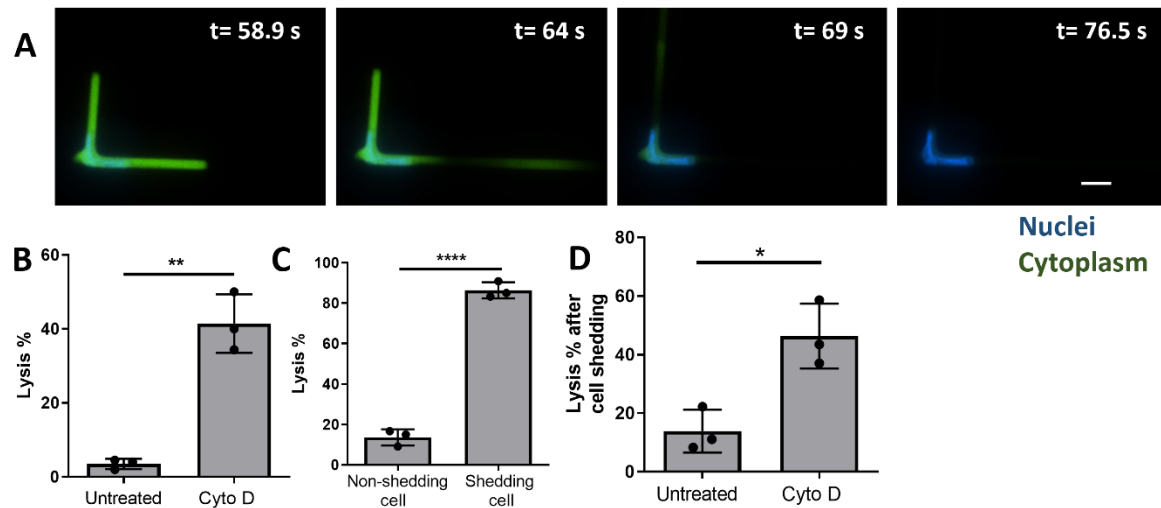


Figure 2-18: Inhibition of F-actin polymerisation promotes cell lysis in capillary bifurcations post cell shedding.

(A-D) Data are obtained by live imaging analysis. (A) Snapshots of a timelapse series of a fluorescently tagged MDA-MB 231 cell during transit in capillary bifurcations that gets lysed. Cytoplasm is depicted green and nuclei blue. Scale bar: 10 μm . (B) Percentage of untreated or Cytochalasin D treated MDA-MB 231 cells that were lysed during transit in capillary bifurcations. (C) Percentage of Cytochalasin D treated MDA-MB 231 cells that were lysed after shedding during transit in capillary bifurcations. (D) Percentage of untreated or Cytochalasin D treated MDA-MB 231 cells that were lysed post-shedding during transit in capillary bifurcations. (B-D) $n=3$ biological replicates for all.

By collecting cells post-transit from microfluidic devices and staining for live/dead cells (**Fig. 2-19A**), treatment with Cyto-D increased significantly the number of lysed cells that were collected post-transit in capillary bifurcations, compared to either untreated cells (Cyto-D: $32 \pm 7.1\%$ vs untreated: $15.1 \pm 2.2\%$, $P < 0.05$) or colchicine-treated cells (Cyto-D: $32 \pm 7.1\%$ vs Colch: $13.2 \pm 4.7\%$, $P < 0.005$) (**Fig. 2-19A-B**). On the other hand, there was no statistical significance between Colch-treated cells and untreated (Colch: $13.2 \pm 4.7\%$ vs $15.1 \pm 2.2\%$, ns) (**Fig. 2-19B**). Moreover, higher mean lysis frequencies were calculated upon Cyto-D treatment both for non-bifurcated capillaries (Cyto-D: $9.33 \pm 2.76\%$ vs untreated: $7.75 \pm$

1.51%, ns) and non-constricted geometries (Cyto-D: $18.69 \pm 1.17\%$ vs untreated: $13.65 \pm 2.55\%$, $P < 0.05$) (**Fig. 2-19C-D**), yet statistical significance was achieved only in the latter. As expected, there was no difference in lysis frequency under static conditions (**Fig. 2-19E**).

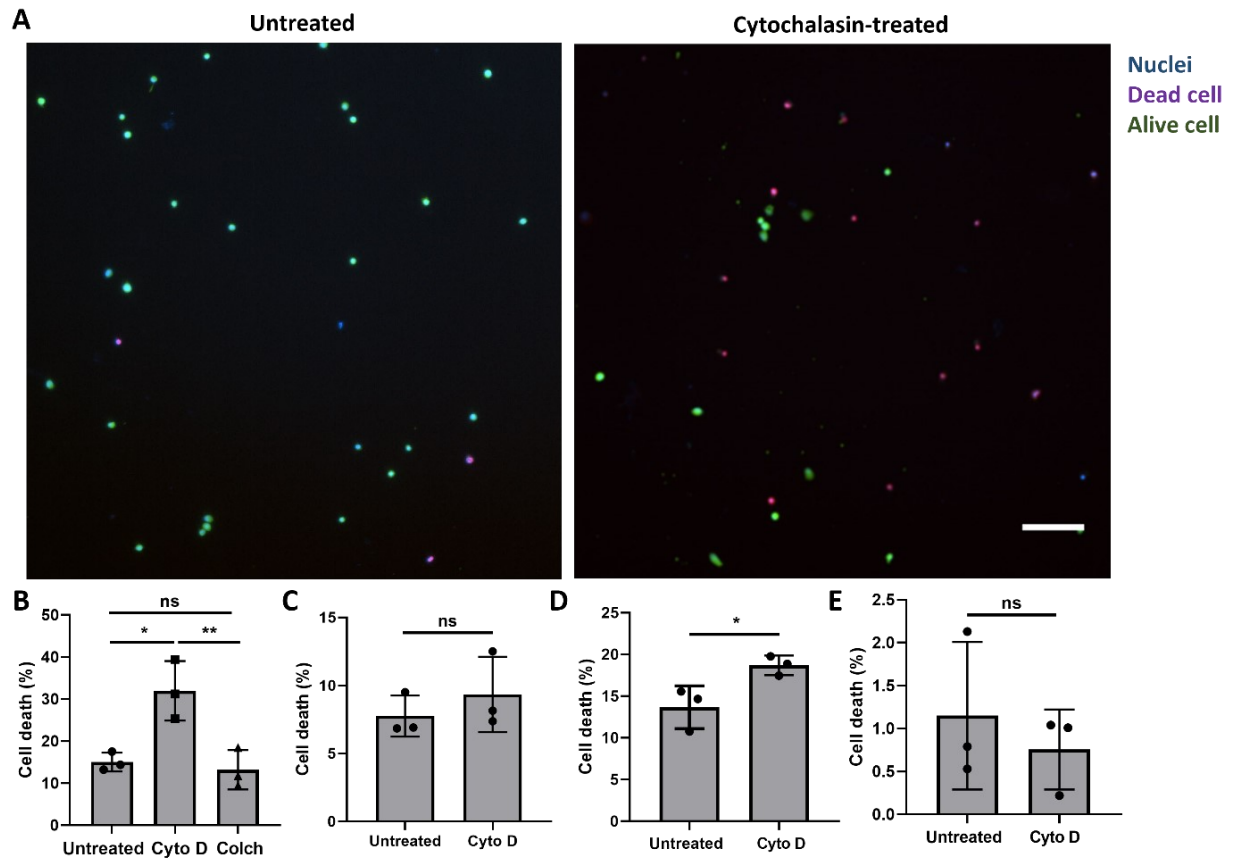


Figure 2-19: Inhibition of F-actin polymerisation promotes cell death post-transit in capillary bifurcations and non-constricted geometries.

(A) Characteristic multifuorescent images of collected MDA-MB 231 cells post transit in capillary bifurcations. Alive cells are depicted green, dead cells are depicted purple (stained as red) and nuclei is depicted blue. Scale bar: 100 μm . (B-E) MDA-MB 231 cells were collected and assessed for viability, based on images generated as in (A), for different conditions. (B) Percentage of untreated, Cytochalasin D treated or Colchicine treated cells that were dead post-transit in capillary bifurcations (C) Percentage of untreated or Cytochalasin D treated cells that were dead post-transit in non-bifurcated capillaries. (D) Percentage of untreated or Cytochalasin D treated cells that were dead post-transit in non-constricted geometries. (E) Percentage of untreated or Cytochalasin D treated cells that were dead in static conditions. (B-E) $n=3$ biological replicates for all.

2.4 DISCUSSION

Navigation of CTCs in the complex environment of the microcirculation is still not fully understood. Although numerous cell intrinsic and blood microenvironment extrinsic factors play a part in cancer metastasis, including arrest, extravasation, endothelium adhesion, shear stress and immune cell recruitment (31,33,34,41,60,62,269), the direct impact of capillary geometries i.e. capillary bifurcations on cancer cell fate remains largely uncharacterised. In this work, I engineered for the first time novel microfluidic models of capillary bifurcation geometries, emulating features such as narrow sizes and diversity of angles and symmetry, representative of *in vivo* capillaries. Furthermore, I report observations of cancer cells shedding Hoechst-negative (nuclei-free) LMPs from their cytoplasm. I have characterised biomechanical aspects of cell shedding during CTC transit in capillary-like geometries and demonstrated that this phenomenon is dependent both on mechanical features of capillaries (presence of bifurcations, presence of narrow angles within a bifurcation, small sizes and shear) and cell properties (cell size, F-actin), that often act in synergy, beyond what was previously established by Headley et al. (71). Moreover, I provide *in vitro* evidence for the first time that shedding of large particles leads to a significant reduction of cell volume, which can impact potentially their downstream journey in the first or distant organs in the course of cancer metastasis.

Nuclei-free LEVs (diameter range $>1\ \mu\text{m}$) have been isolated from the blood of cancer patients previously (75,76), yet they have not been fully verified to be EVs, according to the minimal information for studies of extracellular vesicles (MISEV2018 guidelines) (78). In their mass cytometry imaging approach, Gerdtsen et al. have identified the presence of cytokeratins, heat shock proteins and membrane proteins in cancer patient-isolated LEVs (77), which match the MISEV guidelines (78), suggesting that these are indeed LEVs. Another type of particle, derived from shear and capillary forces *in vitro*, was found to have a size range of $1\text{-}12\ \mu\text{m}$ (**Fig. 2-5D**) and *in vivo* $0.5\text{-}25\ \mu\text{m}$ (71), respectively, both with a mean value around $5\ \mu\text{m}$, presenting an overlapping range with previous estimations from Gerdtsen, who reported LEVs of size from $63\text{-}20.4\ \mu\text{m}$ (77). However, there is much research done on another type of large particle, termed large oncosomes, that share the size range with reported LEVs and LMPs (89,91). Two major differences between shear-derived LMPs and LOs are their biogenesis and the presence of complete or fragmented nuclei based on few reports (91). At

least 2 independent groups have reported large particles that stain positive for Hoechst or DAPI that are nuclear stains (91,121), while several other studies report that LMPs or tumour-derived EVs are negative for Hoechst or DAPI (76,77,90). Moreover, LOs biogenesis has been observed in primary cancer specimens, but LOS have also been identified in the blood of mice (87), yet it remains to be seen whether LOs can intravasate or not or whether they are generated directly in circulation. Current research on LOs in vitro is done by treatment of cultured cells chemically or by depletion of serum, which promotes LOs generation for downstream analyses (87,88). This biogenesis mechanism however is completely distinct from the shear-derived biogenesis of LMPs and considering the differences in nuclei content in some instances, these findings suggest that LMPs and LOs may also differ in functionalities and direct comparison is needed for their subclassification. What is interesting is that both Gerdtsson et al. and Nanou et al. identified a higher count of LEVs compared to CTC counts, suggesting that these LEVs may in fact originate from CTCs transiting the microcirculation of patients. Nonetheless, understanding the similarities and differences among reported LEVs in patients, shear-derived LMPs (in vivo and on vitro) and LOs is crucial, especially in their utilisation in liquid biopsy strategies, where a mix of distinct large particles may be collected. Understanding more holistically these subtypes will ultimately improve the outcome of liquid biopsy applications, which include diagnosis, patient monitoring, therapy progression and prognosis.

In respect to LMPs biogenesis, Headley et al. (71) identified shear as an important mediator of cellular shedding in the lung microvasculature of mice. However, the microvasculature comprises of small arterioles that are not constricted and various constricted geometries of both bifurcated and non-bifurcated narrow vessels. Initially, I showed that there was a greater number of LMPs compared to cells under shear only conditions compared to static, validating the dependency on flow and shear. Moreover, the number of LMPs compared to cells was much higher, when cancer cells transited capillary bifurcations, emphasising that both capillary bifurcations and shear collude to enhance cell shedding significantly. In addition to in vivo work, microfluidic models are good candidates to dissect individual parameters that may have affected cell shedding. Real-time transit of cancer cells in various distinct capillary geometries revealed that several mechanical features increase shedding, namely the presence of bifurcations, the lower values of constriction widths and the narrow angle

between daughter channels. Understanding these contributions is important, since the capillary beds of different organs in a human body are vastly different (227,270) and thus, given the blood route of a CTC, the capillary beds of certain organs may be more prone to promote shedding and trap locally LMPs, which is an interesting future direction. Nonetheless, while these microfluidic devices were a good starting point to mimic CTCs transit and study single parameters in driving cell shedding, they represent very simplistic models of the human microcirculation. These microfluidic devices represent a single channel, while the microcirculation is an intricate network of interconnected capillary vessels and CTCs would encounter potentially dozens of such bifurcations while transiting a single organ's microcirculation (33). In addition, in this series of experiments, cancer cells were added and monitored as a single cellular population, whereas in the human circulation, trillions of red blood cells, trillions of platelets and hundreds of billion immune cells transit at the same time (227), a huge fraction of which will be flowing together with CTCs, potentially applying mechanical pressure through collisions. Altogether, while shedding was greatly prevalent in capillary bifurcations, the datasets indicated a large variance, suggesting that other parameters may have affected cell shedding.

To add more to the complexity of the phenomenon of cellular shedding, cell size represents also a deciding factor in respect to LMPs biogenesis. Quantification of volumes of cells that either shed or did not shed, revealed a large difference, which highlighted that the larger a cell, it tended to shed. Indeed, increased shedding frequency percentages across each experiment were correlated well by an increase in the mean cell volumes across experiments. Comparison between the mean cell volumes of shedding cells between bifurcated and non-bifurcated capillaries, revealed that the cell volume had to be much higher in non-bifurcated geometries, emphasising that cell size is not enough by itself to drive cell shedding, but that capillary bifurcations are also required. Moreover, it is well established that CTCs size can differ between different cancer types or subtypes, between patients and even within the same patient (34,73,74). Therefore, the requirement of size-dependent shedding may have implications in different cancers or patients, where in other cases increased shedding will happen and in other reduced. Whether reduced shedding is beneficial or not, it remains to be seen, given that there is largely no understanding of the role and downstream functionalities of either LMPs or cells post-shedding.

Characterising the large and heterogeneous diameters of shed LMPs made me wonder how shedding would impact cells i.e. cell volume. As expected, the volume of cells post-shedding was significantly lower compared to the cell volume before shedding. These results are in agreement with previous observations of cell size reduction, whereby two independent studies highlighted that patient-isolated CTCs had smaller size when isolated from peripheral compartments i.e. arm veins compared to locations where tumour cells had not infiltrated yet in the bloodstream and thus had not encountered capillary beds (73,74). Based on calculations from the volume loss per cell, there was a large range of volume loss. By plotting cell volumes of cells that had eventually shed with their respective volume loss, it became clear that there was a very weak correlation between larger cell volumes and larger volume loss, whereas there was a strong correlation between cell volumes before and after shedding. These findings suggest that cells' volume is reduced at similar levels, as validated by a relevant histogram. By comparing volume losses across different microfluidic devices, there is compelling evidence that the volume loss is somewhat modulated by how small the width gap between two daughter channels is, which increased the volume loss. This likely occurred, because in equal bifurcations where cells face the same haemodynamic resistance in both daughter channels, the cell is positioned more symmetrically, leaving exposed a larger area of the cell to be shed, but that requires more experiments to be proven. Last but not least, while cell volumes post shedding were reduced, their mean volume was still larger than non-shedding cells. Nonetheless, this reduction was enough to cause rapider escape of cancer cells from capillary bifurcations, which is likely a reflection on the improved haemodynamics conferred by shedding, which reduces cells' resistance. However, this experiment should be taken with caution, given the largely simplistic nature of the microfluidic geometries that is used. For instance, the in vivo residence times of CTCs differ significantly, as well as the definitions of entrapment and arrest. Despite the proclivity of a more facilitated transit post-shedding, to understand better the implications of cell volume reduction on metastatic steps such as arrest and occlusion, more sophisticated models of the microcirculation are required. Nonetheless, these findings highlight that cell volume losses are a direct consequence of cell shedding and may in fact influence multiple downstream steps of the metastatic cascade.

The importance of cytoskeletal proteins in cancer cell movement and migration is well-established (271,272), yet few studies have examined their role during cell transit in capillary

geometries. For instance, Huang et al. showed that CTCs change their morphology and reorganise F-actin to shift from deformed to circular morphologies inside capillaries, which improved extravasation rates. This suggests that shear and narrow constrictions can affect the F-actin network inside the cell (62). In this Chapter, I examined thus how F-actin affects cell shedding. By inhibiting the polymerisation of F-actin in cells, which evidently disrupts its distribution inside the cell, shedding was greatly promoted both in non-constricted and constricted bifurcations. Interestingly, while non-constricted devices presented low shedding, inhibition of F-actin polymerisation led to enhanced shedding, suggesting the important role of F-actin in retaining cells intact against shear only or in combination with capillary bifurcations. These claims are further backed up by employing an F-actin polymerisation promoter, which reduced cell shedding, further establishing a correlation between the strength of a cell's F-actin polymerisation status and shedding. While disruption of the F-actin network affects shedding, these experiments were performed by chemical treatment. Physiologically though, there are various scenarios in the metastatic cascade that can disrupt F-actin. Firstly, during migration in the primary tumour, cancer cells navigate through a dense fibrotic stroma that applies constant mechanical pressure (273). Secondly, during intravasation, cancer cells need to invade and squeeze past through layers of ECM and endothelial cells that also pressure them mechanically (222,274). Last but not least, the mechanical pressure applied at the first capillary bed can trigger F-actin structural changes (62). Overall, these physiological changes of F-actin may affect the probabilities of cell shedding, during their transit in subsequent capillary beds and thus may enhance the availability of LMPs in distant organs rather than only in the primary ones.

Real-time transit of Cyto-D treated cells in capillary bifurcations revealed an increasing frequency of lysed cells, which when quantified was significantly lower in untreated cells. The lysis frequency of Cyto-D treated cells compared to untreated in non-bifurcated and static conditions was unaffected, whereas only slightly increased in shear-only conditions, but nowhere near the impact on cell lysis in capillary bifurcations. These findings suggest that disruption of cellular F-actin has a devastating impact on cancer cells' viability during their transit in capillary bifurcations, emphasising the cooperation of shear and bifurcations. What was further intriguing was the initial observation and subsequent extrapolation that the majority of cell lysis events upon cellular treatment with Cyto-D emerged post cell shedding.

Pretreatment with Cytochalasin led half of shedding cells in capillary bifurcations to be lysed, while the percentage of untreated cells that shed and then lysed was about 4-fold lower, suggesting that weakening of F-actin network may impact the ability of cells to survive the consequences of shedding, though a specific mechanism remains undiscovered. It is possible that shedding These preliminary observations seem to suggest that the combination of a disrupted F-actin network and shedding in capillary bifurcations, both of which can occur physiologically, drive lysis. On one hand, this paves the way for cancer cell F-actin's targeting as a means to induce greater indirect lysis via transit in capillary beds. On the other hand, cell shedding increases under these circumstances and currently the role of LMPs is unexplored, yet based on Headley et al., shed LMPs can indirectly drive metastasis by indoctrinating macrophage populations to assist in metastasis (71). Therefore, the balance between cancer cell death due to triggered shedding and the impact of shed LMPs in metastasis needs to be addressed in the future, before the integration of a potential Cyto-D dependent therapeutic strategy.

The past decade, the emerging isolation technologies have enabled the identification of clusters of cancer cells, both homotypic and heterotypic (110,242,275). It is generally accepted in the community that clusters are much more aggressive clones compared to single cells (27,110), yet their transit in capillary beds is little understood. As expected, clusters shed at higher frequencies compared to single cells, given the larger number of cells within a cluster and released more LMPs compared to singlets. Treatment with Cytochalasin also had no additional significant effect on clusters shedding in capillary bifurcations, highlighting the dominance of capillary bifurcations. Interestingly, when clusters transited non-constricted geometries (shear only), their shedding was similar to single cells, however upon Cyto-D treatment, clusters resisted the large increase of shedding that single cells underwent. These findings highlight the existence of a potential protective mechanism of clusters against shedding in shear only conditions. Given previous findings, whereby promoting F-actin polymerisation reduced cell shedding (observed by Colchicine treatment), F-actin staining proved that Cyto-D clusters retained high staining intensity, whereas F-actin on single cells had been compromised upon exposure to Cyto-D. The maintenance of an uncompromised F-actin network by clusters is likely due to the presence of intercellular junctions such as E-cadherin among others, which have been associated connecting to F-actin (276) and possibly

strengthening the cytoskeleton network. This protective mechanism could not be explored in capillary bifurcations fully, because the shedding frequency of untreated clusters was already too high, suggesting that in capillary bifurcations the overall larger size of clusters and narrow constricted bifurcations are more dominant than F-actin. Altogether, while clusters have been reported to occlude capillaries faster in vivo (110), shedding of multiple LMPs from their surface may in fact reduce significantly their overall volume, as observed for single cells and thus enable their transit in distant organs rather than merely their entrapment in the first capillary bed they encounter.

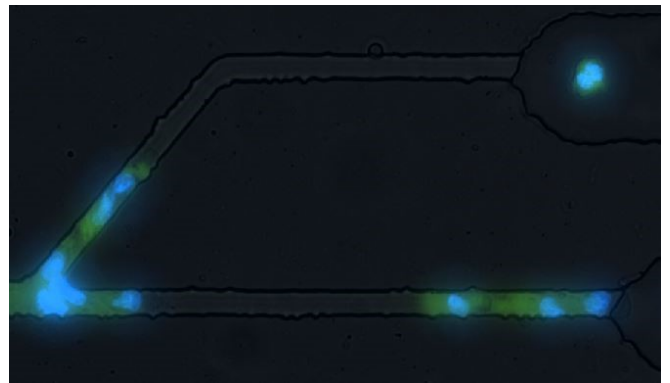
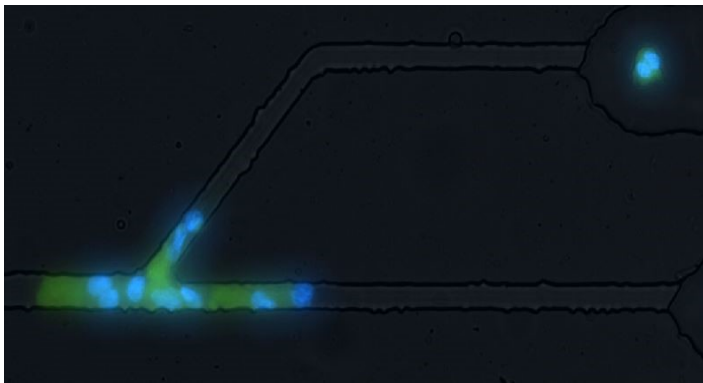
To conclude, despite unveiling multiple factors that promote LMPs biogenesis, the nature and downstream functionalities of shed LMPs remain unexplored. LMPs resemble in nature EVs, yet it is unclear whether they are in fact EVs. Smaller EVs such as exosomes are known to contain biomolecules (277), therefore it is sound to assume that LMPs that originate from the membrane of cells (reducing their volume) will likely contain biomolecules such as proteins, nucleic acids and lipids. Furthermore, the presence of similar large EVs isolated from the blood of patients from distinct cancer types (75,77), suggests that they may interact with various cell types found in the bloodstream, such as endothelial cells that line the blood vessels and immune cells. Headley et al. indeed demonstrated in vivo the uptake of shear derived LMPs by various immune cells (71). Therefore, it is crucial to investigate the interactions of LMPs with other cell types and how this interaction may lead to phenotypic changes. For instance, exosomes uptake by macrophages promotes an immunosuppressive phenotype (214), suggesting that LMPs, potentially carrying biomolecules, will possess capabilities to induce activation of endothelial cells and immune cells, which may influence cancer metastasis.

2.5 CONTRIBUTIONS & ACKNOWLEDGEMENTS

I generated the hypothesis, designed experiments, conducted experiments, collected and analysed all data. Aisher Chen helped with some experiments collecting part of data of cluster transit through some microfluidic devices.

CHAPTER 3

Clusters of cancer cells dissociate during transit in bifurcated capillary geometries



3.1 INTRODUCTION

Circulating tumour cell (CTC) clusters are potent inducers of metastasis (110). Among the different types of clusters, homotypic clusters can be formed by aggregates of tumour cells only or heterotypic clusters can be formed by aggregates between cancer cells and cancer-associated fibroblasts, neutrophils, macrophages or platelets among others (27,208). Both homotypic and heterotypic clusters have been isolated from different cancer types, including breast, prostate and pancreatic cancer types among others (49,110,208,242,278–280). Compared to their singlet counterparts, clusters readily present faster metastasis formation, greater proliferation rates, reduced apoptosis and ROS-dependent death, anti-metastatic immune evasion, gain of stemness and acquisition of hybrid phenotypes (49,67,110,223,281–283). Differences between homotypic and heterotypic clusters have also been identified, with heterotypic clusters presenting higher survival or faster proliferation rates (49,284). Furthermore, the larger size of clusters in tandem with the favourable conditions of the blood microenvironment i.e. capillary beds or microcirculation (34) favours their physical occlusion and eventual metastases, as evidenced by Aceto et al. (110). Coupled together, these advantages highlight that clusters may represent a durable population that needs to be targeted to improve therapeutic options. Indeed, in numerous studies, it was shown that disruption of cluster formation has led to major reduction in metastases formation and increase of overall survival (49,67,110,281,284). On the one hand, increasing knowledge on clusters highlights their importance as a therapeutic target, including the study of a. factors that lead to cluster formation i.e. hypoxia (285), b. the molecular and phenotypic changes that cells within clusters undergo (281), c. extravasation patterns (225) and d. post-extravasation survival and proliferation (49,110). On the other hand, it remains largely unexplored how clusters behave in the numerous and diverse microvessel geometries of the microcirculation.

Previously, Au et al. have demonstrated that the majority of cancer cell clusters could transit undisturbed through non-bifurcated (linear) capillary geometries, as small as 5 μm (45). However, the microcirculation or capillary beds found in the human circulation and in vivo display extensive branching, diverse sizes and numerous distinct geometric configurations (33). For example, real time imaging of injected cells in vivo allowed Kienast et al. to record that capillary bifurcations were capable of slowing down CTCs compared to linear capillaries

(33), suggesting that bifurcated capillaries may influence differently the ability of clusters to navigate through. Moreover, several studies report a consistent pattern of clusters isolation from cancer patient's arm veins, after having passed finger's capillaries, with predominantly low cell counts per cluster (49,242,286). Szczerba et al. have investigated this more in depth, by comparing directly cell counts within clusters from different vascular locations. By collecting samples from arm veins and from tumour-draining vessels (before release in circulation), the authors identified significantly larger cell counts in clusters purified from tumour-draining vessels (49). These studies, coupled together, support the idea that clusters, albeit larger ones (higher counts of cells within a cluster), are less capable of passing through and occlude at capillary beds, partially explaining the predominant isolation of small clusters or singles from arm veins, a location that clusters would encounter capillary beds at least once before being drawn. Interestingly, cell cluster transit in non-bifurcated capillary geometries monitored by Au et al. revealed dissociation of clusters to smaller clusters or to single cells, albeit at low levels of dissociation frequency (45). Given the differences between non-bifurcated and bifurcated capillary geometries, it is sound to assume that the extensively bifurcated network of the microcirculation in humans leads to cluster dissociation and thus explaining partially the collection of small clusters and singles from the blood of cancer patients. Therefore, I sought out to explore the relationship between cluster dissociation and the numerous geometries of the microcirculation.

In this Chapter, following up on the results of cluster shedding, a live imaging approach of clusters transit in microfluidic devices revealed the phenomenon of cluster dissociation to smaller clusters or entirely single cells. Different types of vessels i.e. bifurcated capillaries evoked different responses in respect to dissociation, with bifurcation geometries favouring dissociation, compared to non-bifurcated geometries. Then, I demonstrated that various biomechanical features of capillary bifurcations i.e. constriction width further govern dissociation patterns. Finally, Cytochalasin D, a F-actin inhibitor (262) was found to enhance significantly dissociation frequency of clusters during transit in bifurcated capillary geometries, but also induced cell lysis. Altogether, I hypothesise that while cluster dissociation is largely governed by features of capillary beds, treatment with Cytochalasin D can affect their fate in the microcirculation and be utilised as a potential therapeutic strategy to reduce metastatic burden in patients.

3.2 MATERIALS & METHODS

3.2.1 Cell culture & reagents

MDA-MB 231 breast cancer cells were used throughout this work and were maintained, cultured, prepared and stained as detailed in Chapter 2. Briefly, cells were harvested using 0.25% (v/v) Trypsin and were resuspended in fresh media of DMEM, supplemented with FBS and antibiotics. All reagents were purchased from Sigma Aldrich (UK), unless noted otherwise.

3.2.2 Cell cluster preparation

Harvested single breast cancer cells were incubated either with red or green Calcein-AM (ThermoFischer Scientific, UK) at a final working concentration of 5 μ M for 1 hr at 37°C/5% CO₂. Cells were centrifuged to remove the dye and then 10000 cells/100 μ l were enumerated via a haemocytometer and added in each well of a 96-well round plate. To prevent cell attachment, the well plate was pre-treated with 1% Pluronic F-127 (ThermoFischer Scientific, UK) for 1hr at room temperature. Once coating was finalised, cells were added and left to form cell aggregates for 24 hr. Then, Hoechst 33342 was added directly in the wells, diluted 1000 times at a final working concentration of 16.2 μ M for 5 min at room temperature, directly inside the well with the formed aggregates. Aggregates were then gently resuspended several times to break down to small aggregates of cells, termed as clusters. Multifluorescent images of the spheroids before and after their disruption were obtained using the Nikon microscope, whose use was detailed in Chapter 2.

3.2.3 Cluster transit & processing

The outlets of microfluidic devices (designed and elaborated in Chapter 2-**Table 2-1**) were connected to a 1 m long 2 mm diameter tubing (Qosina, US) that was attached in a needle (Needlez, UK) inserted in 10 ml syringes (henceforth termed as syringe/tubing set-up). 20 μ l of either stained or unstained (dependent on downstream experiment) cluster suspensions were added in the inlet of microfluidic devices and pressure was applied by lowering the syringe/tubing set-up to create a height difference of 30 cm, which represents physiological values found in capillaries (45,266). Cluster transit in microfluidic devices (only for bifurcated and non-bifurcated devices) was imaged directly and multifluorescent images were obtained at high speed (3 frames/sec) and compiled together to form video series, using the Nikon

microscope. Alternatively, stained clusters that transited microfluidic devices were collected either from the outlets of the microfluidic devices (bifurcated, non-bifurcated and non-constricted) or by emptying the liquid in the syringe/tubing directly in wells of a 96-well plate and metrics (expanded later) were analysed. Additionally, unstained clusters were transferred in microfluidic devices and collected as described and were succumbed to a viability assay (elaborated below). Last but not least, clusters were transferred in the inlets of microfluidic devices but no pressure was applied via the syringe/tubing set-up to mimic static (no shear or flow) conditions.

3.2.4 Cell treatment

Single or clustered suspensions were cultured as described earlier, for subsequent treatment with Cytochalasin D (Cyto D), a F-actin polymerisation inhibitor. Cells were treated at a final working concentration of 10 μ M for 3hr at 37°C/5% CO₂. Cyto D was added in adherent MDA-MB 231 cell monolayer that were then harvested to single cells, whereas in respect to clusters, Cyto-D was added directly in the wells after 1 day of aggregate formation.

3.2.5 Viability assay

Unstained clusters or single cells were collected post-transit from microfluidic devices and were transferred in wells of a 96-well plate. Live/dead viability assay was performed by preparing a staining solution, including Calcein-AM (live cell marker), Hoechst 33342 (nuclei) and Propidium Iodide (PI) (dead cell marker), at final working concentrations of 5 μ M, 16.2 μ M and 1 μ g/ml, respectively, diluted in serum-free media. The staining solution was initially prepared at 2-fold higher concentration for each reagent and 100 μ l of staining solution was added in wells with cells filled with 100 μ l already. Several multifuorescent images were obtained at 20x magnification per sample and condition using Nikon microscope. Calcein-positive, Hoechst-positive but PI-negative cells were considered alive cells, whereas Calcein-negative, Hoechst-positive and PI-positive cells were considered as dead cells. Lysis frequency for single cells was extrapolated by dividing the number of dead cells to the overall number of Hoechst-positive single cells. In respect to clusters, enumeration of the total number of dead cells in clusters was divided by the overall number of Hoechst-positive cells within all imaged clusters.

3.2.6 Analysis of cluster transit

Video series were analysed manually by monitoring the transit of clusters in the microfluidic devices. Primarily, clusters were observed to dissociate in smaller clusters and/or single cells. Additionally, clusters also remained intact while transiting through the devices, whereby all cells in a single cluster remained attached to each other. Dissociation was verified by detachment of cells from the cluster and the presence of a gap or a distance between detached Hoechst- and cytoplasm-positive cells and the initial cluster. Dissociation frequency was extrapolated by dividing the number of clusters that presented at least one dissociation event to the overall number of clusters that transited through the microfluidic devices. To exclude the possibility that single cells appeared as clusters by simply being next to each other, clusters were monitored from before their entry to capillary bifurcations. Dissociation events and their frequency were also quantified based on the number of cells within a cluster e.g., 2-cell clusters, 3-cell clusters or clusters comprised of at least 4 cells. Finally, the percentage of cluster transit in a particular direction was evaluated. Since all microfluidic devices tested were bifurcated capillary devices, the quantification of transit direction frequency was calculated in respect to transit in the d_2 daughter channel. Dissociated clusters were also evaluated in regard to the transit of dissociated cells in the same or different daughter channel.

3.2.7 Analysis of collected cells

Suspensions of clusters were prepared and stained as described elsewhere. Then, they were transferred to the inlets of microfluidic devices (bifurcated, non-bifurcated and non-constricted) but no pressure was applied initially. Multifluorescent images at 20x magnification were obtained using Nikon microscope. Then, pressure was applied, as described elsewhere and clusters transited the microfluidic devices. Upon collection in wells of a 96-well plate, the wells were imaged directly and multifluorescent images at 20x magnification were observed. From these images, the number of clusters, the number of single cells and the cell count within a cluster were quantified manually, before or after transit through the devices. As metrics, the ratio of cluster and single cell numbers was calculated before and after collection from microfluidic devices, by dividing the number of clusters to the number of single cells. Moreover, cell counts within clusters were enumerated before and

after transit in microfluidic devices. Data obtained from images from the same well were added together.

3.2.8 Immunofluorescence

100µl of cluster suspensions (100000 cells/1ml) were prepared as previously and were either treated with Cyto-D or remained untreated for 3 hr. Then, both untreated and Cyto-D treated clusters were transferred to a flat bottom 96-well plate and were left for 3 hr. This timepoint was chosen for two reasons. First, it represents a sufficient time period for initiation of cell attachment to allow the performance of immunofluorescence without washing clusters off the bottom of the well plate. Moreover, in three hours of culture, the cells within clusters have not stretched fully in the surface of the well, allowing cells within clusters to retain their more spherical morphology, as the ones in suspension. Then, immunofluorescence staining was performed. Initially, the media was removed and cells were fixed with 4% (v/v) formaldehyde (diluted in PBS) for 12 min at room temperature. After 1x wash with PBS for 3 min, cells were further treated with 0.1% (v/v) Triton X (diluted in PBS) for 5 min at room temperature. After 1x wash with PBS for 3 min, cells were incubated at room temperature for 1 hr with Phalloidin iFluor 647 (Abcam, UK/ab176759), diluted 1000 times in PBS directly from the vial. Finally, cells were further incubated with Hoechst for 5 min at a final concentration of 16.2 µM. Multifluorescent images were obtained using Nikon microscope at 20x magnification.

3.2.9 Graphs & Statistical Analyses

All data were plotted using GraphPad version 9. Comparisons between experimental groups and statistical significance was analysed, as elaborately described in Chapter 2. Illustrations were designed using BioRender. All experiments were run at least three times.

3.3 RESULTS

3.3.1 Clusters of cancer cells dissociate in capillary bifurcations

To mimic the transit of clusters in microvessels in the human microcirculation (**Fig. 3-1A**), bifurcated capillaries (or capillary bifurcations) (**Fig. 3-1C**), non-bifurcated capillaries and non-constricted geometries were fabricated and prepared (**Table 2-1**) and were used to mimic CTCs cluster transit. The formation of large cancer cell aggregates was verified via fluorescent

microscopy (**Fig. 3-1B**). Disruption of these led to the formation of clusters, including small clusters i.e. doublets or triplets to much larger clusters i.e. up to 30-cell clusters, verified positively for both cytoplasm and nuclei staining that are attached to each other (**Fig. 3-1B**). Formed clusters were imaged on real-time during their transit in capillary bifurcations (**Fig. 3-1B-D**). Initially, clusters quickly deformed and reorganised from spherical to more rectangular-like flattened cell morphologies (**Fig. 3-1D**), as described previously by Au et al. (45), enabling transit through the initial non-bifurcated constricted part of the microfluidic device. Then, upon encountering the bifurcation, within seconds, the individual cells within a cluster deformed and squeezed further either in the same or in different daughter channels (more details in upcoming sections) (**Fig. 3-1D**). The detachment of a 3-cell cluster (in the lower daughter channel) from the initial cluster that is trapped in the capillary bifurcation was visualised (**Fig. 3-1D**). In **Video 2 (Appendix 11)**, while a 2-cell trapped cluster is trapped in a capillary bifurcation, slowly cells dissociate and transit in opposite daughter channels. In contrast to Au et al. findings, where they recorded and quantified small dissociation frequencies during clusters transit in non-bifurcated geometries, there was a large tendency of clusters to dissociate in preliminary experiments in capillary bifurcations.

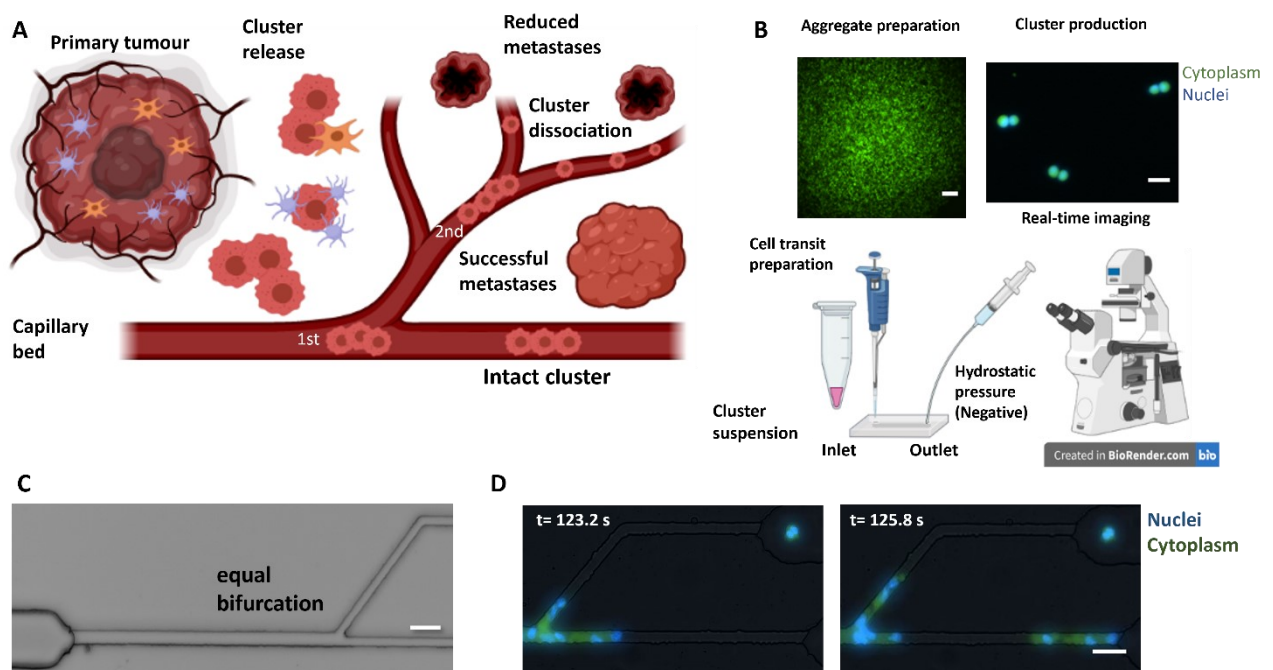


Figure 3-1: Clusters of MDA-MB 231 cells dissociate in capillary bifurcations.

(A) Illustration of clusters navigation in capillary beds. (B) Methodology approach. Large aggregate formation (Scale bar: 100 μm) that were later disrupted to form small clusters (Scale bar: 20 μm) and

then were added in microfluidic devices and live imaged during their transit. (C) Brightfield image of an equal microfluidic device (ENA_7/7) at 20x. Scale bar: 20 μm . (D) Snapshots of a timelapse series of a cluster encountering capillary bifurcations (ENA_7/7) and dissociating to smaller clusters. Cytoplasm is stained green and nuclei blue. Scale bar: 20 μm .

3.3.2 Clusters resist cell lysis in distinct microvessel geometries

Previous observations of single cell transit in capillary bifurcations revealed patterns of cell lysis. This prompted me to investigate whether clustering conferred any protective effects during their transit in microfluidic devices. To investigate this, initially, I loaded independently either single cells or clustered cells through non-constricted geometries to characterise their response to conditions of shear stress only (**Fig. 3-2A**). Upon collection, live/dead staining was performed. Enumeration of PI-positive cells (dead cells) revealed a significant decrease of cell death in clusters (singlets: $13.6 \pm 2.6\%$ vs clusters: $1.7 \pm 1\%$, $P < 0.005$), suggesting that clustering protects against shear-induced cell death (**Fig. 3-2B-C**). Then, I repeated the experiment under the same conditions using capillary bifurcations to explore whether there was a similar response (**Fig. 3-3A**). Indeed, enumeration of PI-positive cells (dead cells) revealed again a significant decrease of cell death in clusters ($15.1 \pm 2.2\%$ vs $4.7 \pm 0.3\%$, $P < 0.005$) (**Fig. 3-3B-C**), suggesting that altogether clustering protects consistently against cell death during transit both under the impact of shear only or the cooperation of shear and capillary forces.

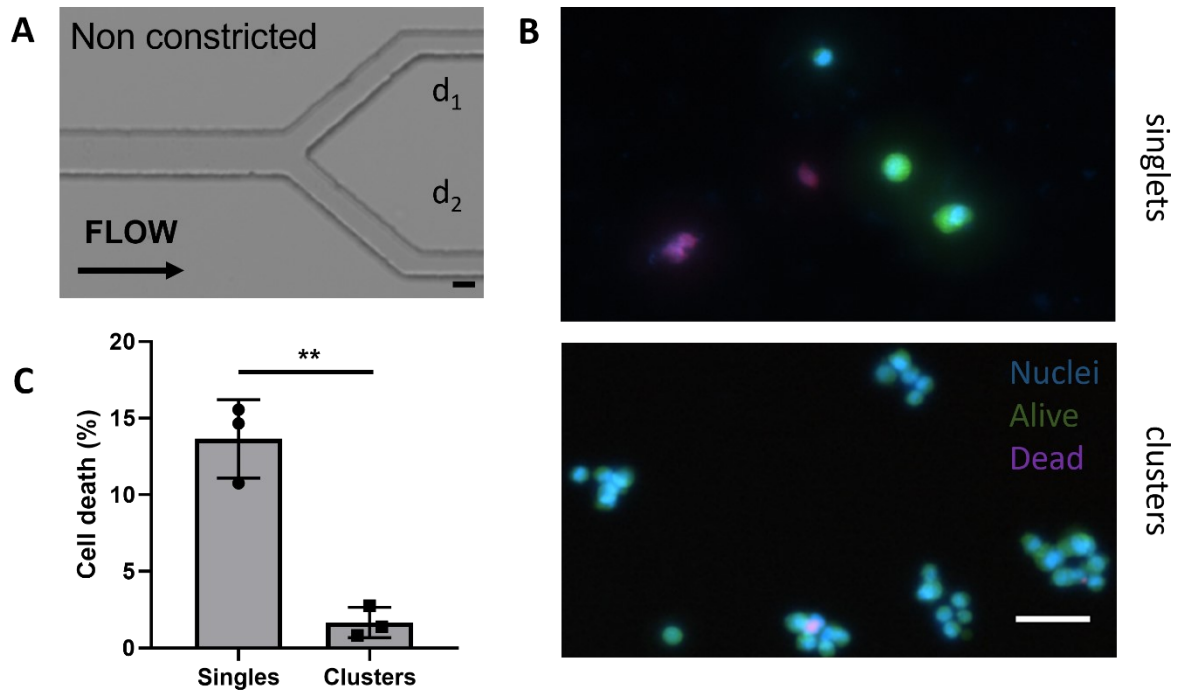


Figure 3-2: Clusters of MDA-MB 231 cells resist cell death post transit in non-constricted geometries under shear conditions only.

(A) Brightfield image of non-constricted geometry at 20x. Scale bar: 20 μm . (B) Multifluorescent images of single or clustered cells, collected post transit from non-constricted geometries. Nuclei was stained blue, alive cells as green and dead cells as red (depicted as purple). Scale bar: 50 μm . (C) Quantification of percentage of dead cells (n=3 biological replicates).

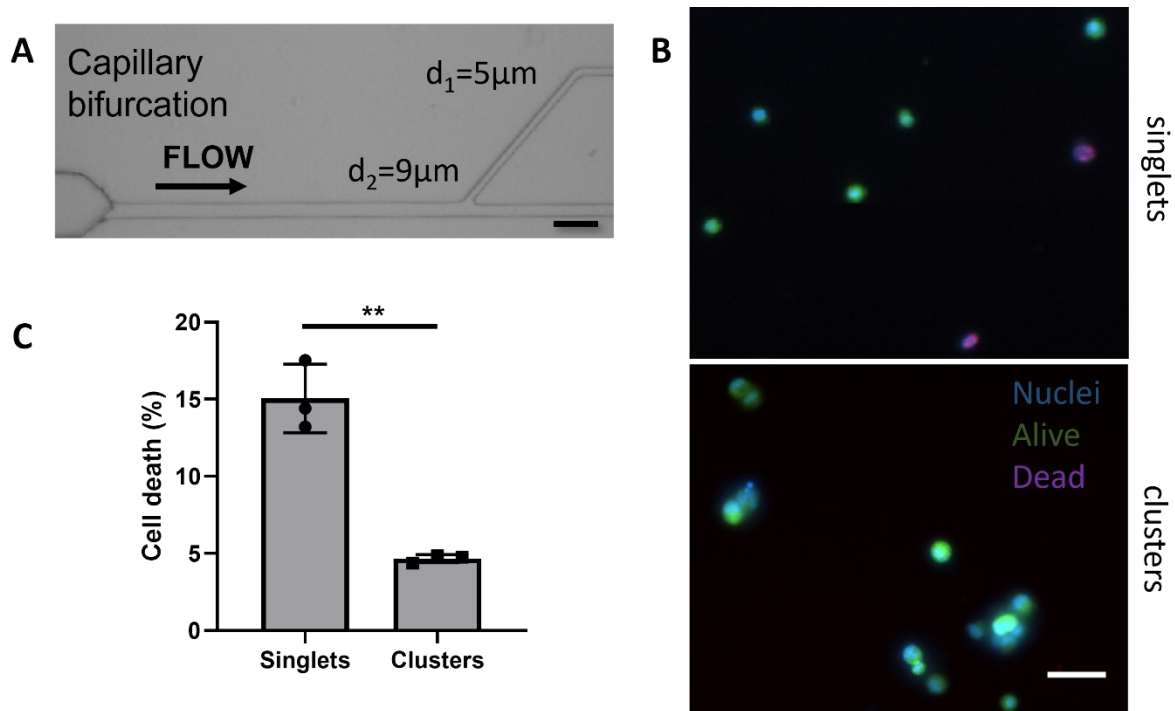


Figure 3-3: Clusters of MDA-MB 231 cells resist cell death post transit in capillary bifurcations.

(A) Brightfield image of a capillary bifurcation geometry at 20x. Scale bar: 20 μm . (B) Multifluorescent images of single or clustered cells, collected post transit from bifurcated capillary geometries. Nuclei was stained blue, alive cells as green and dead cells as red (depicted as purple). Scale bar: 50 μm . (C) Quantification of percentage of dead cells ($n=3$ biological replicates).

3.3.3 Clusters dissociate to smaller clusters or singlets predominately in capillary bifurcations

As shown in **Fig. 3-1D**, clusters tended to dissociate in capillary bifurcations. Thus, this prompted me to investigate the dissociation in various geometries, including bifurcated capillaries, non-bifurcated capillaries and non-constricted geometries. By visualising and recording images of clusters before and after transit (not during transit in this experiment), I used two metrics to characterise efficiency of cluster dissociation; namely i. cell counts within clusters and ii. cluster : singlet ratio. Firstly, in non-constricted geometries, there was a significant decrease in the means of cell counts within clusters (6.4 ± 5.2 vs 3.5 ± 1.9 , $P<0.0001$) and the cluster : singlet ratio (1.3 ± 0.2 vs 0.5 ± 0.2 , $P<0.0005$), before and after (Fig. 3-4A-C).

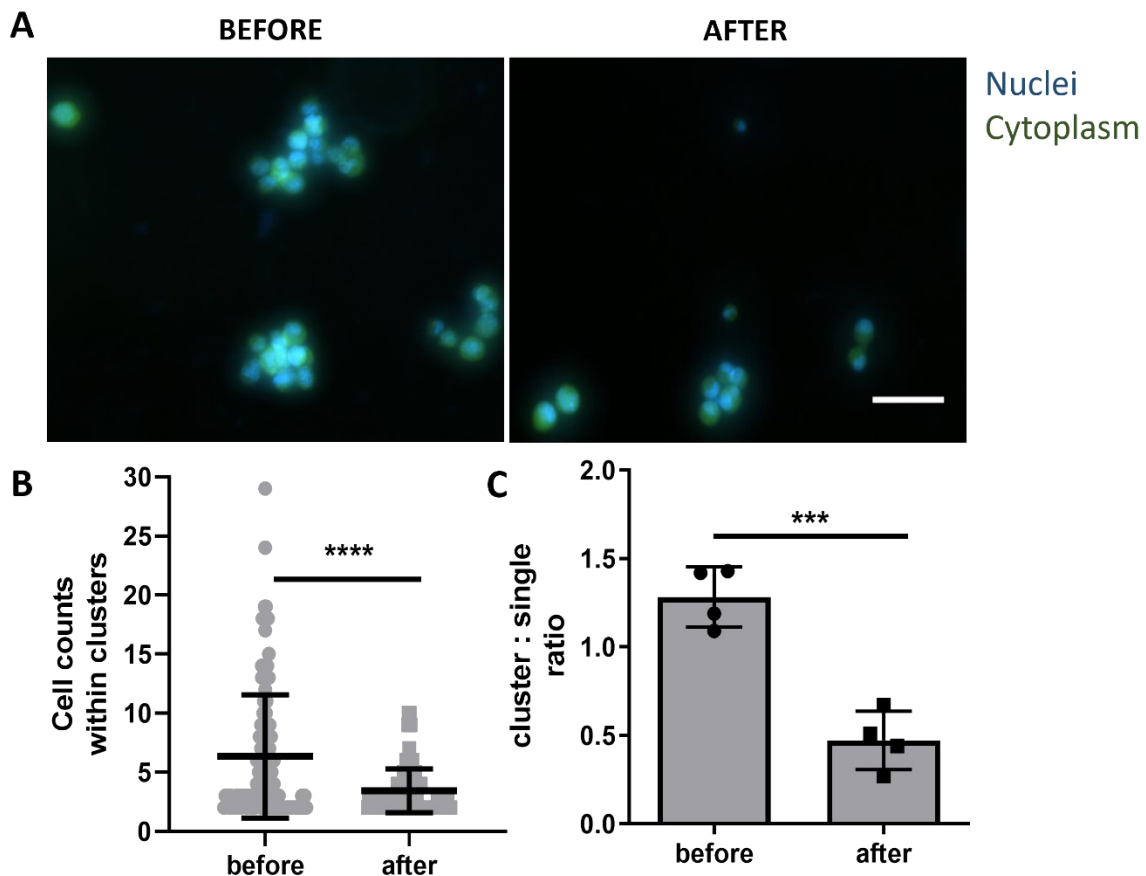


Figure 3-4: Clusters of MDA-MB 231 cells dissociate in non-constricted geometries in shear only conditions.

(A) Multifluorescent images of clusters before and after transit (collected) in non-constricted geometries. Cytoplasm is stained green and nuclei blue. Scale bar: 50 μ m. (B) Cell number within clusters, before (n=140) and after (n=106). Each data point represents the number of cells per individual cluster. (C) Ratio of number of clusters and singles, before and after (n=4 biological replicates).

Secondly, in bifurcated capillary geometries, a similar pattern was observed, accompanied by a significant 2-fold decrease in the means of cell counts, compared to before and after (5 ± 3.9 vs 2.4 ± 0.8 , $P < 0.0005$) and the cluster : singlet ratio (1.1 ± 0.1 vs 0.5 ± 0.1 , $P < 0.005$) (Fig. 3-5A-C).

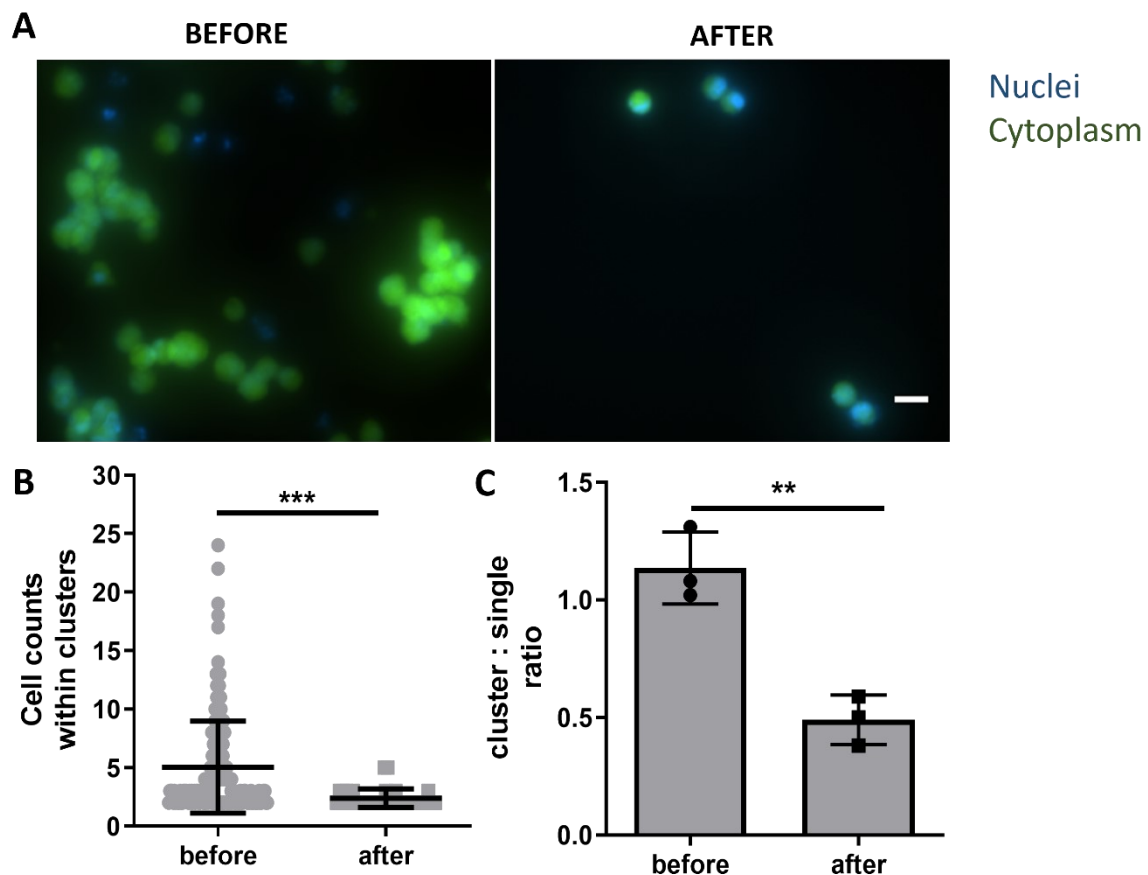


Figure 3-5: Clusters of MDA-MB 231 cells dissociate in bifurcated capillary geometries.

(A) Multifluorescent images of clusters before and after transit in bifurcated capillary geometries. Cytoplasm is stained green and nuclei blue. Scale bar: 20 μ m. (B) Cell number within clusters, before

(n=182) and after (n=33). Each data point represents the number of cells per individual cluster. (C) Ratio of number of clusters and singles, before and after (n=4 biological replicates).

Thirdly, in non-bifurcated capillary geometries, there was again a significant reduction in the means of cell counts within clusters, compared to before and after (5.2 ± 4.8 vs 3.1 ± 1.6 , $P < 0.005$) and the cluster : singlet ratio (0.89 ± 0.1 vs 0.3 ± 0.1 , $P < 0.005$) (**Fig. 3-6A-C**). Finally, comparison of the total cell counts within clusters, exhibited a noticeable difference on the number of cell counts in clusters collected, after transit of each of the three microfluidic devices, with bifurcated capillary geometries imposing the smallest mean of cell counts. Statistical significance was achieved by comparing bifurcated capillary to non-constricted (2.3 ± 0.8 vs 3.5 ± 1.9 , $P < 0.005$) and bifurcated capillary to non-bifurcated capillary geometries (2.3 ± 0.8 vs 3.1 ± 1.6 , $P < 0.05$), but there was no such significance between non-constricted and non-bifurcated capillary geometries (3.5 ± 1.9 vs 3.1 ± 1.6 , ns) (**Fig. 3-6D**). However, it remains unclear whether the small cell count of clusters collected post-transit from capillary bifurcations is due to dissociation or simply a reflection of smaller clusters (in cell number per cluster) passing through. To address these questions raised, a live imaging approach was required.

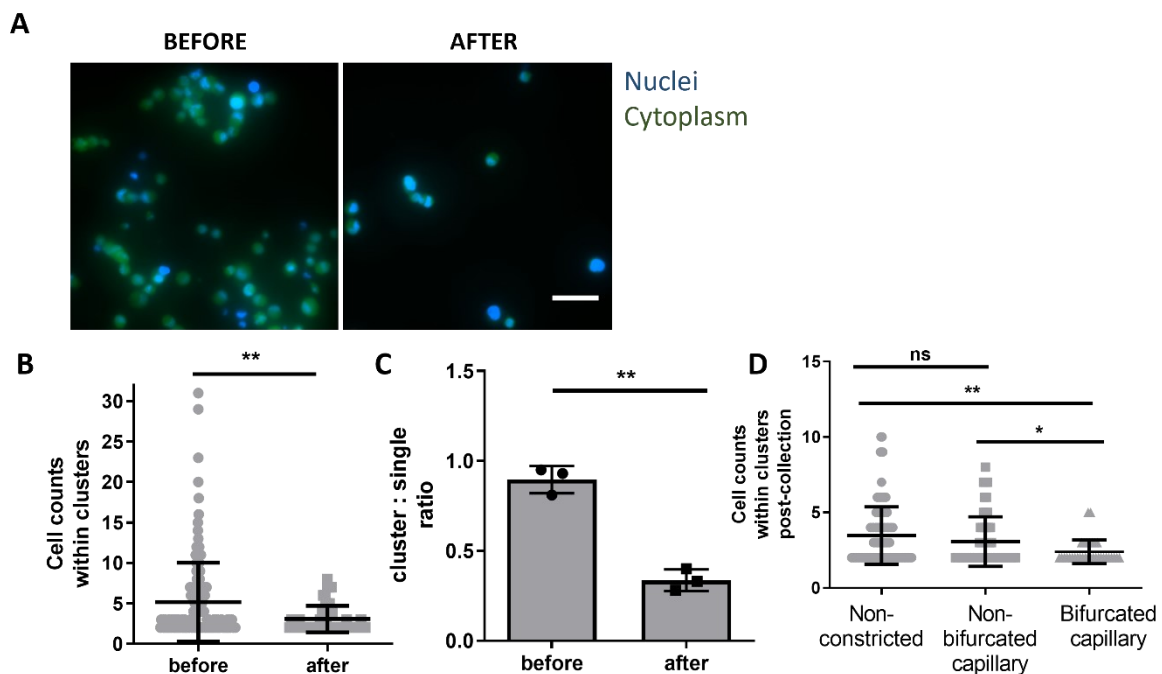


Figure 3-6: Clusters of MDA-MB 231 cells dissociate in non-bifurcated capillary geometries.

(A) Multifluorescent images of clusters before and after transit in non-bifurcated capillary geometries. Cytoplasm is stained green and nuclei blue. Scale bar: 50 μm . (B) Cell number within clusters, before (n=164) and after (n=53). Each data point represents the number of cells per individual cluster. (C) Ratio of number of clusters and singles, before and after (n=4 biological replicates). (D) Compiled data of cell counts within clusters collected post-transit in non-constricted geometries (n=106), non-bifurcated capillary geometries (n=53) or bifurcated capillary geometries (n=33). Each data point represents an individual cluster.

3.3.4 Larger cell counts in clusters drive dissociation

Cluster dissociation events were visualised and verified by detachment of cells, positive for both cytoplasm and nuclei (distinct from cell shedding, as described in Chapter 2) in **Fig. 3-1D**. However, there are various parameters that may influence or contribute to cluster dissociation. Primarily, I enumerated the total count of 2-cell clusters (doublets), 3-cell clusters (triplets) and larger cell clusters (4+) that encountered and transited past a capillary bifurcation via live imaging. There was clearly a dominance of doublets and triplets passing through the capillary bifurcations (**Fig. 3-7A**), which could explain the low cell counts within clusters post-transit from bifurcated capillary geometries (**Fig. 3-5**). However, when I enumerated the count of clusters that dissociated depending on number of cells within clusters, 70% (85/120) of doublets dissociated, 81% (27/33) of triplets dissociated and 93% (14/15) of 4+ cell clusters dissociated (**Fig. 3-7B-C**), suggesting that the accumulation of small clusters post-transit matches a dissociation profile of larger clusters such as triplets and those at least with 4 cells that break down in smaller ones. Furthermore, the increasing percentage of dissociation frequency among doublets, triplets and clusters with at least 4 cells, suggests that dissociation frequency is partially a function of cell counts within clusters (**Fig. 3-7C**). Even very large clusters, consisting of 9 or 10 cells were able to squeeze through, while rarer, yet nonetheless were dissociated in smaller clusters (**Fig. 3-7D-E**).

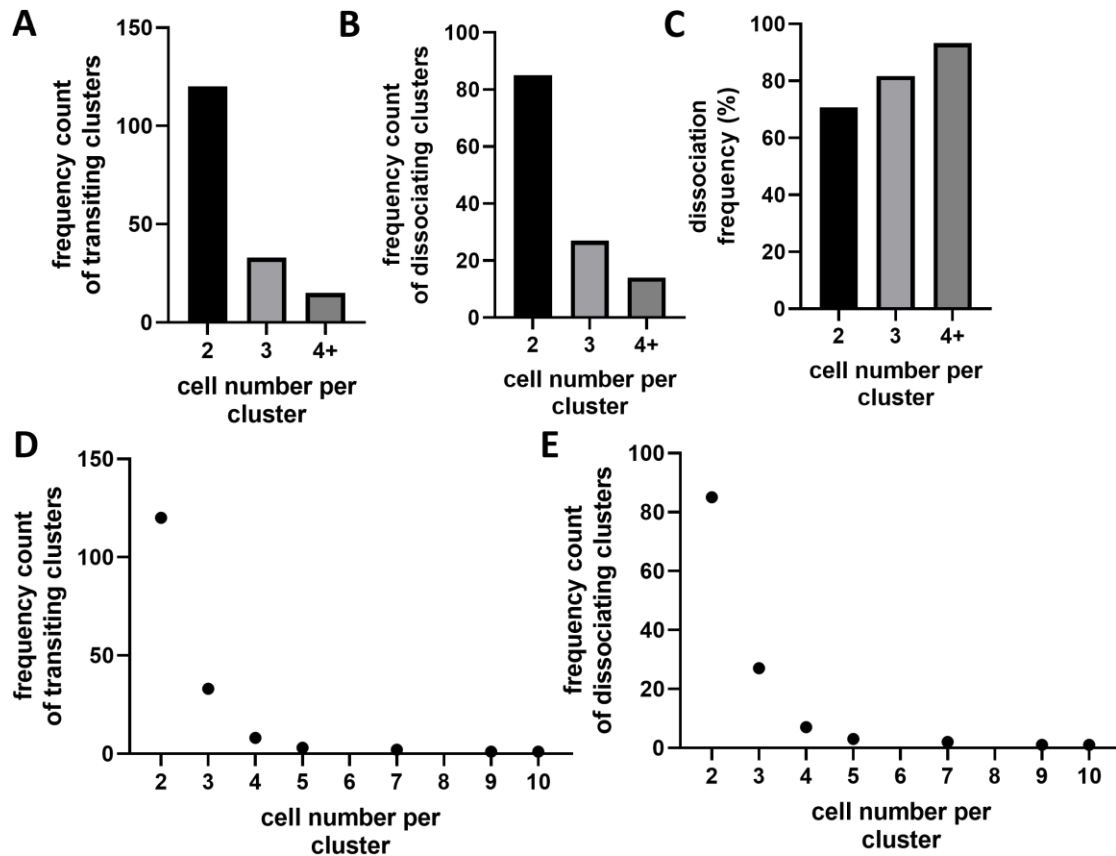


Figure 3-7: Cell counts within clusters affect cluster dissociation in capillary bifurcations.

(A) Number of 2-cell, 3-cell or 4+ cell clusters that transited capillary bifurcations during live imaging. (B) Number of 2-cell, 3-cell or 4+ cell clusters that dissociated at least once in capillary bifurcations during live imaging. (C) Quantification of percentage of clusters that were dissociated per number of cells within clusters. (D) Number of clusters that transited capillary bifurcations during live imaging, showing all distinct clusters based on number of cells within them. (E) Number of clusters that were dissociated during live imaging, showing all distinct clusters based on number of cells within them.

3.3.5 Capillary bifurcations modulate cluster dissociation

Live imaging of cluster transit in capillary bifurcations revealed that clusters could transit and dissociate or remain undisturbed (described as intact) (**Fig. 3-8A-B**), with initial observations that there were different tendencies for dissociation across distinct microfluidic devices. To explore the general contribution of the presence of bifurcations in cluster dissociation, clustered cells' dissociation frequencies were compared between bifurcated capillary and non-bifurcated capillary geometries (same constriction width), revealing higher dissociation frequency mean in the presence of bifurcations ($73.3 \pm 4.2\%$ vs $42 \pm 5.6\%$), (**Fig. 3-8C**). Given

the contribution of larger cell counts in a cluster to enhanced cluster dissociation, the data were replotted only for doublets, revealing again a consistent increased dissociation frequency mean in the presence of bifurcations ($63.5 \pm 2.7\%$ vs 31 ± 5.6) (**Fig. 3-8D**), highlighting that capillary bifurcations contribute to cluster dissociation.

Then, particular characteristics of distinct capillary bifurcations were investigated by comparing initially capillary bifurcations with either equal or unequal constriction width between the two daughter channels (**Table 2-1**). By comparing clusters of all cell numbers, equal geometries greatly promoted cluster dissociation compared to unequal ones ($83.1 \pm 13.3\%$ vs $27.1 \pm 22.3\%$, $P < 0.0001$) (**Fig. 3-8E**), an increase which was reflected on doublets' dissociation frequency too ($80.1 \pm 17\%$ vs $20.5 \pm 16.1\%$, $P < 0.0001$) (**Fig. 3-8F**). Interestingly, both datasets (**Fig. 3-8E-F**) revealed a large variance, especially in the case of unequal bifurcations, suggesting additional features of capillary bifurcations that influence dissociation.

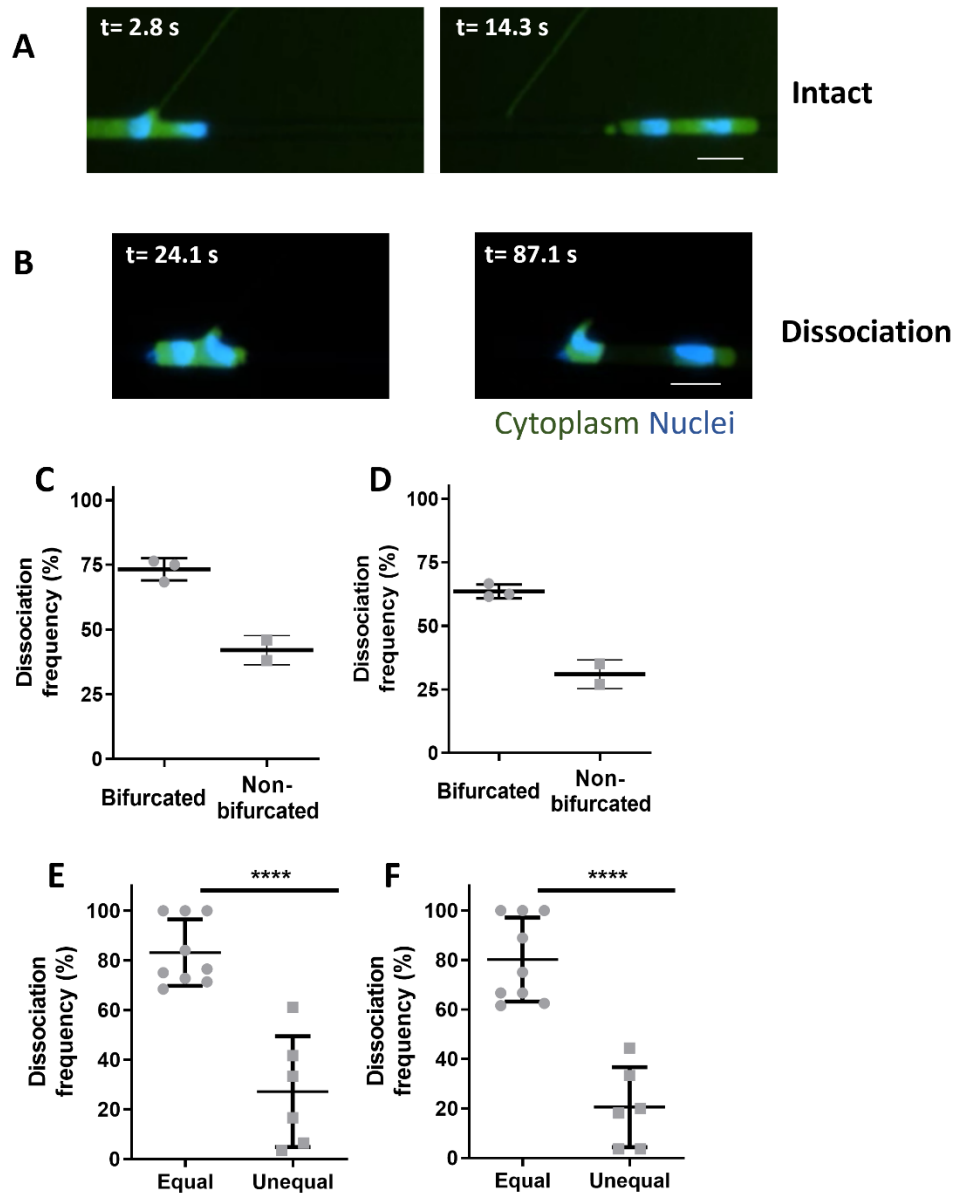


Figure 3-8: Features of bifurcated geometries influence cluster dissociation.

(A-B) Snapshots of fluorescently tagged clusters encountering capillary bifurcations and either remaining intact (A) or dissociating (B). Scale bar: 10 μ m. (C) Percentage of clusters of all cell counts that dissociated in bifurcated capillary (n=3) or non-bifurcated capillary geometries (n=2). (D) Percentage of 2-cell clusters that dissociated in bifurcated capillary (n=3) or non-bifurcated capillary geometries (n=2). (E) Percentage of clusters of all cell cluster counts that dissociated in equal (n=9) or unequal capillary bifurcations (n=6). (F) Percentage of 2-cell clusters that dissociated in equal (n=9) or unequal capillary bifurcations (n=6).

By further dissecting the different capillary bifurcation geometries, those initially grouped as unequal bifurcations revealed differences in dissociation frequency. Lowering the channel

width difference between two daughter channels affected significantly the mean dissociation frequency of clusters of all cell counts upwards about 5-fold (UNA_5.5/7: $45.3 \pm 14.2\%$ vs UNA_5/9: $8.9 \pm 6.9\%$, $P < 0.05$) (**Fig. 3-9A**). When only doublets were accounted for, the mean of dissociation frequency of doublets was still higher in the bifurcated capillary geometries with smaller channel width difference, as expected, (32% vs 9.1% , ns) (**Fig. 3-9B**), but no statistical significance was reached. Similarly, the large variance observed in equal bifurcated capillary geometries, prompted the dissection of equal bifurcations to their distinct constriction widths, comparing thus equal daughter bifurcations of 6.3-, 7- and 9 μm . Increasing the constriction width led to a downward trend of cluster dissociation frequency both for doublets and clusters of all cell counts (**Fig. 3-9C-D**), suggesting that the narrower the constriction, the higher the probabilities of cluster dissociation to occur. Altogether, these findings provide evidence that distinct features of capillary bifurcation geometries, which can be found in great diversity in the human microcirculation, can affect cluster transit and dissociation patterns in various ways.

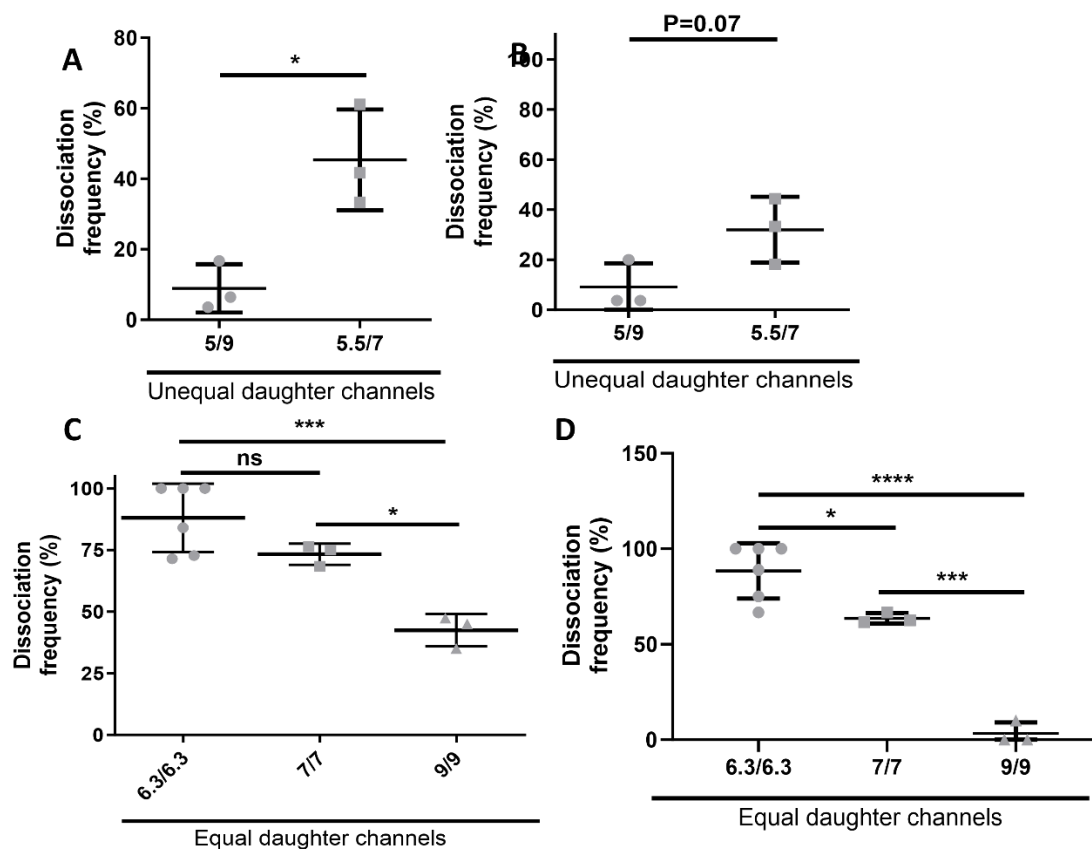


Figure 3-9: Differences in diameters of capillary bifurcations influence cluster dissociation.

(A) Percentage of clusters of all cell counts that dissociated in distinct unequal capillary bifurcations (UNA_5/9 vs UNA_5.5/7) (n=3 for all). (B) Percentage of 2-cell clusters that dissociated in distinct unequal capillary bifurcations (UNA_5/9 vs UNA_5.5/7) (n=3 for all). (C) Percentage of clusters of all cell counts that dissociated in distinct equal capillary bifurcations (ENA-n=6 vs ENA_7/7-n=3 vs ENA_9/9-n=3). (D) Percentage of 2-cell clusters that dissociated in distinct equal capillary bifurcations (ENA-n=6 vs ENA_7/7-n=3 vs ENA_9/9-n=3).

3.3.6 Patterns of cluster transit direction in capillary bifurcations

Then, I sought to explore the transit haemodynamics of clusters in respect to their direction in the bifurcated capillary geometries, based on their dissociation or not. To identify how intact or dissociated clusters transit, the datasets were split accordingly. Given the small number of clusters for example that remained intact in equal bifurcations per experimental sample, the data were pulled together, to compare directly between equal and unequal bifurcation geometries. From the numbers of clusters that remained intact transiting unequal bifurcations, 98.95% (94/95) of these clusters transited through the d_2 channel in the bifurcation, which was the one with the larger width (**Fig. 3-10A**). In contrast, in respect to intact clusters that transited equal bifurcations, 62.96% (17/27) of these transited in the d_2 channel (**Fig. 3-10A**), highlighting that clusters transit preferably in daughter channels with a larger width.

To investigate this time the direction of dissociated clusters, the datasets were compared by determining the number of clusters that dissociated and transited in different daughter channels or in the same. In equal bifurcations, as expected, 87.38% of clusters (90/103) dissociated and eventually transited in different daughter channels, whereas in unequal bifurcations only 40.74% of clusters (11/27) behaved similarly (**Fig. 3-10B**), suggesting that equal bifurcations promote transit of dissociated clusters in different daughter channels. By further segregating the dissociated cluster events according to those that transited in the same direction post-dissociation in d_2 daughter channel, it appears that transit direction reflects the behaviour of intact clusters (**Fig. 3-10A**). In unequal bifurcations, 100% (16/16) of dissociated clusters transited in the channel with the larger width (d_2), whereas in equal bifurcations, 59.1% (13/22) of clusters transited in the d_2 daughter channel (**Fig. 3-10C**), highlighting once again that the differences in channel width in unequal bifurcations and their haemodynamics dictate largely the transit direction of both intact and dissociated clusters.

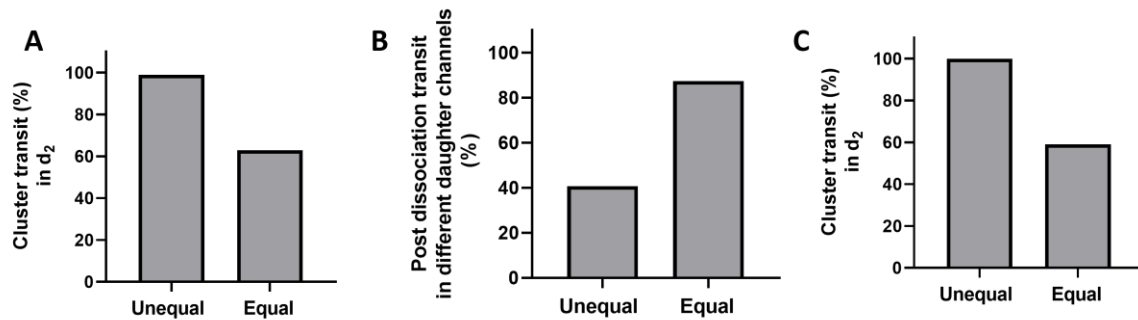


Figure 3-10: Transit direction of clusters in capillary bifurcations.

(A-C) All data were pooled together under the subclassification of equal vs unequal bifurcated capillary geometries. (A) Percentage of intact clusters that transited in d₂ daughter channel in unequal or equal capillary bifurcations. (B) Percentage of clusters that dissociated and transited in different daughter channels in unequal or equal capillary bifurcations (C) Percentage of clusters that dissociated and transited in d₂ daughter channel in unequal or equal capillary bifurcations.

3.3.7 Inhibition of F-actin increases cluster dissociation and cell death

To induce further cluster dissociation, the ability of a F-actin polymerisation inhibitor, Cytochalasin D (Cyto-D) was tested. On that end, the potential of Cyto-D was evaluated during clusters transit in a capillary bifurcation geometry that showcased previously low dissociation frequency, UNA_5/9 (**Fig. 3-9A**). Considering initially clusters of all sizes, Cyto-D increased their mean dissociation frequency about 8-fold ($66.7 \pm 17.5\%$ vs $8.9 \pm 6.9\%$, $P < 0.05$) (**Fig. 3-11A**). However, the mean number of cells within clusters was larger in the Cyto-D treated cluster cohort (3.1 ± 1.6 vs 2.1 ± 0.4 , $P < 0.0001$) (**Fig. 3-11B**) and having established already that cell number within clusters affects dissociation frequency (**Fig. 3-7C**), the data were replotted only for doublets this time. Indeed, Cyto-D induced about 6-fold higher mean of dissociation frequency (57.7% vs 9.1% , $P < 0.05$) (**Fig. 3-11C**), excluding the possibility that the effects of Cyto-D on cluster dissociation was merely a reflection of the higher cell counts within clusters. Altogether, Cyto-D treatment of clusters led to high dissociation frequencies in capillary bifurcations that can potentially disrupt their ability to proliferate and metastasise aggressively.

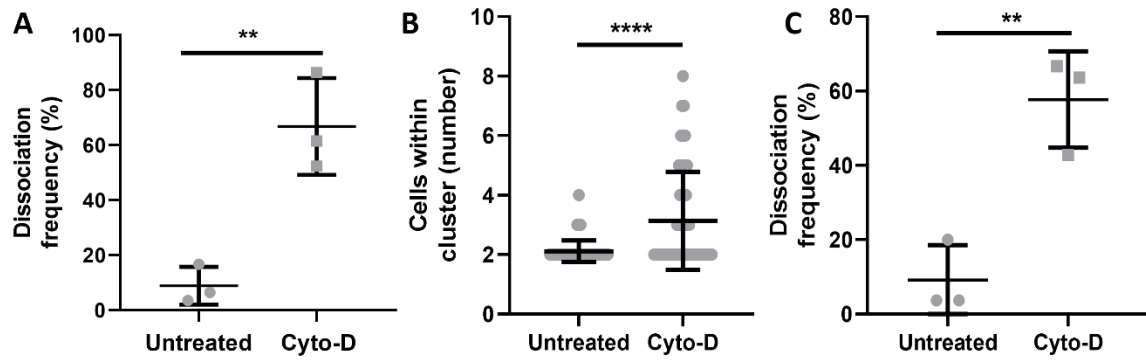


Figure 3-11: Cytochalasin D treatment increases cluster dissociation in capillary bifurcations.

(A) Percentage of untreated or Cytochalasin D treated clusters of all cell counts that dissociated in capillary bifurcations (n=3 for all). (B) Cell numbers per untreated (n=78) or Cytochalasin D treated cluster (n=51). Each data point represents an individual cluster. (C) Percentage of untreated or Cytochalasin D treated 2-cell clusters that dissociated in capillary bifurcations (n=3 for all).

Cluster dissociation was not the only observed effect upon treatment with Cyto-D. Established from previously, clusters presented reduced cell lysis both in capillary bifurcations and non-constricted geometries, compared to singlets (**Fig. 3-2 & 3-3 & 3-12A-B**). When single cells were treated with Cyto-D, the number of single cells was increased in both capillary bifurcations and non-constricted geometries (**Fig. 3-12A-B**). On the other hand, treatment of clusters with Cyto-D led to a small 2-fold increase of cell lysis in non-constricted geometries (untreated: $1.6 \pm 1\%$ vs Cyto D: $3.6 \pm 0.5\%$, $P < 0.05$) (**Fig. 3-12D**), whereas a large 6-fold increase in capillary bifurcations (Cyto D: $27.4 \pm 4.5\%$ vs untreated: $4.6 \pm 0.3\%$, $P < 0.05$) (**Fig. 3-12E**). Clusters retained their ability to survive better compared to single cells in non-constricted geometries even upon treatment with Cyto-D (**Fig. 3-12B&F**), but this protective effect had disappeared in capillary bifurcations (clusters: $27.3 \pm 4.5\%$ vs singlets: $32 \pm 7.1\%$, ns) (**Fig. 3-12A&G**). The increased cell death in Cyto-D treated clusters collected post-transit from capillary bifurcations compared to untreated clusters can be visualised in **Fig. 3-12C**, by the increased number of dead cells (depicted as purple). The effect of cell death is unlikely to be directly because of Cyto-D, given the high viability of single cells attained in static conditions in **Fig. 2-9C** (Chapter 2), suggesting that it's the cooperation of shear and capillary that affect cells within Cyto-D treated clusters, rendering them more prone to cell death. Altogether, these findings highlight that Cyto-D affects clusters transit in capillary bifurcations, by

inducing both their dissociation to smaller clusters and/or singlets as well as cell death, which can ultimately affect the fate and role of clusters in metastasis.

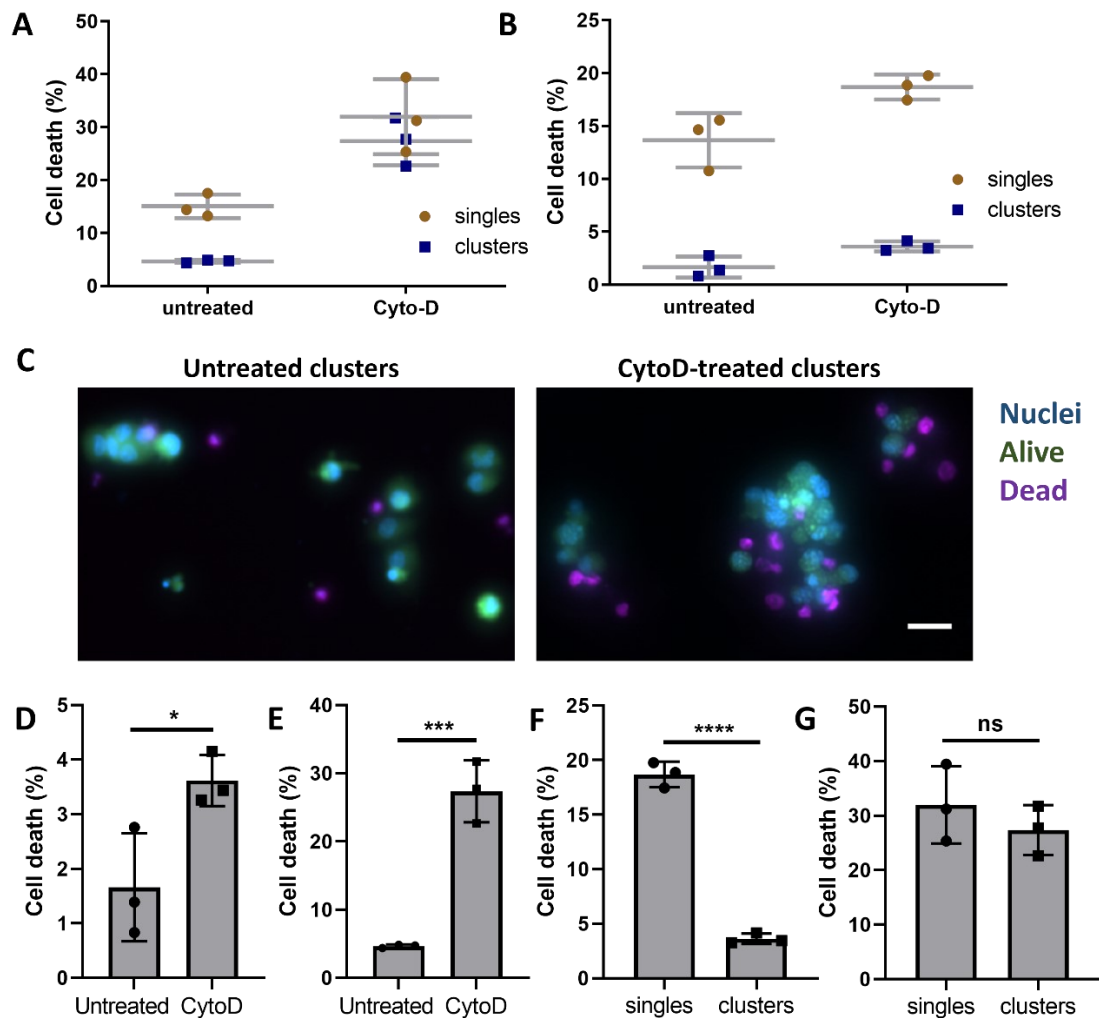


Figure 3-12: Cytochalasin D treatment increases death of cells within clusters in capillary bifurcations.

(A-B) Percentage of dead cells within clusters or dead single cells, untreated or Cytochalasin D treated, post transit in capillary bifurcations (A) or non-constricted geometries (B). (C) Multifluorescent images of untreated or Cytochalasin D treated clusters, collected post transit from capillary bifurcations. Nuclei is stained blue, alive cells are stained green and dead cells are stained red (depicted as purple). Scale bar: 50 μ m. (D) Percentage of dead cells within clusters that were either untreated or treated with Cytochalasin D and collected post transit from non-constricted geometries. (E) Percentage of dead cells within clusters that were either untreated or treated with Cytochalasin D and collected post transit from capillary bifurcations. (F) Percentage of dead cells within clusters or dead single cells that were treated with Cytochalasin D and collected post transit from non-constricted geometries. (G)

Percentage of dead cells within clusters or dead single cells that were treated with Cytochalasin D and collected post transit from capillary bifurcations. (A-G) n=3 biological replicates for all.

3.4 DISCUSSION

Transit of cancer cell clusters in capillary bifurcations is largely underexplored. Although several aspects during their transit in capillary beds are being investigated, including arrest, extravasation, immune cell escape and vascular remodelling (34,223,225), the direct impact of capillary geometries on cluster's fate is not understood. Many of these phenomena described in vivo are observed not on real-time, but after long time intervals post cluster injection (34). This likely enhances the possibility that behaviours of clusters or their cellular responses when encountering capillary bifurcations may be missed, go unnoticed and remain unreported, hindering thus the understanding of phenomena that may be of relevance.

In this Chapter, the implementation of a live imaging approach during cluster transit in novel microfluidic models of capillary bifurcations, which has not been done previously, revealed that the phenomenon of cluster dissociation, defined as detachment of cells from a cluster, is governed by numerous features of capillary bifurcations. Primarily, I demonstrated that multiple factors including the presence of bifurcations, lower constriction widths and the inequality of daughter channel widths promoted cluster dissociation. Moreover, I showed that the number of cells within clusters promotes cluster dissociation e.g. 4-cell cluster vs doublets. Last but not least, I discovered a way to put a halt to the metastatic capabilities of clusters by further inducing their dissociation in certain capillary geometries that inhibited cluster dissociation. By treating clusters with Cytochalasin D, I showed for the first time that during transit of Cytochalasin-treated clusters in capillary bifurcations (with natural low dissociation frequency), not only was their dissociation increased but I quantified elevated cell death, supporting the utility for a therapeutic approach against CTCs clusters.

Primarily, the microcirculation is considered a harsh microenvironment that is often credited as responsible for halting the journey of CTCs towards metastasis (31,32). Several studies report the advantages of clusters over singlets, rendering them more aggressive clones (49,110,284), suggestive of the strong potential for targeting them therapeutically. In this Chapter, experimental results validate the notion that clusters are superior to singlets, given that clusters resisted cell lysis compared to singlets, both during transit in capillary

bifurcations and non-constricted geometries (shear only), suggesting that clusters may be more competent at surviving in the harsh environment of the human circulation and eventually succeed in seeding metastases. Interestingly, the combination of shear and capillary bifurcations increased slightly cell lysis in clusters, as opposed to shear only, suggesting that the narrow constrictions affect clusters viability further, yet still largely protected compared to single cells. In respect to a possible mechanism explaining this enhanced protection on clusters, it is plausible that through intercellular junctions, cells within clusters are able to strengthen the cell cytoskeleton (276), which supports structural integrity and thus protect cells against damage. Additionally, clustering could confer the upregulation of mechanoresistant or anti-apoptotic proteins that further protect against cell death. These findings represent the first reported discovery on the ability of homotypic clusters to protect themselves better compared to singlets in conditions mimicking the microcirculation and strengthens the wide-held belief that clusters are more durable and advantageous over single cells in the journey towards metastasis.

Experiments of clusters' collection post transit from bifurcated capillaries, non-bifurcated capillaries and non-constricted geometries revealed the reduced cell counts within clusters collected. These findings are in agreement with several reports on cluster isolation from cancer patients, showing that the cell counts within collected clusters were predominately doublets, triplets and smaller clusters, composed of smaller than 4-5 cells (49,242). A difference between these studies on patients' isolated clusters results and the in vitro results obtained in this Chapter, is the lack of live imaging in the former. While Szczerba et al. identified reduced cell counts in the blood of cancer patients post-transit in capillaries compared to pre-capillary isolation locations, there is no direct visualisation evidence (49). This opens up the debate whether clusters dissociate to small clusters or whether only small clusters manage to pass through capillary beds. In this Chapter, the use of non-constricted devices, which excludes the possibility of cell entrapment revealed indeed dissociation, suggestive of the fact that at least shear can induce dissociation of clusters to smaller ones. However, in respect to the use of constricted geometries, this debate remains unresolved. For this reason, in vitro microfluidic models that mimic the blood vessels are great tools to visualise the transit of clusters in capillary geometries.

Live imaging of cluster transit in capillary bifurcations revealed the dissociation of all types of clusters, from small ones, doublets and triplets, to larger ones, consisting up to 10 cells. While the number of clusters tested was not huge, only one cluster with at least 4 cells did not dissociate. On the other hand, the fact that 70% of doublets dissociated while a significant proportion remained intact, further explains partially why cancer patients consistently present doublets in their blood (242), given that doublets remain intact at higher percentages and that can also pass easier capillary beds due to their smaller size. The role of cell counts within clusters as a determining factor in dissociation is not surprising, given the established knowledge that individual cells within clusters present phenotypic heterogeneity (287). For instance, previous research highlights that cells within clusters can be epithelial or mesenchymal or hybrid (288), which means that cells with more mesenchymal features will be less prone to remain firmly adhered to each other. Complementary data from Au et al. (45), suggest that adhesion forces within cells in a cluster affect their dissociation. In fact, the strength of adhesion forces is somewhat correlated to epithelial or mesenchymal phenotypes (289). Overall, the higher the number of cells in a cluster, the higher the potential diversity in individual single cell phenotypes which can affect dissociation in ways, many of which remain unexplored.

Moreover, live imaging revealed additional distinct patterns of cluster dissociation to smaller clusters and/or singlets. Several factors have been corroborated as strong contributors of modulating cluster dissociation. Namely, the presence of bifurcations, equality of daughter channel widths and lower constriction widths promoted cluster dissociation, while non-bifurcated geometries, large differences in daughter channel widths and larger constriction widths supported preferably the integrity (intactness) of clusters. The microcirculation in vivo consists of various and diverse microvessels, comprising narrower or larger constrictions, with or without bifurcations and with different width differences between two daughter channels (33,34). Thus, the different dissociation frequencies across all distinct microfluidic models that were utilised, supports the notion that clusters can behave differently in the diverse geometries in the human microcirculation. On one hand, cluster dissociation can be interpreted as a natural defence mechanism of the microcirculation against aggressive cluster-directed metastasis, given the fact that many studies highlight clusters as the most aggressive clones. On the other hand, the complexity of the microcirculation and the diverse

responses of clusters, emphasise that many clusters may escape dissociation or simply dissociate to smaller clusters, that can still act aggressively. In fact, Szczerba et al. have shown that even a few clusters can elicit aggressive metastases and compromise overall survival in mice (49), rendering the existence of cluster dissociation agents crucial to maximise this phenomenon.

Several studies focused on the prevention of cluster formation as a strategy to demonstrate that clusters are aggressive metastatic precursors (49,67,110,281–284,290). For instance, Szczerba et al. showed that disrupting the formation of heterotypic clusters reduces the incidence of metastases and the faster death of mice (49). However, there is no report focusing on how pharmaceutical treatment of clusters affects their disruption, after intravasation while transiting in the blood vessels, before seeding metastases in a distant organ. Previously, Au et al. formulated a simulation model of clusters transit in non-bifurcated capillaries, identifying that adhesion forces dictate their cohesion (45). They found out that lower adhesion forces between neighbouring cells in a cluster led to dissociation of cells. This finding suggests that the forces of shear and constrictions apply mechanical pressure to the intercellular adhesion forces, lowering them potentially below a certain threshold, resulting thus in dissociation. These findings further suggest that weakening of the adhesion forces chemically may be a good strategy to induce further dissociation (45). To this end, the ability of Cyto-D to induce dissociation of clusters during transit in capillary bifurcations at higher frequencies appears promising. Given the influence of Cyto-D on inhibiting F-actin polymerisation, which in turn could lead to reduction of intercellular junctions and adhesion forces (291), this disruption could be a plausible explanation that leads to cluster dissociation. However, F-actin staining on clusters was retained strongly after Cyto-D treatment, suggestive of clusters' F-actin network remaining undisturbed. Thus, it is debatable whether intercellular junctions are affected regardless or whether Cyto-D affects clusters in other ways, which eventually leads to their dissociation. Nonetheless, the increase in cluster dissociation and lysis of multiple cells within clusters during their encounter of capillary bifurcations, suggests the potential use to target clusters using Cytochalasin. Conferring thus dual effects against cluster-directed metastasis.

According to preliminary findings, clusters could survive better compared to single cells, during their transit in either non-constricted (shear only) or capillary bifurcation microfluidic

devices. On the one hand, treatment with Cyto-D, as expected, revealed a slight increase of cell death both in single cells and clusters in non-constricted devices compared to untreated, yet clusters succeeded again at surviving better compared to single cells. While this is an important finding, it should be taken with caution, given the simplicity of the microfluidic devices that represent one single channel. Physiologically, CTCs clusters, as their singlet counterparts, experience in seconds rapid changes in shear stress, as they transit from the larger arteries to smaller arterioles, before encountering capillary beds (37). Such findings would need to be reproduced in more sophisticated models, that will incorporate a heterogeneity of shear stress profiles, longer exposure times to shear stress and distinct fluid velocities to simulate more accurately single cell and cluster transit. For example, it is already established that single cells are less prone to entrapment compared to clusters (110), suggesting that single cells may be exposed for significantly longer periods to shear stress, increasing further their death. On the other hand, clusters that were treated with Cytochalasin, died off at similar levels to single cells post-transit from capillary bifurcations, losing completely their protective mechanism. Along with the increase in dissociation, cytochalasin may have weakened the ability of cells to survive the synergy between shear and capillary forces. Similarly, as previously, the mechanism of F-actin polymerisation inhibition through which cell death is induced particularly in capillary bifurcations remains unresolved, given the fact that clusters resist loss of F-actin, upon treatment with Cyto-D, which will need to be addressed in the future. Nonetheless, Cyto-D elicits dual effects on clusters' fate during their transit in capillary bifurcations, by causing both dissociation and lysis, which could be utilised and explored further as a therapeutic strategy.

The rapid expansion on technological advancements on CTCs isolation technologies has revealed the presence of several distinct types of heterotypic clusters formed in various cancer types, including clusters formed between tumour cells and cancer-associated fibroblasts, neutrophils, platelets or macrophages (49,292). Given the dissociation patterns recorded for homotypic clusters, it is safe to presume that this phenomenon will be observed for heterotypic clusters alike. Exploring how the dissociation patterns of clusters formed for instance by tumour cells and neutrophils differs from those of homotypic clusters, may indicate for example distinct mechanisms of protection against dissociation i.e. via stiffness modulation, compared to homotypic clusters. For instance, neutrophils may activate distinct

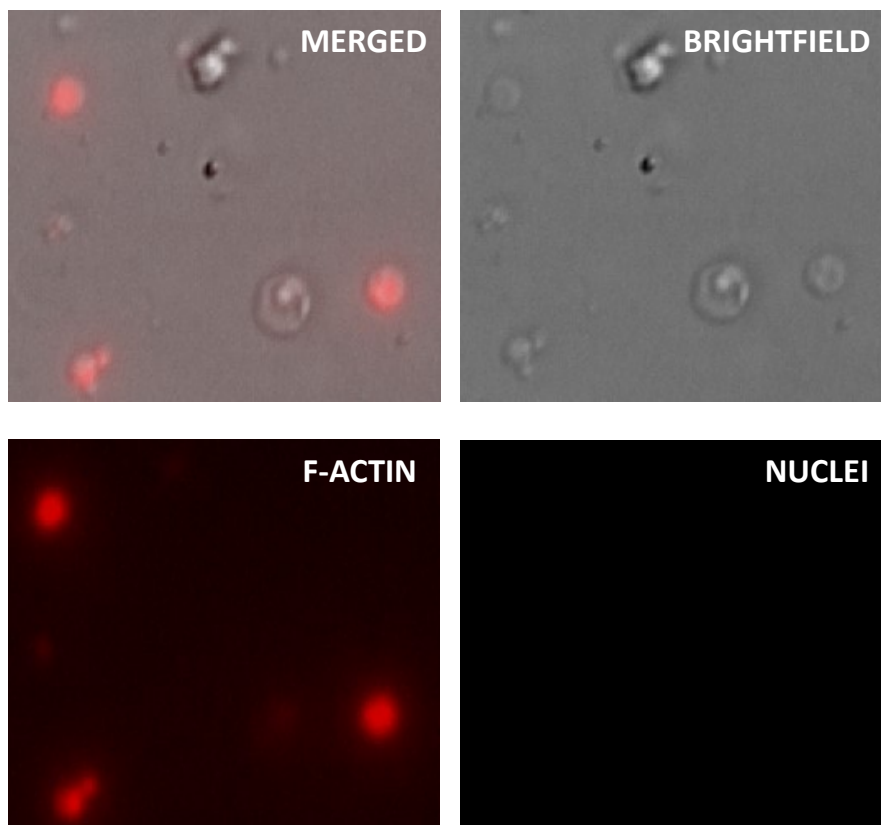
pathways in co-travelling CTCs that will alter their response in capillary beds against dissociation and thus require a different therapeutic approach. Furthermore, understanding better the cell properties that modulate the cohesion of clusters upon transit i.e. cell adhesion forces, will grant insight in the complete repertoire of clusters that enables them to remain adhered. Discovery of such mechanisms will allow the testing of numerous compounds as drug candidates to induce and maximise dissociation as a means to reduce the metastatic burden. To achieve that in a more accurate manner, more sophisticated in vitro models of the microcirculation need to be created, including multiple interconnected bifurcated pathways, with diverse geometries in respect to bifurcation angle, constriction widths and daughter channel width differences to evaluate more successfully the dissociation patterns of clusters. Altogether, clusters, as the most aggressive cancer clone towards metastasis, have weaknesses that can be exploited therapeutically to reduce metastasis and prolong the survival of patients.

3.5 CONTRIBUTIONS & ACKNOWLEDGEMENTS

I generated the hypothesis, designed methodologies and experiments, conducted most of the experiments, collected and analysed all data. Aisher Chen and Georgia Kontaxi helped by performing some experiments on cluster dissociation observations.

CHAPTER 4

Cancer cell-shed large extracellular vesicles contain proteins



4.1 INTRODUCTION

Extracellular vesicles (EVs) are an integral part of cell biology in health and disease alike (79). They are defined by the International Society of Extracellular Vesicles as nuclei-free particles that are shed from cells, unable to replicate (78). EVs are largely mediating intercellular interactions and particularly in cancer, there is a large body of work that highlights their activities in cell reprogramming, angiogenesis, immunosuppression, cell signalling, invasion, metabolism, proliferation and chemoresistance among others (80,214,293–298). Among EVs, the most well-established so far are exosomes and microvesicles, with sizes of 30-120 nm and 50-1000 nm (85). However, there are various other EVs smaller than 1000 nm, elaborated extensively elsewhere (92,299,300). In respect to particles larger than 1000 nm, the literature reports such particles under numerous names, including large EVs (LEVs), large oncosomes (LOs), tumour-derived EVs and shear-derived large microparticles (LMPs) (71,75–77,88,89,92,94), extensively described in Chapter 1. Many studies that utilise these larger particles for downstream analysis, use either LOs or LEVs, obtained via chemical treatment or serum-depletion in vitro (88,89), fundamentally different than the observations of shear-derived LMPs, as introduced in Chapter 2, which have also been observed in vivo by Headley et al. (71). These large particles differ clearly from smaller EVs due to their large size and particularly shear-derived LMPs biogenesis is completely different compared to exosomes, microvesicles and LOs (85,87,92,100) (**Table 4-1**). Nonetheless, all these types of large particles have been documented in a small number of studies compared to exosomes, to induce cell phenotype reprogramming, stimulating invasion and proliferation, disrupting the endothelial barrier, transferring biological material and interacting with various immune cells, suggesting that they are not fragments of cells with no functionality (71,88,89,94,99,120,121). However, the nature of LMPs that are derived prevalently from the combination of shear and capillary bifurcations and whether if they are EVs, remains largely unknown.

Table 4-1 Types of cell-secreted particles

TYPE	LMPs (71)	EXOSOMES(85)	MICROVESICLES (85)	LOs (85)
SIZE	0.5-25 μm	30-120 nm	50-1000 nm	1-10 μm
BIOGENESIS	SHEAR & CAPILLARY DERIVED	MULTIVESICULAR BODIES	PLASMA MEMBRANE	PLASMA MEMBRANE

Currently, investigation of the nature of EVs occurs by ensuring the purity of the population of interest. Various enrichment and purification techniques of small or large EVs during in vitro generation are reviewed, with centrifugation being the most common for exosomes and microvesicles, whereas both filtration and centrifugation are commonly employed for purification of LEVs or LOs (100,301). For instance, LOs are often generated from serum-free cancer cell cultures that drives the shedding of these large particles from the membrane of cells. Collected media, defined as conditioned media (CM) undergoes a series of centrifugations to remove any floating cells and/or debris and at last enables the congregation of large oncosomes as a pellet, by centrifugation at 10000 x g for 30 min (89). In another study, where large and small-sized EVs were compared, a similar approach was utilised, except the supernatant collected post-centrifugation at 10000 x g was succumbed to an additional step of ultracentrifugation at 100000 x g for 1 hr, which allowed exosomes to be pelleted (88). The lack of nuclear stain can be often used as a distinguishing criterion for cell contamination (78), along with routine staining of traditional markers that pertain to the molecular verification of exosomes or microvesicles (302). However, when it comes down to reported LEVs in the literature, protein markers validation according to MISEV guidelines (78) is not fully complete from many studies and only size or partial requirements are met and used as their classification to LEVs.

Minimal information for studies of extracellular vesicles (MISEV 2018) is a canon of guidelines, put forward by the International Society for Extracellular Vesicles (78). This document represents an update of the guidelines that were introduced back in 2014 (303) and is a position statement that facilitates researchers on classifying, characterising and studying different types of EVs in a uniform way compared to other researchers' findings, enabling

reproducibility and verification of common observations (78). For instance, Huang et al. have observed the generation of large particles in vivo by CTCs transit in the microcirculation of zebrafish models, yet they refer to them in their work as apoptotic fragments (62), when in fact these could be LMPs similar to Headley's observed particles (71) or the ones from Chapter 2 observations. In two independent studies, large particles have been identified in the blood of cancer patients and were termed as tumour-derived extracellular vesicles, without proof of meeting all relevant protein markers (75), as set by the MISEV2018 guidelines. These paradigms solidify that to maximise the identification of these large particles and connect unbridged observations by various researchers, following the MISEV2018 guidelines is crucial. Furthermore, this position statement includes suggestions on methodologies, tools and equipment to be used in correct assessment of the nature of EVs (78). For instance, flow cytometry is suggested as the best tool to enumerate LEVs. Moreover, their guidelines suggest particular protein assays to measure the total protein levels of an EV population. Last but not least, the position statement sets out specific guidelines for an observed particle to be characterised and corroborated as an EV. Validation of the presence of at least three proteins is required, including at least one transmembrane and one cytosolic protein. Transmembrane proteins are proof that a lipid bilayer exists in EVs. Similarly, cytosolic proteins presence inside an EV is proof that EVs are capable of encompassing biological material and are not thus fragments of cells. Along with the protein composition, EVs must be analysed at single-vesicle level by at least two complementary techniques. One such is microscopy that provides high-resolution images, such as scanning electron microscopy (SEM) and information on size and morphological features. Another complementary technique should be based on estimation of biophysical features of EVs, other than high-resolution imaging techniques, by employment of light properties, fluorescence properties or electric field displacement. Overall, the MISEV2018 guidelines represent an accurate statement to study EVs in a reproducible manner (78).

EVs contain many types of biomolecules, including proteins, nucleic acids and lipids (95). Current studies have explored more extensively through transcriptomic, proteomic or lipidomic approaches the specific molecular cargo that exosomes carry (96,104). In fact, in certain instances, exosomes display the presence of proteins or RNA molecules that are enriched in exosomes compared to their cells of origin (96). Interestingly, bioinformatic

analyses have revealed how EVs can be involved in multiple molecular function and cell pathways (96), highlighting their diversity and contribution to various stages of metastatic cancer. Less studies though have explored the protein profile of LOs, that were isolated from cultures of prostate cancer cells, yet identified differences between LOs and exosomes in respect to protein enrichment patterns and functionalities (88). These preliminary findings highlight that large particles such as LOs contain a vast array of proteins with a potential for influencing the metastatic cascade. Nonetheless, there is no information whatsoever on the diversity of shear-derived LMPs' molecular content, on their correlation to molecular functions or biological pathways, from which cell compartment they originate from and the molecular content of cells post shedding.

In this work, the molecular content of shear-derived LMPs was explored for the first time. Primarily, a pure population of LMPs was successfully purified from their originating cells post-transit in microfluidic devices. Then, these shear-derived LMPs were verified to match the MISEV2018 guidelines on EVs nature in respect to protein composition, size and morphological features, enabling the acquisition of the LEVs terminology. By comparing total protein levels, loss of protein mass from cells post-shedding was identified, part of which was retained in shed LEVs. Last but not least, the proteome of shear-derived LEVs was characterised widely, connecting the presence of certain biomolecules to cancer-related processes and functions. Altogether, I hypothesise that shear-derived LEVs are rich in biomolecules, namely proteins that can confer them abilities to influence metastasis.

4.2 MATERIALS & METHODS

4.2.1 Cell culture & reagents

MDA-MB 231 breast cancer cells (purchased from ATCC) were cultured, harvested, prepared and stained (where relevant) for downstream experiments, as described in Chapter 2. All reagents were purchased from Sigma Aldrich (UK), unless noted otherwise. All antibodies were purchased from Abcam (UK), unless noted otherwise.

4.2.2 LEVs generation and purification

Microfluidic devices (**Table 2-1**) were prepared as introduced in Chapter 2. Overall, 2 ml of MDA-MB 231 cells (1×10^6 cells/ml) were stained with a red Cell Tracker (ThermoFischer

Scientific) or a green Cell Tracker (ThermoFischer Scientific, UK) for 1 hr at a final concentration of 5 μ M. Then, cells were added in microfluidic devices in continuous volumetric steps of 100 μ l (since the device inlet fills full at 200 μ l) and the PBS pre-filled syringe reservoir whose tubing end was connected to the end of microfluidic devices was lowered to generate pressure across the inlet and the outlets of microfluidic devices. The effluent mix containing cells and LMPs was collected by emptying the content of the syringe in a 15 ml falcon tube.

Two distinct methodologies were applied to purify and separate LEVs from cells. In the first methodology, the collected volume was passed through a Whatman Polydisc 10- μ m pore filter, which upon pressure from the syringe's plunger, the liquid content was pushed in a clean tube through the filter, allowing only LEVs to pass through. In the other methodology, the collected volume that contained cells and LEVs was first centrifuged at 180 x g for 5 min to pellet the cells and debris but not LEVs. The supernatant was collected and transferred to a 2 ml Eppendorf tube, which was further centrifuged at 10000 x g for 30 min to pellet LEVs. The supernatant was removed and fresh media was added in the Eppendorf tube to resuspend LEVs, which were used directly for downstream analyses.

To verify purification of LEVs from cells, cells and LEVs were stained with a cell tracker, as described previously and further incubated with Hoechst 33342 at a final concentration of 16.2 μ M, before the application of a purification methodology. Hoechst was used as a separation criterion, given the lack of nuclear signal in LEVs. Before and after each purification methodology was applied, 100 μ l were added in separate wells of a 96-well plate and were imaged at 20 x magnification using the Nikon microscope (as detailed in Chapter 2). Several characteristic multifuorescent images of each well were obtained. For quantitative analysis, samples were examined via flow cytometry, which will be expanded subsequently. Quantitative analysis was performed on three different conditions: a. fluid volume containing cells and LEVs before centrifugation, b. pelleted cells only after centrifugation and c. purified LEVs only after centrifugation. 500 μ l of each condition was transferred in 2 ml Eppendorf tubes and was loaded in an Amnis CellStream flow cytometer (US).

4.2.3 Immunofluorescence

100 µl of MDA-MB 231 cell suspensions (100000 cells/ml) were transferred in each well of a 96-well plate and were cultured for 24 hr, until 90% confluency. In the next day, cell media was removed and cells were fixed with 4% (v/v) formaldehyde (diluted in PBS) for 12 min at room temperature or with 100% (v/v) Methanol for 5 min at room temperature. When the fixative was removed, cells were washed with PBS thrice for 3 min per wash and further incubated with 0.1% (v/v) Triton X (diluted in PBS) for 5 min at room temperature. Then, cells were washed again thrice with PBS for 3 min per wash. Then, cells were incubated at 4°C overnight with primary rabbit monoclonal antibodies, including Heat-shock protein 90 (HSP90), Cytokeratin 18 (CK18) and cluster of differentiation 81 (CD81). The antibodies were diluted with PBS at final working concentrations of 1.74 µg/ml, 1 µg/ml and 0.66 µg/ml, respectively. The next day, antibody-filled PBS was removed from each well and cells were washed 1x with PBS for 3 min. Secondary goat-anti-rabbit antibody AlexaFluor 488 (Abcam, UK) was diluted in PBS at a final working concentration of 2 µg/ml and was transferred to all wells for 2 hr at room temperature. Finally, after the last washing step, cells were incubated with Hoechst for 5 min at room temperature. All wells were imaged and 3-4 multifuorescent images were obtained across each well, using the Nikon microscope.

Immunofluorescence on purified LEVs was done on the same proteins but with a modified staining protocol. All the chemicals and washing steps were the same as above, except LEVs were suspended in media and so were treated as a suspension line. After every incubation, LEVs suspension was centrifuged at 10000 x g for 30 min. Alternatively, LEVs were stained with Phalloidin that was diluted 1000 times in PBS from purchased vial (Abcam, UK/ ab176759) and incubated with LEVs for 1 hr, without secondary antibody incubation. To image the stained LEVs, 100 µl of LEV-containing media was transferred to a single well in a 96-well plate and multifuorescent images were obtained at 20 x magnification, using Nikon microscope. For flow cytometry studies, 500 µl of LEVs suspension was transferred in a clean 2 ml Eppendorf tube and loaded in an Amnis CellStream flow cytometer.

4.2.4 Flow cytometry

500 µl of either stained cells or stained LEVs, for each appropriate antibody was transferred in the flow cytometer. Unstained cells or single-colour stained cells (or LEVs) were used for

downstream gating. All samples were run at a flow rate of 20 μ l/min 1 ml/ml and at least 1000 events were recorded per condition and replicate.

Quantification of the flow cytometry results required proper gating. LEVs that were pre-stained with a red cell tracker, were initially run without antibody staining to set gating and a threshold for staining intensity that allowed their distinction from debris. To quantify the expression of tested proteins on LEVs, LEVs that were pre-stained as red, were stained as described above with relevant antibodies. Based on the gating criteria of the single-colour controls, the area encompassing the double positive events was delimited and both the frequency of double positive events and the mean green fluorescence intensity were quantified. Similarly for cells, cells were stained with Hoechst (blue signal) and with the respective antibodies, independently expressing green signal. The double positive events were calculated as detailed above.

To quantify LEVs purification efficacy, cells and LEVs were prepared as described elsewhere. The three independent experimental conditions were prepared based on purification methodology of centrifugation, setting three conditions: a. before centrifugation, b. after centrifugation (cell pellet) that was resuspended in equal volume as the other 2 conditions and c. after centrifugation (LEVs suspension). 500 μ l from each sample was run in the flow cytometer, as above. Based on the results of the first condition, two populations appeared. One was positive for nuclear signal, representing the cells and one was negative, representing LEVs. The first gating criterion was to set a low threshold for cytoplasmic staining intensity (Green cell tracker), retained both by cells and LEVs, based on the fluorescence intensity of the unstained sample. Single-colour controls for either cytoplasmic or nuclear signal were also used to enable the formation of two areas, one positive both for cytoplasmic and nuclear stains (cells) and one positive only for cytoplasm staining (LEVs). Quantification of cell numbers in each condition was calculated by dividing the overall number of cells to the overall number of cells and LEVs, as provided by the software. Additionally, for relevant experiments, the number of purified LEVs was enumerated using the flow cytometer, by quantifying the number of fluorescent LEVs.

4.2.5 RNA extraction and real-time PCR

1 ml of LEVs suspension counted with flow cytometry (200.000 LEVs/ml) were centrifuged at 10000 x g for 30 min and total RNA was isolated using a RNeasy Plus Mini Kit (Qiagen, US), according to manufacturer instructions. The purity and quantity of harvested total RNA was assessed and quantified using Nanophotometer (IMPLEN, USA). 500 ng of extracted RNA was reverse transcribed using iScript cDNA synthesis kit (Bio rad laboratories, UK). 20 ng of reverse transcribed cDNA was loaded in 96-well pcr plates Real-time PCR was performed on Applied Biosystems™ StepOne™ Real-time PCR System (Fischer Scientific, UK) using SYBR Green PCR master mix (ThermoFischer Scientific, UK). Extracted RNA was tested for the presence or absence of several mRNA molecules, including Interleukin-6 (IL-6), Fibronectin (FN), Vimentin, cluster of differentiation 44 (CD44), C-X-C motif chemokine ligand 10 (CXCL10), Aldehyde dehydrogenase 1 (ALDH1), B-cell lymphoma 2 (BCL2) and Ribosomal protein large P (RPLO), which was used as a housekeeping gene. Designed forward and reverse primers can be seen in **Table 4-2**.

Table 4-2 Forward and reverse primer sequences

Primer name	Forward primer	Reverse primer
IL-6	GGTCCAGTTGCCTTCTCCCTG	TGCCCATGCTACATTGCGG
CXCL10	TGAGCCTACAGCAGAGGAACC	GCCTCTGTGTGGTCCATCCTT
FN	GATGCACCATCCAACCTGCG	GATTGAGTCCCGGACCGTGT
ALDH1	CCCATCACAGGAGAGAAC	CCCTTAAATGCCATCCTG
BCL2	CAAGAACTTCTACGACAGC	AAGCCATTTTCCTCTTCTTG
CD44	TTATCAGGAGACCAAGACAC	ATCAGCCATTCTGGAATTTG
Vim	GGAAACTAATCTGGATTCACTC	CATCTCTAGTTTCAACCGTC
RPLO	CGGTTTCTGATTGGCTAC	ACGATGTCACTTCCACG

4.2.6 Protein concentration assay

Cells post-shedding and LEVs were separated, as previously described. Control cells that had not gone through microfluidic devices were also used at equal numbers with post-shedding cells. Cells or LEVs were centrifuged at their respective speeds at 180 x g for 5 min or 10000 x g for 30 min, respectively, to remove media. Each fraction was washed 1x with cold PBS (4°C)

and after PBS removal by centrifugation (as above), 300 µl of radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fischer Scientific, UK) was used to resuspend the pellets. The lysis buffer was incubated with cells or LEVs for 15 min on a box filled with ice, while shaken in a plate shaker (200 rpm). All lysed suspensions were further centrifuged separately at 14000 x g for 15 min to ensure that the samples were clear of debris. The protein lysate was collected from the supernatant and stored at -80 °C or was used directly for downstream analyses.

Total protein levels of each sample of interest were quantified using Bicinchoninic acid (BCA) assay kit (LIFE TECHNOLOGIES, UK). A standard solution of Bovine serum albumin (BSA) was used at various concentrations, as per manufacturer's instructions. 25 ml of either standard solution with known concentrations or of samples of interest with unknown concentrations were transferred in separate wells of a 96-well plate. The working reagent was formulated by mixing 9.8 ml of part 1 and 0.2 ml of part 2 (from the kit). 200 µl of working reagent were transferred across each well. The 96-well plate was shaken for a few seconds and then was transferred in an incubator for at least 30 min at 37°C. Then, the plate was cooled down for 10 min and the optical density of samples was measured at 562 nm, using a Varioskan Flash absorbance plate reader (ThermoFischer Scientific, UK). The standard curve was generated, by plotting the absorbance values and known concentrations from the BSA samples, as a polynomial second order curve. According to the obtained equation, the concentrations of samples of interest were extrapolated, using the absorbance readings.

4.2.7 Gel electrophoresis

The complete gel preparation for gel electrophoresis required the preparation of two distinct gels, the separation and the stacking gel. For the separating gel, 1.6 ml PBS, 1.3 ml 1.5 M Tris (Life Technologies, UK), 50 µl 10% (v/v) Sodium Dodecyl Sulfate (Thermo Fischer Scientific, UK), 2 ml Acrylamide/Bis-acrylamide 30% (v/v) solution, 2 µl tetramethylethylenediamine (Bio Rad, UK) and 50 µl 10% (v/v) Ammonium Persulfate (Bio Rad, UK) were mixed. For the stacking gel, 1.4 ml PBS, 0.25 ml 1.5 M Tris, 20 µl 10% (v/v) Sodium Dodecyl Sulfate, 0.33 ml Acrylamide/Bis-acrylamide 30% (v/v) solution, 2 µl tetramethylethylenediamine and 20 µl 10% (v/v) Ammonium Persulfate were mixed. Both gels were polymerised for about 20 min at room temperature. A glass cassette (BioRad, UK) was used to enclose both gels and was then transferred and loaded into an electrophoresis chamber (BioRad, UK). The chamber was filled with Tris-Acetate-Ethylenediaminetetraacetic acid (EDTA) buffer, until the area of the

gel was fully covered. 15 μ l of protein lysate (250 μ g/ml concentration) was mixed with 5 μ l Laemmli protein buffer (Bio Rad, UK) and resuspended well. Then, the mixed solution was heated at 95 $^{\circ}$ C for 5 min and 12 μ l of each sample was released in each well of the gel. 5 μ l of a protein ladder solution (BioRad, UK) was also added, as a standard indicator of proteins' molecular weight in kDa. The electrophoresis chamber was connected to power and the samples were run for 2 hr at 125 V and 400 A. At the end of the electrophoresis, the glass cassette was removed from the chamber and the gel was removed carefully to evade damage. The gel was placed in a box and 50 ml of Coomassie Blue (BioRad, UK) were added for 1 hr at room temperature on a plate shaker. After removal of the staining solution, the gel was destained using an equal amount of Coomassie brilliant blue R-250 (BioRad, UK) in a similar manner. Images of the gel were obtained via a cell camera.

4.2.8 Post capillary cell growth & proliferation

0.5×10^6 cells were added in microfluidic devices and collected as described above. Cells were pelleted at 180 x g after 5 min centrifugation and were counted using a haemocytometer. 250 μ l of cells (0.4×10^6 cells/ml) were poured in separate wells of a 48-well plate to observe growth. Control cells (no transit through devices) were also added in equal numbers in separate wells. Images of the entire well were obtained using the Nikon microscope. Moreover, 3-4 representative images were obtained at 20 x magnification over the course of 7 days. In addition, cells were harvested from the wells, as described earlier and were counted using a haemocytometer.

4.2.9 Sample preparation and mass spectrometry-based proteomics

500,000 control cells, cells post-shedding and purified LEVs were prepared as previously. Cells and LEVs were centrifuged at 180 x g for 5 min or at 10,000 x g for 30 min, respectively, so that media was removed and then they were washed once with cold PBS for 5 min. Samples that were pelleted were then lysed by 200 μ l addition of freshly prepared 8M urea (SIGMA-ALDRICH, USA). Samples were lysed on ice for 20 min and were vortexed briefly throughout. Samples were then stored at -80 $^{\circ}$ C. Protein quantification was performed using Pierce™ Protein Assay Kit (Thermo Scientific, USA), as described elsewhere.

150 μ l of 0.4 μ g/ μ l protein was used per sample for downstream processing and digestion. Disulfide bonds were initially reduced by adding Dithiothreitol (DTT, SIGMA-ALDRICH, USA) at a working concentration of 10 mM. Samples were incubated for 40 min at 50 °C. Then, samples were alkylated using 50 mM working concentration of iodacetamide (IAA, SIGMA-ALDRICH, USA) and further incubated at room temperature in the dark for half an hour. After this step, samples were diluted with 100 mM ammonium bicarbonate (ABC), so that a final concentration of 2 M urea would be reached. Trypsin digestion followed by formulating a trypsin solution (Pierce™ Trypsin Protease MS-grade, Thermo Scientific, UK) at 100 mM ABC solution. 1 μ g/ μ l of trypsin was used per 100 μ g of total protein. Protein digestion using the trypsin solution occurred for 18 hours at 37 °C. At the end of this timeframe, trypsin was saturated with 0.5% (v/v) Trifluoroacetic acid (TFA, Fisher Scientific, UK) and all digested protein samples were desalted using SepPak C18 Plus cartridges (Waters, Milford, USA). As the last step, obtained peptides were dried using a speedvac concentrator (Savant™ SC250EXP SpeedVac™, Thermo Scientific) and then stored at -80°C for later use.

For mass spectrometry analysis, peptides were initially dissolved in trifluoroacetic acid (TFA) at a concentration of 200 ng/ μ L (0.1%) for liquid chromatography–mass spectrometry (LC–MS) Data Independent Acquisition (DIA) analysis. DIA analysis was done using an UltiMate 3000 system that was coupled with the Orbitrap Ascend Mass Spectrometer (Thermo Scientific, UK). A 25 cm capillary column (Waters, nanoE MZ PST BEH130 C18, 1.7 μ m, 75 μ m $\times$ 250 mm) was used. Across all samples, 1 μ g of peptide was analysed. DIA analysis was performed over a 100 min gradient, 5%-35% mobile phase B composed of 80% acetonitrile 0.1% formic acid. MS1 spectra were collected with mass resolution of 60K in the m/z range of 380-985, with Maximum Injection Time 100 ms and AGC 4 $\times$ 10⁵. DIA MS2 spectra were collected with higher energy collisional dissociation (HCD) fragmentation CE 32%, orbitrap resolution 15K with isolation window 10 m/z and 1 m/z overlap, Maximum Injection Time 40 ms and AGC 1 $\times$ 10⁵. Raw data were processed in the DIA-NN software version 1.8.1 for protein identification and quantification using a fasta file containing reviewed UniProt human proteins (Rosenberger et al., 2014). Carbamidomethylation of C and oxidation of M were defined as fixed and variable modifications respectively. Retention time (RT)-dependent cross-run normalization was selected with match between run (MBR) enabled and proteins were filtered at 1% False discovery rate (FDR) Gene identifier names (Gene IDs) were filtered to only include proteins

identified in at least 75% of samples and missing values were imputed using half minimum value per protein. Dataset was log2 transformed, mean centred across samples and median centred within each sample. All analysis was performed using R (version 4.3.1) unless otherwise stated.

4.2.10 Graphs & Statistical analyses

Graphs and data were plotted and statistically compared as described elaborately in Chapter 2. BioRender was used for generating illustrations. All experiments were run at least three times.

4.3 RESULTS

4.3.1 Purification of LMPs through filtration and centrifugation

To study the population of generated LMPs, primarily I sought out to purify them from cells. Thus, I devised a methodology of continuous introduction of MDA-MB-231 breast cancer cells in microfluidic devices to collect large numbers of both cells and shear-derived LMPs. The effluent collection sample, which comprised both cells and LMPs was passed through 10 μm pore filters to purify LMPs from cells. Fluorescent images before and after filtration were obtained and enumeration of cells in both conditions revealed that while the number of cells was large before filtration, there were no cells identified post-filtration (**Fig. 4-1A-C**), observed and quantified by lack of nuclei-positive cells.

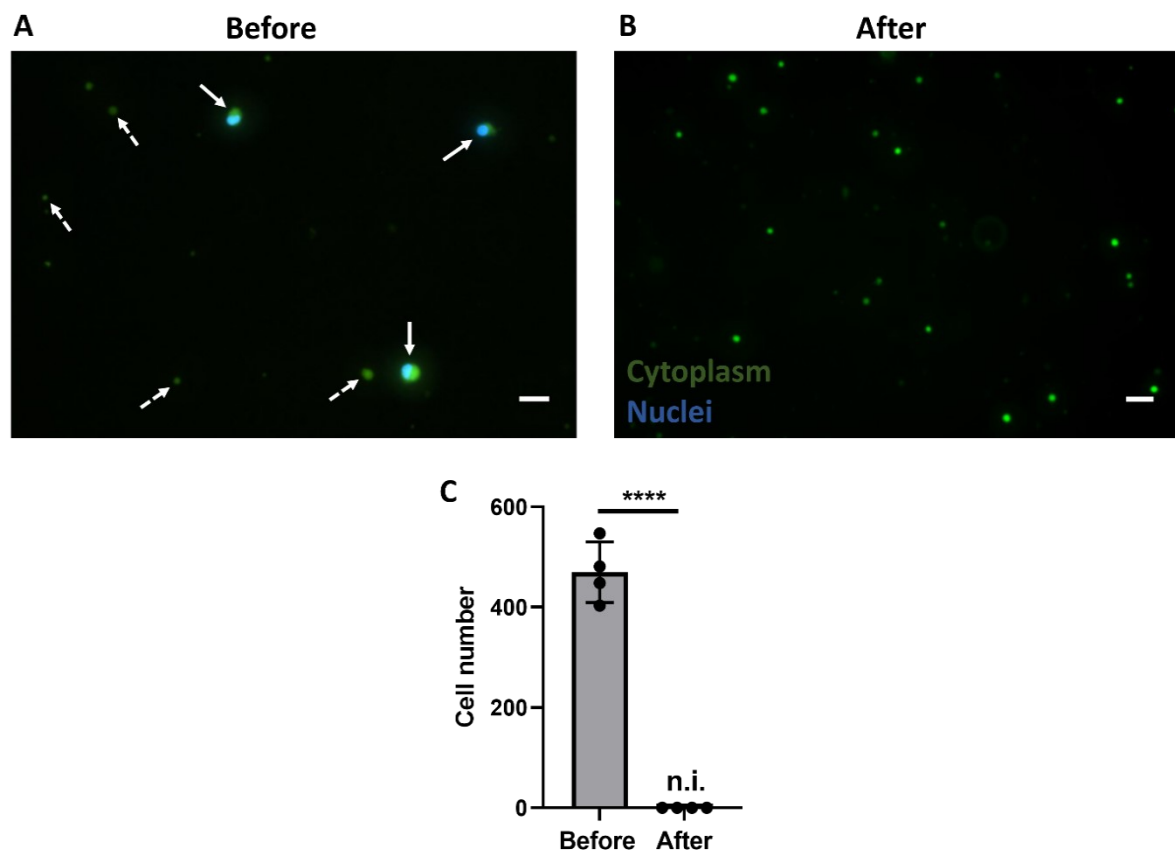


Figure 4-1: Purification of LMPs via filtration.

(A) Multifluorescent image of MDA-MB 231 cells and shed LMPs collected post transit from microfluidic devices. Cytoplasm is stained green and nuclei blue. Image is before filtration. Complete arrows indicate nuclei-positive cells and dashed arrows indicate nuclei-free LMPs. Scale bar: 20 μm . (B) Multifluorescent image of MDA-MB 231 cells and shed LMPs after filtration. Cytoplasm is stained

green and nuclei blue. Scale bar: 20 μm . Image is after filtration. (C) Quantification of cell number in images before and after (n=4 biological replicates for all).

Alternatively, centrifugation was used to purify and separate cells and LMPs post-centrifugation (**Fig. 4-2A**). Post-centrifugation, cells were retrieved in the pellet, whereas LMPs in the supernatant (**Fig. 4-2A**). Via flow cytometry, cell/LMPs populations were gated and only events above a gated cytoplasmic staining intensity were further selected, revealing two distinct populations (**Fig. 4-2B-C**). Then, using nuclear staining, cells and LMPs presence was calculated: a. before centrifugation, b. after centrifugation (pellet only) and c. after centrifugation (supernatant only) (**Fig. 4-2H-I**). Dot plots and histograms before centrifugation reveal the presence of both nuclei-positive and nuclei-negative populations (**Fig. 4-2D-E**), whereas in the supernatant post-centrifugation only one nuclei-negative population was identified (**Fig. 4-2F-G**). In the pellet, cell presence was increased compared to before centrifugation ($93.1 \pm 1\%$ vs $80.7 \pm 1.8\%$, $P < 0.0001$), whereas LMP presence was significantly decreased almost by two thirds ($19.3 \pm 1.8\%$ vs $6.8 \pm 1\%$, $P < 0.0001$) (**Fig. 4-2H-I**). On the other hand, in the supernatant fraction, cell presence was decreased almost entirely compared to before centrifugation ($80.7 \pm 1.8\%$ vs $0.21 \pm 0.3\%$, $P < 0.0001$), whereas LMPs presence was increased about 8-fold ($19.3 \pm 1.8\%$ vs $99.8 \pm 0.3\%$, $P < 0.0001$), highlighting that LMPs had overall a purity $>99.8\%$ in the collected supernatant (**Fig. 4-2H-I**).

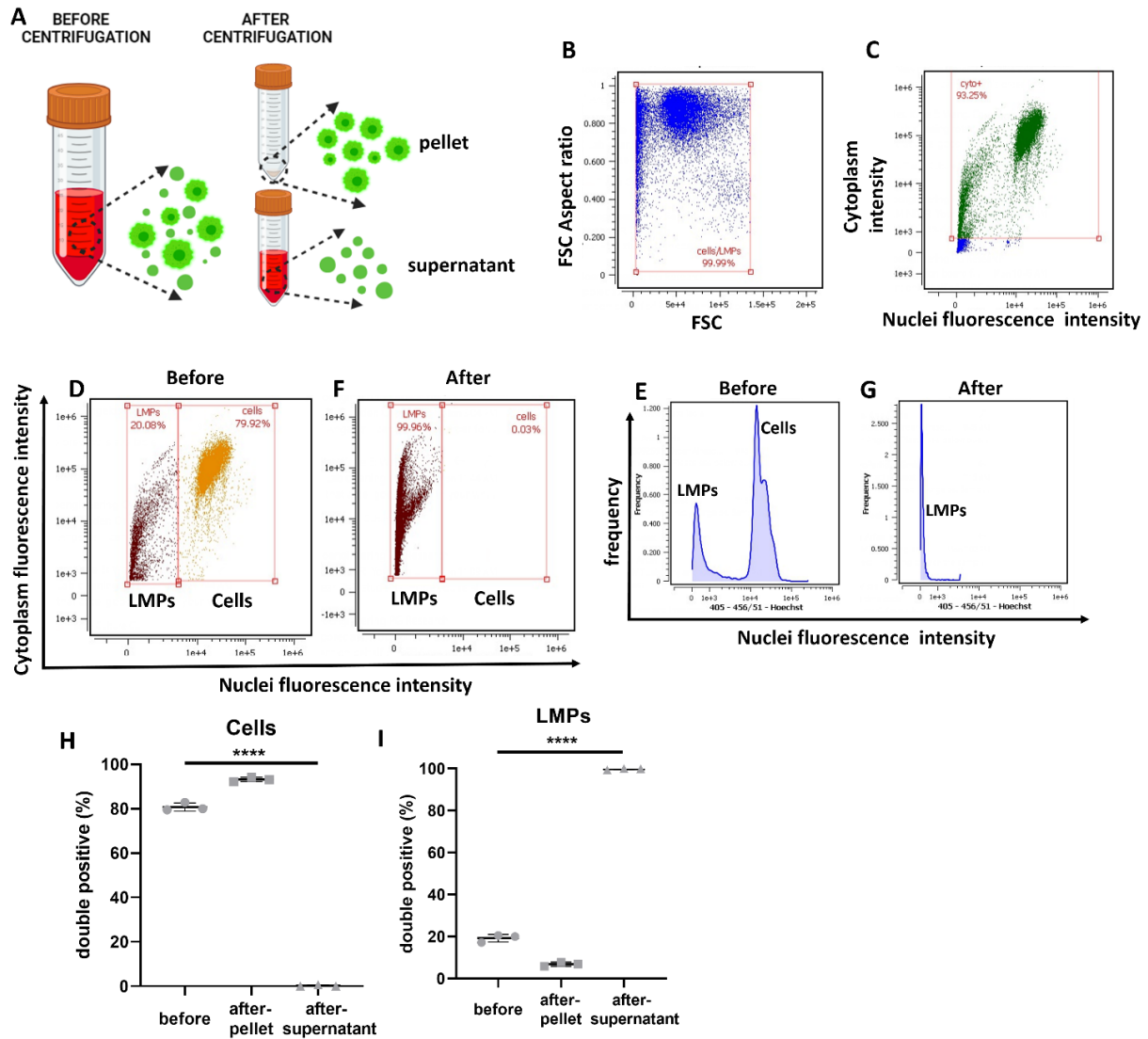


Figure 4-2: Purification of LMPs via centrifugation.

(A) Illustration of centrifugation (180x g for 5 min) as a LMPs purification strategy. (B-I) All data are obtained via flow cytometry. (B) Dot plot of cells and LMPs analysed. Forward scatter indicating size on x axis and forward scatter aspect ratio on y axis. (C) Dot plot of gated cells and LMPs that were positive for cytoplasm stain. (D) Dot plot of cells and LMPs based on nuclei gating criterion before centrifugation. (E) Histogram of cells and LMPs based on nuclei gating criterion before centrifugation. (F) Dot plot of cells and LMPs based on nuclei gating criterion after centrifugation. (G) Histogram of cells and LMPs based on nuclei gating criterion after centrifugation. (H) Quantification of percentage of cells present before centrifugation, after centrifugation in the pellet and after centrifugation in the supernatant (n=3 biological replicates for all). (I) Quantification of percentage of LMPs present before centrifugation, after centrifugation in the pellet and after centrifugation in the supernatant (n=3 biological replicates for all).

4.3.2 LMPs are large extracellular vesicles

LMPs visualisation resembles the definition criteria for extracellular vesicles, denoted by both a lack of a nuclei organelle and their release from cells. Therefore, to determine whether these LMPs are indeed LEVs, I compared the biomolecular constituents of LMPs to the guidelines and classification criteria that were established in the minimal information for studies of extracellular vesicles (MISEV 2018) (78). Firstly, the large size and morphology of LMPs was verified by obtaining a high-resolution scanning electron microscopy-based image of an LMP and by measuring the size of LMPs via fluorescence images, as shown in Chapter 2 (Fig. 2-2E&H).

To complete the set of criteria for LEVs classification, MISEV2018 guidelines recommend the general characterisation of protein markers (78). Thus, I stained LMPs for both transmembrane (CD81) and cytosolic (HSP90, CK18) proteins to verify the lipid bilayer structure. LMPs expressed higher means of fluorescence intensity for all 3 tested proteins compared to non-antibody stained LEVs (**Non-antibody**: 729 ± 297 vs **CD81**: 9600 ± 1243 , $P < 0.0005$; **Non-antibody**: 729 ± 297 vs **CK18**: 20431 ± 4136 , $P < 0.005$; **Non-antibody**: 729 ± 297 vs **HSP90**: 35936 ± 16158 , $P < 0.05$) (Fig. 4-3A-B). To quantify the expression of these proteins on individual LEVs, the relevant histograms were generated (Fig. 4-4A). The double positive events were significantly higher for all 3 tested proteins compared to non-antibody stained LEVs populations (**Non-antibody**: $0.72 \pm 0.47\%$ vs **CD81**: $71.5 \pm 2.1\%$, $P < 0.0001$; **Non-antibody**: $0.72 \pm 0.47\%$ vs **CK18**: $89.5 \pm 4.1\%$, $P < 0.0001$; **Non-antibody**: $0.72 \pm 0.47\%$ vs **HSP90**: $83.5 \pm 3.4\%$, $P < 0.0001$) (Fig. 4-4B), allowing the acquisition of the LEV terminology, upon fulfilment of all EV classification criteria. Moreover, cells were both imaged and analysed via flow cytometry, validating the expression of the tested proteins on cells as well (Fig. 4-3C-F & Fig. 4-4C-D). Nuclei-negative LEVs were additionally visualised for cytoskeletal (F-actin and Cytokeratin 18) and cytosolic (HSP90) proteins, whose presence bolsters the notion that LEVs may in fact contain several proteins from their cell of origin (Fig. 4-5A-C).

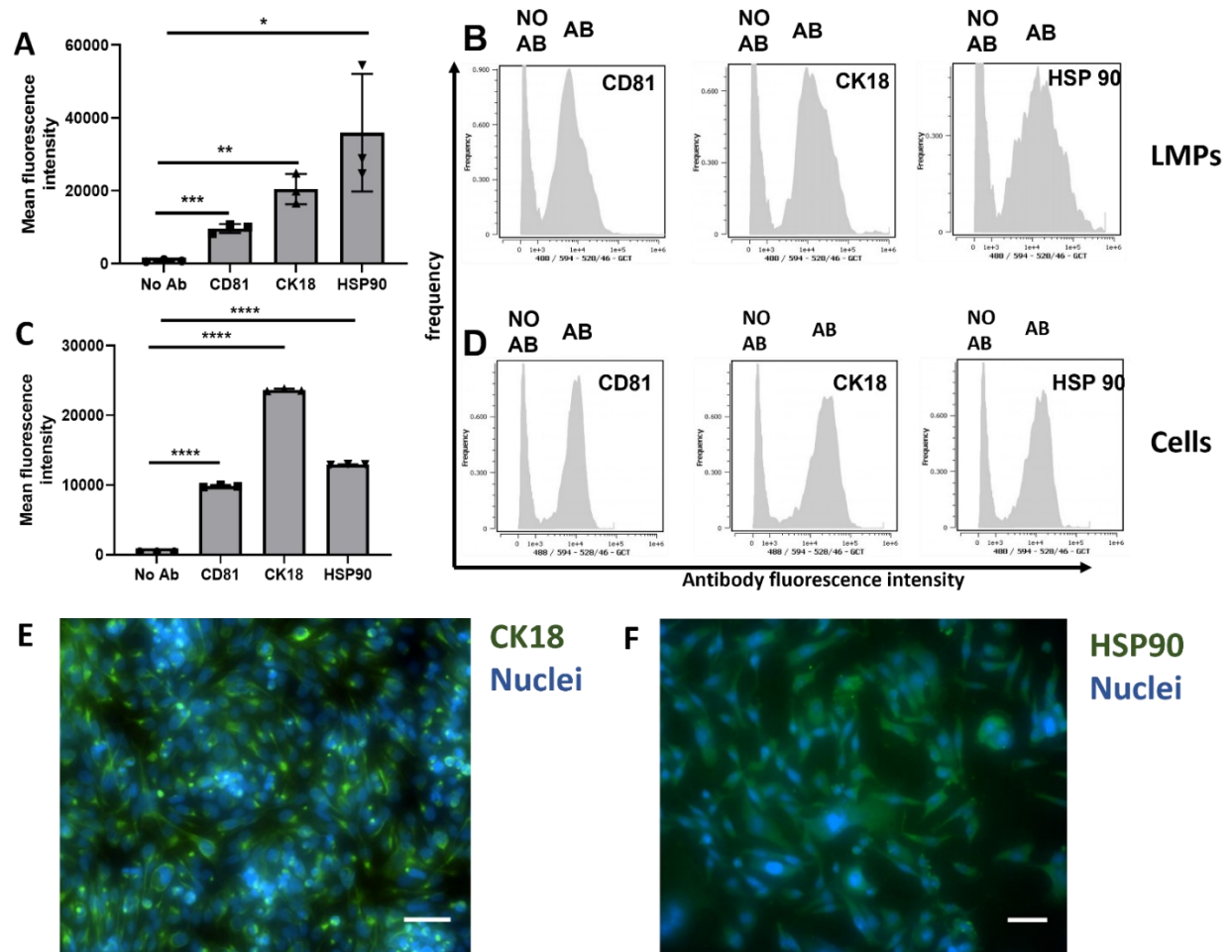


Figure 4-3: Expression of proteins on LMPs and cells.

(A) Quantification of mean fluorescence intensity of non-antibody stained or CD81, CK18 and HSP90 stained LMPs (n=3 biological replicates for all). No antibody is designated as NO AB. (B) Relevant histograms of (A). (C) Quantification of mean fluorescence intensity of non-antibody stained or CD81, CK18 and HSP90 stained cells (n=3 biological replicates for all). No antibody is designated as NO AB. (D) Relevant histograms of (C). (E) Multifluorescent images of cells stained for CK18 (green) and nuclei (blue), obtained at 20x. Scale bar: 50 μ m. (F) Multifluorescent images of cells stained for HSP90 (green) and nuclei (blue), obtained at 20x. Scale bar: 50 μ m.

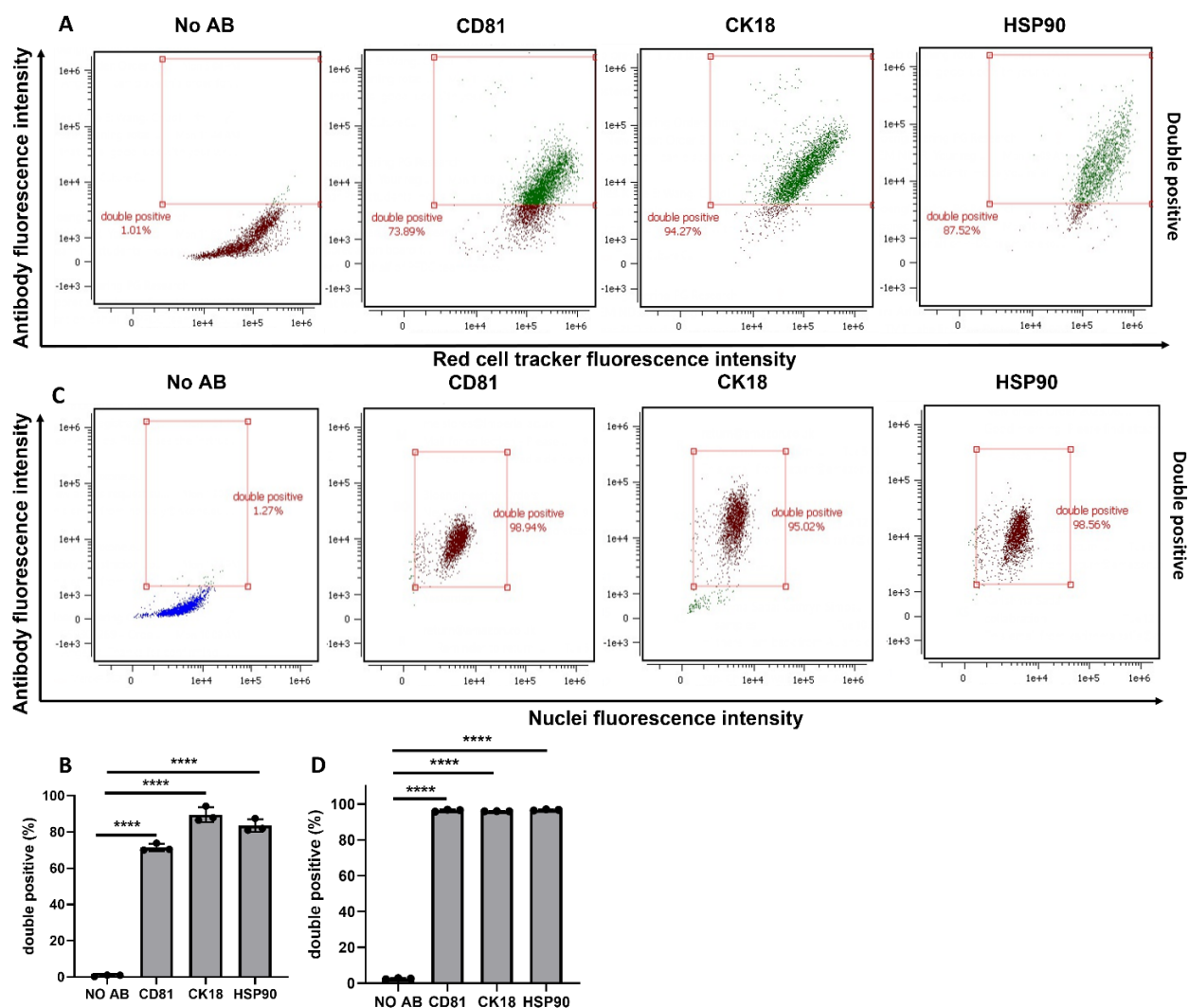


Figure 4-4: Expression of proteins on single-LMP and single cell level.

(A) Percentage of pre-stained LMPs that were positive for CK18, CD81 or HSP90, depicted in dot plots generated by flow cytometry (n=3 biological replicates for all). No antibody is designated as NO AB. (B) Quantitative plots of (A). (C) Percentage of nuclei-positive cells that were positive for CK18, CD81 or HSP90, depicted in dot plot generated via flow cytometry (n=3 biological replicates for all). (D) Quantitative plots of (C).

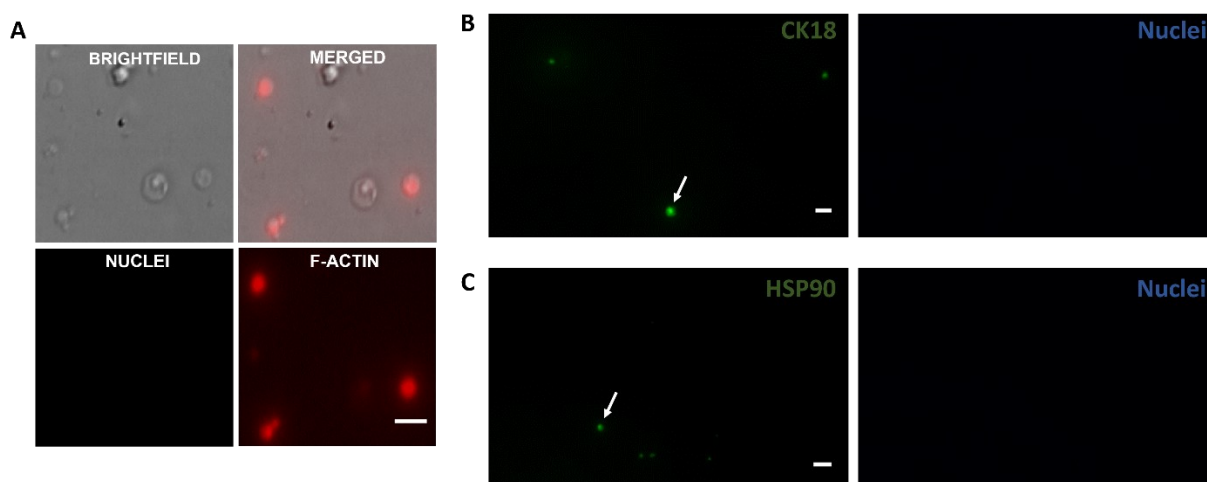


Figure 4-5: Fluorescent imaging of LEVs' proteins.

(A-C) Immunofluorescent staining of purified LEVs. (A) F-actin was stained red and nuclei blue. Scale bar: 10 μ m. (B) CK18 was stained green and nuclei blue. Scale bar: 10 μ m. (C) HSP90 was stained green and nuclei blue. Scale bar: 20 μ m.

4.3.3 Cells lose protein content during shedding but retain proliferation ability

As previously shown in Chapter 2, the volume of cells post-shedding was reduced significantly. Thus, I wondered whether this reduction affects further cells by reducing their protein content. As previously described, the separation and retrieval of both LEVs and post-shedding populations, allowed me to compare their total protein levels, compared against control cells. Primarily, I measured a significant loss (~18%) of protein concentration between control cells and cells post-transit (802.8 ± 8.65 μ g/ml vs 658.8 ± 7.63 μ g/ml, $P < 0.0001$) (**Fig. 4-6A-C**), matching the average cell volume loss calculated previously, being about 20% (**Fig. 2-8E**). As anticipated, purified LEVs also carried a significant amount of protein content, representing about 10% of the original protein content of control cells (83.63 ± 0.75 μ g/ml) (**Fig. 4-6A-C**). Running the protein samples in a protein gel, verified the results, with both cell samples presenting a vaster array of protein bands compared to LEVs, while control cells appeared to have higher intensity for several protein bands compared to cells post-transit (**Fig. 4-7A-B**). LEVs were also rich in diverse protein bands, especially at lower molecular weights (**Fig. 4-7B**), with some distinct from their cell counterparts at 10 or 75 kDa, (**Fig. 4-7C**), suggesting potential enrichment patterns.

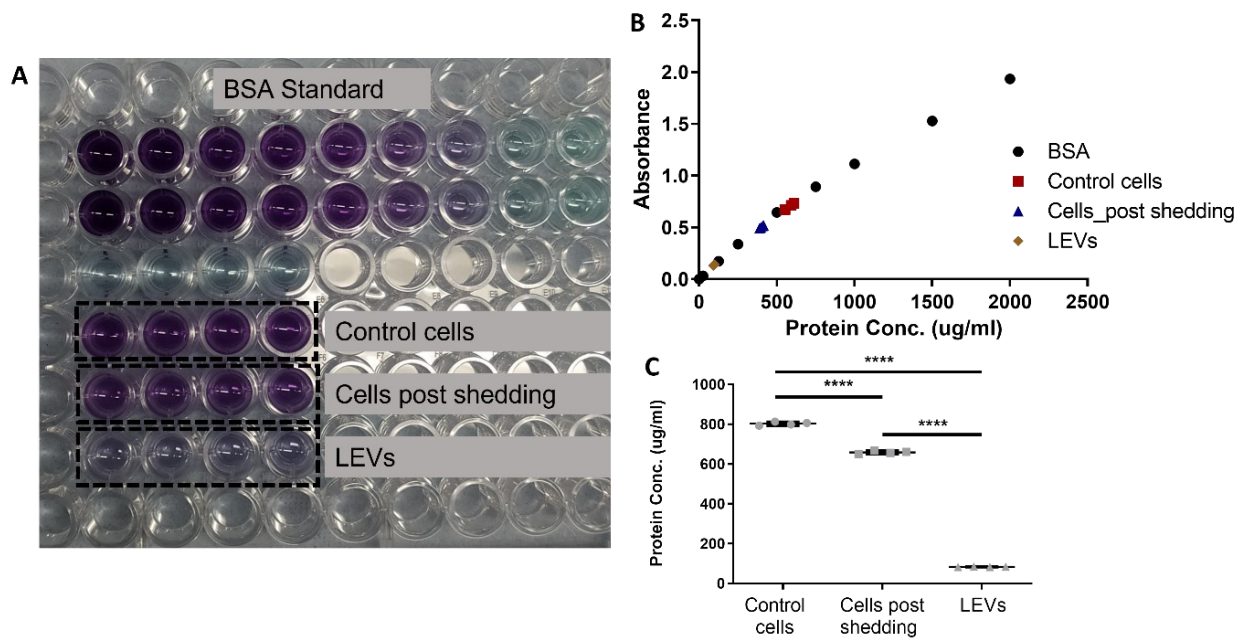


Figure 4-6: Total protein levels of cells and LEVs.

(A) BCA assay for estimation of protein concentration across each sample. (B) Standard curve of BSA and unknown samples: control cells, post-shedding cells and LEVs, plotted as protein concentration ($\mu\text{g/ml}$) in x axis vs absorbance in y axis ($n=4$ biological replicates for all). (C) Quantification of protein concentrations of all 3 above experimental conditions ($n=4$ biological replicates for all).

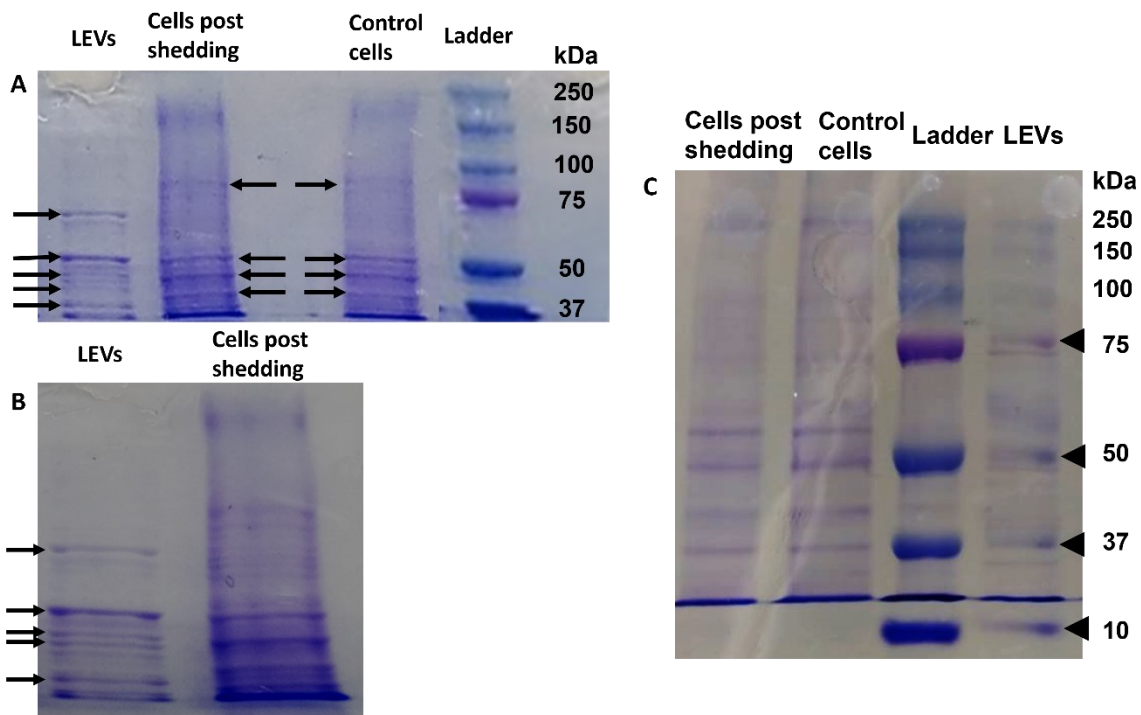


Figure 4-7: Protein blots of cells and LEVs.

(A) First protein blot of control cells, post shedding cells and LEVs, including a ladder for the estimation of molecular weight of proteins. (B) Zoomed image of first protein blot of post-shedding cells and LEVs. (C) Second protein blot of control cells, post shedding cells and LEVs.

Then, I investigated how the ability of cells post-shedding to proliferate is affected. By comparing images of control cells (no transit through devices) and cells post-shedding, there seems to be no difference in growth (**Fig. 4-8A-B**). Moreover, harvesting cells after 72 hr of proliferation, revealed that the absolute numbers of both conditions were not statistically different (170000 ± 5395 vs 139600 ± 30300 , ns) (**Fig. 4-8C**).

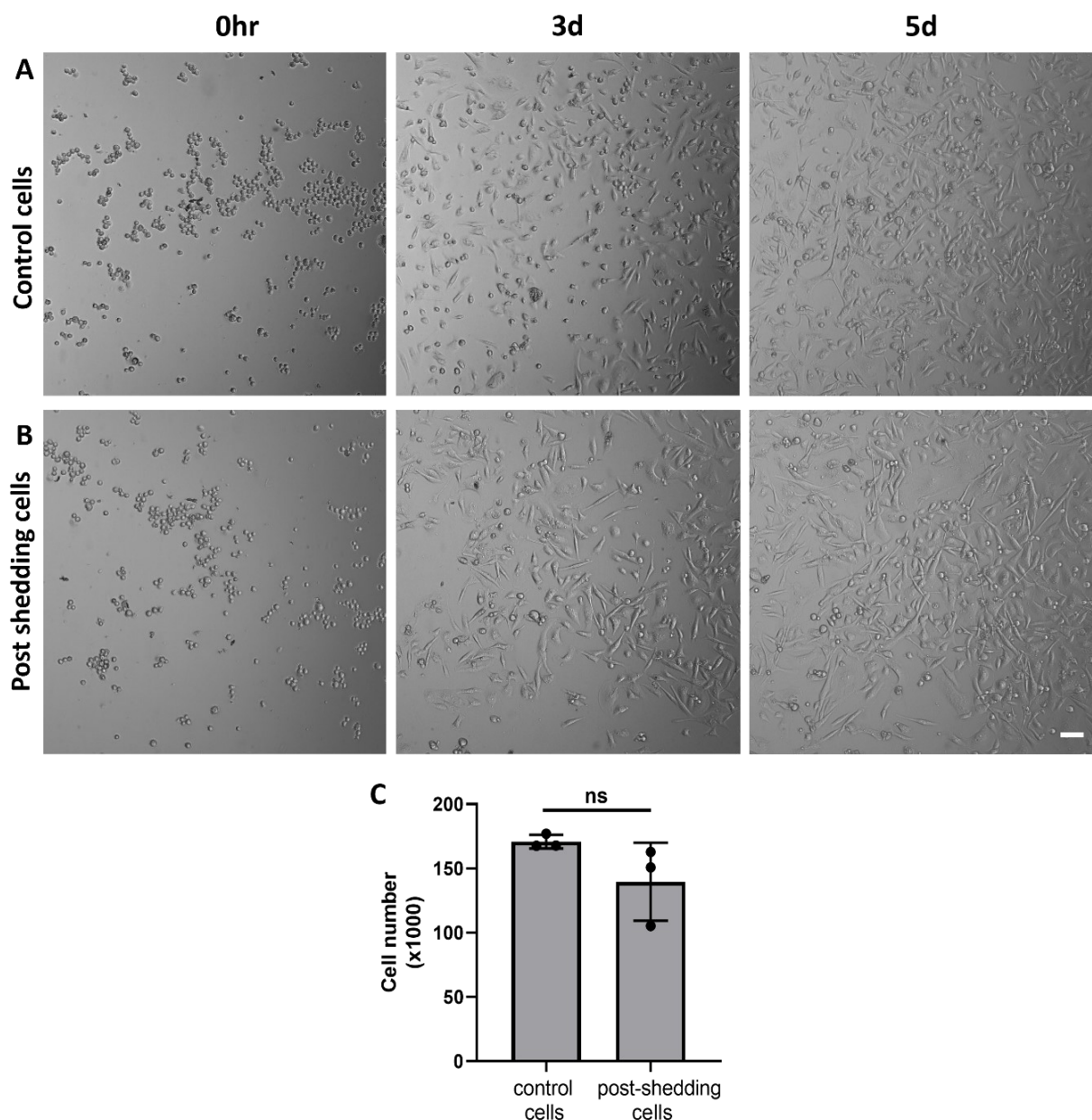


Figure 4-8: Cell growth & proliferation.

(A-B) Scale bar applied for all images: 100 μm . (A) Brightfield image (20x) of control cells (no transit in microfluidic devices) at 0 hr, 3 days and 5 days. Tracking of same spot. (B) Brightfield image (20x) of post-shedding cells (after transit in microfluidic devices) at 0 hr, 3 days and 5 days. Tracking of same spot. (C) Quantification of control or post-shedding cells after harvesting and enumeration at 3 days (n=3 biological replicates for all).

4.3.4 LEVs contain RNA and are positive for various mRNA molecules

Then, I investigated whether LEVs contain other biomolecules, such as nucleic acids, for which other EVs such as exosomes, microvesicles and LOs are (91,96,304). Primarily, I harvested the total RNA from 200000 LEVs, identifying a concentration of 14.4 ng/ μl or overall 432 ng (30 μl overall volume), identifying the presence of total RNA at a high mass for downstream analyses. In addition, the RNA purity was high with a value of A260/A280 at 1.986, which indicates no DNA contamination (**Fig. 4-9A**). Moreover, by analysing the harvested RNA using real-time PCR, several genes, as enlisted in **Table 4-2 & Fig. 4-9B**, were examined, whose presence in the RNA of LEVs was validated against a housekeeping gene. Values close or lower to the ones in the housekeeping gene validate the presence of the specific mRNA molecules (**Fig. 4-9B**). Altogether, these findings highlight that LEVs contain at least two major types of cell biomolecules, proteins and nuclei acids, suggesting that cell volume losses via LEV biogenesis enables the separation of biological material from the cells via LEVs.

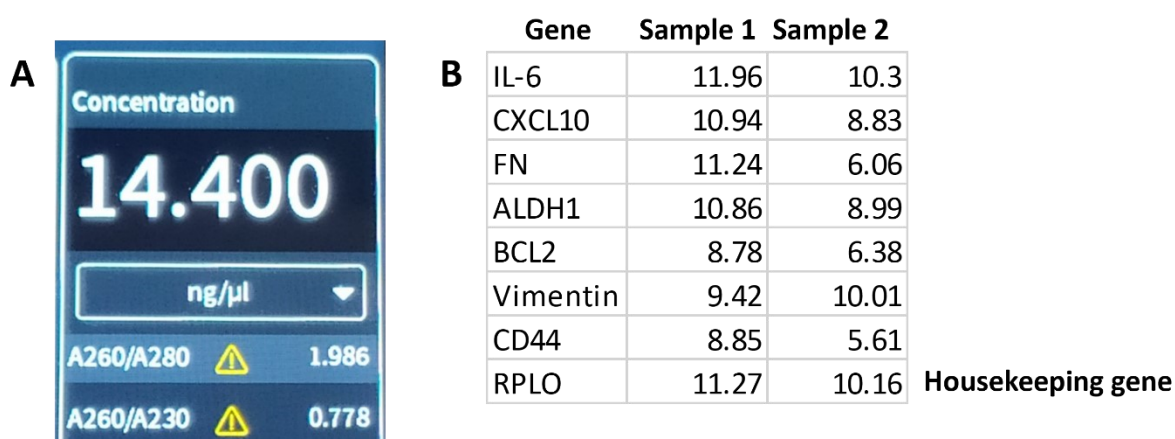


Figure 4-9: Total RNA and mRNA identified in LEVs.

(A) Measurement of quantity and purity (A260/280) of RNA isolated from LEVs. (B) Reverse transcription polymerase chain reaction to validate the presence of several mRNA molecules (n=2 biological replicates for all).

4.3.5 LEVs contain multiple proteins, reflecting their cell of origin

Previous findings on protein incorporation by LEVs, as shown via flow cytometry and protein blots, suggest that LEVs most likely contain a vast array of proteins. Based on the proteome analysis, comparing cells and LEVs proteomes, from the 5569 proteins identified in cell samples, 59.3% (3306/5569) of proteins were found present in LEVs samples (**Fig. 4-10A**). LEVs samples were compared with cells' samples, as depicted in the relevant heatmap for all proteins (**Fig. 4-10B**), highlighting the reduced expression of proteins in LEVs samples, sensible given the LEVs smaller size and their shedding from cells. Interestingly, in **Fig. 4-10C**, a small fraction of proteins, 1.3% (43/3306) of proteins identified in LEVs and cells alike present statistically significant differences, highlighting the enrichment of certain proteins in LEVs, a notion backed in **Fig. 4-7C**.

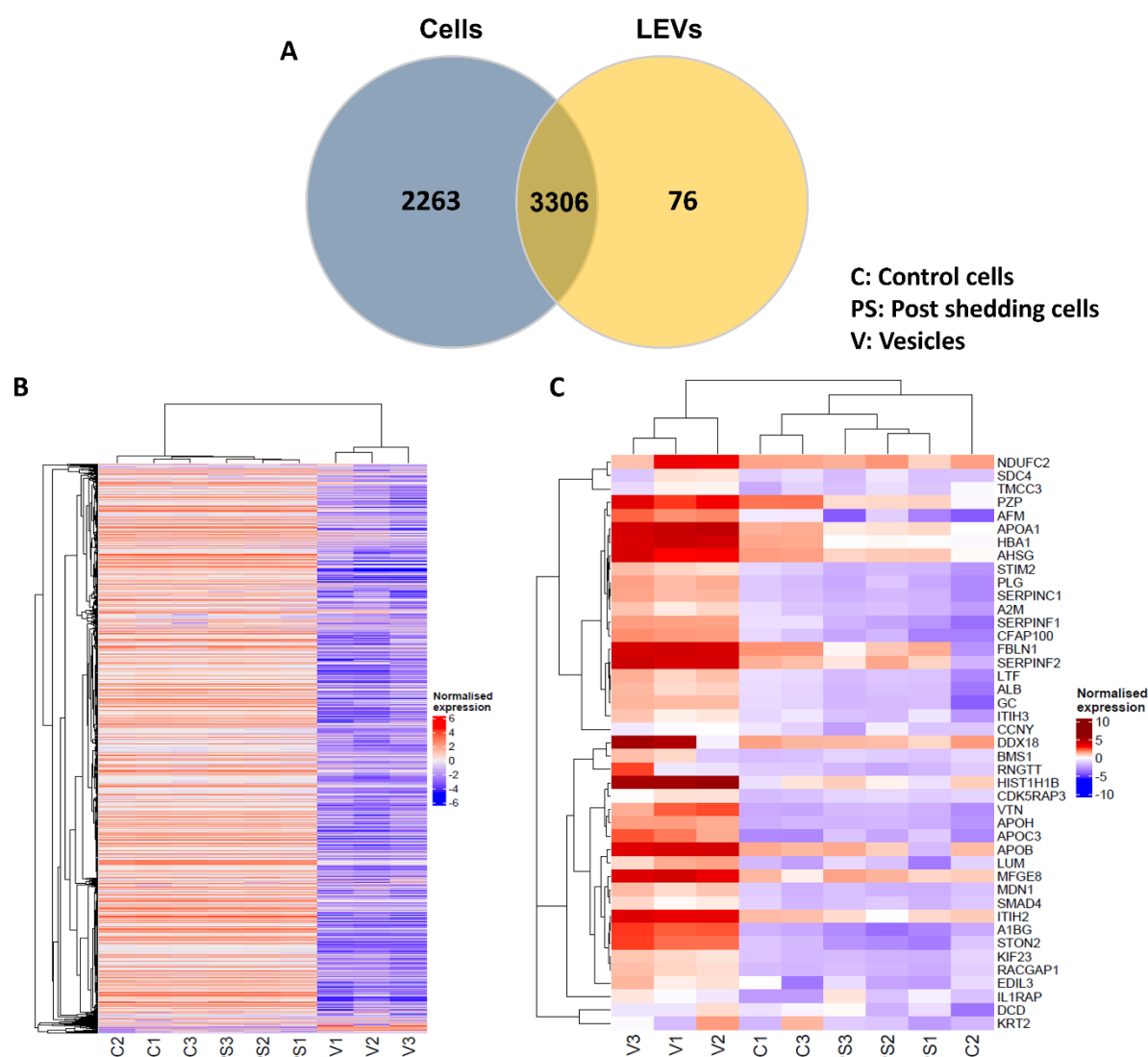


Figure 4-10: Cells and LEVs' proteins overlap.

(A) Venn diagram of overlap of gene IDs between LEVs and cells of origin. Each sample type (n=3) was processed separately using raw data from DIA-NN. (B) Hierarchical clustering using imputed and log2 transformed data (n=3 biological replicates for all). (C) Significance analysis of microarray applied on (B) to receive positive hits (significant) when comparing LEVs to rest of samples (False discovery rate<1%) (n=3 biological replicates for all).

Next, the MISEV2018 guidelines were re-evaluated on a proteome scale for LEVs (**Fig. 4-11**) and for cells (**Appendix 5**). The list of transmembrane or cytosolic proteins per MISEV2018 guidelines was checked against LEVs proteome list. In respect to transmembrane proteins, 75% (27/36) of proteins listed in Category 1A and 31.5% (6/19) of proteins listed in Category 1B, were identified in the LEVs proteome list (**Fig. 4-11A**). In respect to cytosolic proteins, 81%

(26/32) of proteins listed in Category 2A and 100% (25/25) of proteins listed in Category 2B, were identified in the LEVs proteome list (**Fig. 4-11B**).

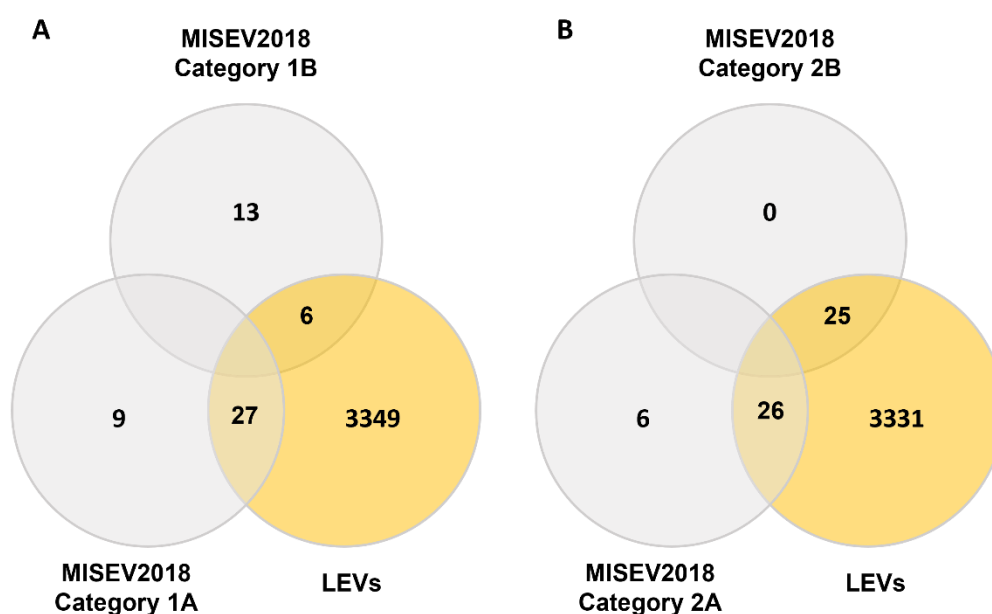


Figure 4-11: LEVs contain multiple proteins per MISEV2018 guidelines on proteome scale.

(A) Venn diagram of number of transmembrane proteins in LEVs that are highlighted in MISEV2018 guidelines, either in Category 1A (non-tissue specific) or category 1B (cell/tissue specific). (B) Venn diagram of number of cytosolic proteins in LEVs that are highlighted in MISEV2018 guidelines, either in Category 2A (proteins with lipid or protein binding ability) or category 2B (promiscuous incorporation in EVs).

4.3.6 LEVs promote cell metabolism and signalling

The large number of proteins found in LEVs suggested the possibility that LEVs may be linked to multiple and distinct biological phenomena. Using a small gene set (50 gene sets), the proteins in LEVs were linked to biological processes, relevant mostly to metabolism (e.g. glycolysis or fatty acid metabolism), signalling, inflammation and protein secretion (**Fig. 4-12A**), with the latter three highlighting potential signalling capabilities to affect their environment. In particular, 19% (38/200) of proteins in LEVs were identified to overlap with proteins relevant in inflammatory responses, as highlighted by the gene ID annotations (**Fig. 4-12B-C**).

Then, by utilising a much larger gene library in respect to biological processes (7647 gene sets), LEVs proteins revealed links again to metabolic pathways, such as generation of

precursor metabolites and energy (**Fig. 4-13A**). In addition, protein targeting and protein localisation were frequent processes, highlighting the nature of EVs (**Fig. 4-13A**). Last but not least, LEVs proteins were linked to immune cells e.g. neutrophils (**Fig. 4-13A**), and altogether these findings insinuate that LEVs are capable for interactions with other cell types. Looking at cell components of LEVs' proteins, investigation of 1015 gene sets, revealed that their proteins are associated with vesicles and extracellular region, suggesting not only the nature of shed LEVs but also LEVs' secretory abilities (**Fig. 4-13B**).

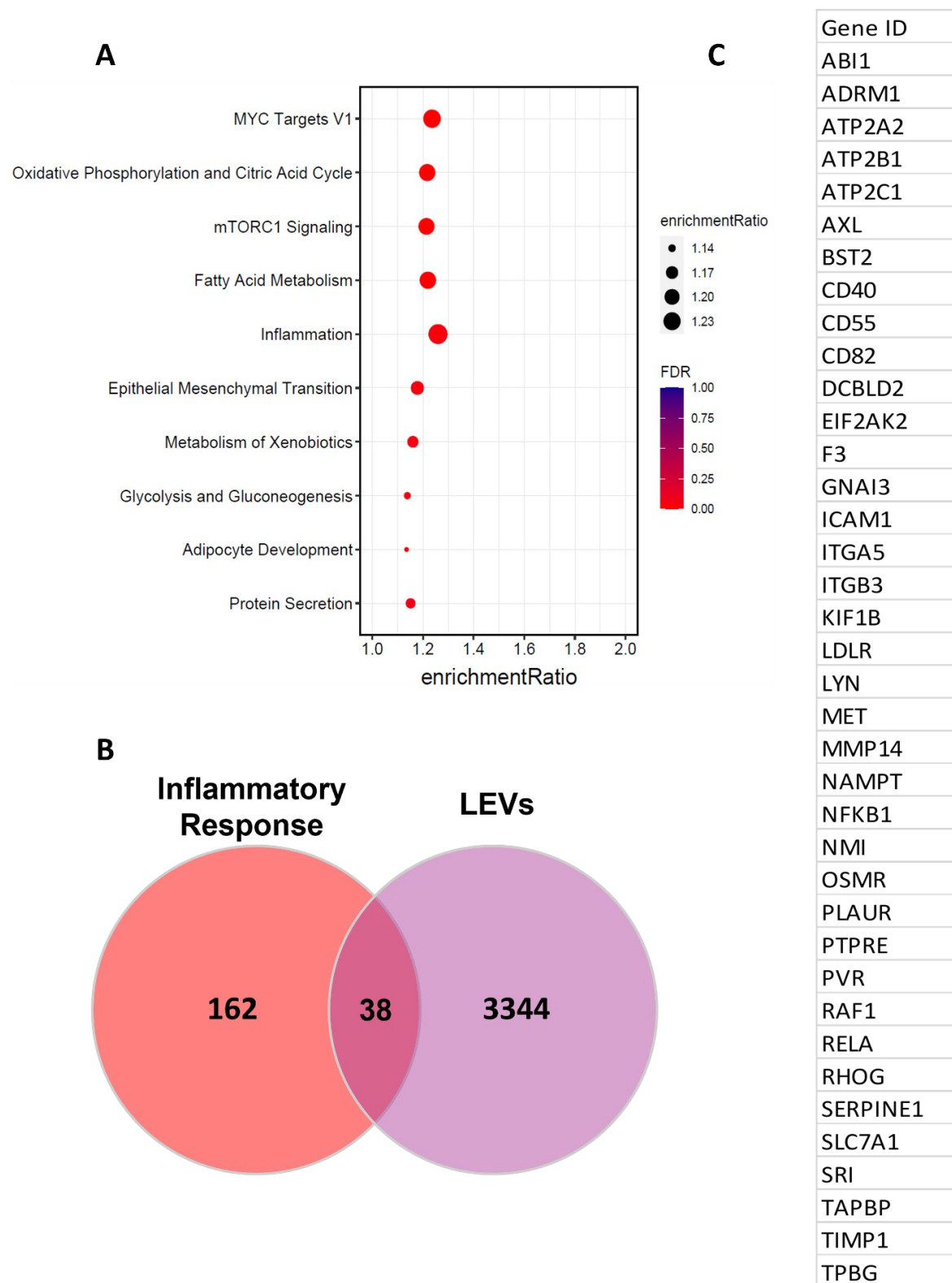


Figure 4-12: LEVs are involved in multiple pathways.

(A) Identification of biological processes enriched in LEVs, using Hallmark H1 gene sets (50 gene sets).
 (B) Venn diagram of number of LEVs proteins that overlap with proteins involved in inflammatory response. (C) List of overlapping proteins of (B).

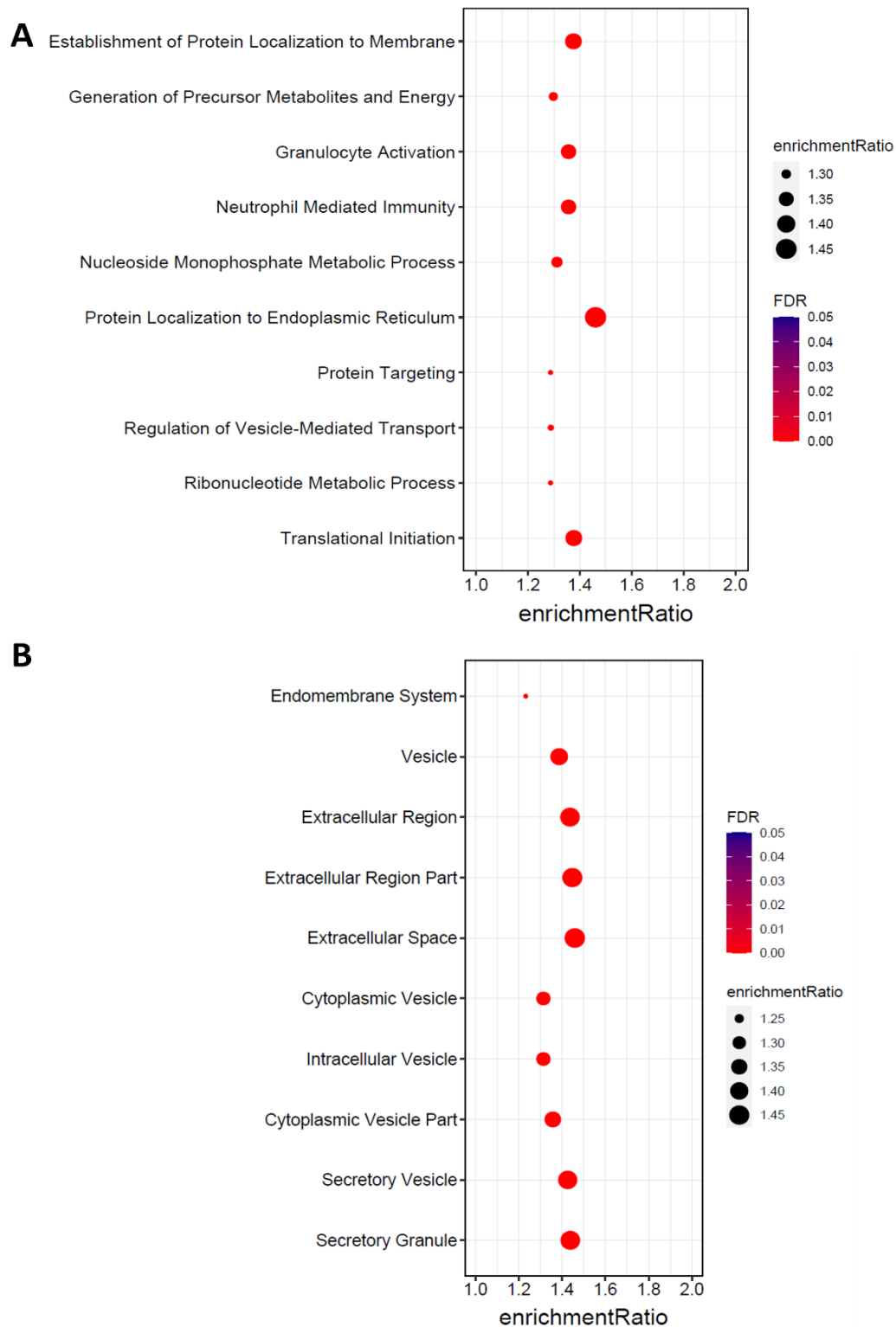


Figure 4-13: Gene ontology analysis on LEVs.

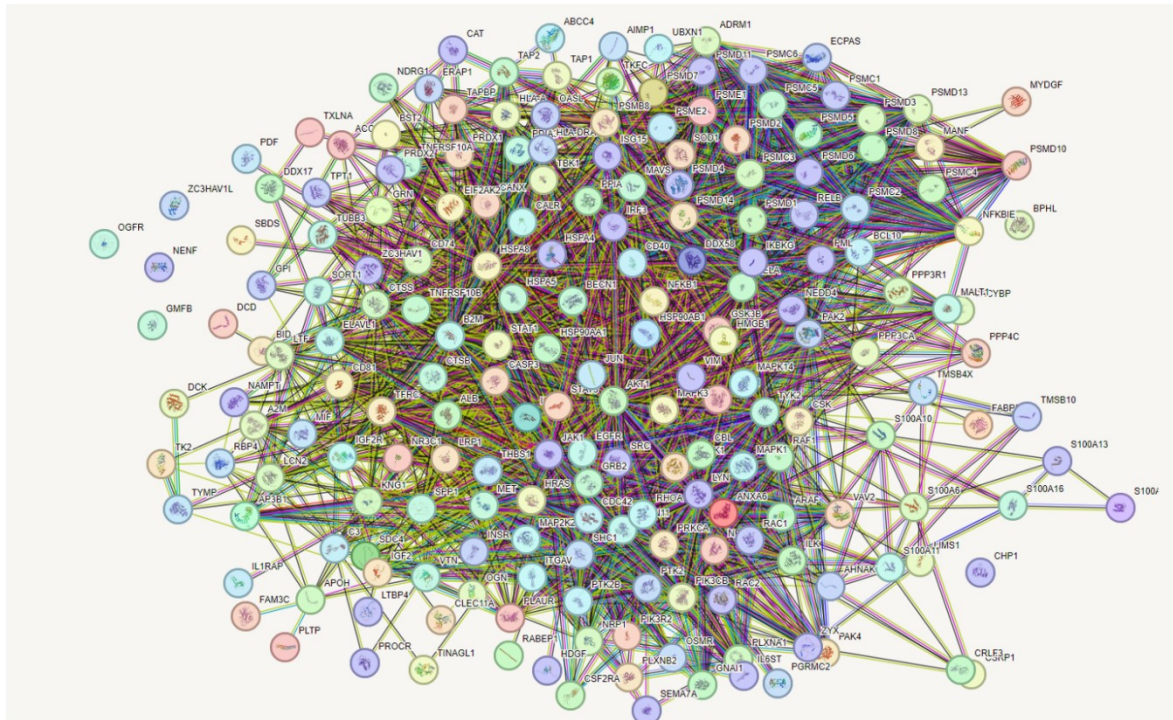
(A) Identification of biological processes enriched in LEVs, using Gene Ontology C5 biological process gene sets (7647 gene sets). (B) Identification of cellular component enriched in LEVs, using Gene Ontology C5 cellular component gene sets (1015 gene sets).

4.3.7 LEVs proteins link to immune-related events

Given the previous suggestions on LEVs potential role on interactions with other cell types and particularly immune cells, more specific analysis was performed. Proteins from LEVs that were identified and correlated to immune-related events (matched to IMMPORT gene lists) were brought together in a STRING protein network, depicting that the majority of proteins found in LEVs samples cooperate with many other proteins inside that network (**Fig. 4-14A**). Many of these LEVs proteins are connected to cytokines and chemokines that can influence immune cells, emphasising the secretory nature of LEVs (**Fig. 4-14B**). Other biological processes on immune cells linked to LEVs proteins were T cell signalling and antigen presentation, strengthening the idea that LEVs can interact with immune cells (**Fig. 4-14B**). More specifically, functional associations of LEVs proteins based on the STRING network, reveal that LEVs contain many proteins that are connected to biological and reactome pathways, relevant to signal transduction (**Fig. 4-15A**). As a paradigm, the reactome pathway 'Cytokine signalling in immune system' contains overall 706 gene IDs, and more than 10% (79 out of 706) of these are found in LEVs (**Fig. 4-15A**). In addition, looking at molecular functions, based on gene ontology data sets, LEVs contain a large number of proteins that are linked to protein and receptor binding, highlighting their capabilities to adhere and interact physically with immune cells (**Fig. 4-15A**).

Lastly, based on the current literature on factors that can stimulate immune cells, specifically monocytes, a list of candidate factors was put together and run against the LEVs proteome list (**Fig. 4-15B**). 57% (16/28) of the candidate factors were identified in LEVs samples, while 11 of these candidate factors were absent from LEVs samples. While microfibril associated protein 1 was not found in LEVs, a very similar protein, actin filament associated protein was identified in LEVs (**Fig. 4-15B**).

A



B

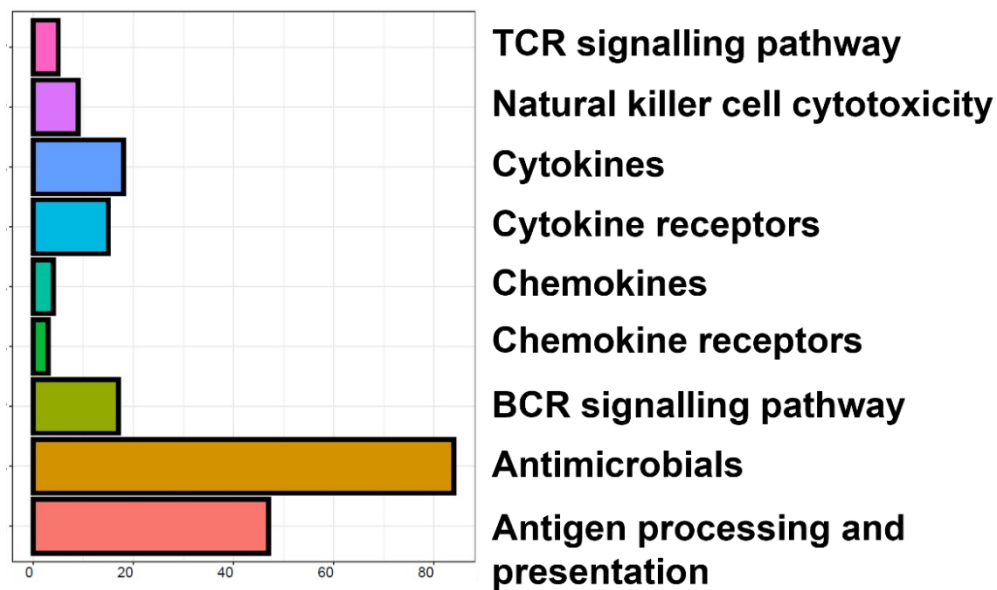


Figure 4-14: LEVs proteins linked to immune-related events.

(A) STRING network visualisation of LEVs proteins that are relevant to immune-related events via checking against IMMPORTE gene lists (4723 genes). (B) Identification of biological pathways enriched in LEVs via STRING enrichment analysis.

A

Functional enrichments in your network

[explain columns](#)

Biological Process (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:1902531	Regulation of intracellular signal transduction	84 of 1726	0.68	4.50e-31
GO:0042221	Response to chemical	122 of 4010	0.47	6.70e-31
GO:1902533	Positive regulation of intracellular signal transduction	65 of 997	0.8	1.06e-29
GO:0002376	Immune system process	89 of 2121	0.61	2.44e-29
GO:0048584	Positive regulation of response to stimulus	89 of 2131	0.61	2.44e-29
(more ...)				
Molecular Function (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0005515	Protein binding	165 of 7242	0.35	5.05e-35
GO:0005102	Signaling receptor binding	72 of 1499	0.67	7.23e-26
GO:0019899	Enzyme binding	80 of 2084	0.57	2.45e-23
GO:0042802	Identical protein binding	72 of 2144	0.52	3.41e-17
GO:0044877	Protein-containing complex binding	53 of 1261	0.61	1.34e-15
Reactome Pathways				
pathway	description	count in network	strength	false discovery rate
HSA-168256	Immune System	118 of 1979	0.76	1.70e-59
HSA-1280215	Cytokine Signaling in Immune system	79 of 706	1.04	9.90e-55
HSA-168249	Innate Immune System	83 of 1041	0.89	2.61e-47
HSA-449147	Signaling by Interleukins	61 of 453	1.12	2.81e-45
HSA-2454202	Fc epsilon receptor (FCER1) signaling	42 of 131	1.5	9.97e-44
(more ...)				

B

Input	Match type	Approved symbol	Approved name	HGNC ID	Location	LEVs overlap
IL-4	Alias symbol	IL4	interleukin 4	HGNC:6014	5q31.1	NOT FOUND
IL-10	Alias symbol	IL10	interleukin 10	HGNC:5962	1q32.1	NOT FOUND
TGFB	Previous symbol	TGFB1	transforming growth factor beta 1	HGNC:11766	19q13.2	TGFB1I1
IL-6	Alias symbol	IL6	interleukin 6	HGNC:6018	7p15.3	IL6ST
IL-8	Alias symbol	CXCL8	C-X-C motif chemokine ligand 8	HGNC:6025	4q13.3	NOT FOUND
M-CSF	Alias symbol	CSF1	colony stimulating factor 1	HGNC:2432	1p13.3	NOT FOUND
GM-CSF	Alias symbol	CSF2	colony stimulating factor 2	HGNC:2434	5q31.1	GM-CSFR
GC	Approved symbol	GC	GC vitamin D binding protein	HGNC:4187	4q13.3	GC
AMP	Alias symbol	MFAP1	microfibril associated protein 1	HGNC:7032	15q15.3	AFAP1
ITG	Unmatched	integrins				ITGA1,ITGA2,ITGA3,ITGA5,ITGA6,ITGAV,ITGB1,ITGB3,ITGB4,ITGB5
FN1	Approved symbol	FN1	fibronectin 1	HGNC:3778	2q35	FN1
CD106	Alias symbol	VCAM1	vascular cell adhesion molecule 1	HGNC:12663	1p21.2	NOT FOUND
CD44	Approved symbol	CD44	CD44 molecule (Indian blood group)	HGNC:1681	11p13	CD44
VEGF	Previous symbol	VEGFA	vascular endothelial growth factor A	HGNC:12680	6p21.1	NOT FOUND
CCL	Previous symbol	CRYGEP	crystallin gamma E, pseudogene	HGNC:2412	2q33.3	NOT FOUND
CCL2	Approved symbol	CCL2	C-C motif chemokine ligand 2	HGNC:10618	17q12	NOT FOUND
CXCL1	Approved symbol	CXCL1	C-X-C motif chemokine ligand 1	HGNC:4602	4q13.3	NOT FOUND
CXCL5	Approved symbol	CXCL5	C-X-C motif chemokine ligand 5	HGNC:10642	4q13.3	NOT FOUND
IFNG	Approved symbol	IFNG	interferon gamma	HGNC:5438	12q15	NOT FOUND

Figure 4-15: LEVs contain proteins that interact and act as signalling agents towards immune cells

(A) Identification of biological processes, molecular function, or reactome pathways, enriched in LEVs, using STRING network visualisation from Figure 4-14 and functional enrichment analysis. (B) Identification of candidate factors in LEVs proteins in the proteome list, potent at stimulating monocytes.

4.4 DISCUSSION

The focus of this Chapter was to delve deeper in the nature of the previously observed LMPs, which were generated during their transit in microfluidic devices i.e. capillary bifurcations.

After purifying LMPs from contaminating MDA-MB 231 cells post microfluidic transit experiments, I demonstrated for the first time that LMPs derived from shear- and capillary forces represent a new type of large extracellular vesicle, by fulfilling imaging and protein inclusion requirements, as set by the MISEV2018 guidelines (78). Given that shear- and capillary-derived LEVs were never examined before for their molecular cargo, I used molecular assays, namely protein staining, protein blots and PCR to demonstrate the presence of a large array of proteins and of several mRNA transcripts. Furthermore, we examined for the first time the proteome of shear- and capillary derived LEVs more widely using proteomics, identifying that they contained thousands of proteins, relevant to biological pathways such as metabolism, cell reprogramming, inflammation, protein secretion and immune interactions. Altogether, these novel findings push the evolution of the field of EVs, particularly of LEVs, to pave the way for a more holistic understanding of their role in cancer metastasis.

Previously, several methods have been described for the purification of EVs of all sizes, with the most prevalent ones being centrifugation and filtration techniques (100,301). For instance, purification of exosomes requires several centrifugation steps to remove cells, cell debris, larger vesicles to retrieve a final product of pure exosomes for long incubation times. When it comes to larger vesicles, traditionally, centrifugation values of 10000 x g are usually performed to allow the pellet formation of large vesicles (88). However, shear-derived LEVs whose size varies from 1-11 μm can have different responses to these centrifugation speeds. For example, I showed that this speed was enough to pellet LEVs that contained almost no cells (>99.8% purity), yet the initial speed to pellet cells contained a small fraction of LEVs, lowering the yield of purified LEVs. This suggests that larger LEVs may be collected in the pellet as larger and heavier particles, compared to smaller LEVs. Nonetheless, in respect to LEVs purity, both filtration and centrifugation yielded a pure LEVs population. Results from visual quantification revealed that there was 0% cell contamination post-filtration, but in flow cytometry, this result was 99.8%. This small difference is likely due to autofluorescence that is picked up by the high sensitivity of flow cytometers or perhaps during the collection of the supernatant post-centrifugation by gentle disruption of the pellet and collection of a few contaminating cells. It is highly unlikely that cells resisted centrifugation speed of 180 x g to

remain in the supernatant. Nonetheless, successful acquisition of a pure LEVs population paves the way for unbiased downstream analyses.

In this Chapter, LMPs that were previously generated upon shear and capillary bifurcations exposure were identified as LEVs. This validation was performed by matching features and protein composition of LMPs to the respective criteria of the MISEV2018 guidelines (78). In respect to protein composition, LMPs were positive for CD81, a transmembrane protein and two cytosolic proteins (HSP90 and CK18), sufficient to validate the EV nature of a particle, yet our proteomic approach revealed numerous proteins in LEVs that are enlisted in MISEV2018 guidelines, further strengthening their biological nature. MISEV2018 guidelines require the presence of transmembrane proteins to support the lipid bilayer structure that EVs possess generally (78). In addition, the presence of cytosolic proteins further supports not only the lipid bilayer, since without one, proteins would leak out, but also the ability of EVs to carry inside them biological material. The presence of a lipid bilayer and the enclosing of biological material suggest cumulatively that LMPs are not open cell fragments and can thus be considered as EVs with potential functionalities. Interestingly, quantification of protein expression on LEVs via flow cytometry was done both in a bulk manner, by measuring the mean fluorescence intensity and in a single vesicle level, by quantifying the number of vesicles that express the protein. While almost the majority of LEVs presented each protein, the 75% of vesicles expressing CD81, suggests that LEVs may present heterogeneity or potentially emerging from different cell compartments, enriched for different proteins. Furthermore, CD81 is also used as an exosome marker and previous studies have discerned protein level differences in respect to CD81 between exosomes and LOs (88). However, it appears that shear-derived LEVs have a high expression of CD81, which introduces the need for more direct comparison studies between all these types of EVs. In addition, MISEV 2018 guidelines require analyses of single vesicles (78). The use of high-resolution images, generated from scanning electron microscopy are an ideal methodology to observe morphological features and visualise the size of EVs. Both fluorescence visualisation and electron microscopy highlight the large size of these LEVs, which is distinguishably larger compared to other smaller EVs, such as exosomes (30-120 nm) and microvesicles (120-1000 nm) (85). The membrane of shed LEVs resembles the surface of cancer cells, which is expected, given their release from the membrane of cancer cells. Previously, Nanou et al. have isolated large tumour-derived

extracellular vesicles from patients with castration resistant prostate cancer and show a gallery of scanning electron microscopy images (75) that resemble morphologically this work's LEVs and further strengthen the notion that such LEVs are derived from the transit of CTCs in the microcirculation.

The presence of several proteins within LEVs begged the question as to how rich these LEVs are in proteins. Preliminary results, by measuring the total levels of protein revealed a reduction in the protein content of cells that transited through microfluidic devices, compared to control cells, reflecting that shedding and the volume reduction lead to reduction in proteins. The mean volume reduction of cells post-shedding was about 20%, which matches well the mean reduction in total protein levels in cells post-shedding (about 18%). This reduction is qualitatively reflected also in protein blots that reveal the lower intensity of protein bands in cells post-shedding compared to control cells. However, it appears that the same protein bands appear in the two populations, suggesting that cells post-shedding do not lose necessarily proteins rather these are reduced. This is also reflected in the proteome analysis, whereby control cells present higher intensity compared to post-shedding cells in many instances (**Fig. 4-10C**). Nonetheless, reduction of protein levels may impact the journey of CTCs. Osmani et al. demonstrated that reduction of integrin proteins in the surface of cancer cells impacts their ability to adhere stably to the endothelium in zebrafish models (60), suggesting that volume loss due to shedding and the subsequent protein reduction levels may affect many downstream metastatic steps.

Shed LEVs contained a significant percentage of protein mass, which represented about 10% of total protein content of control cells. Given the previous calculation on post-shedding cells loss of about 18%, it would be expected that this fraction would have been retained in LEVs. Several scenarios could explain this drop or loss in protein mass, including protein leak or even active secretion from LEVs. Moreover, the yield of LEVs post-centrifugation was not 100%, which could have explained the underrepresentation of its protein quantity. On the other hand, qualitatively, LEVs were positive for about 3300 proteins, representing about 60% of all proteins identified in cells, reasonable given the fact that LEVs were secreted by the membrane of cancer cells. Only 76 proteins positive in LEVs were not identified in cells (1.3%), which should not be the case, given the shedding nature of LEVs from cells. This might be explained by 'noise' during the normalisation of the data or potentially some impurities,

collected in the LEVs samples. Nonetheless, the large similarity of LEVs and cells protein composition, suggests that LEVs can be used in liquid biopsy applications, for early diagnosis, prognosis, patient monitoring and even extracting protein information. What needs to be mentioned though is that this proteomic analysis is done in a bulk manner, meaning that not every individual LEV will contain all these proteins. In fact, comparison of individual LEVs may reveal that they contain different protein composition eg. proteins concentrated from different cell compartments.

Bioinformatic analyses revealed many interesting insights in the capabilities of LEVs, based on their protein composition. First of all, biological processes like metabolism were referenced multiple types, resembling previous findings on shear-derived LMPs or large oncosomes, as shown previously (71,88,220), emphasising that LEVs (as shown in this project) share similarities with large oncosomes and LMPs. More interestingly, Headley et al. demonstrated that their observed LMPs were positive for mitochondria, explaining the correlation to multiple metabolic pathways, discovered in this Chapter. Second of all, there was a clear correlation of LEVs proteins to inflammatory and cell reprogramming (Epithelial-to-Mesenchymal transition) pathways, suggesting that LEVs can interact and potentially reprogram cell types such as endothelial cells and immune cells, which would be readily available to LEVs, which can be secreted in the microcirculation. Last but not least, LEVs proteins were linked to signalling events and protein binding functions, especially towards immune cells. Headley et al. have previously observed the uptake of shear-derived LMPs by several immune cells such as neutrophils and monocytes or macrophages (71), corroborating the relevance of the proteomic findings on the roles of LEVs.

In this work, the attempt to explore fully the molecular content of shed LEVs revealed the richness in protein content. This is an exciting finding, emphasising that work should be done to implement LEVs in liquid biopsy techniques to improve early diagnosis and patient prognosis. The expression of RNA by LEVs paves the way for the application of other omics techniques on LEVs, such as transcriptomics and lipidomics. Furthermore, little work so far indicates direct differences between LEVs and smaller vesicles, exosomes, in respect to their molecular content, yet there is largely any direct comparison between their downstream functionalities. The proteomic data highlighted that LEVs are linked to functions and molecular pathways on metabolism, inflammation, signalling and cell reprogramming,

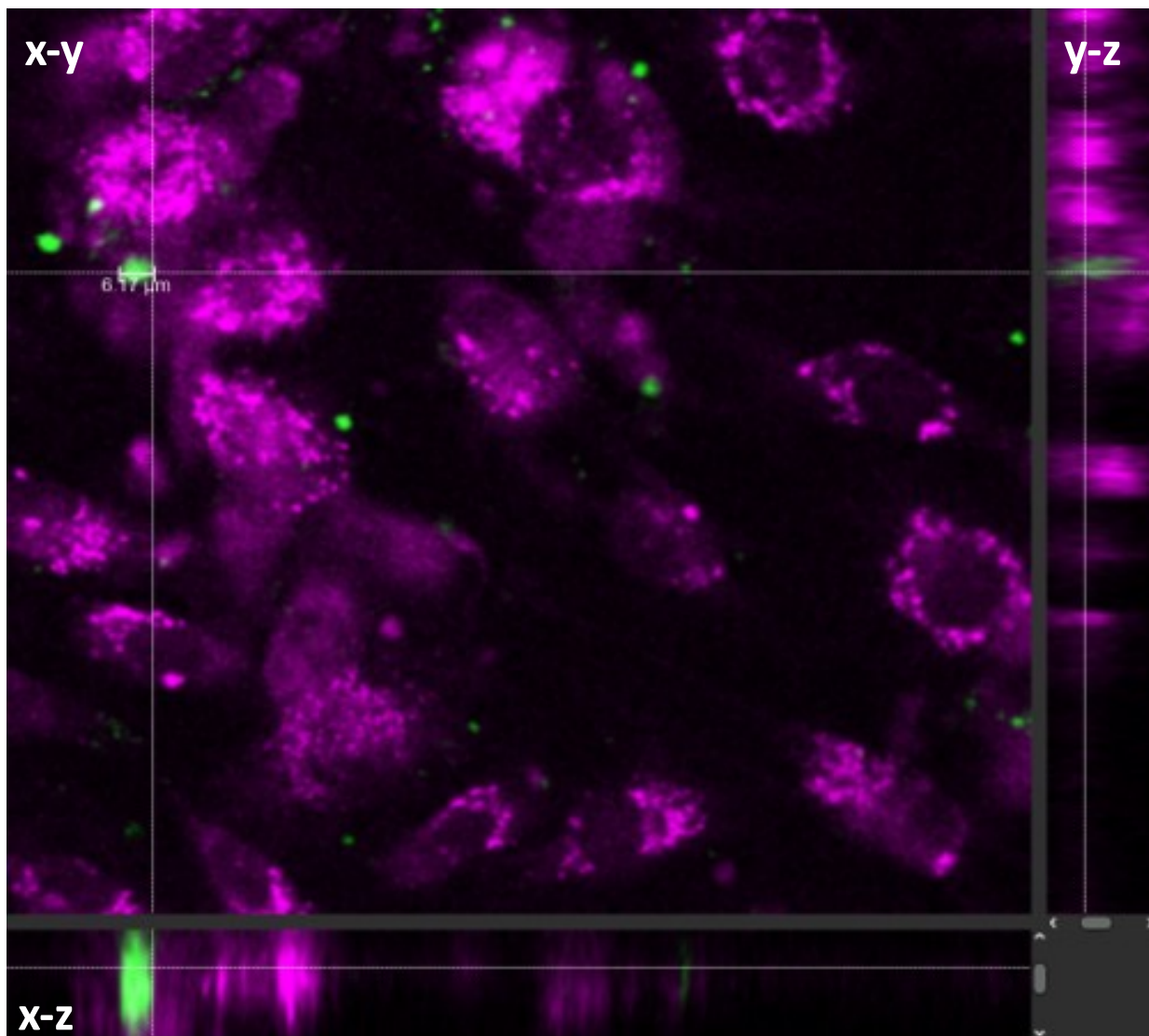
highlighting the need to evaluate interactions of LEVs with other cell types. Thus, it is sound to hypothesise that LEVs are capable of interacting with multiple cell types that influence them towards an activation state to induce the formation of a pre-metastatic niche and metastasis.

4.5 CONTRIBUTIONS & ACKNOWLEDGEMENTS

I generated the hypothesis, designed methodologies and experiments, conducted all the experiments, collected and analysed all data. Sara Arfan performed the proteomic experiment and respective bioinformatic analyses.

CHAPTER 5

Large extracellular vesicles disrupt the endothelium integrity to facilitate cancer metastasis



5.1 INTRODUCTION

The relationship between cancer metastasis and the vascular endothelium is crucial (59,305). The endothelial cells that line the blood vessels of all organs and tissues regulate blood homeostasis and the balanced exchange of nutrients and waste between the bloodstream and tissues. Furthermore, the endothelial cell lining acts as a cohesive physical barrier but also functions as a regulatory unit, by sustaining a normal blood flow, preventing immune cell recruitment and coagulation (122). However, upon incident of an injury in a tissue, endothelial cells become activated to promote wound healing and repair (306). Thus, the activated endothelial cells recruit immune cells, enable their attachment via upregulation of adhesive molecules, become more permeable to facilitate immune cell extravasation and infiltration of immune cells, induce blood clot formation to prevent further bleeding and lead the genesis of new blood vessels to ensure the proper supply of the damaged tissue (59,123,306,307). While this is a series of physiological responses to injury repair, perpetual activation of endothelial cells is hijacked during cancer, allowing thus the formation of a premetastatic niche (150,308–310) that favours cancer metastasis.

During cancer development and progression, the endothelium is involved in many steps of the metastatic cascade, including intravasation, endothelium arrest and adhesion and extravasation of circulating tumour cells (CTCs) (33,34,59,60,62,222,311–313). For instance, Follain et al. have corroborated that while favourable flow profiles promoted arrest of CTCs in the microcirculation (34), stable arrest to the endothelium is regulated via interactions of CTCs with adhesion molecules from endothelial cells (60). The authors have also demonstrated how trapped CTCs can either squeeze through neighbouring endothelial cells or remodel the vasculature to allow their engulfment and pass to the outer part of the blood vessels (34). Relevant findings support the notion that soluble factors secreted from CTCs or leaked from the primary tumour, such as interleukins can activate the endothelium and enhance CTCs-endothelium interactions and their extravasation (48,63,314). Evani et al. demonstrated that while breast cancer cells (MDA-MB 231) were not able to adhere to activated endothelium alone, when monocytes were implicated, aggregates of tumour cells and monocytes were able to adhere at significantly higher percentages to the activated endothelium compared to non-activated endothelial cells under flow. These findings suggest that not only the presence of monocytes but also the activated endothelium contribute

synergistically to promote cancer cell adhesion (315). In fact, several reports have investigated the ability of the activated endothelium to attract and promote monocyte adherence in inflammatory conditions (123,148,316). In respect to vascular permeability, Tichet et al. showcased how conditioned media from melanoma cells promoted the increase in intercellular gaps in endothelial cell monolayers, which promoted permeability and melanoma cell extravasation (317). Altogether, these findings consolidate the various and diverse downstream effects of activated endothelial cells and how they are utilised from cancer cells to succeed in their metastatic journey.

Endothelial cell activation is often stimulated by pro-inflammatory cytokines, such as TNF- α or interleukins (318). Conditioned media collected from cultured cancer cells is often used as an activator of endothelial cells (150), suggesting the importance of soluble factors influencing this process. However, it is well accepted that the conditioned media of cultured cancer cells, especially when cells are under some type of stress i.e. serum-free media, can be rich in small EVs i.e. exosomes or microvesicles and/or larger extracellular vesicles (LEVs) i.e. large oncosomes (80,88). Hood et al. harvested and purified exosomes from melanoma cell line B16-F10 and cocultured them with mouse endothelial cells. By using cytokine arrays, they identified secreted cytokines, responsible for angiogenesis and inflammatory roles, including interleukins and immune-stimulating factors that were not expressed in high levels compared to untreated endothelial cells (163). Wang et al. also studied the impact of isolated exosomes from melanoma cells on brain microvascular endothelial cells, identifying the reduction of vascular endothelial cadherin (VE-cad) and enhanced permeability. Moreover, brain microvascular endothelial cells released significantly higher amounts of IL-6 compared to unexposed cells, which is a pro inflammatory cytokine (165). While several other studies ascribe endothelial cell activation and their downstream effects to promote cancer (164,319–322), there is little understanding of the interactions between LEVs and endothelial cells. Spurling et al. identified large vesicle-like bodies in the culture of melanoma cell lines that lacked intact nuclei yet some were positive for nuclei fragments and tested their impact on endothelial cells. As expected, they observed similar effects as those imposed by exosomes, whereby these large vesicles were taken up by endothelial cells and disrupted their endothelial barrier, validated by disruption of VE-cad junction marker, F-actin reorganisation and reduced barrier resistance (121). It should be noted that given the presence of nuclei in

some of these large vesicles, it remains unclear by the authors whether these were apoptotic fragments (with fragmented nuclei), large oncosomes or LEVs with no nuclei. Nonetheless, to the best of my knowledge this is the only study that investigates the interactions between large vesicle-like structures and endothelial cells, while there are no reported findings whatsoever on the relationship between shear-derived LEVs and the activation of endothelial cells.

In this Chapter, shear-derived LEVs were utilised to investigate the interactions with human umbilical vein endothelial cells (HUVECs), their activation and downstream functional effects. Primarily, I demonstrated that LEVs were internalised upon co-culture with endothelial cells, which utilised predominantly macropinocytosis as an internalisation mechanism. Then, by inspecting activation markers, I identified a decrease in VE-cad which was induced both by co-culture with LEVs but also through soluble factors, secreted by LEVs. Disruption of VE-cad led subsequently to increase in the free/empty space between neighbouring endothelial cells, disrupting the monolayer integrity and cell connections, which further promoted endothelium permeability and invasion of cancer cells. Matching a pro-inflammatory phenotype, activated HUVECs were capable of stimulating monocytes polarisation to a pro-tumour M2 macrophage phenotype and also supported enhanced attachment of monocytes, both features exhibited by activated endothelial cells that reportedly shape the pre-metastatic niche. Altogether, I hypothesise that LEVs contribute to activation of endothelial cells, promoting systemic inflammation and rendering the activated endothelium as a favourable location that will promote cancer metastasis.

5.2 MATERIALS & METHODS

5.2.1 Cell culture & Reagents

MDA-MB 231 breast cancer cells were cultured, maintained, prepared and stained as described in detail in Chapter 2. THP-1 monocytes were purchased from ATCC (UK) and behave as a suspension cell line. THP-1 monocytes were cultured at 37°C/5%CO₂ and passaged/maintained in Roswell Park Memorial Institute (RPMI) 1640, that was supplemented with 1% (v/v) Penicillin/Streptomycin, 10% (v/v) heat-inactivated fetal bovine serum (FBS), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Gibco, US), 2.5 g/l Glucose (Merck, UK), 1 mM sodium pyruvate (Gibco, US) and 50 pM β -

mercaptoethanol (Gibco, US) at final concentrations. Human umbilical vein endothelial cells (HUVECs) were cultured at 37°C/5%CO₂ in EGM™-2 Endothelial Cell Growth Medium-2 BulletKit™ (Lonza, Switzerland), supplemented further with 10% (v/v) FBS. HUVECs were trypsinised using 0.25% (v/v) Trypsin and after centrifugation at 180 x g for 5 min, the pellet was resuspended in fresh serum-filled media to inactivate trypsin and cells were transferred in new T25 tissue flasks. HUVECs were sub-cultured and expanded every 3 days. All reagents were purchased from Sigma Aldrich (UK), unless noted otherwise. All antibodies were purchased from Abcam (UK), unless noted otherwise.

5.2.2 Cell & LEVs coculture

MDA-MB-231 originating LEVs from transit in microfluidic devices were generated, purified and enumerated, as elaborated in Chapter 4. HUVECs and purified LEVs were stained with a red Cell Tracker and a green Cell Tracker, respectively or vice versa for downstream imaging. Cell trackers were diluted 1000 times in the respective media and incubated with cells or LEVs at a final working concentration of 5 µM for 1 hr at 37°C/5%CO₂. In experiments that did not require downstream analysis of imaging, neither HUVECs nor LEVs were stained. In respect to co-culture, HUVECs and LEVs were always mixed at a 3 : 1 ratio, a ratio that was kept constant across all different experiments or conditions tested. However, the co-culture time differed according to the respective experiment. For internalisation studies, HUVECs and LEVs were co-cultured for at least 16 hr. In contrast, for PCR, immunofluorescence or other assays, co-culture lasted 30 hrs to allow sufficient time for interactions as well as reflection on molecular changes in HUVECs. Finally, the co-culture between LEVs and HUVECs occurred in distinct well plates, dependent on the downstream experiment.

5.2.3 Collection of conditioned media

Conditioned media (CM) was used in many assays. Media from adhered MDA-MB 231 breast cancer cells was collected, centrifuged at 180 x g to remove contaminating cells and the supernatant was collected, described as CM. CM was similarly collected from untreated HUVECs. In HUVEC-LEVs co-culture, after the incubation time of 30 hrs, media was removed entirely, removing thus non-internalised LEVs and fresh HUVEC media was added. After a day, CM from HUVECs that were treated with LEVs, was collected as described above. Finally, CM was also collected from LEVs. Purified LEVs were initially centrifuged at 10000 x g for 30 min

and were resuspended in fresh media. LEVs were then cultured for 1 extra day and then pelleted again at 10000 x g for 30 min. The supernatant was collected as LEVs' CM.

5.2.4 Cell treatment

For internalisation mechanism studies, 150000 or 20000 HUVECs were cultured for 1 day in wells of 24- or 96-well plates, respectively. HUVECs were stained with a red Cell tracker (ThermoFischer Scientific, UK) for 1 hr at 37°C/5%CO₂ and then treated either with Cytochalasin D (Cyto-D) or 5-(N-Ethyl-N-isopropyl)-Amiloride (EIPA, MedChemExpress, Sweden) for 3 hr at 37°C/5%CO₂ at final working concentrations of 10 µM and 50 µM, respectively. Untreated HUVECs were also used as a negative control. After the compounds were removed with the media, HUVECs were treated with fresh media that contained LEVs, pre-stained with Green Cell tracker (ThermoFischer Scientific, UK) as above and incubated at 37°C/5%CO₂ for 16 hrs. Then, media with non-internalised LEVs was removed and HUVECs in 96-well plates were imaged directly in confocal microscope (as will be expanded below). Alternatively, HUVECs cultured in 24-well plates were harvested using a Cell Scraper (FischerScientific, UK) and 500 µl of cell-filled media was transferred to an Eppendorf tube and subsequently used for flow cytometry studies, as will be elaborated later. In some experiments, tumour-necrosis factor alpha (TNF-α) was incubated with HUVECs for 30 hr at a final working concentration of 0.1 µg/ml. TNF-α served as a positive control for HUVEC phenotypic changes (318).

5.2.5 Confocal microscopy

96-well plates were loaded in the stage of a Leica SP8 Inverted scanning confocal microscope. The range of emission and excitation wavelengths was selected, based on the emission and excitation range of both red and green Cell Tracker, that were used to stain HUVECs and LEVs, respectively. Z-stack upper and lower thresholds were set and images were obtained at the Z-axis range with a step of 2 µm. Images were obtained at a resolution of 1024 x 1024 pixel. Internalisation of LEVs was verified using the software of the microscope, by applying orthogonal cross sectioning to image HUVECs in all pairs of coordinates i.e. x-y, x-z and y-z. Alternatively, by applying the same settings, images were obtained for 2 hr with 1min intervals to compile video series. Time lapse videos were recorded in an attempt to observe potential mechanisms for LEV internalisation or 'catch' on real time other interactions

between HUVECs and LEVs. Finally, the diameter of internalised LEVs was calculated via the software, only after visually validating that a particular LEV was internalised by orthogonal cross sectioning.

5.2.6 Immunofluorescence

15000 HUVECs and 5000 purified LEVs were cocultured as described elsewhere in wells of a 96-well plate for 30hr. HUVECs were further treated with LEVs CM or MDA-MB 231 CM for 30 hr. Untreated HUVECs served as a negative control. 4% (v/v) formaldehyde, diluted in PBS was added in each well for 12 min at room temperature. Wells were washed three times with PBS for 3 min per wash and further incubated with 0.1% (v/v) Trypsin (diluted in PBS) for 5 min at room temperature. After three washes with PBS for 3 min per wash, all samples were incubated overnight at 4°C with primary vascular endothelial cadherin (VE-cad) antibody or vascular cell adhesion molecule (VCAM) rabbit anti human primary antibodies that were diluted in PBS at final working concentrations of 1 µg/ml and 2 µg/ml. The next day, after the final 1x PBS wash for 3 min, all wells were incubated with goat anti rabbit secondary antibody Alexa Fluor 488 for 2 hr at room temperature that was diluted in PBS at a final working concentration of 2 µg/ml. After secondary antibody removal, cells were incubated with Hoechst for 5 min at room temperature and imaged directly in the Nikon microscope. 3-4 characteristic multifuorescent images were obtained at 20 x magnification for each condition and per replicate.

Additionally, immunofluorescence on THP-1 monocytes was performed as described above, after their treatment with either untreated or LEV-treated HUVECs' CM. THP-1 as a suspension cell line required centrifugation at 180 x g for 4 min at every step of immunofluorescence. THP-1 were stained by using primary cluster of differentiation (CD206) rabbit monoclonal antibody (diluted 100 times from the vial's volume-ab270682) and primary TNF-α rabbit monoclonal antibody at final working concentration 0.09 µg/ml overnight at 4 °C. The next day, cells were washed 1x with PBS for 3 min and then further incubated for 2 hr at room temperature with secondary goat-anti-rabbit antibodies, using Alexa Fluor 488 and Alexa Fluor 647, respectively for TNF-α and CD206, at final working concentrations of 2 µg/ml. 3-4 characteristic multifuorescent images were obtained using the confocal microscope per condition and replicate.

5.2.7 Flow cytometry

150000 HUVECs and 50000 purified LEVs were cocultured as described elsewhere in wells of a 24-well plate. Untreated HUVECs were used as a negative control. Immunofluorescence staining for VE-cadherin was performed as elaborated above. HUVECs were scraped using Cell scrapers and 500 µl were transferred in Eppendorf tubes for flow cytometry. Samples were resuspended well and added to the Amnis CellStream flow cytometer (Luminex, UK), run at 1 µl/min. At least 10000 cells per condition were analysed. Cells were initially gated based on a low threshold for antibody staining intensity, which was set according to the unstained sample. The mean fluorescence intensity of the gated population was provided by the software.

Alternatively, 150000 THP-1 monocytes were treated either with untreated or LEV-treated HUVECs' CM for 30 hr and were subsequently stained for CD206 and TNF-α as above. Unstained and single-colour controls were used to gate the areas for CD206-expressing events. The mean fluorescence intensity and the frequency of CD206 positive events were calculated by the software.

Additionally, flow cytometry was used to investigate the internalisation mechanisms by HUVECs co-cultured with LEVs. Unstained HUVECs, untreated HUVECs (stained as red) and LEVs alone (stained green), were used as single controls to set the thresholds for red and green staining intensity. In respect to the co-culture conditions, HUVECs were stained and treated with either Cyto-D or EIPA and then co-cultured with LEVs. LEVs- co-culture without any chemical treatment was also used as a positive control. Enumeration of the double positive events was used as a metric to quantify how many HUVECs had ingested LEVs under the different conditions. At least 10000 events were recorded per replicate and condition.

5.2.8 Real-time PCR

300000 HUVECs and 100000 purified LEVs were cocultured in wells of 12-well plate. Untreated HUVECs as negative control. After 30 hr incubation, cells were harvested using lysis buffer (Qiagen, US) and total RNA was harvested using the RNeasy Mini kit (Qiagen, US). Quantity and purity of extracted RNA was evaluated and quantified using Nanophotometer (IMPLEN, USA). 1 µg of RNA was reverse transcribed by using an iScript cDNA synthesis kit (Bio rad laboratories, UK). Using Applied Biosystems™ StepOne™ Real-time PCR System

(Fischer Scientific, UK) with SYBR Green PCR master mix (ThermoFischer Scientific, UK), 50ng of cDNA was amplified and real-time PCR was performed. Relative mRNA expression of VE-cad was extrapolated using the $2^{-\Delta\Delta C_t}$ method. The forward and reverse VE-cad primers were designed as GCCCACAGGCACGATCTGTT and CATCCGGTTCTGGGGCTCAT, respectively (ThermoFischer Scientific, UK). Ribosomal protein large P (RPLO) was used as housekeeping gene, with forward primer sequence (CGGTTTCTGATTGGCTAC) and reverse primer sequence (ACGATGTCACTTCCACG).

5.2.9 Monolayer permeability

15000 HUVECs and 5000 purified LEVs were cocultured as described elsewhere in wells of a 96-well plate. Brightfield and fluorescent images of confluent HUVEC monolayers were obtained at 0 hr using Nikon microscope at 10 x and 20 x magnification. After incubation with LEVs for 30 hr, images were obtained again to compare the impact of LEVs on disrupting the monolayer. Untreated HUVECs were used as negative control. Alternatively, HUVECs were stained with VE-cad and Hoechst as described elsewhere and multifuorescent images were obtained at 20 x magnification. The cell-free area was calculated by analysing the images in ImageJ.

Dextran permeability assay was further used to investigate HUVEC monolayer disruption. Millicell hanging cell culture inserts with 0.4 μm pores (Merck, UK) were loaded in wells of a 24-well plate. 900 μl of media was added in the well and 200 μl of HUVECs (75000 cells/ml) were then transferred to the inserts treated with 5000 purified LEVs for 30hr. Untreated HUVECs on cell hanging inserts were used as negative control. Media was washed and replenished with fresh media, that contained 10000 molecular weight Dextran Alexa Fluor™ 647 (ThermoFischer Scientific, UK). Media was collected from the bottom of the wells at 0, 6 and 24 hr and red fluorescence was analysed using Varioskan Flash plate reader (ThermoFischer Scientific, UK). Red fluorescence was analysed using excitation and emission values of 650- and 668 nm.

Alternatively, millicell hanging cell culture inserts with 8 μm pores (Merck, UK) were placed in wells of a 24-well plate. 900 μl of media was added around the cell insert and 200 μl of HUVECs (75000 cells/ml) were then transferred on top of the inserts. Confluent HUVEC monolayers were treated with 5000 LEVs for 30 hrs. Untreated HUVECs were used as a negative control.

MDA-MB 231 breast cancer cells were harvested and stained with a green Cell Tracker for 1 hr at 37°C/5% CO₂. 100 µl of MDA-MB 231 cells (100000 cells/ml) were transferred in the LEV-treated or untreated HUVECs in cell hanging inserts for 1 day, incubated at 37°C/5% CO₂. Media from the wells around the cell inserts was collected at 0 and 24 hr and fluorescence was read using the plate reader, with excitation and emission values set at 492 and 517 nm. The fluorescence measured at 24 hr was normalised initially based on blank samples (cell-free media) and then based on the 0 hr values.

5.2.10 Monocyte stimulation

15000 HUVECs and 5000 LEVs were co-cultured as described elsewhere. Untreated HUVECs were also cultured as a negative control. CM was collected from both conditions, as described above. 15000 THP-1 monocytes were added in wells of a 96-well plate and were treated for 30 hr with CM from LEV-treated or untreated HUVECs. Monocytes cultured on monocyte media was used as a negative control. Immunofluorescence for CD206 and TNF-α markers was performed as described previously and multicolour images at 20 x magnification were obtained as described elsewhere. Alternatively, 100000 THP-1 monocytes were added in wells of a 24-well plate and were again treated either with untreated or LEV-treated HUVECs CM. Untreated monocytes were used as a negative control. Finally, monocytes were collected and analysed via flow cytometry, as described previously.

5.2.11 Monocyte attachment

15000 HUVECs were added in separate wells of a 96-well plate and left to attach and grow for 1 day. 30000 THP-1 monocytes were cultured in separate wells of a 48-well plate and were left to grow for 1 day and were then stained with a green Cell Tracker, as described elsewhere. HUVECs or THP-1 monocytes were independently treated with 5000 or 10000 purified LEVs for 30 hr, respectively. Untreated HUVECs and untreated THP-1 monocytes were used as a negative control. LEVs were removed from the media from both cell populations and three experimental conditions of co-culture were set: a. Untreated HUVECs with untreated THP-1 monocytes b. LEV-treated HUVECs with untreated THP-1 monocytes and c. LEV-treated HUVECs with LEV-treated THP-1 monocytes. Green fluorescence was quantified at 0 hr of co-culture using a Varioskan Flash plate reader with excitation and emission values set at 492 and 517 nm, respectively. Fluorescent images of the entire well were obtained of all

conditions at 0hr. After 4hrs of co-culture, media from every well was removed and replaced with fresh media. Media without fluorescent cells was used as a blank measurement. Green fluorescence was quantified again at 4 hr and fluorescence images were obtained, as described above. To quantify these results, first, the 'blank' value was subtracted from each measurement, obtained from the plate reader. To further quantify the remaining fluorescence of attached THP-1 monocytes percentage-wise, initially the difference between fluorescence values before and after media removal was divided by the fluorescence value before media removal and then this value was subtracted from 100% to reflect the remaining fluorescence of attached cells.

5.2.12 Graphs & Statistical Analyses

GraphPad version 9 was used to plot all data in graphs and perform statistical comparisons between two or at least three different experimental groups, respectively. Illustrations were designed using BioRender. All experiments were repeated at least three times.

5.3 RESULTS

5.3.1 Endothelial cells internalise LEVs

To begin understanding the interactions between endothelial cells and LEVs, human umbilical vein endothelial cells (HUVECs), a traditional cell line used to study endothelial cells, were co-cultured with enumerated LEVs at a cell : LEVs ratio of 3:1. Initially, co-cultured HUVECs with LEVs were imaged directly (**Fig. 5-1A**) or at a span of several hours in an attempt to record any physical interactions. HUVECs were observed to extend part of their membrane via various mechanisms, including what appears to be lamellipodia-like extensions, circular ruffles or large bleb formation in the surface of endothelial cells in an attempt to interact with LEVs (**Fig. 5-1B-D**). In video 3 (**Appendix 11**), endothelial cells (stained green) and LEVs (stained red) were recorded to interact. The cell of interest (full arrow) uses membrane protrusions (dashed arrow) around LEVs, possibly in an attempt to interact or internalise it (**Video 3-Appendix 11**). All these mechanisms, according to the literature were found to correlate with macropinocytosis mechanisms, a form of endocytosis and large particle internalisation, which all cells can perform (323). Several LEVs could be visualised inside an intracellular structure that resembles a vacuole (**Fig. 5-1E**), resembling *in vivo*

macropinocytosis, suggesting the formation of a macropinosome (324). These structures were not universally observed in all HUVECs that were positive for LEVs.

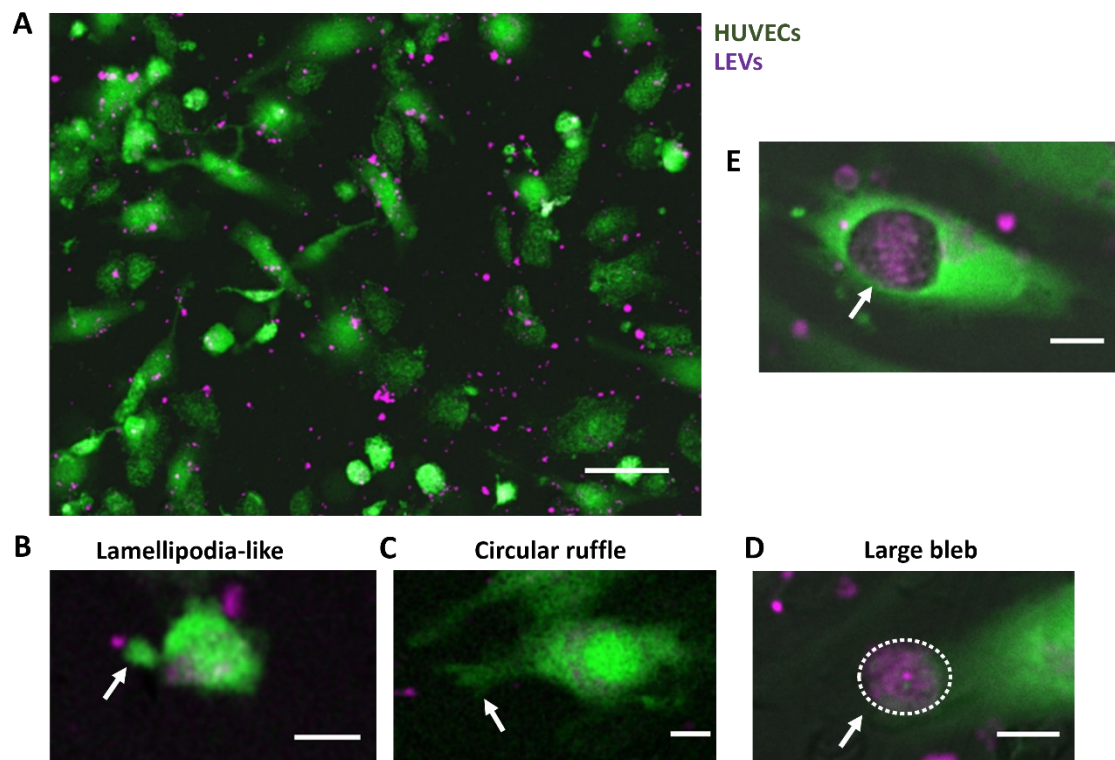


Figure 5-1: Interactions of HUVECs with LEVs.

(A-E) HUVECs were stained green and LEVs red (depicted as purple). (A) Co-culture of HUVECs with LEVs. Scale bar: 50 µm. (B) HUVECs trigger lamellipodia formation (arrow) to interact with LEVs. Scale bar: 10 µm. (C) HUVECs form circular ruffles (arrow) to interact with LEVs. Scale bar: 10 µm. (D) HUVECs form large blebs (arrow) on their surface to interact with LEVs. Scale bar: 10 µm. (E) Formation of large vacuoles (arrow/dashed circle) within HUVECs that potentially encompass LEVs. Scale bar: 10 µm.

To verify whether LEVs were internalised, pre-stained HUVECs (red) and LEVs (green) were co-cultured for at least 16hr and Z-stack images were obtained to validate internalisation of LEVs, by visually corroborating that LEVs were inside HUVECs. In a gallery of such images (**Fig. 5-2A-E**), orthogonal cross-sectioning allowed the visualisation of double-positive events in all three plane pairs (xy, xz and yz), verifying that LEVs were successfully internalised. The mean diameter of internalised LEVs was 2.8 ± 1.2 µm (**Fig. 5-2F**). In other instances, LEVs were outside the cell as orthogonal cross sectioning revealed where cells and LEVs don't colocalise in all three planes (lack of co-localisation in the x-z plane(**Appendix 7**)). In time-lapse videos,

LEVs appeared to be very motile (**Video 3-Appendix 11**), covering large distances and moving around cells. However, it was not possible to capture an internalisation event in live imaging.

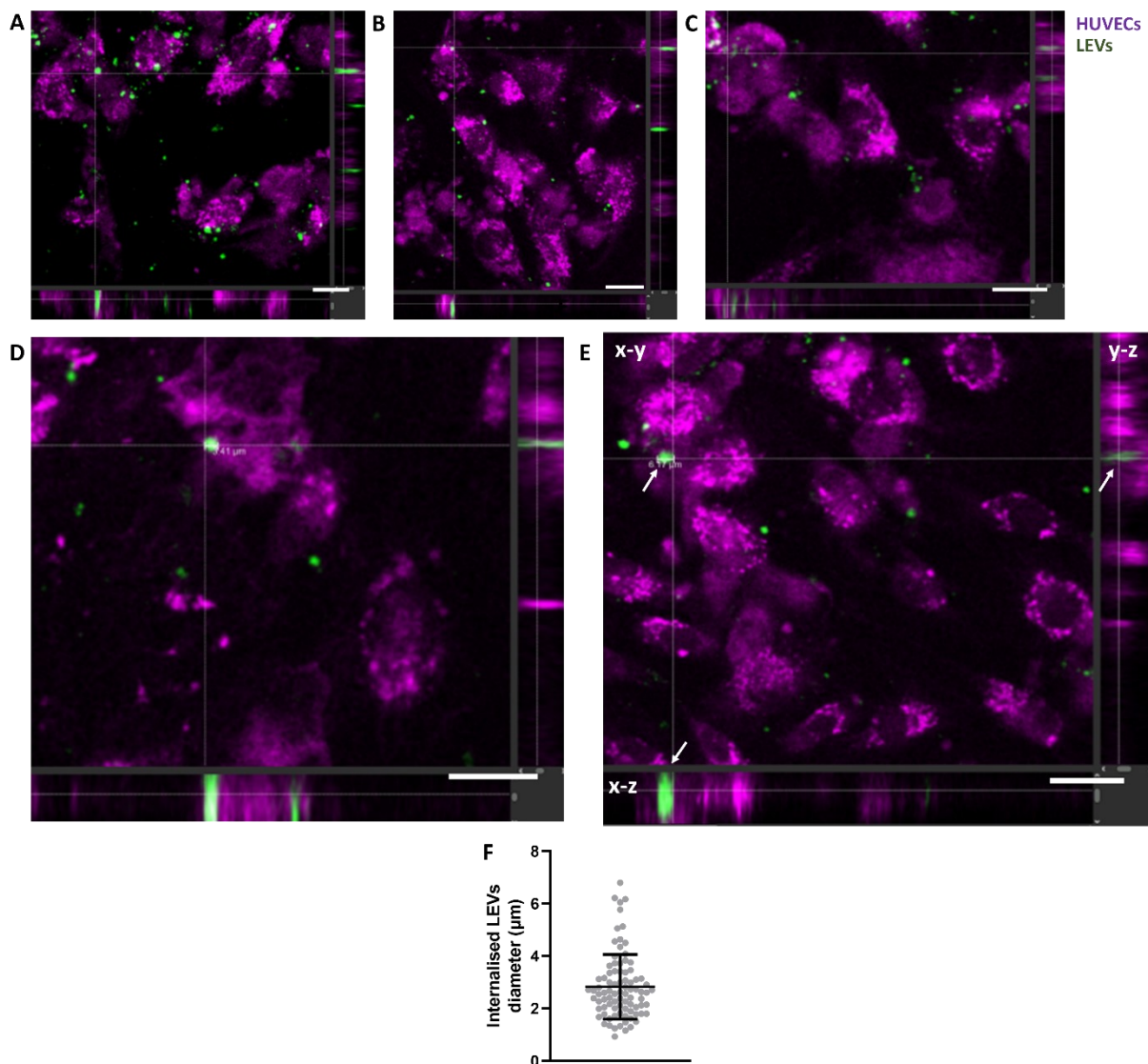


Figure 5-2: Internalisation of LEVs by HUVECs.

(A-E) Gallery of images of red-stained HUVECs (depicted as purple) having internalised green-stained LEVs after co-culture of at least 16hr. Scale bar: 20 μm. (F) Diameter of internalized LEVs (μm). Each data point represents a single LEV (n=87).

To verify the generalities of these observations, flow cytometry was used to quantify the frequency of HUVECs that had internalised LEVs. Based on the mono-cultured HUVECs, the gating threshold permitted the distinction between HUVECs that had internalised or not LEVs (**Fig. 5-3A**). Indeed, the mean frequency of HUVECs that had internalised LEVs was $32.9 \pm 1.6\%$ (**Fig. 5-3B**). When fluorescent beads of 5 μm were co-cultured with HUVECs at the same ratio,

as HUVECs : LEVs, the mean internalisation frequency by HUVECs was very low, similar to HUVECs alone (beads: $0.43 \pm 0.2\%$ vs HUVECs alone: $0.34 \pm 0.42\%$, ns) (**Fig. 5-3F-G**). In combination with the visualisation data through microscopy, it is evident that LEVs can be consistently taken up by endothelial cells.

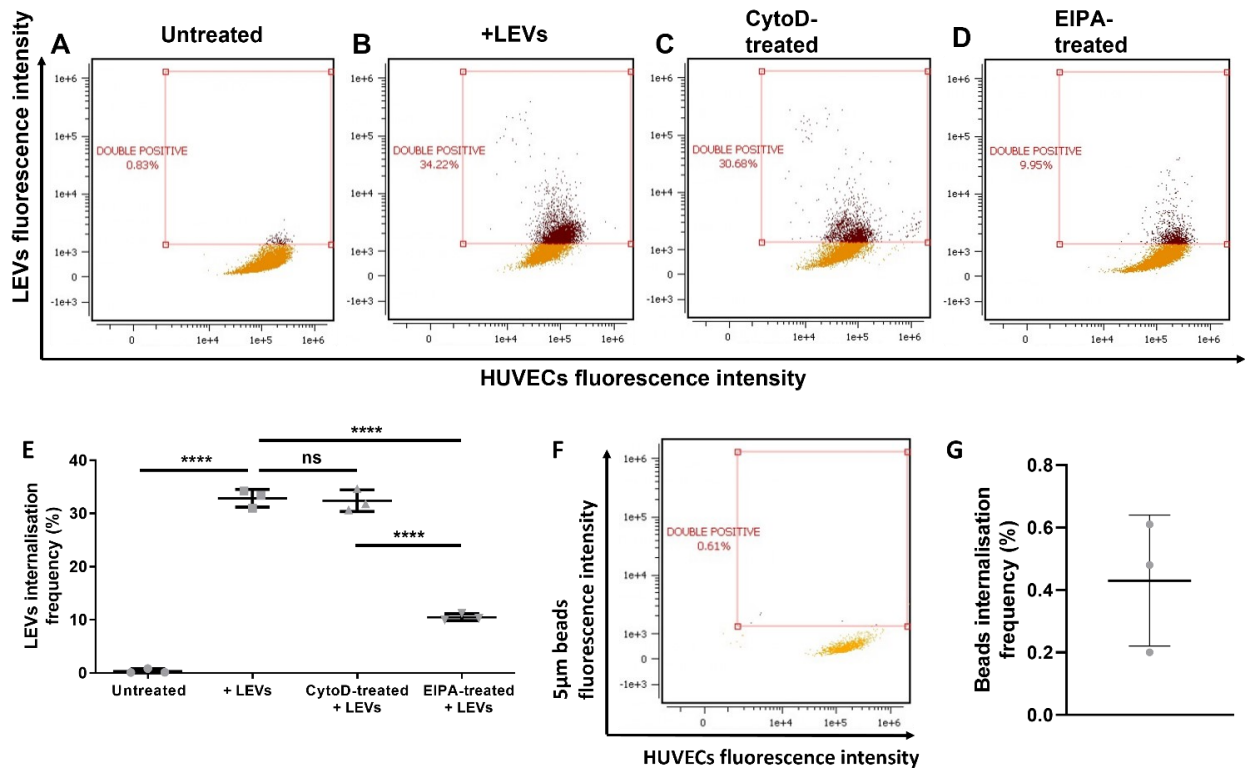


Figure 5-3: Internalisation mechanisms of LEVs by HUVECs.

(A-D) Dot plots of double positive events of HUVECs that have internalised LEVs under different experimental conditions. HUVECs were initially pre-treated for 3 hrs with either Cytochalasin D or EIPA and then co-cultured with LEVs for at least 16hr. (E) Quantification of double positive events of (A-D) (n=3 biological replicates for all). (F) Dot plot of double positive events of HUVECs that have internalised 5 µm beads after 16hrs co-culture. (G) Quantification of double positive events of (F) (n=3 biological replicates).

5.3.2 Mechanisms of LEVs internalisation by HUVECs

Endocytosis mechanisms vary for large or smaller particles, as described extensively by Chen et al. (323). Large microparticles such as the observed shear-derived LEVs, can potentially be internalised usually by either phagocytosis, which is restricted almost exclusively to ‘professional’ phagocytes i.e. macrophages or macropinocytosis, performed by most cell types (323). Phagocytosis requires physical interaction with LEVs, while macropinocytosis

involves the ‘drinking’ of extracellular fluid (323). To explore which mechanism allows LEVs internalisation by endothelial cells, HUVECs were pre-treated with either Cytochalasin D (Cyto-D) or 5-(N-Ethyl-N-isopropyl)-Amiloride (EIPA) to inhibit each endocytosis mechanism (325,326), respectively. As expected, Cyto-D had no noticeable effect on reducing the mean of LEVs internalisation frequency in co-cultures (untreated: $32.9 \pm 1.6\%$ vs Cyto-D treated: $32.4 \pm 2\%$, ns) (**Fig. 5-3B-C&E**). On the other hand, treatment with the EIPA inhibitor resulted in a major reduction of about 70% in the mean of LEVs internalisation frequency in co-cultures (untreated: $32.9 \pm 1.6\%$ vs EIPA treated: $10.4 \pm 0.6\%$, ns) (**Fig. 5-3B&D&E**), compared to untreated co-cultures, highlighting the prevalent role of macropinocytosis in internalisation of LEVs.

5.3.3 LEVs promote VE-cad⁻ VCAM⁺ activated endothelial phenotypes

Next, I wondered how LEVs affect endothelial cell phenotypes. In the first two hours of live imaging during HUVECs and LEVs co-culture, the morphology of endothelial cells appeared different compared to monoculture of endothelial cells (**Fig. 5-4A-C**). Cells had become smaller and appeared more contracted and less stretched, features which suggest the beginning of an acquisition of an activated phenotype at 2 hrs (**Fig. 5-4A-B**). In addition, the empty areas around the cells were much wider, suggesting disruption of the endothelial integrity (**Fig. 5-4C**).

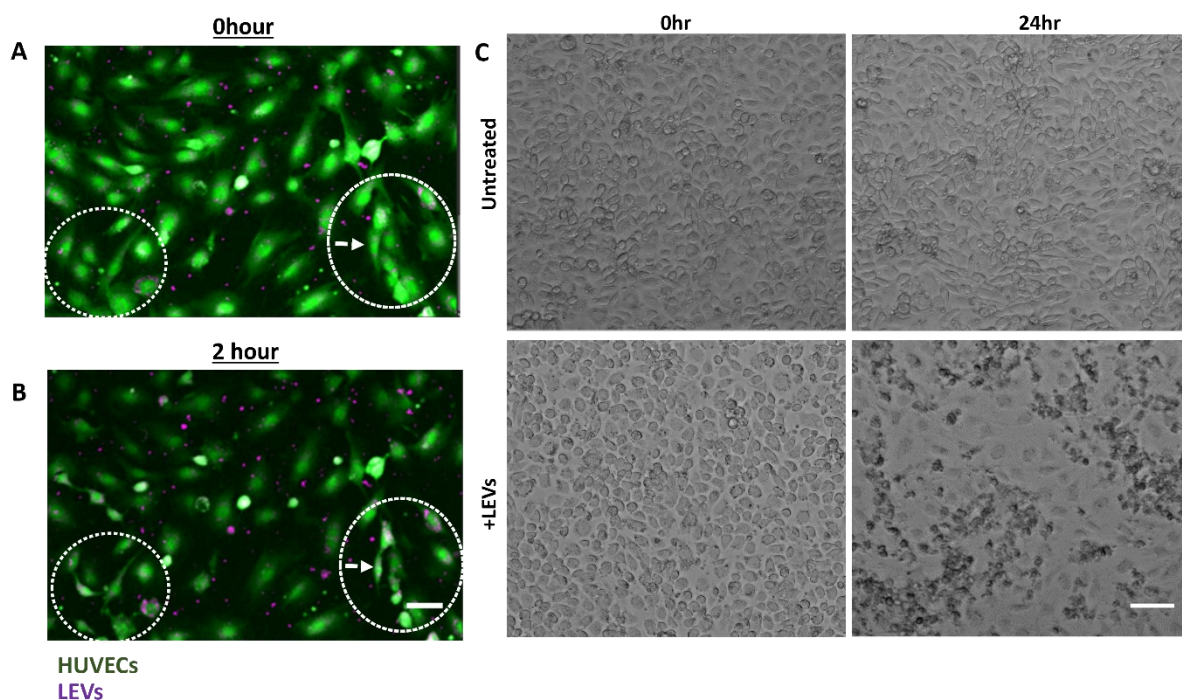


Figure 5-4: LEVs induce morphological changes in HUVECs.

(A-B) Multifluorescent images of co-cultured HUVECs (green) and LEVs (red/depicted as purple) at 0 and 2hr. Scale bar (applies to both): 50 μ m. (C) Brightfield images of untreated or LEV-treated HUVECs at 20x at 0 and 24hr. Scale bar: 50 μ m.

To confirm these suspicions, endothelial cells were stained initially for VE-cadherin (VE-cad), a junction protein that strengthens intercellular adhesions between neighbouring endothelial cells. Untreated HUVECs presented high intensity of VE-cad staining (green) around the periphery of the cell as well as in the areas that connect cells, whereas treatment with LEVs resulted in complete abrogation, visually, of the VE-cad staining patterns (**Fig. 5-5A**). The intensity of VE-cad was quantified additionally using flow cytometry, highlighting a large difference in mean fluorescence intensity after HUVECs were co-cultured with LEVs (untreated: 10694 ± 1612 vs LEV-treated: 7602 ± 183 , $P < 0.05$) (**Fig. 5-5B-C**). Treatment with conditioned media from MBA-MD 231 breast cancer cells, which contains soluble factors and smaller vesicles i.e. exosomes resulted too in disruption of the VE-cad staining patterns but not to such a great extent, as visualised by LEVs treatment (**Fig. 5-5A**), suggesting a dominant effect presented by LEVs. Interestingly, treating HUVECs with LEVs CM, also disrupted VE-cad staining intensity and junctions, but again not to the extent that LEVs directly co-cultured with HUVECs had (**Fig. 5-5A**), suggesting that LEVs secrete soluble factors to affect HUVECs. VE-cad

was downregulated also in respect to mRNA expression, plummeted at about 70% (untreated: 1 ± 0 vs LEV-treated: 0.3 ± 0.1 , $P < 0.0005$) (**Fig. 5-5D**). Overall, these findings emphasise the strong contribution of LEVs in VE-cad disruption.

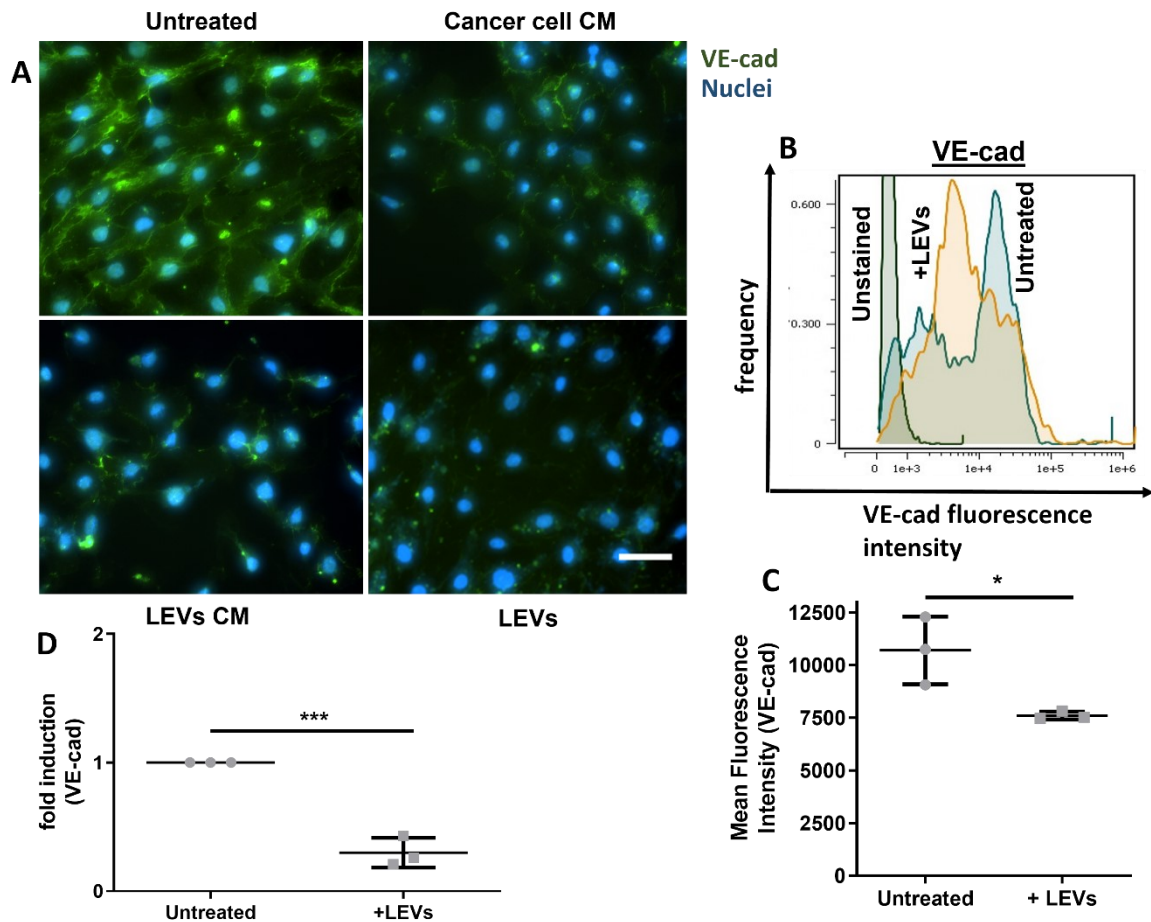


Figure 5-5: LEVs reduce disrupt VE-cadherin intercellular junction on HUVECs.

(A) Fluorescent images of stained HUVECs for VE-cad at 20x. HUVECs were treated either with LEVs, LEVs CM or MDA-MB 231 cell CM for 30hr. VE-cad is stained as green and nuclei as blue. Scale bar: 50 μ m. (B) Histogram of mean fluorescence intensity of VE-cad on unstained, untreated or LEV-treated HUVECs, analysed via flow cytometry. (C) Quantification of (B) ($n=3$ biological replicates for all). (D) VE-cad fold induction of untreated or LEV-treated HUVECs (30hr), calculated via reverse transcription quantitative polymerase chain reaction ($n=3$ biological replicates for all).

Another protein that is universally accepted as a marker of endothelial activation is vascular cell adhesion molecule (VCAM) (327). Ample evidence demonstrates its upregulation on endothelial cells, when stimulated with cytokines or inflammatory factors, such as tumour-necrosis factor (TNF- α) (327). Immunofluorescent analyses revealed the absence of VCAM expression from untreated endothelial cells, where there is complete lack of VCAM⁺ green

signal around the cell nuclei, as seen in **Fig. 5-6A**. In contrast, HUVECs treatment with TNF- α led to acquisition of VCAM-positive signal around the cell nuclei (**Fig. 5-6A**), as expected. Similarly, HUVECs treatment with LEVs had a profound increase in green VCAM-positive signal around the cell nuclei (**Fig. 5-6A**).

Another HUVEC activation marker is nitric oxide (NO), whose generation has been established on TNF- α treated HUVECs (134). As seen in **Fig. 5-6B**, the intracellular staining for NO is largely enhanced in HUVECs-LEVs co-culture, compared to monoculture. Quantification of mean fluorescence intensity via flow cytometry revealed the increase in NO generation (untreated: 3297 ± 321 vs LEV-treated: 5994 ± 1321 , $P < 0.05$) (**Fig. 5-6C**). Altogether, the reduction of VE-cad protein, increase of VCAM protein expression and the increased NO generation emphasise the changes of HUVECs to an activated VE-cad⁻ VCAM⁺ phenotype.

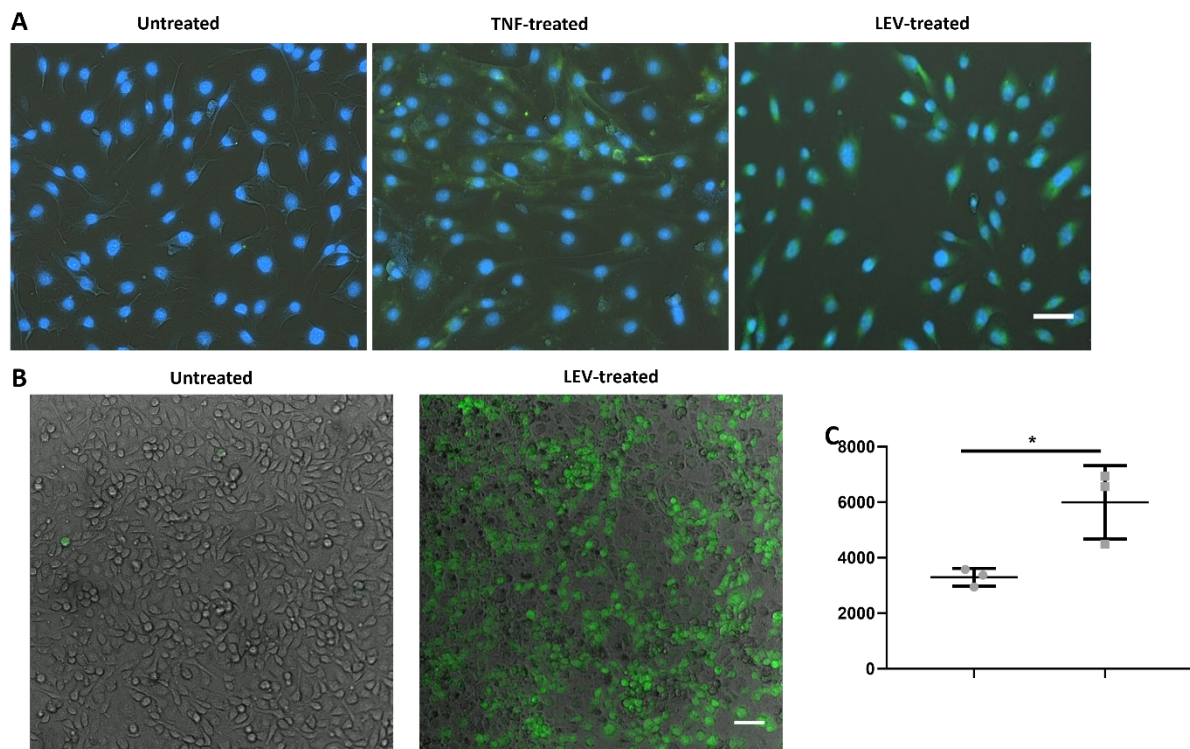


Figure 5-6: LEVs induce the expression of VCAM and generation on HUVECs.

(A) Untreated, TNF α -treated or LEV-treated HUVECs were stained for VCAM after coculture of 30hr. VCAM was stained green and nuclei blue. Scale bar: 50 μ m. (B) Untreated or LEV-treated HUVECs were stained for nitric oxide after coculture of 30hr. Nitric oxide was stained green. Scale bar: 50 μ m. (C) Quantification of nitric oxide's mean fluorescence intensity via flow cytometry (n=3 biological replicates for all).

5.3.4 LEVs induce changes in endothelial monolayers permeability

Disruption of VE-cad junctions between neighbouring endothelial cells leads reportedly to enhanced permeability (59). To explore the idea that shear-derived LEVs can enhance permeability, confluent monolayers of HUVECs were treated with LEVs, revealing the formation of large gaps (**Fig. 5-7A**). Quantification of the cell-free area (black areas) established the extent of disruption between HUVECs, caused by LEVs (untreated: $11.5 \pm 4\%$ vs LEV-treated: $43.2 \pm 5.1\%$, $P < 0.0001$) (**Fig. 5-7B**), suggestive of enhanced permeability. To validate this notion, confluent monolayers were loaded in transwell inserts with $0.4 \mu\text{m}$ pores. In LEV-treated HUVECs, there was a 30% increase in the mean absorbance of the fluorescent dextran that passed through compared to untreated HUVECs (untreated: 60.6 ± 2.8 vs LEV-treated: 78.7 ± 2.7 , $P < 0.005$) (**Fig. 5-7C-D**). The increased values of absorbed dextran in the media are accompanied by a change of colour in the media around the transwell insert, appearing more blue (reasonable given the emission spectra of the fluorescent dextran used) (**Fig. 5-7C**). These findings were further reproduced by using a transendothelial migration assay to quantify the number of invading MDA-MB 231 breast cancer cells. In a similar format as previously, confluent HUVEC monolayers were cultured this time in hanging cell inserts with pores of $8 \mu\text{m}$. Invasion of MDA-MB 231 breast cancer cells in LEV-treated HUVEC monolayers was higher compared to untreated HUVECs, reflected by the higher fluorescence values of collected cancer cells from the media around the cell inserts (untreated: 0.28 ± 0 vs LEV-treated: 0.42 ± 0 , $P < 0.0001$) (**Fig. 5-7E**). Altogether, these findings corroborate the notion that LEVs by disrupting VE-cad junctions affect indirectly permeability of the endothelium, which facilitates cancer cells and potentially other factors to pass through and extravasate.

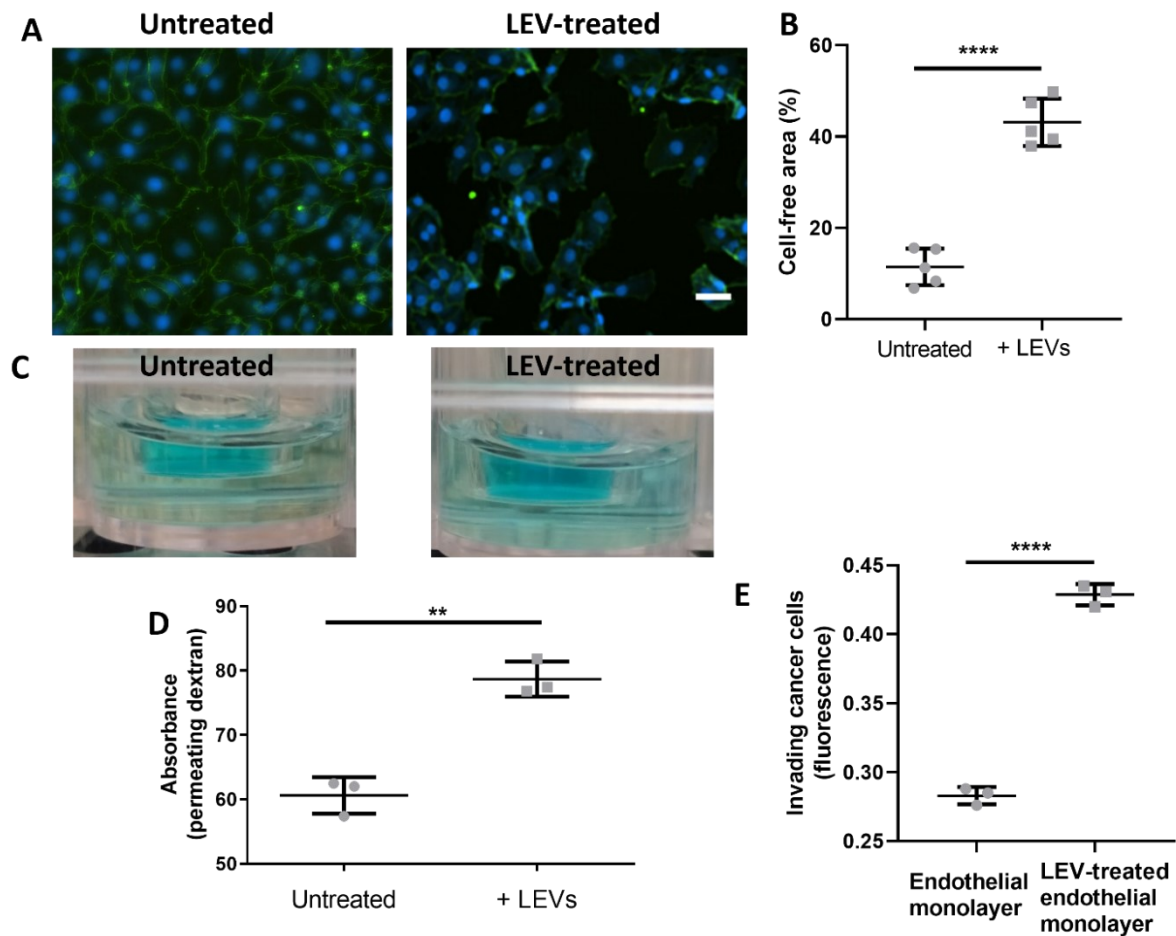


Figure 5-7: LEVs promote HUVECs monolayer permeability.

(A) Untreated or LEV-treated HUVEC monolayers after 30 hr of co-culture, stained for VE-cad. Scale bar: 50 μ m. (B) Quantification of cell-free area of (A) (n=5 areas). (C) Images of wells that untreated or LEV-treated HUVEC monolayers were cultured on cell hanging inserts with 0.4 μ m pores for 30 hr, and that were later incubated with fluorescent dextran 647. (D) Quantification of permeated fluorescent dextran through untreated or LEV treated HUVEC monolayers (n=3 biological replicates for all). (E) Fluorescence of MDA-MB 231 cells that invaded and were collected after co-culture with untreated or LEV-treated HUVEC monolayers that were cultured in cell hanging inserts with 8 μ m pores (n=3 biological replicates for all).

5.3.5 LEVs-induced activated HUVECs stimulate monocyte differentiation and attachment

Another hallmark of endothelial activation is the acquisition of an inflammatory phenotype, which can be identified partially based on the ability of activated endothelial cells to stimulate monocytes (123). CM from untreated HUVECs or LEV-treated HUVECs (LEVs were removed 1

day before collecting CM) was added to THP-1 monocytes, a traditional cell line used to study monocytes, for 30 hr. By testing both M1-associated (TNF- α /green) and M2-associated (CD206/red) markers of monocytes differentiation (180), the conditioned media of LEV-treated endothelial cells (activated cells) induced upregulation of CD206 (M2 marker) instead of TNF- α (M1 marker), whereas both markers were absent or very weakly expressed from either untreated monocytes or monocytes treated with untreated HUVEC's CM (**Fig. 5-8A**). Quantification via flow cytometry revealed that the mean fluorescence intensity of CD206 was significantly higher when monocytes were treated with CM from LEV-treated HUVECs compared to HUVEC CM treated (4429 ± 53 vs 584 ± 22 , $P < 0.0001$) or compared to untreated monocytes (4429 ± 53 vs HUVEC CM: 577 ± 1.7 , $P < 0.0001$), whereas there was no statistical significance between untreated monocytes and HUVEC CM treated ones (577 ± 1.7 vs 584 ± 22 , ns) (**Fig. 5-8B-C**). Analyses of CD206 on single cell level, revealed the same pattern, as expected (**Fig. 5-9A-B**), supporting that LEVs can influence HUVECs, which in turn can further affect other cell populations, matching the acquisition of a pro-inflammatory role.

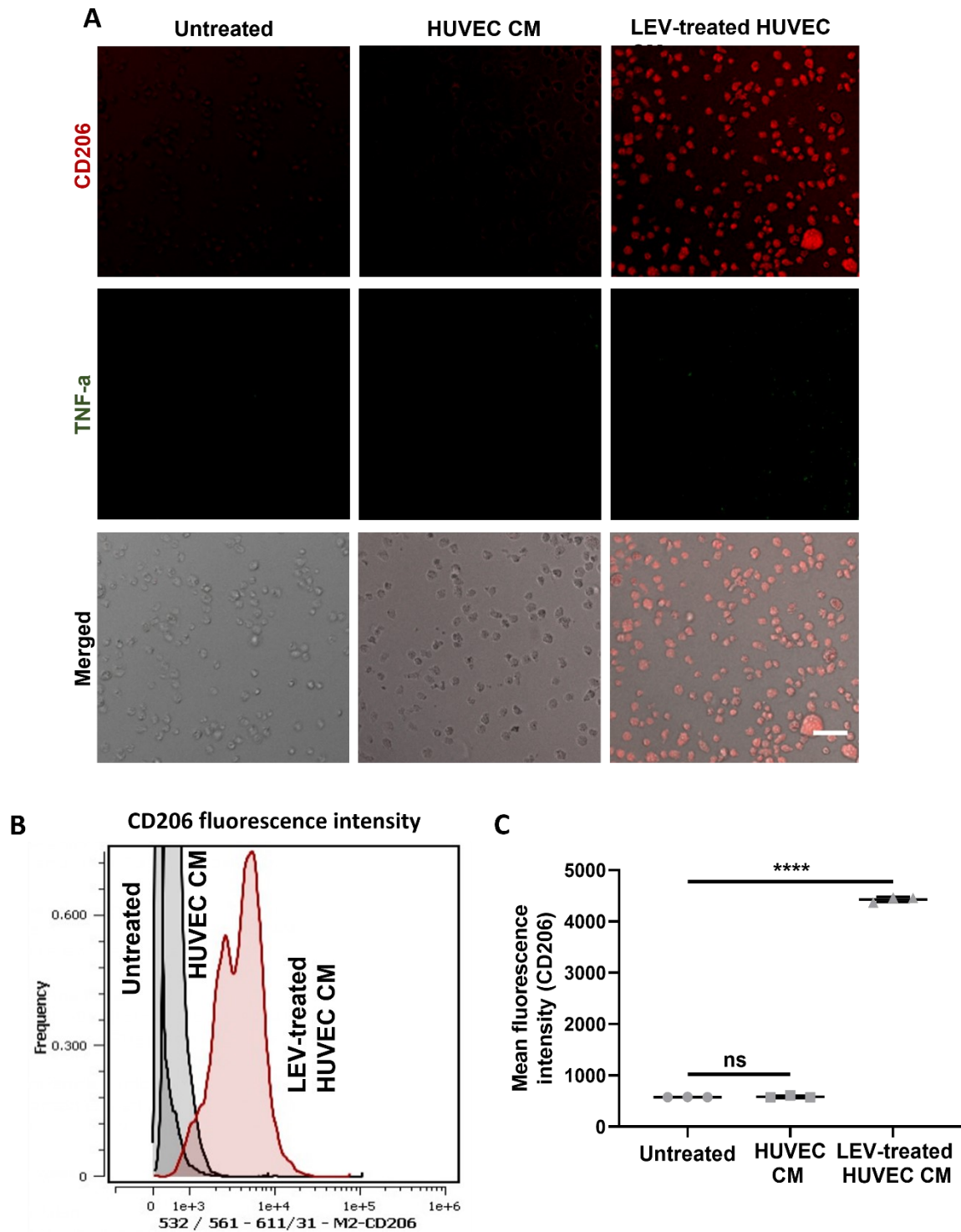


Figure 5-8: LEV-treated HUVECs stimulate via soluble factors monocyte differentiation to M2 macrophages.

(A) Immunofluorescent staining of untreated, HUVEC CM or LEV-treated HUVEC CM monocytes. CD206 was stained red and TNF-α was stained green. Scale bar: 50 μm. (B) Histogram of CD206 mean fluorescence intensity for above 3 experimental conditions. (C) Quantification of mean fluorescence intensity of CD206 (n=3 biological replicates for all).

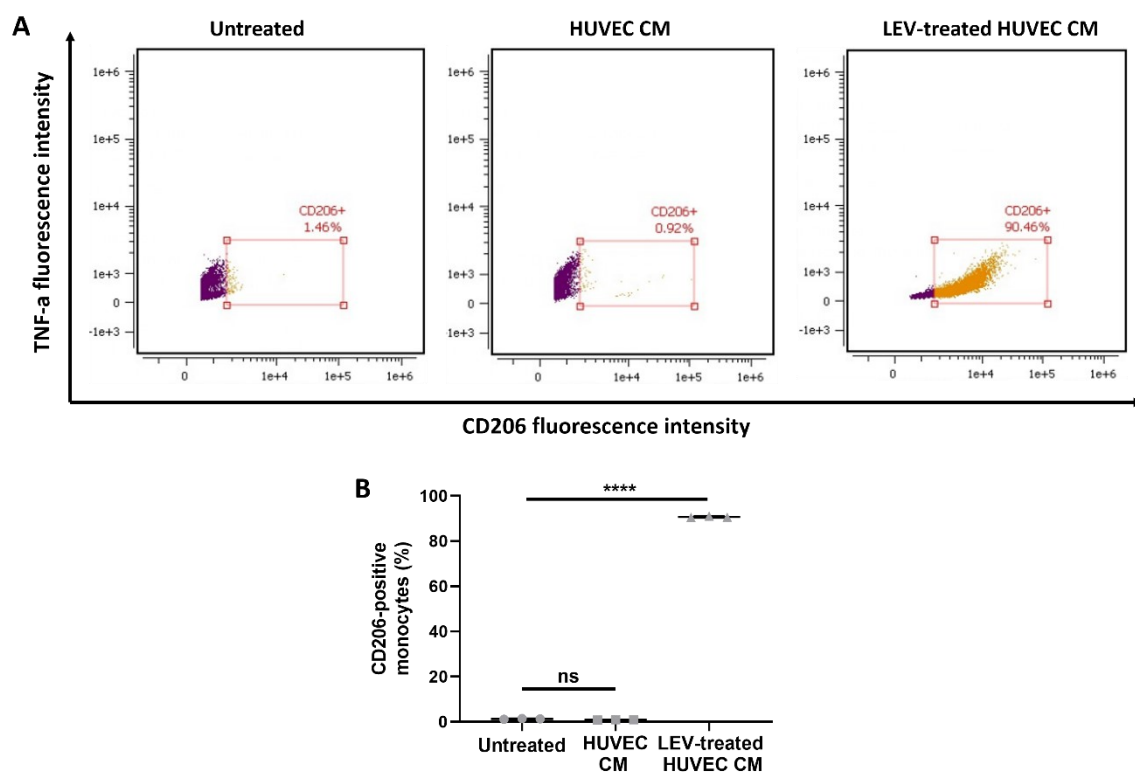


Figure 5-9: LEV-treated HUVECs stimulate via soluble factors the majority of monocytes to differentiate to M2 macrophages.

(A) Dot plots of untreated or HUVEC CM-treated or LEV-treated HUVEC CM-treated monocytes expressing CD206 on single cell level. (B) Quantification of CD206-positive monocytes in above conditions (n=3 biological replicates for all).

Along with monocyte stimulation, I wondered whether LEV-stimulated HUVECs could promote monocyte attachment (123). To explore this idea, LEV-treated endothelial cells were cultured with fluorescently labelled monocytes and promoted monocyte attachment compared to untreated HUVECs ($7.9 \pm 1.5\%$ vs $2.2 \pm 1\%$, $P < 0.005$), as seen both by the increased number of attached monocytes after media removal and the fluorescence values of adhered monocytes (**Fig. 5-10A-D**). Similarly, when both HUVECs and monocytes were pre-treated with LEVs, the attachment of the now differentiated monocytes was even larger compared to both previous conditions ($15.47 \pm 1.3\%$ vs $7.9 \pm 1.5\%$, $P < 0.0005$; $15.47 \pm 1.3\%$ vs $2.2 \pm 1\%$, $P < 0.0001$) (**Fig. 5-10A-D**).

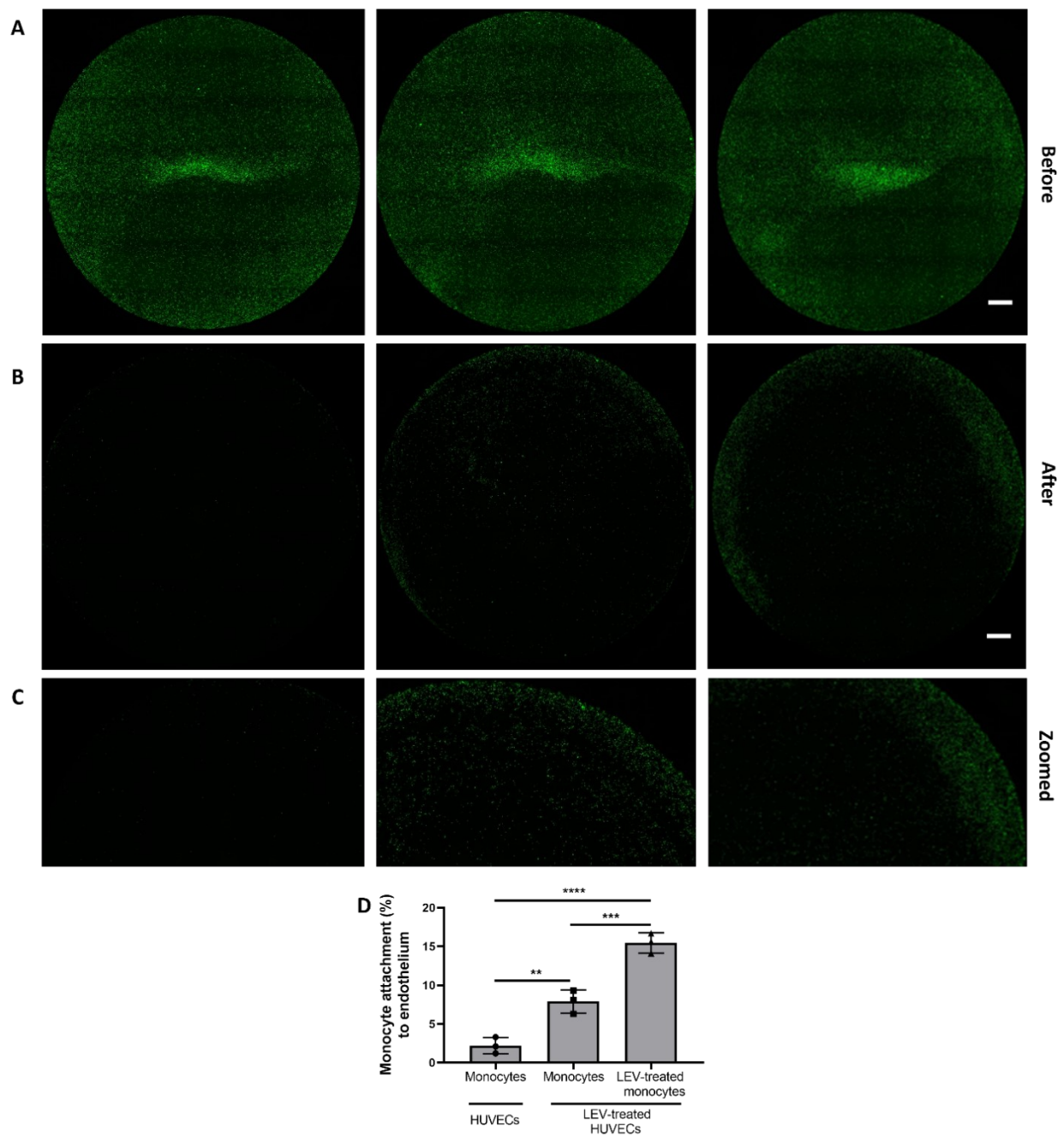


Figure 5-10: LEVs promote enhanced adhesive interactions of HUVECs with monocytes.

(A) Images of fluorescently tagged monocytes added to monolayers of HUVECs at 0hr. From left to right, monocytes are added to HUVECs, monocytes are added to LEV-treated HUVECs and LEV-treated monocytes are added to LEV-treated HUVECs. Scale bar: 500 μ m. (B) Images of fluorescently tagged monocytes captured after co-culture with HUVEC monolayers for 4 hr and media was removed. From left to right, monocytes that remained adhered to HUVECs, monocytes that remained adhered to LEV-treated HUVECs and LEV-treated monocytes that remained adhered to LEV-treated HUVECs. Scale bar:

500 μm . (C) Zoomed part of images from (B). (D) Remaining fluorescence percentage after media removal (n=3 biological replicates for all).

5.4 DISCUSSION

In this Chapter, the interactions between shear- and capillary-derived LEVs and endothelial cells are described for the first time. More specifically, I demonstrated using confocal microscopy and flow cytometry that LEVs can be internalised by endothelial cells by micropinocytosis and not phagocytosis. Co-culture of LEVs with endothelial cells further led to a series of changes, whereby LEVs disrupted endothelial cells' intercellular junctions and their barrier integrity, facilitating the passage of tumour cells. Moreover, I proved that LEVs triggered activation of endothelial cells, which was measured by a. the increase in protein expression of VCAM, an endothelial cell activation marker and b. the manifestation of an activated phenotype, where activated endothelial cells stimulate monocyte to macrophage differentiation and promoted their attachment. Overall, LEVs shed by CTCs and their impact on endothelial cells suggests that LEVs have the capacity to contribute to the formation of a pre-metastatic niche, which involves activation of endothelial cells and thus promote cancer metastasis.

Internalisation of EVs from endothelial cells has been documented previously (325,328). While more studies have shown that exosomes or microvesicles, originating from cancer cells, can be internalised by endothelial cells (157,160), there is only one study that shows the internalisation of LEVs. Spurling et al. identified a large vesicular population that was collected upon static culture of melanoma cells depleted with serum, a biogenesis mechanism that is fundamentally distinct from LEVs, derived due to transit in capillary bifurcations. In fact, a fraction of this large vesicular population was positive for Hoechst signal, opposed to complete absence from shear-derived LEVs, but did not display an intact nuclei (121). Based on this Chapter's findings, HUVECs could internalise regularly shear-derived LEVs. While these preliminary findings are very promising, the experiments were performed in static conditions. An attempt to perform this experiment using a peristaltic pump to set up continuous flow of LEVs on vascularised microfluidic chips (Appendix 8) was done once, but the experiment did not reveal any uptake, potentially due to detachment of HUVECs from the device that affected the results. Nonetheless, Hyenne et al. demonstrated *in vivo* that smaller EVs such as

exosomes can flow closer to the walls of the endothelium, as opposed to red blood cells, and were internalised by endothelial cells (221). It is possible that due to their larger size, LEVs will maximise their interaction area with endothelial cells, which can favour their internalisation. Furthermore, Headley et al. also demonstrated the internalisation of shear-derived large microparticles in vivo, during their generation in lung microvessels by various local immune cells (71), boosting the notion that LEVs can potentially be taken also by endothelial cells under flow, though this warrants further research.

In respect to internalisation, phagocytosis and macropinocytosis are the two major endocytosis mechanisms for particles of large sizes (323). In respect to smaller EVs such as exosomes and microvesicles, multiple internalisation pathways exist and have been shown to affect particle uptake by endothelial cells (323), however there are no reported internalisation mechanisms of shear-derived LEVs by endothelial cells. Treatment with a phagocytosis inhibitor, Cytochalasin D, had no effect whatsoever on internalisation by endothelial cells, reasonable given the exclusively documented phagocytic ability of macrophages and monocytes. However, inhibition of macropinocytosis led to a massive decrease of 70%, highlighting the dominance of macropinocytosis as an internalisation mechanism. The lack of internalisation of fluorescent beads of 5 μm by HUVECs, which is also the mean size of shear-derived LEVs (**Fig. 2-2H**), suggest that some type of initial interaction of chemical recognition between LEVs and HUVECs may be required to enable internalisation. Furthermore, revisiting the diameter of internalised LEVs, their mean value was around 2.8, about half the mean size of LEVs, suggesting that endothelial cells preferably internalise smaller LEVs. Thus, it is possible that if the distribution of LEVs' diameter shifts downwards, that the internalisation frequency by HUVECs will go up in these experimental conditions. Last but not least, micropinocytosis is regulated via different mechanisms (lamellipodia, circular ruffles or large blebs) and it is thus possible that the EIPA inhibitor does not block all 3 mechanisms, thus explaining the incomplete reduction of LEVs internalisation frequency. The combination with other micropinocytosis inhibitors such as imipramine may in fact abolish it entirely.

Investigation of the effects of LEV treatment of endothelial cells revealed a significant downregulation of VE-cad staining and disruption of the intercellular connections. This was accomplished not only via LEVs coculture with HUVECs but also by applying conditioned

media from purified LEVs, suggesting that LEVs secrete factors that can affect the endothelium, another proof that shear-derived LEVs are indeed EVs (78). However, in the co-culture, the disruption of VE-cad was more profound, indicative of both LEV's uptake and soluble factors as important mediators in downstream modulation of endothelial junctions. VE-cadherin and in general, intercellular junctions, both adherent and tight junctions are responsible for sustaining a firm network of a vascular endothelium, preventing permeability (59). Several studies have correlated downregulation of junction proteins and a disrupted endothelial barrier integrity with enhanced permeability, by studying the role of EVs for instance (121,165,329). In this Chapter, these same changes in the endothelium occurred by their co-culture with shear-derived LEVs that changed endothelial cells' morphology, leaving empty spaces between them. These results suggest that CTCs shedding LEVs can use it to their advantage to indoctrinate the endothelium barrier and pass through blood vessels and extravasate. Indeed, this was validated in a simplistic manner by using a transendothelial migration assay, whereby MDA-MB 231 breast cancer cells could invade at larger numbers, when HUVECs were treated with LEVs. These experiments did not include live imaging, therefore the invasion mechanism remains inconclusive, nonetheless supports the potential of extravasation. In vivo, it has been previously reported that two of the major mechanisms of extravasation are migration of cancer cells through neighbouring endothelial cells or by causing vessel remodelling (34). In **Fig. 5-4C**, HUVECs upon treatment with LEVs are repositioned and appear remodelled at 24hr, however, these images are generated under static conditions in 2D, warranting further research on more sophisticated models to examine more holistically the influence of LEVs on 3D formed blood vessels.

Changes in endothelial cell junction proteins by LEVs treatment were accompanied by marked upregulation of VCAM and generation of NO, which represent markers of endothelial cell activation (134,327). VCAM protein increased expression on endothelial cells is often linked to enhanced interactions with immune cells (330), particularly with monocytes and macrophages, acting as a molecular bridge for enhanced interactions, which eventually allow immune cells to roll along the endothelium and extravasate at the site of injury (139). It is theorised that cancer cells adapt and utilise a similar mechanism to roll along endothelial cells, until they find a location to extravasate, suggesting that LEVs-induced expression of VCAM can facilitate CTCs. In respect to NO, its generation is linked both to anti- and pro-

tumorigenic activities (331,332). In particular, NO production by HUVECs, instigated by interleukins or other tumour cell factors is linked to enhanced tumour cell adhesion, vascular permeability and angiogenesis (333–335). What is described in the literature seems to align with previous results in this Chapter, where LEVs induced disruption of HUVECs' intercellular junctions and enhanced permeability, which may be aftereffects of NO production. Overall, LEVs potentiate activation of HUVECs and drive many changes, including increase in VCAM and NO, which are linked to several and distinct downstream effects, highlighting that LEVs can convert endothelial cells to activated phenotypes and confer them diverse features towards influencing cancer metastasis.

Another consequence of LEVs induced endothelial cell activation is the ability of activated endothelial cells to stimulate monocytes to pro-tumour M2 macrophages via the secretion of soluble factors. Physiologically, the activated endothelium in response to tissue injury would secrete factors to recruit immune cells or platelets to promote injury healing (123). However, in the context of cancer, where endothelial cells remain constitutively activated, these secreted factors stimulate restlessly other cell types, favouring the formation of a supportive environment for CTCs (150,308). This Chapter's experimental findings highlight that the conditioned media from mono-cultured endothelial cells failed to induce monocyte differentiation, as LEV-treated endothelial cells achieved. These findings highlight that the conversion of endothelial cells to an activated phenotype matches the current literature and serve to establish LEVs as a strong contributor of this phenomenon.

Another hallmark of endothelial cell activated phenotypes is their capacity to increase immune cell attachment (123). Given the increased expression of VCAM protein on HUVECs, this could be one of the reasons that activated endothelial cells promoted monocyte attachment, as shown experimentally in this Chapter and backed up by the literature (139,330). What is even more interesting was that when both endothelial cells and monocytes were pre-treated with LEVs, LEV-stimulated monocytes (that became M2 macrophages) attached to LEV-stimulated endothelial cells at much higher percentages. In fact, the presence of shear-derived LEVs potentially in the blood of patients, suggests that they may interact with multiple cell types. This supports the idea that LEVs can activate independently endothelial cells and monocytes and boost their co-interactions further to promote the formation of a pre-metastatic niche, which supports CTCs metastatic journey.

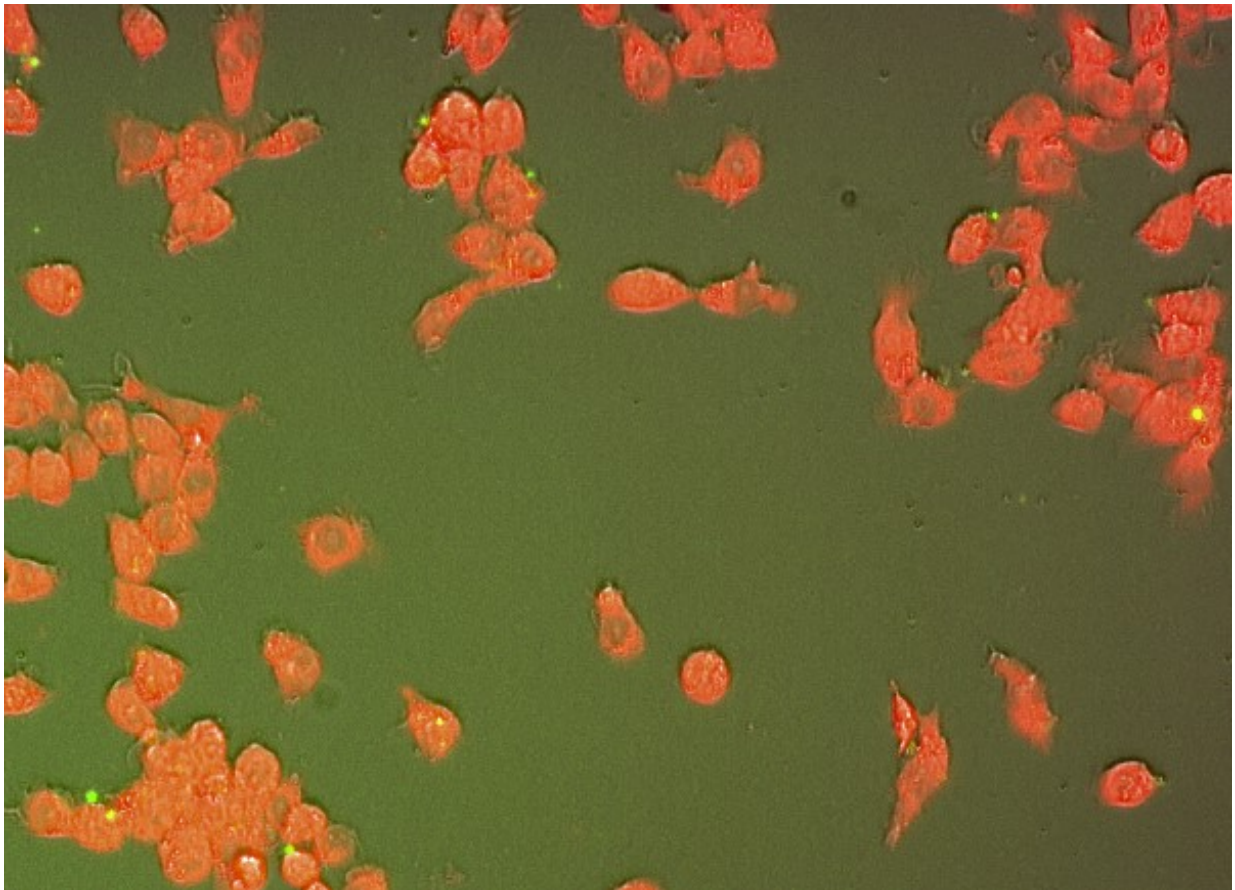
The new mechanism of endothelial cell activation by shear-derived LEVs raises many new possibilities in supporting cancer metastasis. While certain features are similar to effects driven by exosomes, LEVs have previously been shown to affect cancer cells differently than exosomes (88). Thus, it is interesting to compare directly the impact of exosomes vs shear-derived LEVs on endothelial cells. In fact, proteomic and transcriptomic studies on LEV-treated vs exosome-treated endothelial cells will shed insight in the full repertoire of proteins and genes involved, which may be linked to new molecular functions and biological pathways. While LEVs interact and activate endothelial cells in static conditions, more sophisticated mimicking vascularised models of the microcirculation are required to study the internalisation of LEVs in a more physiological manner (under flow) as well as their downstream effects. Such models will further enable a direct correlation between LEV-driven effects on endothelial cells and other metastatic steps such as cancer cell extravasation in live imaging approaches. Altogether, shear-derived LEVs' complete interactions with the endothelium is still at a very nascent stage, yet these results solidify the important contribution of LEVs to systemic inflammation.

5.5 CONTRIBUTIONS & ACKNOWLEDGEMENTS

I generated the hypothesis, designed experiments, conducted experiments, collected and analysed data. Diana Visan helped collecting the data in respect to the internalisation frequency of LEVs (via flow cytometry).

CHAPTER 6

Large extracellular vesicles promote M2 pro-metastatic macrophage phenotypes



6.1 INTRODUCTION

Immune cells play an important role in sustaining health across an organism (168). Immune cells i.e. monocytes are patrolling the bloodstream to inspect for particular pathogens that invade and attack the human body. Monocytes get activated and polarised to specialised types of macrophages, M1 macrophages to defend against a pathogen and M2 macrophages to promote wound healing and injury repair (175,189). For instance, during injury, monocytes are mobilised and recruited by soluble factors to the sites of injury, differentiate into macrophages and perform their relevant tasks. In particular, M2 macrophages are important mediators of anti-inflammatory activity to suppress immune attacks and allow activities such as angiogenesis, ECM remodelling and wound healing (189,194). These are all part of a physiological response and once healing is successful, there is no longer need for either type of macrophage. However, during cancer, which is a multi-year disease that sustains continuous inflammation, M2 macrophage activity persists and their repertoire for anti-inflammatory activity and tissue repair is utilised in a malignant manner (194–196,336,337).

During cancer metastasis, monocytes or macrophages are often polarised to M2 tumour-associated macrophages (TAMs)(194). Several reviews elaborate on the various mechanisms that TAMs contribute to various steps of metastases, including invasion, intravasation, extravasation, formation of pre-metastatic niche, CTCs survival, cytotoxic immune cell responses and vascularisation (197,208,338–341). For instance, Guo et al. (2019) used a microfluidic approach to mimic interactions between non-small cell lung carcinoma cell lines and macrophages. Upon differentiation of monocytes to M2 macrophages by cancer cells, the authors observed that M2 macrophages induced epithelial to mesenchymal transition on cancer cells, increasing their migration and invasion patterns (342). Wang et al. studied in a zebrafish model how macrophages could assist intravasation. The authors isolated macrophages from tumours that were rich in factors that can shift macrophages to M2-specific macrophages and co-implanted these with cancer cells. What they observed was that in the coculture where macrophages had not converted to M2, the dissemination areas were very few compared to co-implantation of M2 macrophages with cancer cells, suggesting the important role of M2 macrophages in intravasation (343). Wei C et al. (2018) demonstrated how M2 macrophages could block colorectal cancer cell death induced by a

chemotherapeutic drug. M2 macrophages were found to inhibit the activation of pro-apoptotic proteins and concurrently increase the expression of anti-apoptotic proteins (206). Altogether, all these reported studies among many others, emphasise the crucial and diverse role of pro-metastatic M2 macrophages on promoting several steps involved in the success of cancer progression and metastasis.

M2 macrophages are often activated by soluble factors secreted from cancer cells (344,345). However, the number of studies that report polarisation towards a M2 phenotype based on cancer EVs influence, has increased in the past decade, especially in respect to exosomes (213,214,346–348). For instance, Jiang et al. demonstrated that exosomes, derived from the conditioned media of bladder cancer cells resulted in polarisation towards M2 macrophages, which led to an immunosuppressive phenotype by reducing the number of cytotoxic T cell populations. The authors further showed in vivo that when exosome biogenesis was inhibited, the percentage of M2 macrophages had been reduced while the numbers of cytotoxic T cells went up, highlighting how exosomes mediate immunosuppression in an indirect manner (214). In another study, Hao et al. showcased that exosomes derived from breast cancer cell lines induced polarisation of M0 macrophages to M2 macrophages, which supported breast cancer cell proliferation, migration and invasion (349). In respect to larger EVs or particles, Headley et al. investigated the influence of shear-derived large microparticles (LMPs) on the course of metastasis, demonstrating initially that these LMPs were internalised by various immune cells of myeloid origin including neutrophils, monocytes, macrophages and dendritic cells. The authors further showed that disruption of monocyte recruitment in vivo, by knockout of C-C motif chemokine receptor 2 (CCR2 gene), reduced the number of macrophages that were positive for LMPs, while the number of dendritic cells positive for LMPs were higher, which correlated with an increase in cytotoxic CD8⁺ T cells. Furthermore, depletion of the dendritic cell population resulted in six-fold higher metastases formation, suggesting overall that a shift in LMPs internalisation by specific immune cells could have protective or lethal effects (71). Despite these compelling preliminary findings, there is currently no knowledge on the interactions and impact of shear-derived large extracellular vesicles (LEVs) on monocytes or M1 macrophages.

Thus, in this Chapter, shear-derived LEVs were co-cultured independently with THP-1 monocytes or anti-tumour THP-1 differentiated M1 macrophages to examine their

differentiation to pro-tumour M2 macrophages. Primarily, it was shown that both immune cell populations can internalise LEVs both under static and flow conditions. Two endocytosis mechanisms, relevant for internalisation of large particles have been identified, namely phagocytosis and macropinocytosis, acting in synergy to enable internalisation of LEVs. Relevant markers of a M2 macrophage phenotype have been identified both in protein and gene expression levels, corroborating the successful conversion of monocytes and M1 macrophages to M2 ones, after interactions with LEVs. Consequently, LEV-derived M2 macrophages promoted their attachment to endothelial cells and exhibited an immunosuppressive phenotype by diminishing the secreted levels of Interferon gamma (IFN- γ) by T cells. Altogether, I hypothesised that LEVs are capable of indoctrinating immune cells to pro-tumour M2 macrophages to contribute to cancer metastasis.

6.2 MATERIALS & METHODS

6.2.1 Cell culture & Reagents

All reagents were purchased from Sigma Aldrich (UK), unless stated otherwise. All antibodies were purchased from Abcam (UK), unless noted otherwise. MDA-MB 231 breast cancer cells were maintained, cultured, harvested and stained as described in Chapter 2. Human umbilical vein endothelial cells (HUVECs) were cultured, maintained, harvested and prepared, as described in Chapter 5. THP-1 monocytes were purchased from ATCC (US) and are a suspension cell line. THP-1 monocytes were maintained in Roswell Park Memorial Institute (RPMI) 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% Penicillin/Streptomycin, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Gibco, US), 2.5 g/l Glucose (Merck, UK), 1 mM sodium pyruvate (Gibco, US) and 50 pM β -mercaptoethanol (Gibco, US). When THP-1 monocytes were thawed for the first time, cells were sub-cultured once a week for a month. Cells started growing as aggregates and gradually became single cells over the time period of the first month. After this initial period, THP-1 suspension monocytes were sub-cultured every 3 days by removing 75% of media and replenishing with an equal volume of fresh media. M1 macrophages were obtained from THP-1 monocytes through a series of chemical treatments. Given our inability to sub-culture obtained M1 macrophages, the differentiation protocol of monocytes to M1 macrophages was applied for every single experiment. Primarily, THP-1 monocytes would be treated with

phorbol-12-myristate-13-acetate (PMA) at final working concentration of 150 nM for 24 hr to obtain M0 macrophages. Subsequently, M0 macrophages would be treated with 20 ng/ml (final concentration) of Interferon gamma (IFN- γ /Biotechne, UK) for 24 hr to obtain M1 macrophages (180). M1 macrophages were used immediately for downstream experiments and were maintained in monocyte media. Last but not least, Sup T1 human T cells (ATCC, US) were maintained as a suspension cell line in RPMI 1640 media, supplemented with 10% FBS, 1% Penicillin/Streptomycin and 1% of Interleukin 2 (Appleton Woods, UK).

6.2.2 Cell & LEVs coculture

LEVs for downstream experiments were generated, purified and enumerated, as described in detail in Chapter 4. THP-1 monocytes, M1 macrophages or T cells were treated with LEVs independently always at a 3 : 1, cell to LEVs ratio. For internalisation mechanism studies, cells and LEVs were pre-stained with a red Cell tracker (ThermoFischer Scientific, UK) and a green Cell tracker (ThermoFischer Scientific, UK), respectively, at a final working concentration of 10 μ M for 1 hr at 37°C/5%CO₂ and then were co-cultured either at wells of a 24- or 96-well plate for at least 16 hours. For imaging of molecular markers, PCR and for all other assays tested, cells and LEVs were co-cultured for 30 hrs.

6.2.3 Collection of conditioned media

THP-1 monocytes or obtained M1 macrophages were treated with LEVs, as described above. After 30 hr of co-culture, media with LEVs was removed and fresh media was added to cells, which was collected after 24 hr and centrifuged to pellet any cells, allowing the collection of the supernatant as conditioned media (CM), designated as LEV-treated monocyte CM. CM was collected also from untreated monocytes. CM from cultured MDA-MB-231 was also collected, as above. Purified LEVs were pelleted at 10000 x g for 30 min and fresh media was used to resuspend them. After being left for 1 day in culture, LEVs were pelleted again at the same conditions to collect the supernatant as CM that contained secreted factors from LEVs.

6.2.4 Cell treatment

To investigate mechanisms of internalisation by monocytes or M1 macrophages, 20000 pre-stained red monocytes or M1 macrophages were cultured independently in wells of a 96-well plate. Initially, monocytes or M1 macrophages were treated independently either with

Cytochalasin D (Cyto D) or 5-(N-Ethyl-N-isopropyl)-Amiloride (EIPA, MedChemExpress, Sweden) or combination for 3 hr at 37°C/5%CO₂ at final working concentrations of 10 µM and 50 µM, respectively. Once compounds were removed, pre-stained green LEVs were added, based on the 3:1 cell : LEVs ratio and co-cultured for at least 16 hrs. Then, media with non-internalised LEVs was removed and fresh media was added. Cells were imaged directly in Confocal microscopy, as will be elaborated below. The above experiment was repeated in wells of 24 well plates, for flow cytometry studies. This time 150000 cells were co-cultured with 50000 LEVs. Cells were scraped, using a Cell Scraper (ThermoFischer Scientific, UK) and 500 µl of cell suspensions were transferred in Eppendorf tubes for downstream analyses in ab Amnis CellStream flow cytometer (Luminex, US). Moreover, M0 macrophages were treated with 20 ng/ml Interleukin-4 (Biotechne, UK) for direct conversion to M2 macrophages. Both monocytes and M1 macrophages were also incubated with fluorescent beads (green) of 5 or 10 µm at a similar timeframe and mix ratio as with LEVs.

6.2.5 Confocal microscopy

Monocytes and M1 macrophages that were co-cultured with LEVs were imaged in a Leica SP8 Inverted scanning confocal microscope. The settings of the microscope were applied similarly, as elaborated in Chapter 5. Several Z-stack images were obtained at a step of 2 µm for each experimental condition, by initially setting the z height range. Orthogonal cross sectioning was applied to validate internalization of LEVs, that enabled visualization on all three coordinates' pairs i.e. x-y, y-z and x-z. Images were also obtained for 2 hrs with 1 min intervals that were then compiled to video series. All images were obtained at 20 x magnification.

6.2.6 Flow setup

To mimic the transit conditions found in the bloodstream, LEVs were added either with THP-1 monocytes or THP-1 derived M1 macrophages in a custom set up, under continuous flow conditions. Firstly, the inlet and outlet of microfluidic devices were connected with separate 50 cm long 2 mm diameter (external) of tubing (Qosina, US). The other end of the tubing that was connected to the outlet of the microfluidic device was added in a 50 ml falcon tube that contained cell media solution. Then, a third piece of tubing (as above) was added inside the solution of the falcon tube and its other end was connected to a 12V adjustable peristaltic dosing pump (Amazon, UK, G628-1-2), that was the inlet of the pump. The other end of the

tubing that was initially connected to the inlet of the microfluidic device was connected to the outlet of the pump. This set up allowed LEVs and cells to flow from the media-filled falcon tube inside the peristaltic pump and into the microfluidic device in a continuous loop. LEVs and either monocytes or M1 macrophages were added in the falcon tube reservoir a few seconds before initiating the power on the peristaltic pump, to avoid any internalisation in static conditions. 100000 LEVs and 300000 cells were stained independently as green and red, respectively, as described elsewhere.

6.2.7 Adhesion assay

15000 pre-stained monocytes with a red Cell Tracker, were added in each well of a 96-well plate and were treated independently with 5000 purified LEVs, LEVs CM and MDA-MB 231 CM for 30hrs. Untreated monocytes were used as a negative control. Images of the entire well as well as 3-4 characteristic images of each well/condition at 20 x magnification were obtained using Nikon microscope, after 24 hrs before and after media removal to evaluate remaining cells in wells. Quantification was performed by analysing the number of monocytes before and after media removal, as recorded in 20x images and extrapolating their difference percentage wise.

6.2.8 Proliferation assay

12000 monocytes were added in each well of a 96-well plate and were treated independently with 4000 purified LEVs or LEVs CM for altogether 48 hrs. Untreated monocytes were used as a negative control. To measure proliferation of each condition, the MTT cell proliferation assay kit (Abcam, UK) was used. Treatment media was removed by centrifugation of monocytes at 180 x g for 5 min and a mix of 50/50 μ l of serum-free media and MTT reagent was used to resuspend the pellet and then transferred again in the respective well. For background measurements, blank controls were used, which consisted of serum-free media and MTT reagent, but without any cells. The 96-well plate was incubated for 3 hrs at 37°C/5% CO₂. Then, 150 μ l of MTT solvent was added directly in each well. The well plate was wrapped in foil and was shaken for 15 mins at room temperature. Absorbance values were read at an optical density of 590 nm, using Varioskan Flash plate reader. For quantification, the 'blank' measurement was subtracted from each measurement, providing the corrected absorbance value.

6.2.9 Immunofluorescence

15000 monocytes and 5000 purified LEVs were cocultured as described elsewhere in wells of a 96-well plate for 30 hr. Monocytes were further treated with LEVs CM. Untreated monocytes served as a negative control. 4% (v/v) formaldehyde, diluted in PBS was added in each well for 12 min at room temperature. Wells were washed three times with PBS for 3 min per wash and further incubated with 0.1% (v/v) Triton X (diluted in PBS) for 5 min at room temperature. After three washes with PBS for 3 min per wash, all samples were incubated overnight at 4 °C with a combination of either C-X-C motif chemokine 10 (CXCL10) recombinant rabbit monoclonal antibody (ThermoFischer Scientific, UK) at 2.5 µg/ml final working concentration and PE/Cy7® Anti-Mannose Receptor antibody, (diluted 100 times from the vial's volume-ab270682). Alternatively, samples were incubated with a combination of PE/Cy7® Anti-Mannose Receptor antibody, (diluted 100 times from the vial's volume-ab270682) and recombinant anti-tumour necrosis factor-alpha (TNF-α) primary antibody at a final working concentration of 0.5 µg/ml. The following day, after a final wash 1x with PBS for 3 min, all samples were incubated for 2 hrs at room temperature, with a combination of goat anti rabbit secondary antibody Alexa Fluor 488 and goat anti rabbit secondary antibody Alexa Fluor 647, at final working concentrations for both at 2 µg/ml. 3-4 characteristic multifuorescent images were obtained at 20 x magnification for each condition and per sample, using either confocal microscopy or the Nikon microscope.

6.2.10 Flow cytometry

150000 THP-1 monocytes were cultured in wells of a 24 well plate and underwent the immunofluorescence protocol, for the same experimental conditions as tested in previous paragraph. 500 µl of each sample was transferred in a 2 ml Eppendorf tube and loaded in a Amnis CellStream flow cytometer, run at 20 µl/min. Unstained and single-colour controls were used to gate the areas for either double positive events (CXCL10 + CD206) or only CD206-expressing events. The mean fluorescence intensity and the frequency of either CD206-only or double positive events were calculated by the software.

Additionally, 150000 of either THP-1 monocytes or THP-1 derived M1 macrophages were cultured in wells of a 24-well plate and one day later were stained and treated with Cyto-D or EIPA or a combination, as described elsewhere. 50000 LEVs were then added in all above

conditions and co-cultured for 16 hrs. LEV-treated monocytes or LEV-treated M1 macrophages, without Cyto-D or EIPA treatment were also used as a positive control for internalisation. Monoculture of stained or unstained THP-1 or M1 macrophages were used to set the gating criteria for enumeration of the double positive events, described as cells that had ingested LEVs. At least 1000 events were recorded per replicate and condition and the frequency of double positive events was calculated. Finally, the same analyses was done for cells that were co-cultured with fluorescent beads.

6.2.11 Real-time PCR

300000 of either THP-1 monocytes or THP-1 derived M1 macrophages were co-cultured with 100000 purified LEVs in wells of 12-well plate. Monocultures of either monocytes or M1 macrophages were used as negative control. After 30 hr incubation, 300 µl of lysis buffer (Qiagen, US) was added to wells and total RNA was harvested using the RNeasy Mini kit (Qiagen, US). The purity and total quantity of the harvested RNA was assessed and extrapolated using Nanophotometer (IMPLEN, USA). 1 µg of RNA was reverse transcribed by using an iScript cDNA synthesis kit (Bio rad laboratories, UK). Then, 50 ng of cDNA was amplified via real-time PCR on Applied Biosystems™ StepOne™ Real-time PCR System (Fischer Scientific, UK), by using SYBR Green PCR master mix (ThermoFischer Scientific, UK). Relative mRNA expression of all tested genes was extrapolated using the $2^{-\Delta\Delta C_t}$ method. The forward and reverse primers were designed for cluster of differentiation (CD206), fibronectin (FN), arginase-1 (Arg-1), interleukin-6 (IL-6), C-X-C motif chemokine 10 (CXCL10) and ribosomal protein large P (RPLP0), detailed in **Table 6-1**. Ribosomal protein large P (RPLP0) was used as housekeeping gene.

Table 6-1 Forward and reverse primer sequences

PRIMER	FORWARD SEQUENCE	REVERSE SEQUENCE
CD206	CGTTCGGTTCACCCACTGGA	ACATCCCATAAGCCCCCTGC
FN	GATGCACCATCCAACCTGCG	GATTGAGTCCCGGACCGTTG
ARG-1	TGGCAAGGTGGCAGAAGTCA	TGGCATGGCCAGAGATGCTT
IL-6	GGTCCAGTTGCCTTCTCCCTG	TGCCCATGCTACATTGCCG

CXCL10	TGAGCCTACAGCAGAGGAACC	GCCTCTGTGTGGTCCATCCTT
RPLO	CGGTTTCTGATTGGCTAC	ACGATGTCACTTCCACG

6.2.12 Monocyte attachment

15000 HUVECs were added in wells of a 96-well plate and left to grow for 24hrs. Additionally, 30000 THP-1 monocytes were cultured in independent wells of a 48-well plate and were grown for 1 day, after which they were stained with a green cell tracker, as described elsewhere. Then, monocytes were co-cultured with 10000 LEVs for 30hrs. Then, LEVs were removed from the treated wells and 10000 LEV-treated monocytes were added to confluent monolayers of HUVECs. Untreated monocytes of the same numbers were added in wells with HUVECs as a negative control. The well plate was loaded in a Varioskan Flash plate reader and green fluorescence was quantified at 0hr, with excitation and emission values set at 492 and 517 nm, respectively. Fluorescent images of the entire well were also obtained at 0hr. After 4 hrs of co-culture, the media was removed to clear away non-adhered monocytes and the green fluorescence and fluorescent images were obtained, as above. Media without fluorescent cells was used as a blank measurement. The value of the blank solution was subtracted from each reading. To extrapolate the fluorescence of remaining attached monocytes, the fluorescence value after media removal was subtracted from the fluorescence value before media removal and the difference was divided by the initial fluorescence value. The result obtained percentage wise was lastly subtracted from 100% to reflect the remaining fluorescence of attached monocytes or LEV-treated monocytes.

6.2.13 T cell proliferation

2000 T cells were added in separate wells of a 96 well plate and were stained with a green cell tracker, as explained elsewhere. Then, cells were cultured with LEVs or monocyte CM or LEV-treated monocyte CM for 48 hr. Untreated T cells served as a negative control. Then, T cells were centrifuged at 180 x g for 4 min and supernatant was removed. The pelleted T cells were resuspended in 500 µl of fresh media and were counted in flow cytometry, by enumerating the cell events that expressed green signal.

6.2.14 T cell activation

50000 T cells were added in separate wells of a 48 well plate and were independently co-incubated with LEVs or LEV-treated monocyte CM for 48 hrs. Untreated T cells were used as a negative control. CM from monocytes or LEV-treated monocytes was also analysed to normalise T cell secretion of IFN γ , when T cells were incubated with either. Cells were removed by centrifugation at 180 x g for 4 mins and then media was collected. The LEGEND MAXTM Mouse IFN γ ELISA kit was used to measure the secreted IFN γ levels by T cells. A standard solution of IFN γ was prepared according to the kits instructions. The plate was washed 4x with wash buffer. Then, 50 μ l of Assay buffer was added in each well and immediately 50 μ l from each sample or standard solution were added in each well. The plate was incubated at room temperature for 2 hrs in a plate shaker (200 rpm). Then, the contents were discarded and plate was washed 4x with wash buffer. Then, 100 μ l of Mouse IFN γ detection antibody was added in each well and plate was incubated at room temperature for 1 hr in a plate shaker (200 rpm). Then, the content was removed and all wells were washed 2x with wash buffer. Then, 100 μ L of Avidin solution was added in each well and the plate was incubated for 30 min at room temperature in a plate shaker (200rpm). Then, all wells were washed again 2x with wash buffer and 100 μ L of substrate solution was added in each well and the plate was incubated for 15 mins in the dark. The reaction was stopped by adding 100 μ l of stop solution. The plate was imaged using a Varioskan flash plate reader at an absorbance of 570 nm and 450 nm. The absorbance values of 570 nm was subtracted from the absorbance values from 470 nm for all samples. Then, the absorbance value of the blank measurement (no cells) was subtracted from each reading. At this point, the standard curve was generated based on the known concentrations of the standard samples. In respect to the unknown samples, the samples where T cells were cultured with either monocyte or LEV-treated monocyte CM were normalised by subtracting the absorbance values of monocyte or LEV-treated monocyte CM that were analysed without any cells' inclusion. Finally, the concentration of IFN γ for all samples were calculated according to the generated standard curve.

6.2.15 Graphs & Statistical Analyses

All data were plotted using GraphPad version 9, elaborated in Chapter 2. Statistical differences were evaluated for significance either between two or among at least 3 experimental conditions, using GraphPad. All experiments were repeated at least thrice. Illustrations were designed using BioRender.

6.3 RESULTS

6.3.1 LEVs interact with monocytes and M1 macrophages

To explore initially how shear-derived LEVs interact with THP-1 monocytes or THP-1 derived M1 macrophages independently, initially THP-1 monocytes were treated with PMA and subsequently with IFN γ to obtain M1 macrophages (**Fig. 6-1A**). Both monocytes and M1 macrophages were co-cultured independently with LEVs and were visualised under static conditions (**Fig. 6-2A&C**). Untreated monocytes appeared highly spherical, given their suspending nature in media, whereas LEV-treated or PMA-treated monocytes assumed more stretched morphologies, appearing adhered (**Fig. 6-1A-B**). IFN γ treatment also resulted in differentiation of M0 macrophages (PMA-treated only) to M1 macrophages that also appeared different morphologically, with a larger size and membrane protrusions compared to PMA-differentiated M0 macrophages (**Fig. 6-1A-B**). As previously established, fluorescent LEVs were enumerated via flow cytometry and were co-cultured with either monocytes or M1 macrophages. Via live imaging I observed that monocytes (suspension cell line) were generally motile near LEVs, but no recording was acquired. Fluorescent images revealed that LEVs appeared co-localised with cells in the x-y plane, suggestive of their internalisation (**Fig. 6-2A&C**).

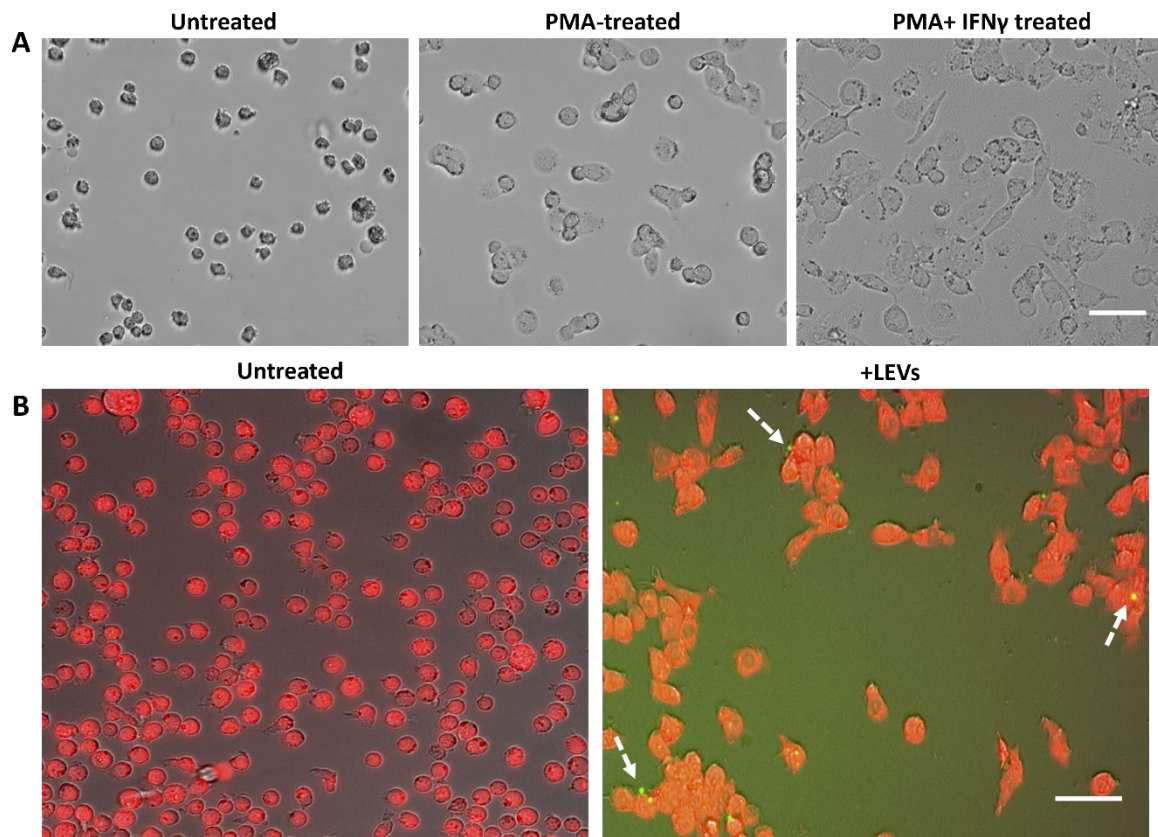


Figure 6-1: LEVs interact with monocytes.

(A) Brightfield images (20x) of THP-1 monocytes, PMA-treated monocytes and of PMA-treated monocytes that were further treated with IFN γ . Scale bar: 50 μ m. (B) Fluorescently tagged monocytes (red) were co-cultured with LEVs (green). Images obtained at 20x. Dashed arrows indicate the location of green-stained LEVs on red-stained monocytes. Scale bar: 50 μ m.

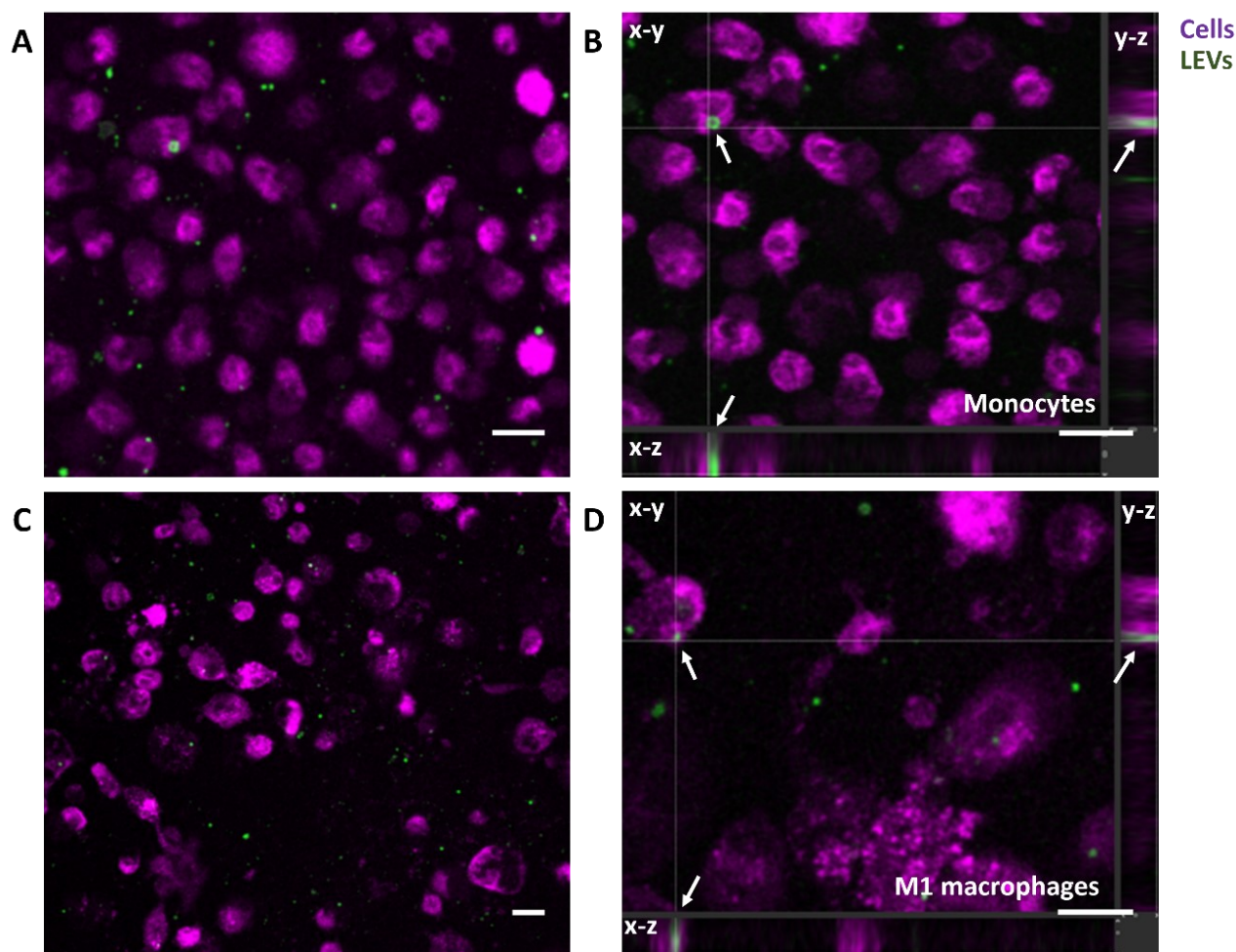


Figure 6-2: LEVs are internalised by monocytes and M1 macrophages.

(A-D) Monocytes or M1 macrophages are stained red (depicted as purple) and LEVs a green. Scale bar: 20 μm . (A) Fluorescent image of monocytes and LEVs co-culture (B) Orthogonal cross sectioning of a fluorescent image of an LEV internalised by monocytes. (C) Fluorescent image of M1 macrophages and LEVs co-culture (B) Orthogonal cross sectioning of a fluorescent image of an LEV internalised by M1 macrophages.

Multifluorescent images of pre-stained cells and LEVs with different colours and careful inspection of the Z-stack slices (orthogonal cross-sectioning of all 3 pairs of coordinates), revealed that LEVs were internalised both by monocytes and M1 macrophages (**Fig. 6-2B&D**). To further quantify the LEVs internalisation frequency, via flow cytometry it was determined that the mean internalisation frequency of monocytes and M1 macrophages were $48.8 \pm 2.8\%$ and $58.5 \pm 5.7\%$, respectively, higher compared to their respective single-colour controls, designated as untreated (**Fig. 6-3A-B, Fig. 6-5A-B**). Co-culture of either monocytes or M1 macrophages with fluorescent beads of either 5- or 10 μm , highlighted that the internalisation

frequency of either beads was similar to mono-cultured monocytes or M1 macrophages (**Fig. 6-4A-B, Fig. 6-6A-B**). Furthermore, the shift of the monocyte : LEVs ratio from 3:1 to a 6:1 ratio led to lower internalisation frequency from 48.8% to 14.2% (**Fig. 6-3A-B** & Appendix 9), suggesting that the availability of either cells or LEVs affects the probabilities of internalisation frequency. When tested for HUVECs, the same HUVEC:LEVs ratio 3:1 was held constant, but the co-culture occurred in 12-well plate instead of 24-well plate, and the internalisation frequency was lowered from 34.2 to 5.2 (Fig. 5-3B & Appendix 10), suggesting once again that reduced space for interactions increased probabilities of internalisation. Altogether, both qualitative and quantitative data support LEVs internalisation by various immune cells at high frequencies in static conditions.

EIPA or combo of compounds was added to monocytes 3 hr prior to LEVs addition. (B) Quantitative data of (A) (n=3 biological replicates for all).

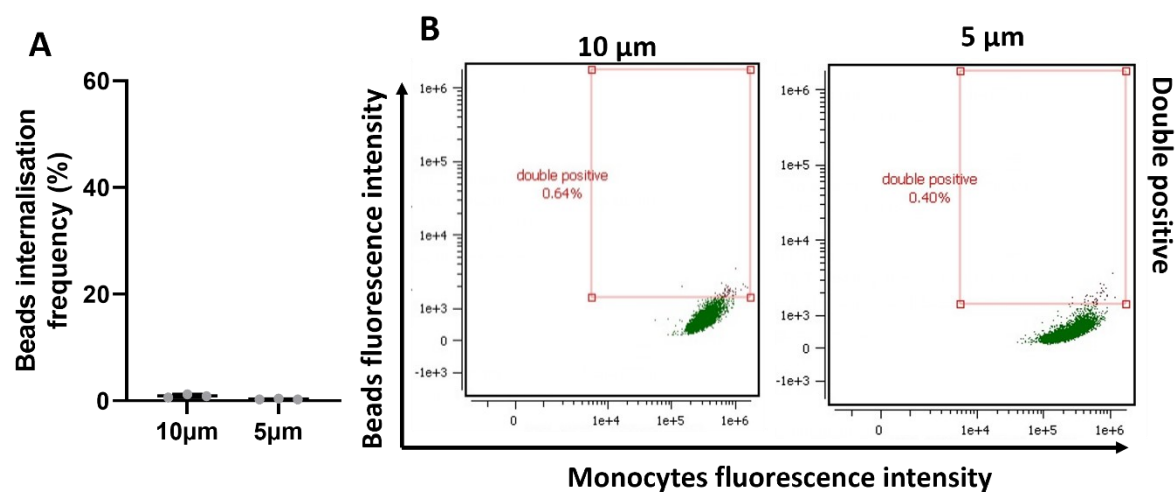


Figure 6-4: Beads' internalisation mechanisms by monocytes.

(A) Quantitative data of beads (10 µm and 5 µm) internalisation by monocytes (n=3 biological replicates). (B) Relevant dot plots representing double positive events.

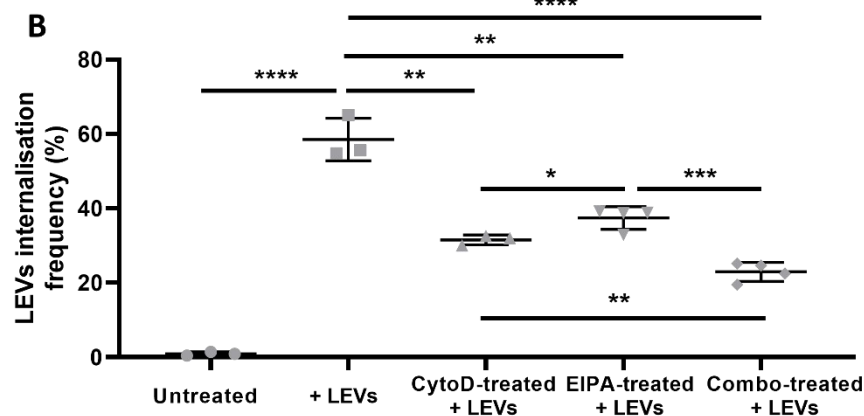
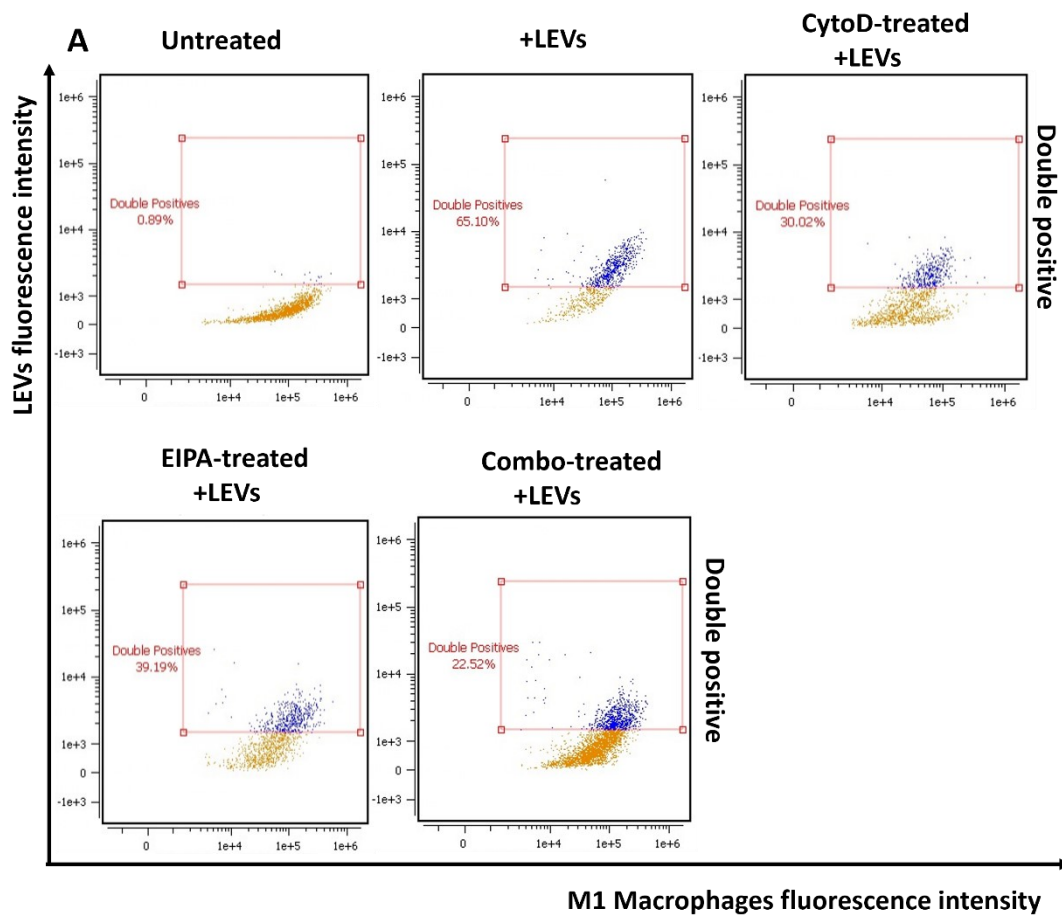


Figure 6-5: LEVs' internalisation mechanisms by M1 macrophages.

(A) Dot plots of double positive events, representing M1 macrophages that have internalised LEVs under different conditions via flow cytometry. LEVs were co-cultured with M1 macrophages for 16hr. Cyto-D or EIPA or combo of compounds was added to M1 macrophages 3 hr prior to LEVs addition.

(B) Quantitative data of (A) (n=3 biological replicates for all).

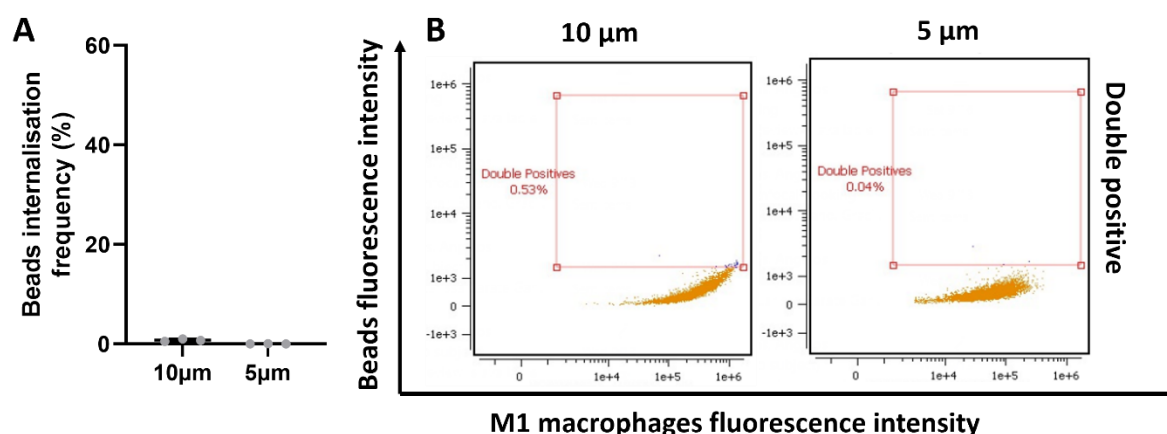


Figure 6-6: Beads' internalisation mechanisms by M1 macrophages.

(A) Quantitative data of beads (10 µm and 5 µm) internalisation by M1 macrophages (n=3 biological replicates). (B) Relevant dot plots representing double positive events.

6.3.2 LEVs influence monocytes

To begin exploring the influence of LEVs on monocytes, the adhesion potential of monocytes was evaluated. Monocytes are a suspension cell line, but exhibition of adherent features is in agreement with differentiation to an adherent macrophage population, as shown by PMA treatment (**Fig. 6-1**). As seen in **Fig. 6-7A**, the number of remaining cells before and after media removal, when monocytes were treated with LEVs, is clearly higher. Quantification revealed that monocytes that were cultured with LEVs CM or LEVs or MDA-MB-231 cancer cell CM, presented significantly higher adhesion percentages, compared to untreated (**untreated**: $1 \pm 0.7\%$ vs **LEVs CM**: $28.3 \pm 12\%$, $P < 0.05$; **untreated**: $1 \pm 0.7\%$ vs **LEVs**: $47.1 \pm 10.6\%$, $P < 0.005$; **untreated**: $1 \pm 0.7\%$ vs **cancer cell CM**: $56.1 \pm 15\%$, $P < 0.0005$), whereas multiple comparisons between the three treatment conditions revealed no statistical significance (**Fig. 6-7B**). Interestingly, LEVs were also able to promote cell proliferation (**Fig. 6-7C-D**). What was further intriguing was that while LEVs promoted monocyte adhesion at about 50% of the population, the majority of cells that were positive for internalised LEVs were actually suspended (adherent: $11.3 \pm 1.4\%$ vs LEVs: $88.7 \pm 1.4\%$, $P < 0.0001$) (**Fig. 6-7E**).

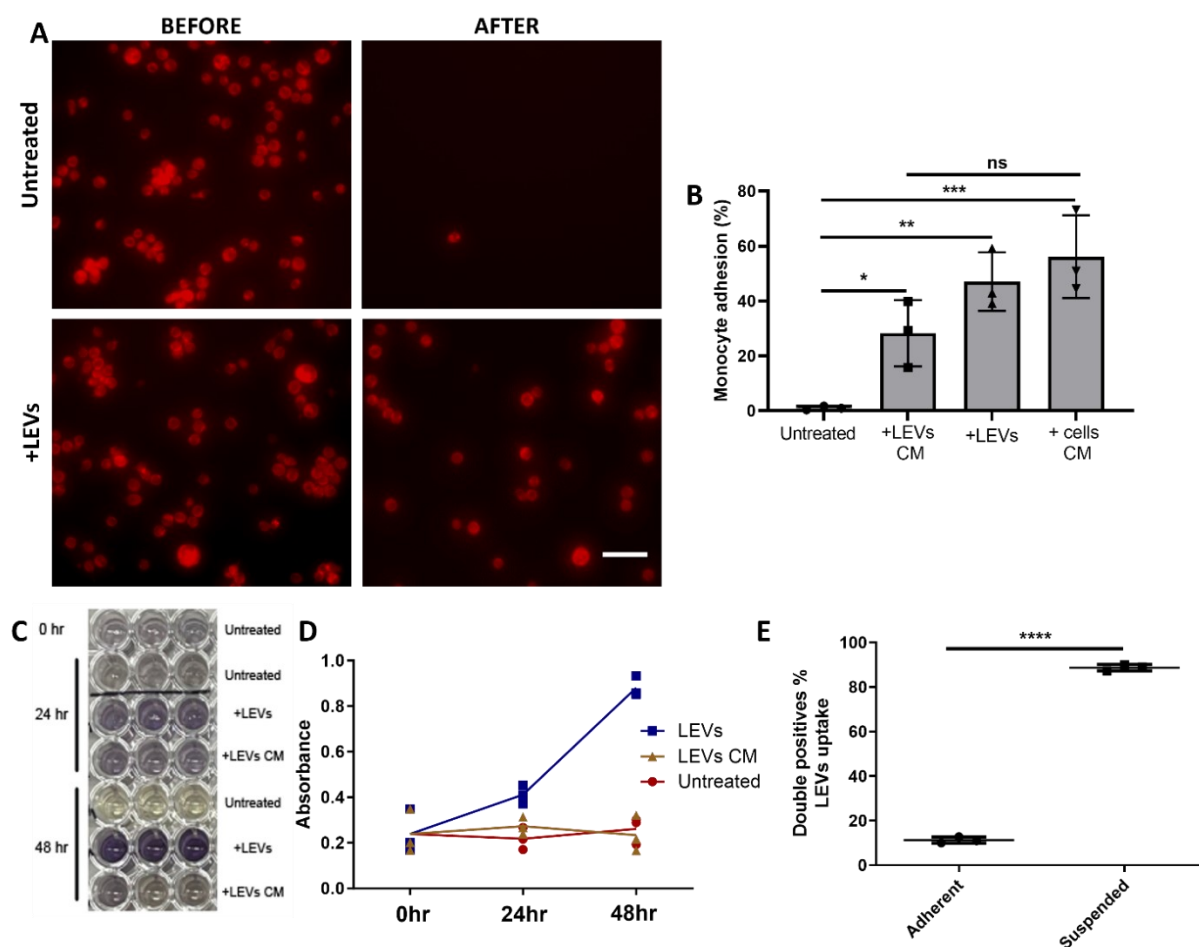


Figure 6-7: LEVs induce changes to monocytes.

(A) Fluorescently tagged monocytes (red) were treated with LEVs, LEVs CM or MDA-MB 231 cell CM for 30hr. Images were obtained before and after media removal to assess monocyte adhesion to wells. Scale bar: 50 μ m. (B) Quantitative data obtained from 20x images (n=3 biological replicates for all). (C) MTT assay for proliferation estimation. (D) Quantitative data of (C) based on absorbance measurements (n=3 biological replicates for all). (E) Percentage of adherent or suspended monocytes that were positive for internalised LEVs, analysed via flow cytometry (n=3 biological replicates for all).

6.3.3 Monocytes and M1 macrophages internalise LEVs under flow conditions

To mimic the transit of LEVs and immune cells in the bloodstream, a flow setup using a peristaltic pump was utilised (**Fig. 6-8A**), that allowed the continuous flow from a reservoir through the pump and through a microfluidic device with a single channel (100 μ m width x 100 μ m height). Upon co-culture for 16hr, both monocytes and M1 macrophages were

verified to have internalised LEVs, albeit M1 macrophages did so at a much lower frequency (Fig. 6-8B-E). In respect to monocytes, preliminary findings suggested that a fraction of monocytes becomes adherent upon LEVs interactions (Fig. 5-5A-B). Therefore, to directly compare internalisation frequencies between static and flow conditions for monocytes, monocytes were cocultured with LEVs in static conditions, but only suspended monocytes were further analysed. Thus, monocytes internalised LEVs at higher frequency means in static conditions compared to flow (static: $72.1 \pm 0.6\%$ vs flow: $55.4 \pm 2.7\%$, $P < 0.0005$) (Fig. 6-8B-C). In respect to adherent M1 macrophages, their LEVs internalisation frequency mean was $12.5 \pm 0.2\%$ significantly higher compared to mono-cultured M1 macrophages (Fig. 6-8D-E). Cumulatively, these findings highlight that LEVs can be consistently internalised by monocytes and macrophages both in static and flow conditions.

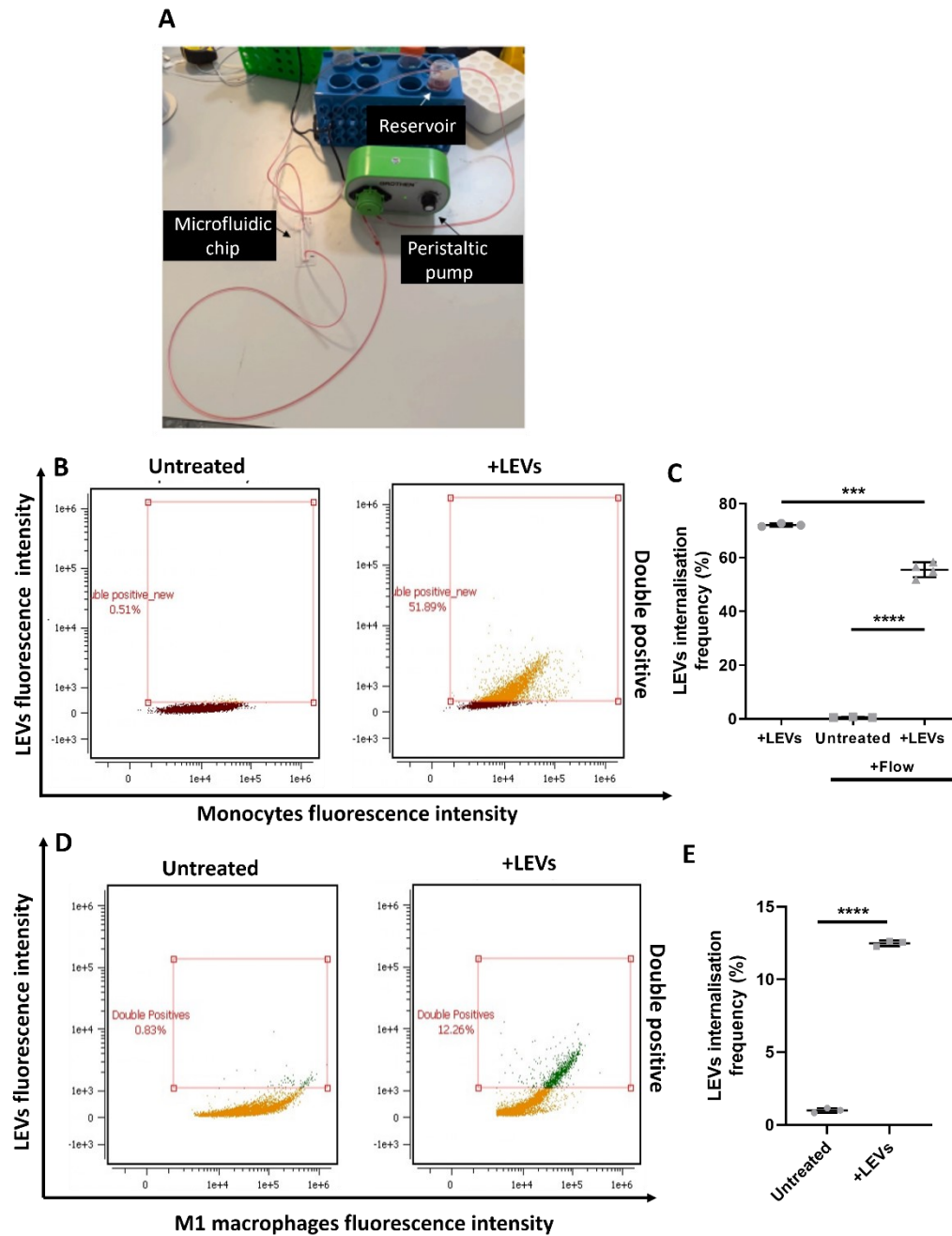


Figure 6-8: Monocytes and M1 macrophages internalise LEVs under flow conditions.

(A) Image of custom set-up to mimic continuous flow conditions. (B) Dot plots of double positive events, representing monocytes that have internalised LEVs under continuous flow. (C) Quantitative data of (B) (n=3 biological replicates for all). Comparison with monocytes that were suspended in static conditions. (D) Dot plots of double positive events, representing M1 macrophages that have internalised LEVs under continuous flow. (E) Quantitative data of (D) (n=3 biological replicates for all).

6.3.4 Monocytes and M1 macrophages combine endocytosis mechanisms to internalise LEVs

To investigate the endocytosis mechanism involved, we used as previously established, Cyto-D and EIPA to inhibit phagocytosis and macropinocytosis, respectively. In respect to monocytes, their internalisation frequency mean was reduced significantly by treatment with Cyto-D, EIPA or the combination (untreated: $48.8 \pm 2.8\%$ vs Cyto-D: $27.6 \pm 1.3\%$, $P < 0.0005$; untreated: $48.8 \pm 2.8\%$ vs EIPA: $22 \pm 0.9\%$, $P < 0.0005$; untreated: $48.8 \pm 2.8\%$ vs combination: $2 \pm 0.3\%$, $P < 0.0001$) (**Fig. 6-3A-B**). Interestingly, treatment with EIPA had a more significant reduction of internalisation frequency mean compared to treatment with Cyto-D (EIPA: $22 \pm 0.9\%$ vs Cyto-D: $27.6 \pm 1.3\%$, $P < 0.005$), whereas the combination of drugs lowered almost entirely internalisation frequency, compared to individual treatments (**Fig. 6-3A-B**).

In respect to M1 macrophages, their internalisation frequency mean diminished significantly by treatment with Cyto-D, EIPA or the combination of compounds (untreated: $58.5 \pm 5.7\%$ vs Cyto-D: $31.5 \pm 1.3\%$, $P < 0.005$; untreated: $58.5 \pm 5.7\%$ vs EIPA: $37.4 \pm 3\%$, $P < 0.005$; untreated: $58.5 \pm 5.7\%$ vs combination: $23 \pm 2.5\%$, $P < 0.0001$) (**Fig. 6-5A-B**). Interestingly, treatment with Cyto-D had a more significant reduction of internalisation frequency mean compared to treatment with EIPA (Cyto-D: $31.5 \pm 1.3\%$ vs EIPA: $37.4 \pm 3\%$, $P < 0.05$), whereas the combination of drugs lowered significantly the internalisation frequency, compared to individual compound treatments (**Fig. 6-5A-B**), but did not abolish it completely. Altogether, evidence was collected that both phagocytosis and macropinocytosis are utilised exclusively by monocytes and predominantly by M1 macrophages to internalise LEVs.

6.3.5 LEVs induce differentiation of both monocytes and M1 macrophages to CD206+ M2 macrophages

To validate molecularly the impact of LEVs on monocytes, validation markers were used both at protein and gene expression levels. A traditional marker associated with a M2 macrophage phenotype (180,350), CD206 was highly expressed in LEV-treated monocytes, compared to untreated (2056 ± 129 vs 438 ± 11 , $P < 0.0001$), whereas CXCL10, a general immune cell marker was retained (**Fig. 6-9A-C**). According to single-cell analysis, the vast majority of monocytes had converted to CD206+ macrophages upon LEVs exposure compared to untreated ($90.3 \pm$

2.5% vs $1.7 \pm 0.5\%$, $P < 0.0001$) (**Fig. 6-10A-B**). To ensure that LEVs did not induce monocyte differentiation to M1 macrophages, the experiment was repeated by comparing TNF- α and CD206 protein expression, that act as M1 macrophage and M2 macrophage markers, respectively (180,350). Multifluorescent images revealed the absence of TNF- α signal in all conditions visualised, suggesting that monocytes differentiate fully to M2 macrophages upon exposure to LEVs (**Fig. 6-11A**). While previous findings on CD206 higher expression were reproduced (**Fig. 6-9A-C**), there was also an increased expression of CD206 by treatment with LEVs CM compared to untreated (**LEVs CM-treated**: 2155 ± 35 vs **untreated**: 577 ± 1.7 , $P < 0.0001$), albeit lower compared to treatment with LEVs (**LEVs CM-treated**: 2155 ± 35 vs **LEVs-treated**: 3924 ± 41 , $P < 0.0001$) (**Fig. 6-11B-C**). Revisiting the data on single-cell level, significantly less monocytes were differentiated to CD206+ M2 macrophages under treatment of LEVs CM compared to LEVs treatment (**LEVs CM**: $66.7 \pm 0.3\%$ vs **LEVs**: $98.1 \pm 0.3\%$, $P < 0.0001$) (**Fig. 6-12A-B**).

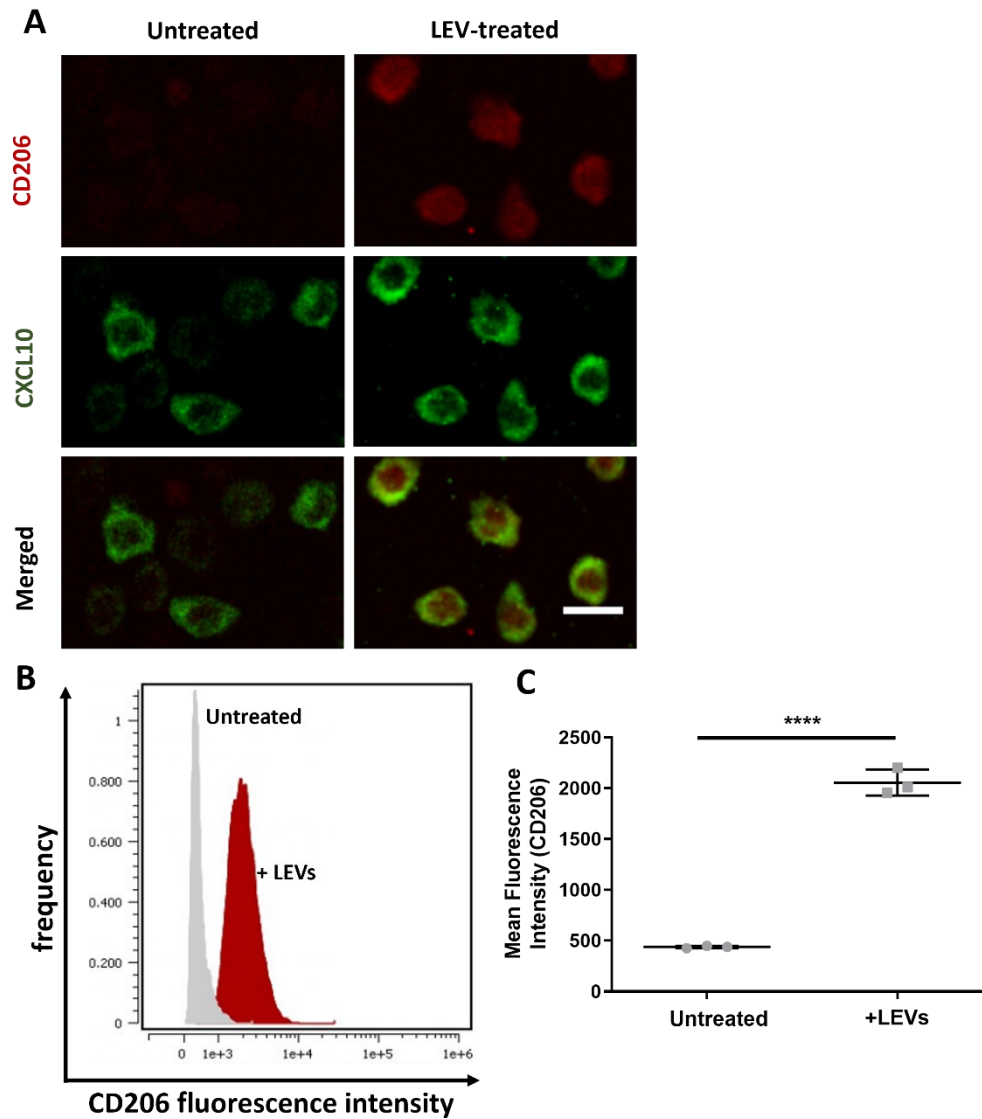


Figure 6-9: LEVs induce differentiation of monocytes to M2 macrophages.

(A) Multifluorescent images of untreated or LEV-treated monocytes for CD206 (red) and CXCL10 (green) at 20x. Scale bar: 20 μ m. (B) Mean fluorescence intensity of CD206 expression by untreated or LEV-treated monocytes. (C) Quantitative data of (B) (n=3 biological replicates for all).

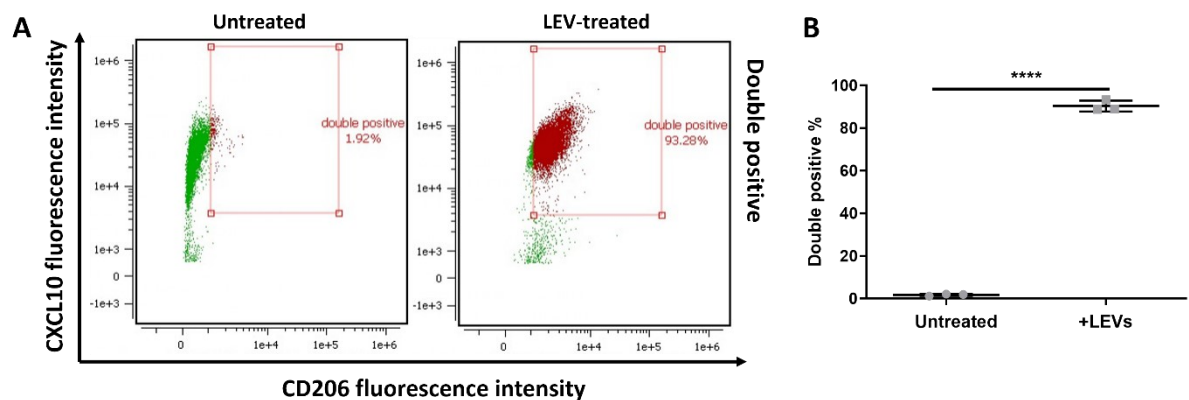


Figure 6-10: LEVs induce differentiation of monocytes to M2 macrophages.

(A) Dot plots of percentage of untreated or LEV-treated monocytes that express CD206. (B) Quantitative data of (A) (n=3 biological replicates for all).

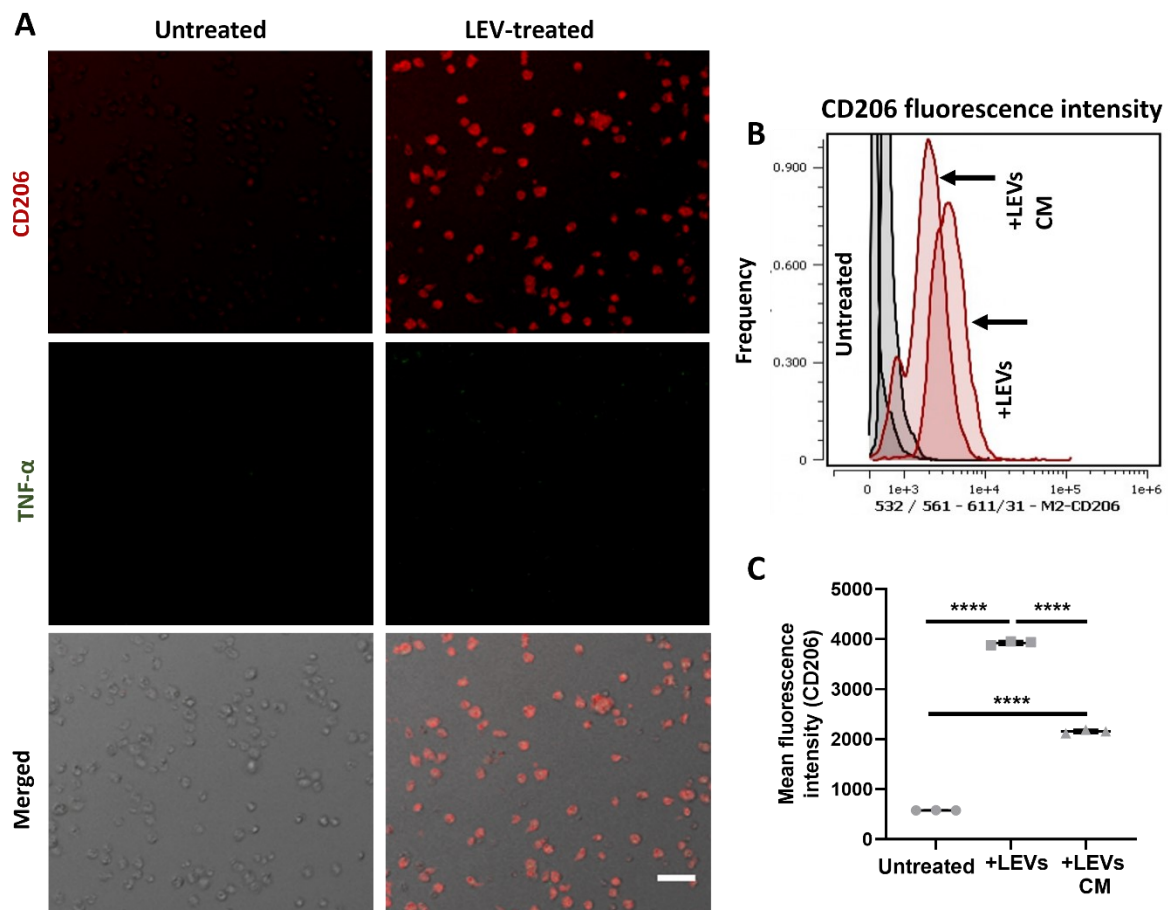


Figure 6-11: LEVs and their CM induce differentiation of monocytes to M2 but not to M1 macrophages.

(A) Multifluorescent images of untreated or LEV-treated monocytes for CD206 (red) and TNFα (green) at 20x. Scale bar: 50 μm. (B) Mean fluorescence intensity of CD206 expression by untreated or LEV-

treated monocytes or monocytes treated with LEVs CM. (C) Quantitative data of (B) (n=3 biological replicates for all).

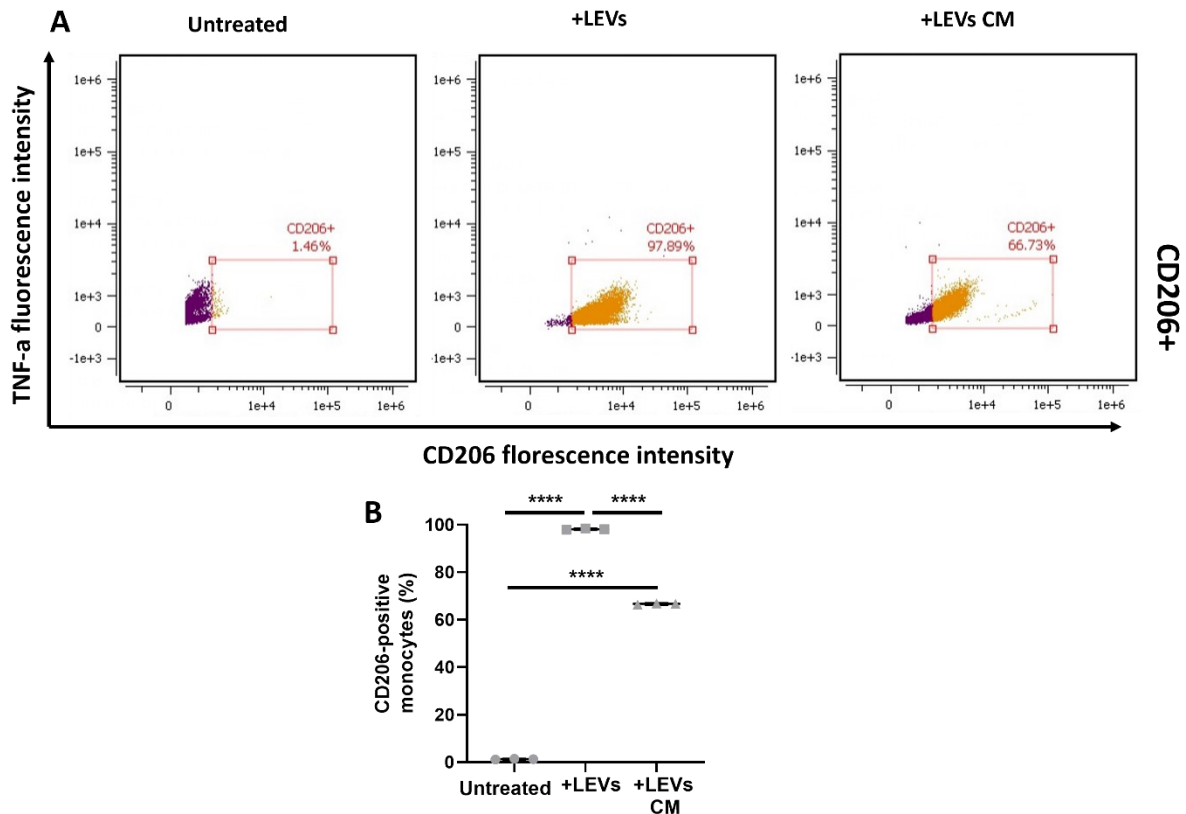


Figure 6-12: LEVs and their CM induce differentiation of monocytes to M2 but not to M1 macrophages.

(A) Dot plots of percentage of untreated or LEV-treated monocytes or monocytes that were treated with LEVs CM that express CD206. (B) Quantitative data of (A) (n=3 biological replicates for all).

To evaluate phenotypic changes in gene levels, real time quantitative PCR was used. LEV-treated monocytes exhibited significantly increased mean expression of both CD206 and FN compared to untreated (CD206: 4.5 ± 1.3 vs 1 ± 0 , $P < 0.05$; FN: 3 ± 1.2 vs 1 ± 0 , $P < 0.05$), whereas expression of other genes such as Arg-1, Il-6 and CXCL10 remained statistically unaltered (**Fig. 6-13A**). On the other hand, treatment of M1 macrophages with LEVs resulted in more clearly distinguishable profiles, with various markers expressed differentially, including CD206, Arg-1 and FN compared to untreated (CD206: 2.1 ± 0.23 vs 1 ± 0 , $P < 0.005$; Arg-1: 2.2 ± 0.5 vs 1 ± 0 , $P < 0.05$; FN: 1.7 ± 0.4 vs 1 ± 0 , $P < 0.05$), whereas expression of genes such as IL-6 and CXCL10 remained unchanged (**Fig. 6-14A**). Altogether, LEVs are capable of

inducing differentiation of both monocytes and M1 macrophages to pro-tumour M2 macrophages.

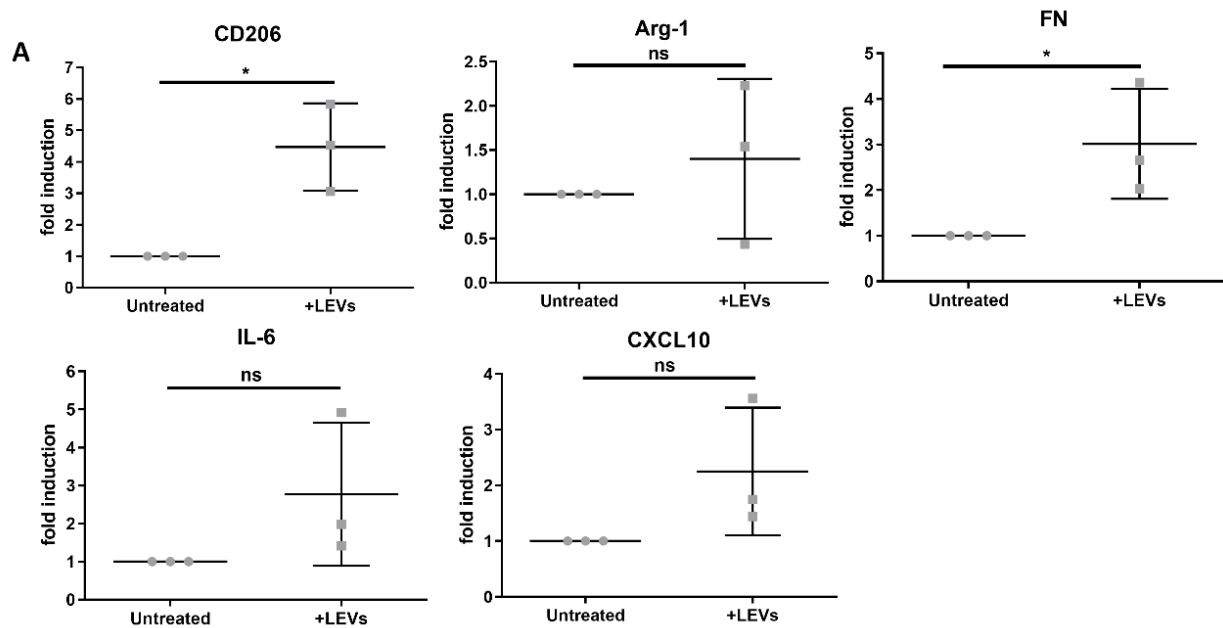


Figure 6-13: LEVs upregulate M2 macrophage gene markers in monocytes and M1 macrophages.

(A) Reverse transcription quantitative polymerase chain reaction between untreated and LEV-treated monocytes for CD206, Arg-1, FN, IL-6 and CXCL10 (n=3 biological replicates for all).

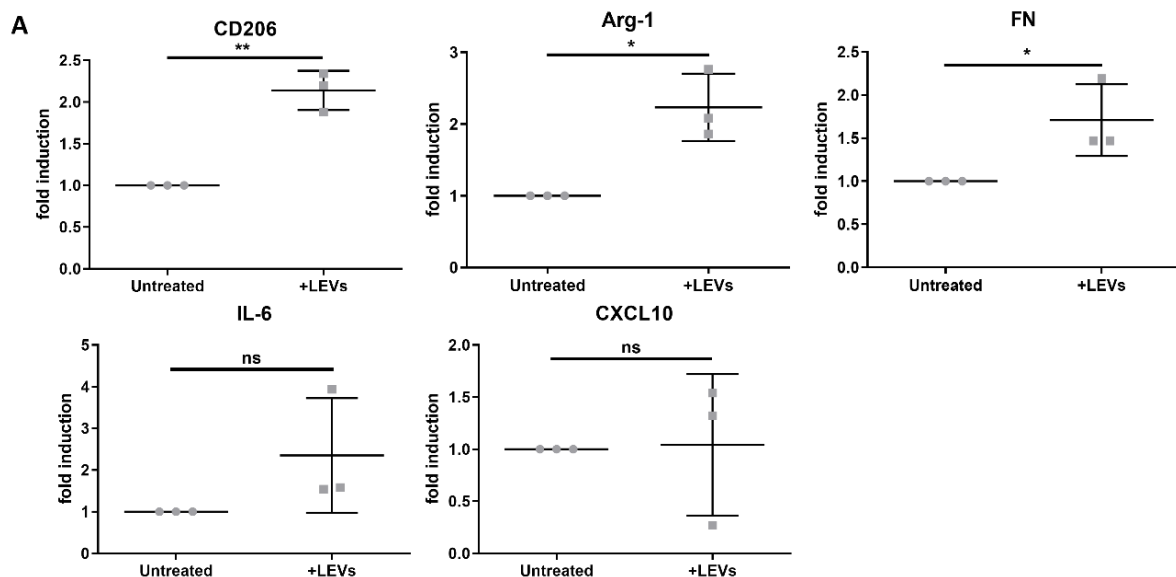


Figure 6-14: LEVs upregulate M2 macrophage gene markers in M1 macrophages.

(A) Reverse transcription quantitative polymerase chain reaction between untreated and LEV-treated M1 macrophages for CD206, Arg-1, FN, IL-6 and CXCL10 (n=3 biological replicates for all).

6.3.6 LEVs induced activation of M2 macrophages promotes endothelium attachment

M2 macrophages are evidently established as both pro-tumoral and pro-metastatic phenotypes, contributing to cancer metastasis (194). One such effect is their ability to interact with the endothelium (351,352). To explore this idea, I further tested the ability of LEV-treated monocytes to attach to HUVEC monolayers. The number of LEV-treated monocytes that remained adhered before and after media removal appeared to be higher compared to untreated monocytes (**Fig. 6-15A-B**). Quantification corroborated that indeed the percentage of adhered monocytes that had been treated with LEVs was higher (LEV-treated: $7.2 \pm 2.1\%$ vs untreated: 2.2 ± 1 , $P < 0.05$) (**Fig. 6-15C**).

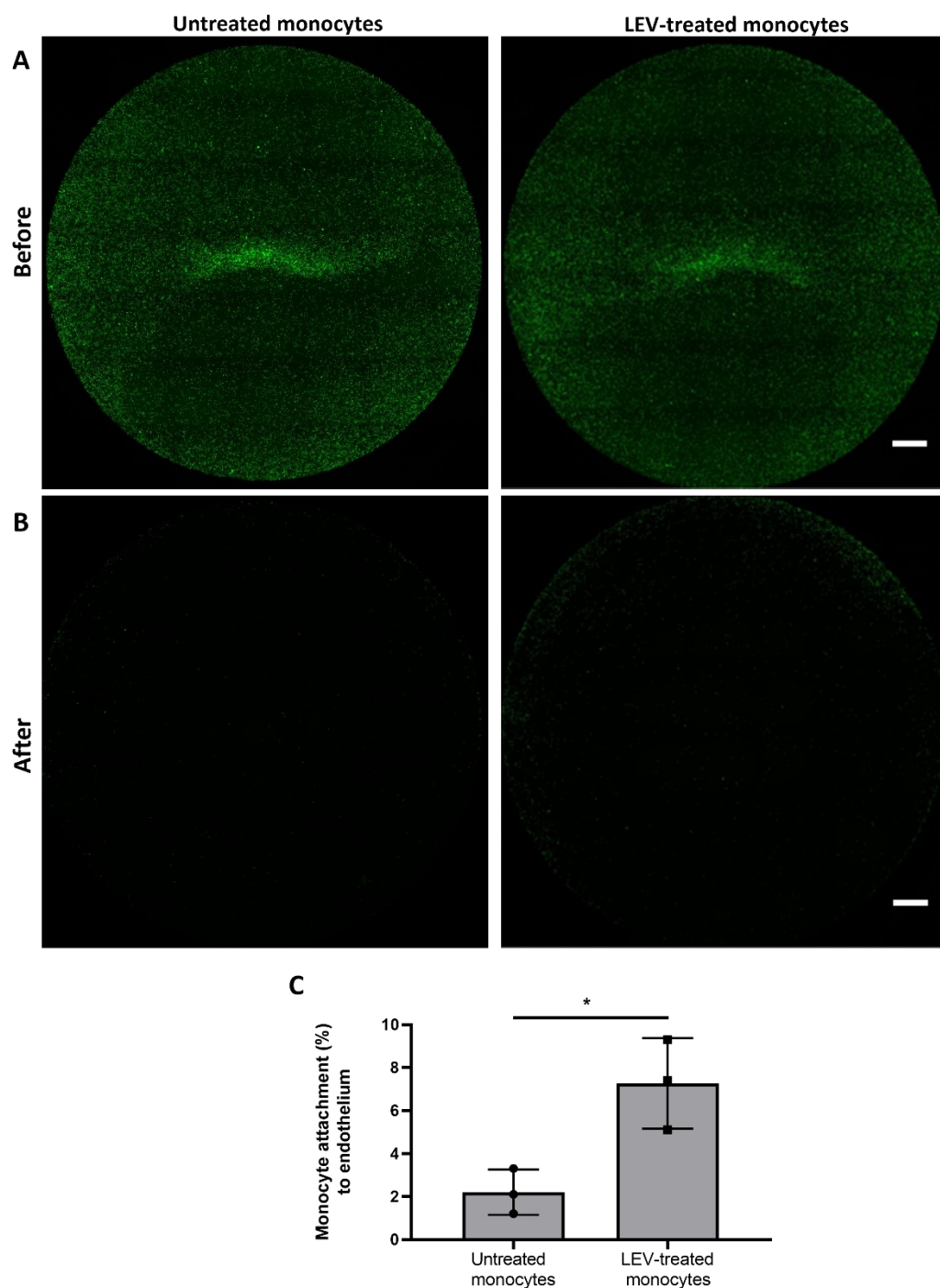


Figure 6-15: LEVs enhance attachment of differentiated monocytes to confluent endothelium.

(A) Images of fluorescently tagged monocytes added to monolayers of HUVECs at 0hr. From left to right, monocytes are added to HUVECs and LEV-treated monocytes are added HUVECs. Scale bar: 500 μ m. (B) Images of fluorescently tagged monocytes on HUVECs at 4 hr after media removal. From left to right, monocytes and LEV-treated monocytes that are adhered to HUVECs. Scale bar: 500 μ m. (C) Remaining fluorescence percentage after media removal (n=3 biological replicates for all).

6.3.7 LEVs-derived M2 macrophages modulate T cells

M2 macrophages contribution and modulation of T cells is well established (189–191,207). Incubation of T cells with monocyte CM did not trigger any significant changes in T cell proliferation (**untreated**: 4656 ± 1065 vs **monocyte CM**: 5658 ± 289 , ns), whereas both LEVs and LEV-treated monocyte CM promoted significantly proliferation of T cells (**untreated**: 4656 ± 1065 vs **LEV-treated monocyte CM**: 7339 ± 231 , $P < 0.005$; **untreated**: 4656 ± 1065 vs **LEV-treated**: 6700 ± 63 , $P < 0.05$), (**Fig. 6-16A**). Furthermore, CM obtained from LEV-treated monocytes increased T cell proliferation significantly compared to T cell treatment with untreated monocyte CM (**monocyte CM**: 5658 ± 289 vs **LEV-treated monocyte CM**: 7339 ± 231 , $P < 0.005$) (**Fig. 6-16A**), suggesting that LEVs evoke distinct downstream responses of stimulated monocytes.

In contrast, all treatments of T cells revealed a significant reduction in the mean secretion levels of IFN γ by T cells, compared to untreated (**Fig. 6-16B**). On the other hand, while the mean secretion of IFN γ by T cells was lower when T cells were treated with LEV-treated monocyte CM from when T cells were treated with untreated monocytes' CM, there was no statistical difference (**LEV-treated monocyte CM**: 172.9 ± 23.3 pg/ml vs **monocyte CM**: 249.6 ± 58.2 pg/ml, ns) (**Fig. 6-16B**).

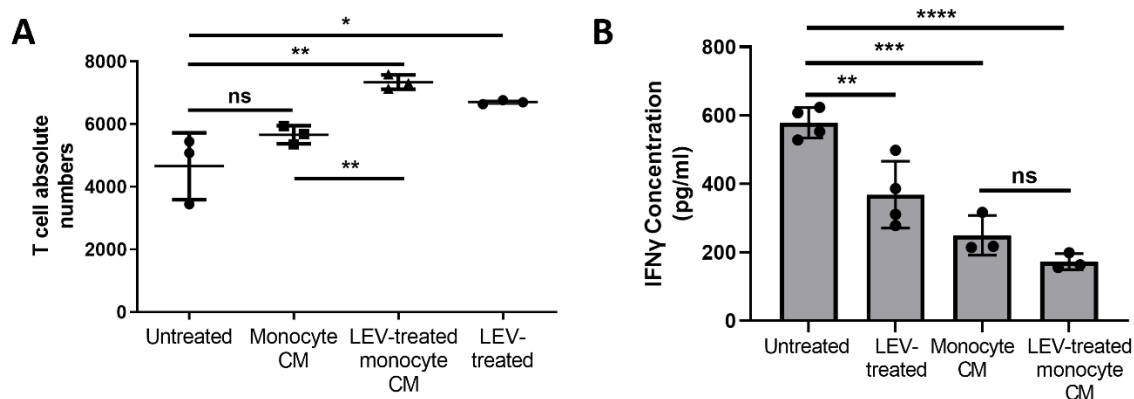


Figure 6-16: LEVs modulate T cells directly or indirectly via stimulation of monocytes

(A) Quantification of absolute T cell (fluorescently tagged) numbers via flow cytometry after 48 hr for all tested conditions (n=3 biological replicates for all). (B) ELISA assay for quantification of IFN- γ secretion levels by T cells after 48 hr for all tested conditions (n=4 biological replicates for untreated and LEV-treated, n=3 for remaining datasets).

6.4 DISCUSSION

In this Chapter, the interactions between shear- and capillary-derived LEVs independently with monocytes and M1 macrophages were investigated further. I show for the first time that shear- and capillary-derived LEVs were taken up via a combination of phagocytosis and macropinocytosis both by monocytes and M1 macrophages. I further demonstrated that LEVs and their secretome could stimulate monocyte differentiation to M2 but not M1 macrophages. Moreover, LEVs also stimulated anti-metastatic M1 macrophages to pro-metastatic M2 macrophages, corroborated my multiple M2-related genes. To further probe the malignant phenotypes of M2 macrophages, formed by LEVs, I showed that LEV-induced M2 macrophages adhered in the endothelium and reduced activation of T cells. Altogether, both monocytes and M1 macrophages are important in cancer immunity, yet their interactions with LEVs renders them to pro-metastatic phenotypes, further contributing to the formation of a pre-metastatic niche.

Previously, Headley et al. identified macrophage-positive populations *in vivo* that had internalised shear-derived LMPs (cytoplasts according to their terminology) among other immune cells of myeloid origin (71). Given the *in vivo* complexity which presents numerous immune cells competing with each other for particle internalisation, the interactions of shear-derived LEVs independently with monocytes or M1 macrophages were studied *in vitro*, in respect to internalisation. As expected, M1 macrophages internalised LEVs at a higher percentage compared to monocytes. This is backed up by the current understanding of phagocytosis efficiency for instance, given M1 macrophages are more specialised ‘eaters’ of particles, due to their specialised role in attacking pathogens and microorganisms that infect a host (353). Their F-actin rich cell protrusions might also allow them to get in contact for longer with LEVs and internalise them via phagocytosis (354).

Two major *in vivo* endocytosis mechanisms of internalisation of large particles or even large viruses by immune cells for example, are phagocytosis and micropinocytosis. Phagocytosis requires physical contact between F-actin driven cell protrusions and particles to be grabbed and internalised, whereas macropinocytosis operates essentially on the imbibement of extracellular fluid, which pulls particles inside the cells, without requiring physical contact (323). While both monocytes and M1 macrophages combined both endocytosis mechanisms

according to this Chapter's findings, monocytes depended more significantly on macropinocytosis, whereas M1 macrophages utilised more dominantly phagocytosis. This supports the general idea that phagocytosis is restricted more to macrophage populations (353), whereas monocytes utilise traditional endocytosis mechanisms, which can be performed by all cell types. Interestingly, the combination of the 2 inhibitors completely abrogated monocyte-dependent internalisation, proving that there are no alternative mechanisms. However, this was not the case in M1 macrophage-dependent internalisation, since there was about 20% of inexplicable internalisation fraction. One possibility is a different response from M1 macrophages compared to monocytes due to the interference of the two chemical inhibitors, though this is not reported in the literature. A second possibility is that the EIPA inhibitor does not inhibit fully macropinocytosis. Macropinocytosis functions via multiple mechanisms and it is plausible that EIPA inhibits only some of them, explaining thus the lack of complete abrogation of internalisation frequency. Another possibility is that the size distribution of the LEVs used in each condition could be heterogeneous across experiments, shifting downwards. Pacheco et al. demonstrated that an increasing size of polystyrene microparticles led to lower numbers of macrophages that had internalised particles (355). Thus, it is plausible that LEVs size is a crucial parameter, suggesting that lower size LEVs may be taken up by other endocytosis mechanisms, relevant for smaller particles, close to the range of 1 μm or simply the passive uptake of 1 μm particles occurs more successfully.

To mimic the transit of LEVs in the bloodstream and their interactions with monocytes or M1 macrophages, a peristaltic pump was used to allow for continuous flow of co-cultured LEVs with cells. While both monocytes and M1 macrophages exhibited uptake of LEVs under flow conditions, M1 macrophages displayed about 5-fold lower uptake frequency. Most probably, M1 macrophages failed to internalise LEVs at a great frequency because they were circulating along with LEVs instead of being adherent. This may have affected their ability to phagocytose LEVs, given the differences in macrophage morphology between static and flow conditions, which could have affected their protrusions and thus minimising their ability to capture LEVs. On the other hand, Headley et al. demonstrated that macrophages could internalise shear-derived LMPs under flow, but these results were recorded during interactions of macrophages and LMPs in lung microvessels (71), which are narrower constrictions with

lower blood flow profiles and may have allowed longer contact times with LEVs that allowed their internalisation, compared to the large diameter of the microfluidic chip used (100 μm) and the tubing (1 mm internal diameter) in the peristaltic pump flow setup.

Monocyte polarisation to macrophages occurs in response to injury (175,189). Their recruitment and subsequent macrophage polarisation are effects induced by various stimuli, including the secretion of soluble factors from activated endothelial cells, as established previously in Chapter 5. In vitro, THP-1 monocytic suspension cell line is the traditional cell line used to investigate monocytes and thus the acquisition of an adherent phenotype suggests differentiation to macrophages. Initial results exhibited the ability of LEVs to induce differentiation of suspended monocytes to adherent macrophages at about 50% conversion rate. During co-culture of 30hr, LEVs secrete soluble factors that can drive differentiation too, as shown by the monocyte-induced adhesion cause under treatment of LEVs CM alone, which was nonetheless lower in mean than LEVs-monocyte co-culture, suggesting that these effects are most likely a combination of physical interactions and internalisation and soluble factors. Interestingly, molecular techniques such as fluorescent protein staining and PCR revealed the conversion of monocytes to CD206+ M2 macrophages, but not to M1 macrophages (absence of TNF- α , a M1 marker).

The findings of this Chapter highlight the conversion not only of monocytes but also of M1 macrophages to M2 macrophages. M1 macrophages are pro-inflammatory and anti-tumorigenic (194). However, various factors from the tumour microenvironment, secreted factors or vesicles can stimulate their conversion to anti-inflammatory and pro-tumorigenic M2 macrophages (356). Similar to LEVs impact on monocytes, LEVs co-culture with M1 macrophages also resulted in their polarisation to M2 macrophages. These findings suggest that LEVs can successfully convert at least 2 immune populations to the same pro-tumour macrophage phenotype. Interestingly, while genes such as CD206 and FN have been found upregulated both in monocytes and M1 macrophages post-treatment with LEVs, validating their conversion to M2 macrophages, Arginase-1, another typical M2 macrophage marker (180), was significantly upregulated only in LEV-treated M1 macrophages. This is likely due to the fact that gene expression was performed at 30hr, while monocytes require likely more time for some of the genes to be upregulated significantly, given their direct conversion from monocytes to M2 macrophages. This suggests that the conversion of monocytes to M2

macrophages is successful, but longer time is required for the complete changes to be expressed and recorded, though longer incubation times before RNA extraction may resolve this inconsistency in one out of three replicates. Furthermore, other markers were tested such as IL-6 and CXCL10, which provided inconsistent results. Analysis of the literature on previous research findings revealed that other research groups have identified increased expression of IL-6 in M2 macrophages but others in M1 macrophages (180,357). In fact, M2 macrophages comprise a large variety of M2 subtypes, including M2a, M2b, M2c, M2d and tumour-associated macrophages, all of which come with expression profiles and downstream functionalities that overlap (178) and is thus increasingly difficult to identify correctly which M2 subtype is existent. Nonetheless, LEVs stimulate successfully polarisation of monocytes and M1 macrophages towards CD206⁺ and TNF- α ⁻ M2 macrophages, which are considered general traits of a M2 macrophage phenotype, that are accompanied by features that enable indirectly cancer metastasis.

As a natural response to injury, M2 macrophages modulate endothelial cells responses through secretion of pro-angiogenic and tissue remodelling molecules to induce proliferation of endothelial cells, sprouting and angiogenesis (351,358). Another important feature that characterises the phenotype of M2 macrophages in respect to the endothelium is their enhanced attachment to it (123). Indeed, LEV-treated monocytes were added to endothelial monolayers and their attachment increased compared to untreated monocytes, which matches the more adherent phenotype of macrophages. Physiologically, M2 macrophages adhere in the endothelium to extravasate and invade tissues to respond to an injury (184), but this process is hijacked in cancer progression and metastasis (194). Thus, M2 macrophages act malignantly, by indoctrinating the endothelium upon their attachment and this enhanced permeability caused, may be one of the reasons for enhanced CTC extravasation, as argued above. Altogether, more studies involving direct imaging on real time between the interactions of LEV-stimulated monocytes with the endothelium are needed to compare if these effects are unique or similar compared to macrophages that are stimulated by other factors.

LEV-stimulated M2 macrophages were able to modulate T cell proliferation and activation status via secretion of soluble factors that triggered these. CM from LEV-treated monocytes (verified as converted M2 macrophages) increased proliferation and reduced T cell activation,

compared to untreated T cells, clearly highlighting that M2 macrophages secrete a repertoire of factors. Interestingly, response of T cell proliferation after exposure to CM from untreated monocytes did not reveal any statistical significance, emphasising that LEVs successfully alter monocyte phenotypes, converting them to M2 macrophages. However, induced T cell proliferation does not align with an immunosuppressive macrophage phenotype necessarily. Having said that, LEV-stimulated M2 macrophages were able to reduce activation of T cells, which does match an immunosuppressive phenotype. What needs to be addressed at this point is the complete changes that LEVs cause. For instance, there are various subtypes of M2 macrophages, others involved in inducing T cell proliferation and others halting T cell proliferation. It is possible that LEVs trigger a mix of M2 macrophage subtypes or even a new subtype, which explains the contrasting results. Moreover, what appears to be inexplicable is why the CM from untreated monocytes reduces significantly the activation of T cells by decreasing the secreted levels of IFN γ , whereas the opposite has been reported. This difference could be attributed to the different T cells used in this study. It is also possible that the monocytes used for this experiment were slightly activated due to some exogenous exposure during their culture, which affected the experiment. Last but not least, this experiment involved the culturing of T cells with CM, whereas physical contact from monocytes may confer different results.

This Chapter describes altogether the impact of LEVs on conversion of monocytes and M1 macrophages to pro-metastatic M2 macrophages. One crucial aspect that needs to be addressed is the exact and wider expression profile of the molecular signature of LEV-induced M2 macrophages, given the diversity of M2 subtypes in respect to molecular composition, as this will increase the understanding of the repertoire of their functionalities. For example, omics techniques such as transcriptomics or proteomics will be very useful to deciphering the full changes of LEV-converted M2 macrophages and through bioinformatic analyses unlock their full potential on molecular functions and biological pathways they influence. Furthermore, direct evidence of LEV-converted macrophages on their ability to support CTCs will be needed. Models mimicking the microcirculation, including flow and capillary geometries will be vital to investigate the multiple interactions between CTCs, macrophages and endothelial cells in more physiological and constrained conditions under flow. Primarily, it remains to be seen whether LEV-stimulated M2 macrophages directly affect the

endothelium to promote CTCs extravasation in real-time imaging and whether they co-extravasate to further support metastases formation. In addition, LEV-stimulated M2 macrophages have been shown to reduce activation of T cells, but it remains unclear whether that translates to reduced T cell cytotoxicity against CTCs. Altogether, LEVs released in the microcirculation from CTCs have the potential to interact and polarise immune cells to perform diverse roles in facilitating CTCs successful journey to metastasis.

6.5 CONTRIBUTIONS & ACKNOWLEDGEMENTS

I generated the hypothesis, designed all experiments, conducted most of the experiments and analysed all data. Aoyu Zhang and Ashna Mymuna helped collect some of the data.

CHAPTER 7

7.1 CONCLUSIONS & STATEMENT OF IMPACT

In this research thesis, evidence was collected on the various responses of cancer cells during their transit in the microcirculation and particularly in capillary bifurcations. The utilisation of microfluidic devices, mimicking the numerous geometries found in the human microcirculation under flow conditions, enabled the dissection of multiple factors that affect tumour cell movement, shedding of large membrane particles and cell survival. While the biogenesis of LEVs from tumour cells during their transit in the microcirculation, appears to be more of a biomechanical driven phenomenon rather than an active decision taken from tumour cells, the shed large particles have the potential to affect their blood environment in many ways. This is insinuated primarily by the vast array of proteins that are present inside these large vesicles, which were correlated to various pathways and molecular functions connected to metabolism, inflammatory response and cell reprogramming. This notion was further bolstered by the interactions of LEVs with endothelial cells, monocytes or M1 macrophages. LEVs were able to induce changes not only via their uptake or physical interactions but also via secretion of soluble factors, highlighting their diverse capabilities. For instance, upon exposure of endothelial cells to LEVs, HUVECs' intercellular junctions (VE-cad), connections and morphological features were disrupted, enhancing permeability. Moreover, increase of VCAM expression and NO generation highlight that HUVECs not only become activated but that they possess distinct 'weapons' to assist CTCs by stimulating immune cells and enhancing their co-interactions. The additional effects observed by LEVs on monocytes and M1 macrophages emphasise that LEVs are capable of interacting with multiple cell types and thus facilitate the formation of a collaborative pre-metastatic niche.

The reinforcement of the current literature on shear- and capillary-derived LMPs that were verified to be LEVs, as shown in this research thesis is crucial for the overall understanding of their direct or indirect impact on metastasis. Cancer cells shedding LEVs impacts significantly their volume, which may influence several metastatic steps such as arrest, endothelium adhesion, long-term survival, extravasation or post-extravasation proliferation. For instance, this cell volume reduction may render cells less capable to arrest and be trapped in narrow capillaries in the human body, reducing thus the number of available cells for extravasation and thus reducing probabilities of metastasis. Moreover, the active manipulation of cell shedding by use of Cytochalasin D and Colchicine paves the way for probing whether

enhanced or compromised shedding correlate to enhanced or reduced metastasis or vice versa, compared to untreated cancer cells. Investigation of these aspects can provide information on how cell shedding can influence probabilities of CTC-directed metastasis and thus encourage the research community to delve deeper in the impact of this phenomenon and whether shedding or lack thereof would be more beneficial.

On the other hand, LEVs generated by CTCs transit in the microcirculation, can continue journeying in the blood of patients, corroborated by isolation of nuclei-free large particles from other research groups. The last 2 experimental chapters of this thesis highlight that LEVs can interact with multiple cell types, such as endothelial cells and immune cells. This obtained knowledge introduces a new mechanism through which CTCs can affect indirectly their surroundings in the bloodstream and shape a pre-metastatic niche. This is a great compelling start towards the investigation of the various LEVs contributions more holistically. The acquisition of a proinflammatory phenotype by endothelial cells, initiated by LEVs, suggests that the co-relationship between CTC-directed metastasis and the inflamed endothelium of a particular organ may in fact be initiated in patients by the generation of LEVs from CTCs. This research thesis boosts this notion and establishes a sound reasoning on why the research community should pursue the field of LEVs and their downstream contributions further.

Finally, cancer-originating LEVs represent tumour material, which can be interrogated. Chapter 4, in particular, focuses and expands on the information we can extract from such LEVs. The vast array of proteins they contain (many of which are shared with their cell of origin), their higher counts than CTCs (as shown previously from others), their large size (compared to exosomes) along with the fact that they are generated from tumour cells that remain alive post-shedding, renders them ideal candidates for liquid biopsy applications, such as early diagnosis, patient monitoring, cancer cell clonal information among others. Identification of numerous proteins encompassed in LEVs opens new directions to devise new or revise current methodologies and optimise their isolation and purification while minimising contamination from non-cancer cells' LEVs from the blood of cancer patients. Altogether, this research thesis emphasises that shed LEVs are not mere apoptotic fragments, rather cell-secreted particles with biomolecules and functionalities that can shape the course of metastasis.

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APPENDIX

Appendix 1: Licence for reuse of published images

Page No.	Type of work:	Name of work	Source of work	Copyright holder and contact	permission requested on	I have permission yes/no	Permission note	Licence link (if needed)
16	Figure	Overview of the metastatic cascade	Molecular principles of metastasis: a hallmark of cancer revisited.	Fares et al.	N/A	yes	Creative Commons Attribution 4.0 International License	https://creativecommons.org/licenses/by/4.0/
17	Figure	Tumour cell adaptation and interactions within the microcirculation	Arresting metastasis within the microcirculation	Varotsos Vrynas et al.	N/A	yes	Creative Commons Attribution 4.0 International License	https://creativecommons.org/licenses/by/4.0/
22	Figure	Intravital imaging of the first hours of lung seeding by B16 melanoma	Visualisation of immediate immune responses to pioneer metastatic cells in the lung	Headley et al.	16.11.23	yes	Requested permission	https://s100.copyright.com/CustomAdmin/PLF.jsp?ref=cdbf8009-c3ab-44b6-81cb-39542a5b67c4
31	Figure	Figure 1	Delineating the role of extracellular vesicles in cancer metastasis: A comprehensive review	Majood et al.	N/A	yes	Creative Commons Attribution License	N/A

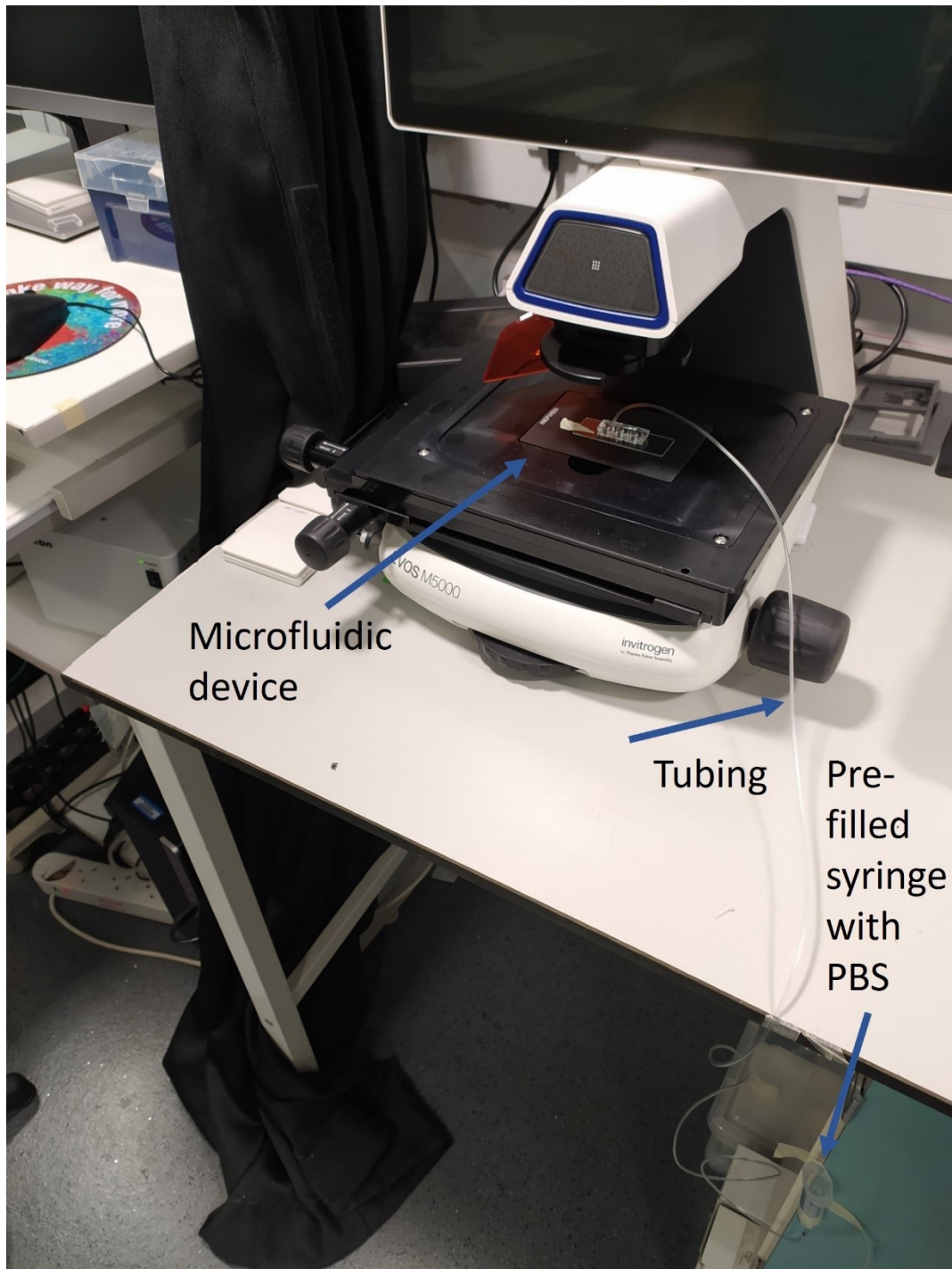
Appendix 2: Video legends

Video 1 caption: A single MDA-MB 231 cell transiting a capillary bifurcation and releasing LEVs. Cytoplasm was stained with CMTPX cell tracker (red) and nuclei with Hoechst-33342 (blue). Scale bar: 9µm.

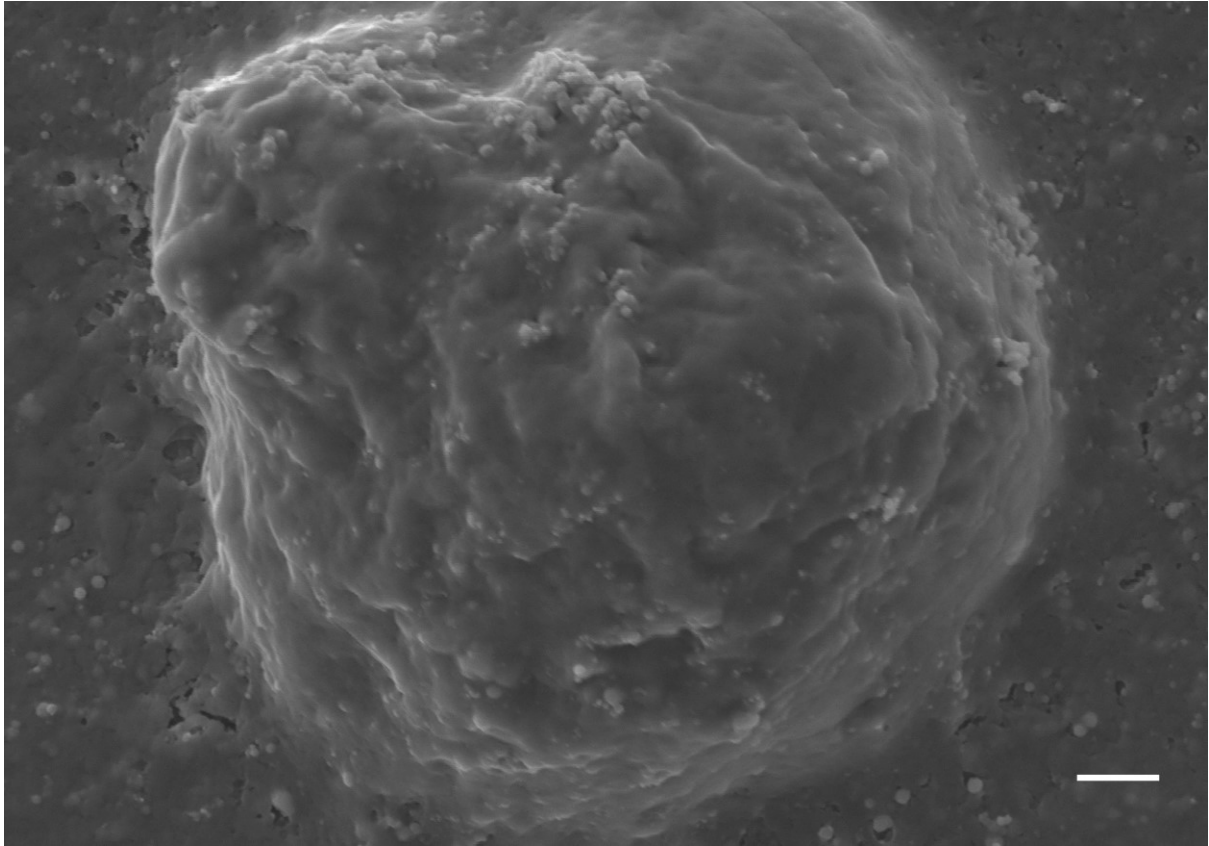
Video 2 caption: A 2-cell cluster of MDA-MB 231 cells transits, and encounters a capillary bifurcation, which leads to dissociation of the cluster to 2 single cells. Cytoplasm was stained with CMFDA cell tracker (green) and nuclei with Hoechst-33342 (blue). Scale bar: 6.3 µm.

Video 3 caption: A human umbilical vein endothelial cell (full arrow) near LEVs. HUVECs were stained with CMFDA cell tracker (green) and LEVs with CMTPX cell tracker (red). Membrane activity i.e. protrusions can be visualised near a LEV (dashed arrow). Scale bar: 20 µm.

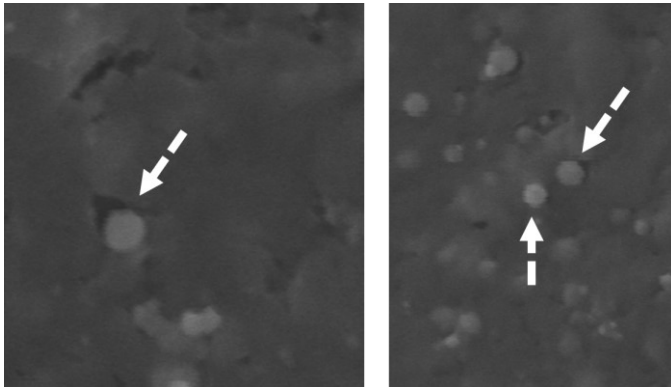
Appendix 3: Experimental setup for live imaging of cell transit in the microfluidic device



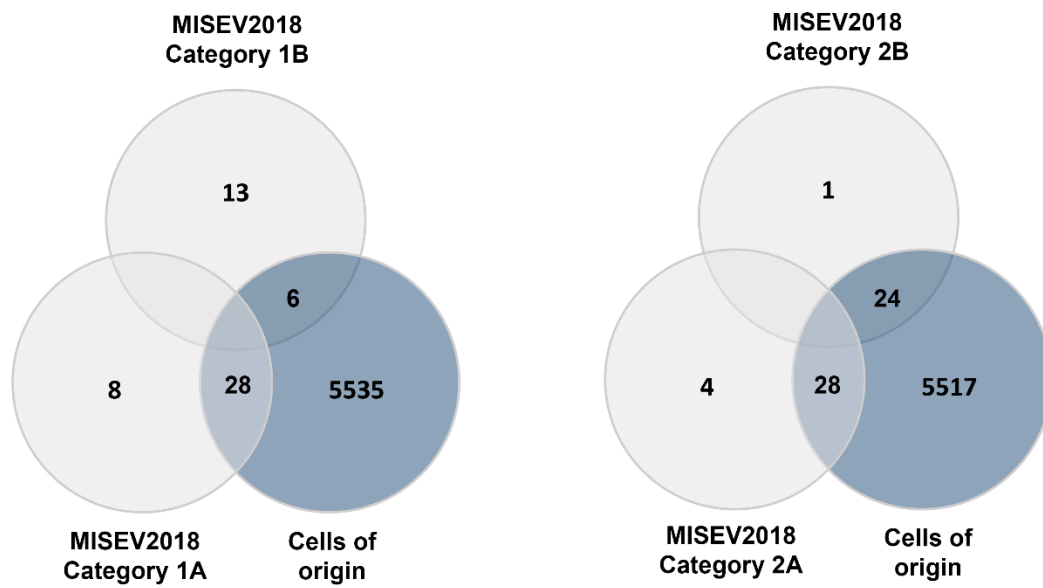
Appendix 4: Scanning electron microscopy image of a MDA-MB231 cancer cell. Scale bar: 1 μm



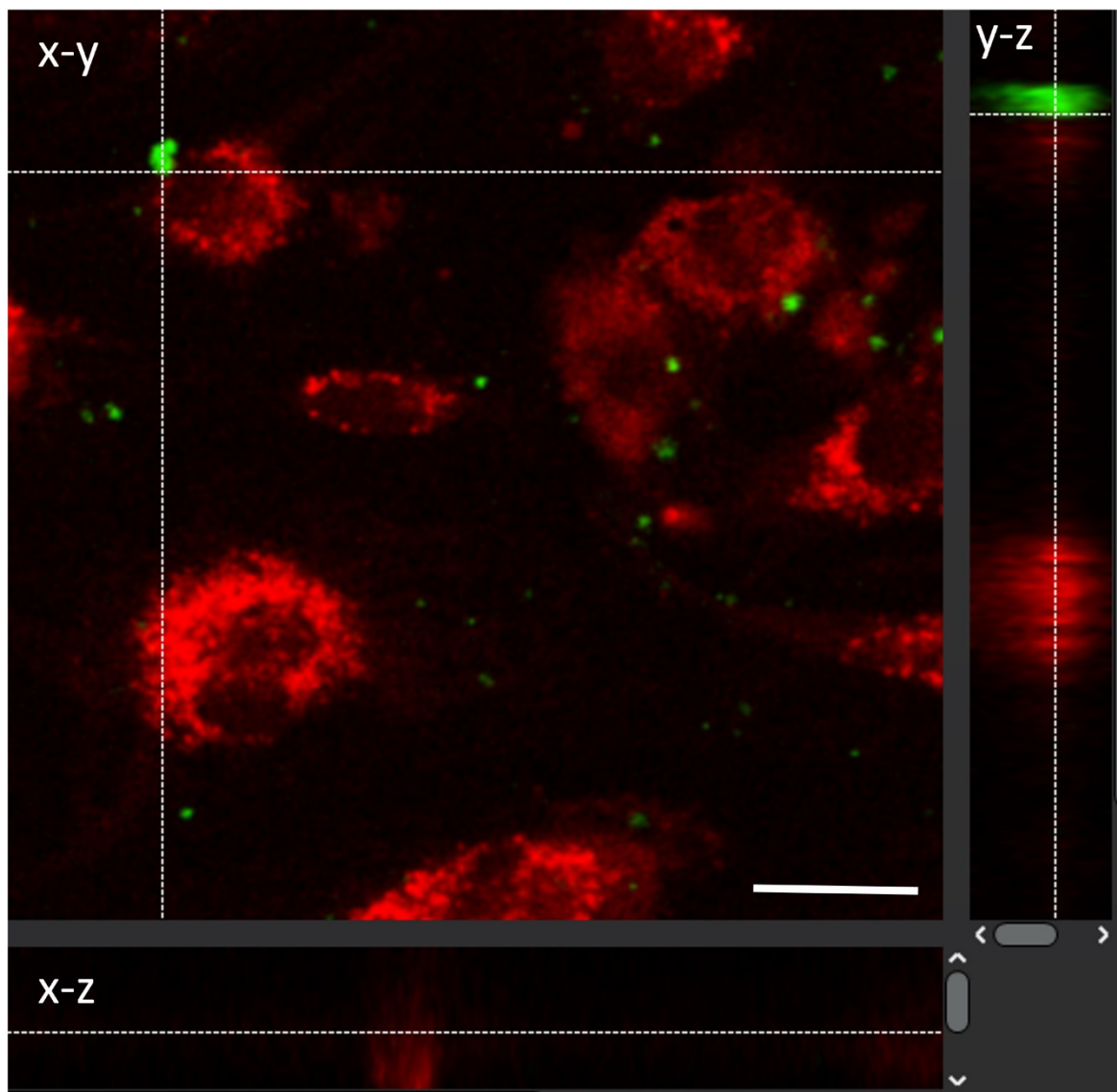
Appendix 5: Zoomed images of exosomes from above image



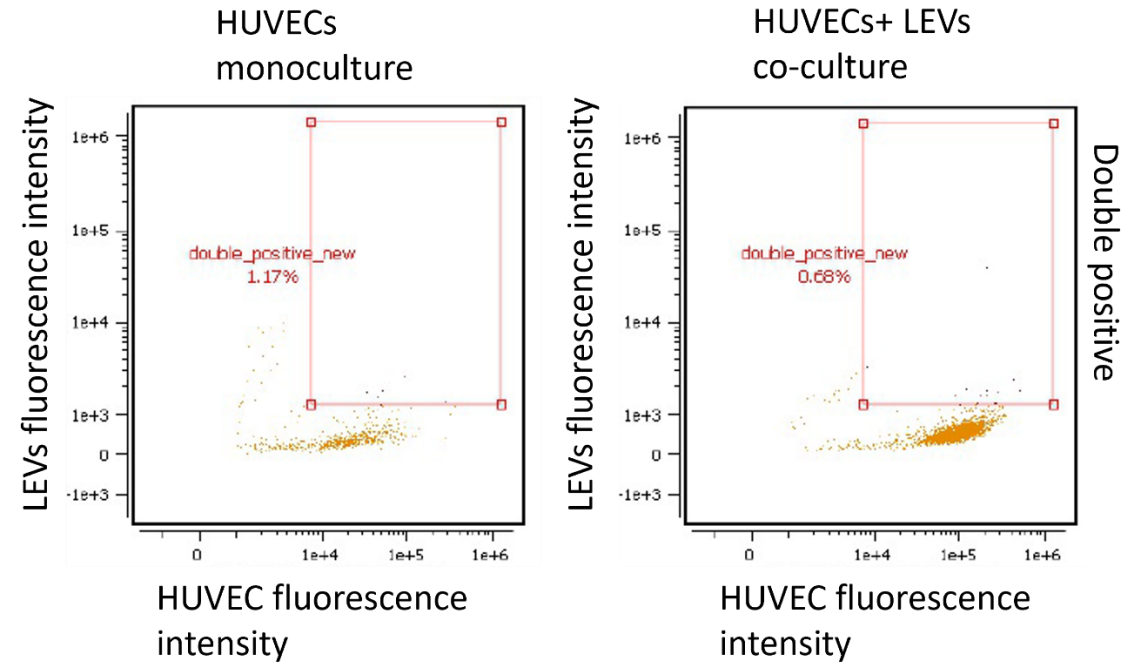
Appendix 6: Overlap of gene IDs between cells' proteome and listed factors from MISEV guidelines



Appendix 7: Orthogonal cross sectioning of a HUVEC (red) and a LEV (green) being outside of the cell. HUVECs were stained with CMTPX cell tracker (red) and LEVs with CMFDA cell tracker (green).

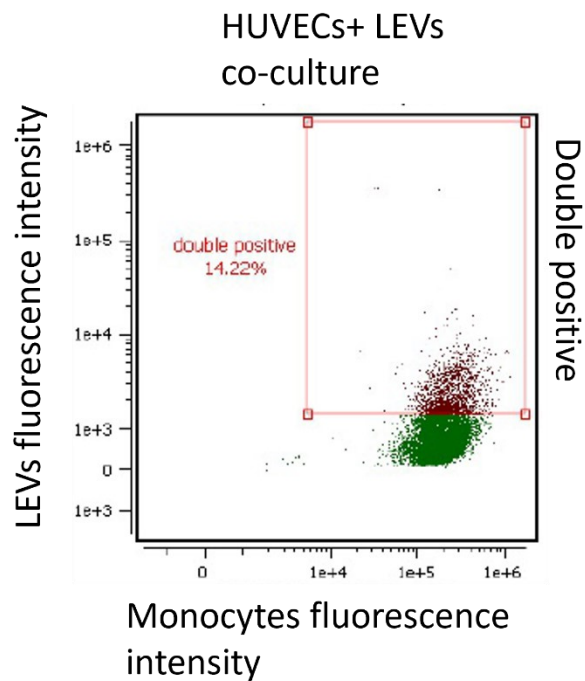


Appendix 8: LEVs uptake by HUVECs under flow conditions. Monoculture of HUVECs (left) and co-culture (right).

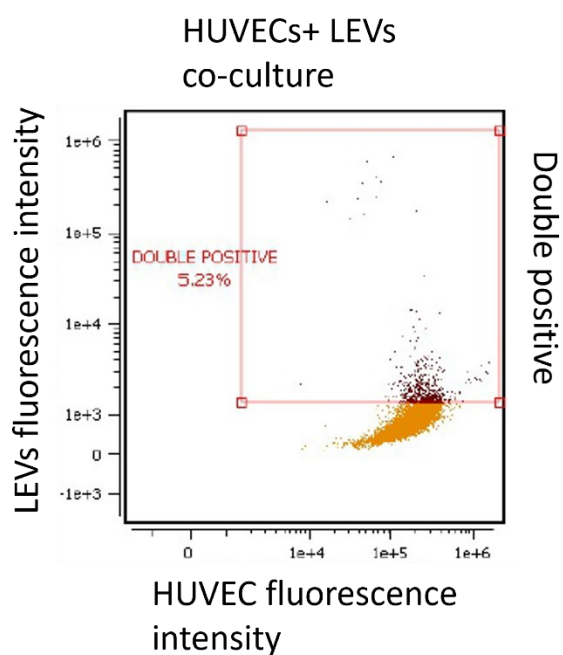


Appendix 9: LEVs uptake percentage by co-culture with monocytes, cultured in a 6:1 monocytes : LEVs ratio.

Add flow cytometry data on low uptake at different ratios/same wells or same ratios/different well sizes



Appendix 10: LEVs uptake percentage by co-culture with HUVECs, cultured in a 3:1 HUVECs : LEVs ratio in a 12-well plate.



Appendix 11: List of video files

<https://zenodo.org/records/10731025>