The role of extracellular vesicles in premetastatic niche formation as well as their value as metastatic biomarkers

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Abstract

Metastasis is defined as the occurrence of secondary cancerous growths appearing elsewhere in the body away from the initial site of the primary tumour, and is well established to considerably and negatively affect disease outcome. Metastatic formation in a foreign host-tissue is preceded by a state of hiked susceptibility to cancerous dissemination. Such a tissue is referred to as a premetastatic niche (PMN). A group of cell secreted lipid enclosed packages containing proteins, RNA and DNA termed extracellular vesicles (EV) have been observed to play an extensive role in PMN formation. The aim of this thesis is to explore the contemporary understanding of the involvement of EVs in PMN formation, and from there judge the feasibility of using EVs to gauge metastatic risk in patients. EVs are known to both transmit and represent their parent cell's phenotype. The primary tumour benefits evolutionary from immune suppression, angiogenesis, vascular leakage and extracellular matrix alterations. EVs evoking such modifications may land elsewhere in the body whereafter they give rise to metastases in four consecutive steps of tissue remodification: priming, licensing, initiation and progression. For these steps to occur immune suppression needs to be elicited by inhibiting and activating anti- and protumoral processes respectively, whereas EV-related markers indicating the exact opposite may be an indication of a better prognosis. Meanwhile, EV markers for angiogenesis and leaky vasculature may signify enhanced metastatic potential. As do those aimed at the deposition or breakdown of specific extracellular matrix components. Methods aimed at EV isolation and the acquisition of big data such as genomic, transcriptomic and proteomic data are being optimised. It is expected that the mechanisms underlying PMN formation will be further unravelled whilst extensive screening of EVs in patients gradually will become possible. When science and healthcare have arrived at that point, I propose establishing a metastatic potential staging system onto which personalised treatment could be based to negate metastatic risk.

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Introduction

Metastasis, the dissemination of cancer to secondary sites distal to the primary tumour, is an indication of poor prognosis for cancer patients. The heterogeneity and multitude of locations of metastatic tumours complicate treatment. Furthermore, complications such as obstructions, paraneoplastic syndrome and direct organ damage can arise as a result of metastasis which eventually may cause death. Indeed, it is estimated that for most cancers between 60-90% of mortalities can be attributed to metastasis as a result, depending on the type of cancer[1–3]. The early detection and negation of metastatic risk may therefore conspicuously contribute to increasing overall survival in patients struck with a variety of cancers.

For metastasis to occur malignant cells need to detach from the main tumour, enter circulation, nest into foreign tissues and not only survive but proliferate into a secondary cancerous mass. Metastatic spread occurs in a biased pattern that cannot be explained by circulation and proximity to the host tissues to the founder tumour alone. Certain tissues are struck by metastasis more frequently than others; with brain, liver, lymph nodes, bone and lungs being frequently compared to other organs and tissues. Based on this apparent organotropism, Paget proposed his "seed and soil" theory. Paget conjectured that this biased spread could be explained by certain tissues ("the soil") being particularly well suited for hosting the tumour cells' ("the seeds") nesting and subsequent proliferation[2].

Interestingly, the primary tumour can cultivate the microenvironment of distant healthy tissues into becoming tumour friendly through intercellular communication, a phenomenon partially attributed to the observed organotropism of metastasis[4]. A non-malignant distant site where the microenvironment is altered in a way that accommodates tumour cell settlement and proliferation is referred to as a premetastatic niche (PMN). Distally nested cancer cells are subjected to challenges similar to those faced by the founder tumour in earlier stages. Consequently, the PMN is reminiscent of the tumour microenvironment. Increased vascular leakage, breakdown or modification of extracellular matrices (ECM), and the presence of surface molecules on the membranes of local cells aiding in cell-cell attachment help circulating cancer cells gain a foothold in the host tissue. Meanwhile, immune suppression, angiogenesis and altered local metabolism aid in the unchecked proliferation of recently integrated tumour cells[5–7].

One of the avenues of systemic intercellular communication utilised by primary tumours is through extracellular vesicles (EVs)[8–11]. EV is an umbrella term denoting phospholipid packages between 50 to 5000nm either secreted by cells (e.g. exosomes or microvesicles), or left over from the cellular debris after apoptosis. EVs can be taken up by other cells and may contain DNA, RNA and proteins apt to tweak target cell phenotype[12]. It has been shown that tumour secreted EVs travel through the vascular system and end up at sites where PMN formation and eventual dissemination occur, hinting at a prominent role in PMN cultivation[8,13]. Furthermore, it has been shown that a whole range of EV associated RNAs and proteins are implicated in cancer progression. Recent developments in omics technology have translated to more and better data that can be used to identify new EV associated prognostic markers and their roles in PMN development[14–18]. New insights obtained with these data could provide clinicians with a valuable prognostic tool, onto which personalised treatment schemes could be provided. The aim of this report is therefore to explore the usability of EVs as biomarkers in the context of PMN formation. To explore this, I will touch on how EV content is able to alter target cell phenotype and the consecutive steps tissues go through to progress into a PMN. After that, the relevance of EV uptake mechanisms in PMN formation will be discussed. The effect of EVs on the antitumoral immune response as well as ECM and vascular remodelling will also be explained. Finally, I will go through the different techniques of isolating EVs, analysing their content and attempt to propose a strategy aimed at ascertaining metastatic risk.

EV content both represents and transmits systemically donor cell phenotype

The human body has evolved to clear out defective cells such as malignant cells through the immune system, as they pose an existential threat to the human body. Macrophages and neutrophils migrate into the site of an insult (such as cancer initiation) and induce further immune recruitment and responses. Meanwhile, immune cells such as CD8+ T-cells and NK-cells patrol tissues and eliminate abnormal cells[19–21]. Should circulating tissue cells manage to evade the immune system to a degree where nesting is possible, proliferation into a secondary mass is still not guaranteed. First, the ECM and local cells must display surface molecules for circulation cancer cells to attach themselves to begin with. Secondly, the microenvironment must contain growth factors aiding in tumour proliferation. And lastly, the local tissues must be supplied with sufficient amounts of nutrients to help sustain rapid cell growth[2,3,6].

Unsurprisingly, PMN formation where the above-mentioned hurdles are nullified appears to be a vital prerequisite for metastatic establishment. In silico mathematical modelling conducted by Qian et al. indeed indicated that without supportive environment parasitic tumour cells are unable to progress and will die off even after nesting into the host tissue as a result[22]. The primary tumour helps educate future metastatic sites by changing healthy tissues into PMNs via secreted factors like EVs. Pioneering research by Kaplan et al. showed that media conditioned with B16 melanoma cells are able to elicit PMN formation in mice, as mice treated with B16 cell conditioned media were struck more often with metastatic formation after being intradermally implanted with Lewis Lung Carcinoma[23]. Later on, Peinado et al. corroborated the idea of EV-induced PMN formation in independent research. Peinado et al. found that melanoma derived EVs were able to transfer the oncoprotein MET to bone marrow derived stem cells which then in turn displayed a pro-metastatic phenotype in-vitro. Mice educated with EV loaded MET were later on found to be suffering from metastatic lesions more often compared to the control when subsequently implanted with tumour cells[24]. Cancer is a term annotated to a variegated set of pathologies, however. And the fluctuating degree of metastatic potential of different cancers reflects this. Looking into EV load is therefore crucial in understanding metastatic risk factors.

Thanks to advancements in bioinformatics, several studies have found ways to fingerprint exosome content and link them to cancer subtype, phenotype and cell origin. Extensive exosomal proteomic research has been conducted on a varied array of cancers; including breast, (non-small cell) lung, colorectal, prostatic, gastric, ovarian and pancreatic cancer[14–16,18,25–27]. Proteomic analysis of exosomes derived from 10 breast cancer cell lines, for example, was able to distinguish the proteomic footprint of triple negative breast cancer from HER2 positive breast cancer cell lines through unsupervised hierarchical clustering, whilst also being able to match enriched pathways to the high metastatic and high proliferative potential often seen in triple negative and HER2 positive breast cancer respectively[16]. Similar results were obtained with regard to non-small cell lung cancer. Proteins related to proliferation were comparatively more abundant in exosomes secreted by non-small cell lung cancer cell lines than those of immortalised normal bronchial epithelial cell lines. Furthermore, protein content of circulating exosomes was suitable for identification of non-small cell lung cancer and could even distinguish between metastatic and non-metastatic variants in patients[15].

RNA is the second important biomolecule found within exosomes. Every species of RNA found in the cell is generally also present in exosomes. And although the function of some exosomal RNA types is disputed (a discussion too complex for the scope of this report), confirmed functional RNAs such as mRNA, long non-coding RNA, siRNA and miRNA can have a markable effect on cell phenotype[18,25,28,29]. Transcriptomic studies with regard to the effect of EV RNA have not been conducted to the same extend as the proteomic studies mentioned earlier. Although protocols for sequencing shorter RNA molecules have already been devised, sequencing longer exosomal RNA molecules (such as mRNA and

long non-coding RNA) has proved problematic up until recently[18]. Notwithstanding the contemporary limited data on the EV transcriptome, down- and upregulation of several specific individual EV miRNAs have been associated with breast, lung and colorectal cancers[25,28]. mRNAs have also been implicated with cancer progression and/or metastasis as demonstrated by Yokoi et al. In that study, researchers demonstrated EVs carrying MMP1 mRNA aided in ovarian cancer dissemination through induction of apoptosis of mesothelial cells and the subsequent breakdown of peritoneal mesothelium barrier in an in vitro study[30].

In recapitulation, EV content reflects the origin and state of the cells that expulse them. Cancer cells continuously evolve to aid proliferation and invasion into local tissues. And whilst eliciting these effects at distal tissues may not directly benefit the primary tumour, these associated phenotypes may aid in tumour survival locally (hence they are selected for). PMN formation is a consequence of the collateral damage primary tumours evoke upon the secretion of protumoral factors, such as cancer associated EVs, which seep out into the circulation. Once these systemically transported factors collectively cultivate foreign tissues into PMNs, the precedent for metastatic spread is set.

Premetastatic Niche formation, a continuous and complex chain of events

Having established global exosome proteomic and transcriptional changes occur in cancer patients that can be linked to cancer associated pathways is the first step in working towards a personalised metastasis disruption approach. Still, this information needs to be translated into actual therapeutics/prognostics and for that the mechanisms underlying PMN formation need to be unravelled. As is indeed a major theme in this report, the PMN niche is reflective of the invasive fronts adjacent to the main malignant mass of the primary tumour[31]. PMN formation is hallmarked by immunosuppression, extracellular matrix remodelling and angiogenesis[5–7]. The metamorphosis of healthy tissue to metastatic site occurs in a consecutive sequence of discrete events, dubbed by Liu et al. as priming, licensing, initiation and progression[7].

The first stage in PMN formation is referred to as priming[7]. Healthy tissues contain a multitude of cell types which together with the ECM make up the stroma. Stromal cells are the sentinels of local tissue homeostasis, and consist of populations of endothelial cells, vascular cells, tissue associated macrophages and fibroblasts. Under normal physiological conditions, stromal cells help respond to insults such as tissue damage or pathogenic presence. Macrophages for example, help eliminate threats to the local tissues and are able to instigate an inflammatory response thereby helping to recruit both innate and adaptive immune cells to the site of the insult. Since this inflammatory chain reaction is inherently damaging to the tissue, they have a dichotomous set of roles as they also serve to aid in wound resolving processes together with fibroblasts. During a chronic inflammatory microenvironment, such as present within the tumour microenvironment, healing-like processes prevail. Such healing-like processes are hallmarked by a supressed immune response, angiogenesis and dysplasia[31,32]. It is probable that these local stromal cells are the first components affected during the priming stages of PMN formation[4]. Fibroblast phenotype has been observed to change to a tumour friendly variant when stimulated with cancer derived exosomes[33]. These so-called cancer associated fibroblasts aid in further immune suppression through the secretion of cytokines, chemokines and fibroblast associated exosomes which, together with additional cancer associated exosomes educate macrophages towards the M2-like (wound healing and tumour friendly) subtype. M2-like macrophages and their N2-like neutrophil counterparts, together with fibroblasts, start in turn rallying the endothelial cells to mount an immune suppressive and tissue remodelling effort of their own[34–38]. Where applicable, cytotoxic immune cells such as cytotoxic T-cells, NK-cells and B-cells may be inhibited in their activation as a result. In all, this culminates into a microenvironment that increasingly becomes more agitated and starts shaping into a PMN[31,32].

Antitumoral is not synonymous to 'immune repulsive' or 'immune devoid', and immune cells may even be drawn to the PMN to consequently further its progression. This recruitment process plays a paramount contribution to the licensing process and forms the link between the priming of local tissues and the establishment of PMN. Most of the premetastatic niche starts to take shape[4]. Fibroblasts, M2-like macrophages and N2-like neutrophil start to become more prominent and aid in ECM remodelling, furthering immune suppression and angiogenesis[34–38]. Myeloid derived suppressor cells (MDSCs) are a group of immature myeloid cells derived from a monocyte or neutrophil origin and are a hallmark of chronic inflammation, and may be recruited to the PMN as well[21]. In the PMN they then further suppress antitumoral responses and remodel the tissue. Some cells are inherently antitumoral and need to either be de-activated or repulsed from the PMN because they are major contributors to cell death. Patrolling monocytes, NK-cells, innate T-cells, DC-cells, B-cells and CD8+ T-cells are either directly or indirectly involved in tumour cell lysing and have to be repressed or excluded [7,31,32]. Indeed, the presence and level of activation of cytotoxic cells such as CD8+ T-cells in the TME is generally associated with a better prognosis for afflicted patients[20]. Furthermore; CD8+ T-cells, NK-

cells and the patrolling monocyte subtype are involved in immunosurveillance, a process where tumour cells are sought out and destroyed effectively eliminating metastatic threat locally and systemically[21,31]. In the end, once the anti-tumoral aspects of the local stroma and recruited immune cells have been dampened and their tissue remodelling wound-healing like properties have been magnified, the stage is set for metastasis to commence [4].

At a certain point, the PMN starts becoming tumour friendly to the extend where it effectively accommodates the settlement of tumour cells into the local tissue without being purged from it by immune players. However, immune suppression and a nutritious soil are not the sole facets contributing to metastatic onset [4,39]. Rationally, access to the site in question must be present in order for tumour cells to settle properly. Vascular permeability is important, and the PMN is generally hallmarked by increased vascular leakage[6,7]. In vivo mouse melanoma, colorectal and breast cancer models have shown loss of vascular integrity does increase metastatic potential[40–42]. Moreover, cancer cells need to attach themselves to or within the PMN to effectively colonise[7]. Stromal cells help alter the ECM in a way that favours this[36,39]. For instance, fibronectin and collagen-crosslinking aids in tumour cell adhesion and has been shown to accumulate at future metastatic sites in vitro breast and colon cancer mouse models respectively[43,44]. Colonisation of (undetectably) small quantities cancer cells is possible through these types of vascular and ECM alterations. Small colonies, referred to as micro-metastasis, are part of the initiation stage. The initiation stage constitutes the transition phase from PMN to fully matured metastatic site[7].

Although in this report the PMN is defined as a tissue with heightened potential for metastasis spawning, it is important to highlight the mechanisms underlying the subsequent outgrowth of micro-metastasis during the progression stage. If the PMN does not offer adequate support for uncontrolled cell growth, dormant metastasis-like colonies of single disseminated cancer cells may form what is referred to as a sleeping-niche[6]. These sleeping-niches can during later stages still form a novel cancerous growth. Therefore, it is of significant diagnostic and therapeutic relevance to unravel the mechanisms and traits necessary for cancerous outgrowth. Evidence suggests certain excreted factors may re-awaken senescence tumour cells nested in micro-metastatic colonies[6]. A striking example is the TGF-β ligand antagonist DAN Domain BMP Antagonist Family Member 5 (referred to Coco in the cited paper) which is associated with lung cancer relapse and dormancy retirement by re-engaging stem cell transcription programs in dormant cancer stem cells[45].

Figure 1 encapsulates these four stages associated with the transformation from healthy tissue, to PMN and eventually metastasis. As nicely illustrated, host tissue evolving from healthy to a full metastatic site is a gradual process that can be classified into the above-mentioned distinct stages priming, licensing, initiation and progression. What is underappreciated in figure 1 is the amount of complexity of the interplay between the different facets of tissue homeostasis that brings about the increased vulnerability for metastatic colonising [5–7]. A recent effort has been made to chart the processes transforming tissues from healthy. As was mentioned previously, this is partially done through proteomics and transcriptomic approaches, as well as by looking at specific EV cargo in in vitro and in vivo models. One of the main challenges in both analysing EV cargo with regard to using PMN formation as a prognostic marker is to couple the EV cargo data to the complex processes underlying PMN formation. Therefore, from here on onward I shall attempt to explore possible fruitful avenues that could aid in PMN related biomarking and therapeutics.
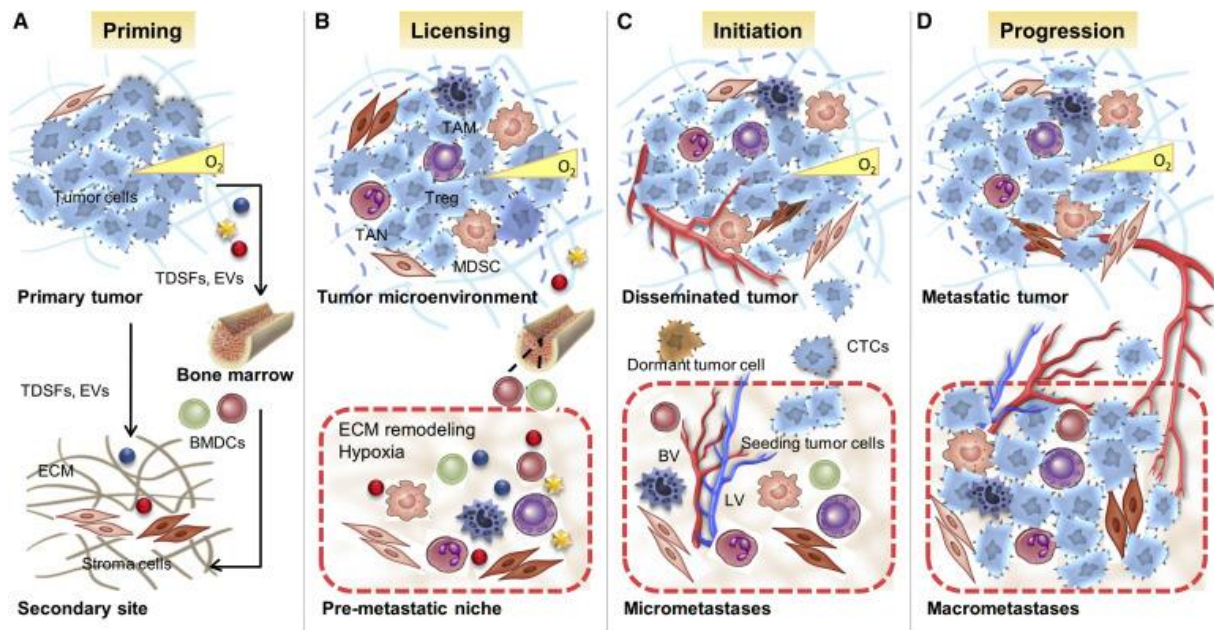
Figure 1 The consecutive steps of metastatic formation, adapted from Liu et al.[7] the transition from healthy tissue, to PMN and eventual metastasis occurs in 4 distinguishable steps. Priming: where the phenotype of the tissue and its residential cells are altered to lay the foundation for PMN formation (A). Licensing: where PMN development is initiated through ECM remodifications, recruitment of auxiliary immune cells and angiogenesis start occurring (B). Initiation: where the PMN has matured to the point where circulating cancer cells are beginning to nest into the host tissue (C). And finally, progression: where said cancer cells are situated in a fully matured niche that is capable of sustaining the rapid cancer cells' growth (D).

The relevance of EV uptake and surface markers

When analyzing PMN forming potential, the route of EV uptake should initially be taken into consideration when trying to deduce which cells affect the above-mentioned metastatic cascade and in what manner. This is of importance since EV content may evoke intracellular effects that could seem discrepant or even blatantly paradoxical. Signal transducer and activator of transcription 3 (STAT3) is a compelling example of what such a phenomenon may look like, and how it may be of relevance when analyzing PMN formation. STAT3, a transcriptional regulator and signal transductor, is able to generally activate cells. As a result, STAT3 activation is able to orchestrates anti-inflammatory responses in suppressive cell populations but at the same time also functions to activate cytotoxic cells of the adaptive arm of the immune system[46]. Since tumour cells need to suppress the immune response, they may evolve to suppress STAT3 in certain cell types but activate it in others. This is exemplified by a pair of studies where one study has shown STAT3 to be inhibited in T-cells by the uptake of TAM associated EVs, whereas an unrelated group of researchers found tumour derived (TD)-EVs did in fact activate the STAT3 pathway for MDSCs[47,48]. This contradiction (STAT3 being inhibited by some tumour friendly EV content, but activated by others) becomes coherent when one starts looking at both the function of STAT3 and the cell types where the EV content respectively ends up. In both cases, STAT3 sets in motion cell activation. But because of the very nature of the target cells (T-cells being able to lyse tumour cells, and MDSCs being able nourish the TME) the emergent outcomes of STAT3 activation in both cell types are radically different. It therefore stands to reason cancers benefit from suppressing STAT3 activity in some cell types (like T-cells) while activating it in others (like MDSCs), since both scenarios eventually aid in immune suppression. It is because of nuances like this, sufficient efforts should be undertaken to probe the road towards phenotype shifts by picking apart the mechanisms involved with EV uptake.

The mechanisms underlying EV uptake are involute to the point where Mulcany et al. decided to dedicate an entire review to contemporary knowledge about the subject. Briefly summarized, the authors described several EV uptake mechanisms to exist, including endocytosis, macropinocytosis, phagocytosis, and lipid raft–mediated internalization. Although a large and varied group of cells have been demonstrated to possess EV uptake capabilities in fluorescent labelling studies, specific means of EV uptake vary and have been shown to exists on a spectrum of specificity[49]. EV uptake facilitated by clathrin-mediated endocytosis and macropinocytosis, two examples of relatively conserved phenomena observed in a large and varied group of cells[49–52]. On the other hand, phagocytosis (which can take up larger fragments such as apoptotic bodies, compared to other types of internalisations) is made use of by a set of specialised cells such as macrophages and DCs[49]. Experiments conducted with pHrodo labelled EVs that produce fluorescent signal in the acidic environment of phagosomes in conjunction with phagocytosis inhibitor wortmannin have indeed demonstrated EV uptake to be possible via phagocytotic routes[49,53]. At the very end of the specificity side of the spectrum, surface markers may designate EV uptake as well. For instance, CD81 and CD9, two members of the Tetraspanin family (a group of EV associated membrane protein), have been demonstrated to be important for EV uptake in DCs as their antagonism hampers this[49,54]. Surface markers have not been researched extensively to date, but an effort is starting to be made to map out what surface markers might bind to specific EV-associated ligands. For example, a collection of receptors that are expressed on endothelial cells (dubbed 'endothelial zip-codes) have been found to bind to specific ligands on EVs[55].

EV uptake is a complicated topic. And although the section above provides some insight into what mechanisms are involved in selective EV-uptake in target cells and a lengthy discussion concerning its exact modi operandi is beyond the scope of this report. It is likely all of the above-mentioned mechanisms play a role in EV uptake in tissues that go on to develop into a PMN. Some of these, like micropinocytosis may by a plethora of cells. Others, like the selective uptake of EVs bearing integrins α6β1 and α6β4 by lung associated fibroblasts, could serve as useful indicators of the eventual destination of circulating EVs[56,57]. Nevertheless, the factors dictating EV uptake have not been studied extensively in the context of PMN development. This field of study therefore should therefore be studied more extensively. By having the tools to investigate EV uptake mechanisms and how they are affected by malignant presence, an estimation can be made with respect to where certain EV content may end up. Self-evidently, knowledge about EV entry has to analysed in conjunction with EV cargo.

The role of EV in immune regulation

EV shedding and uptake are important facets in the intercellular communication between immune players. Since several immune players from both the adaptive and innate immune system affect PMN formation to a significant degree, it can be inferred that immune related EVs play an important role in metastatic escalation[54,55]. In the PMN, tumour associated antigens (TAA), surface molecules (e.g., T- and B-cell receptors, Major histocompatibility complex (MHC), integrins, etc.), miRNA, immune checkpoint inhibitors (ICI) play an important role in dictating the course of the immune response[58]. Immune modulatory EVs can be of both tumour origin (Tumour-derived EVs or TD-EVs) or be part of the immune response itself (immune-derived EVs or ID-EVs). Generally, when looking from the perspective of both ID-EV and TD-EV cargo and/or surface molecules are generally classified as exerting either anti- or protumoral effects on immune players[59]. Besides that, another useful school of thought with regard to their effects on the immune system is subdividing effects into two categories: function activation and function inhibition[60].

As a rule, for PMN formation to come to fruition the antitumoral functions are to be inhibited. A well-researched example of antitumoral functions being inhibited, both in the context of PMN inducing EVs and elsewhere within the field of immunotherapeutics, is through IC axes[61,62]. This is exemplified by studies indicating EV associated PDL1 was responsible for immune evasion in NSCLC, and their increased presence being a statistically significant marker to response to PD1 related therapy for melanoma patients[63,64]. Besides surface interactions intracellular inhibition elicit relevant phenotypical changes as well. For example, ARG1 containing EVs were shown to inhibit CD4+ and CD8+ T-cell function after being consumed by DCs in an ovarian cancer mouse model[65]. Similarly, TD-EV associated survivin (a TAA) uptake by NK-cells obstructed both their cytotoxicity and proliferation[66]. Besides TD-EVs, these types of inhibitory EVs have been uncovered for ID-EVs. M2-like tumour associated macrophages (TAMs) secrete EVs containing the miRNAs miR-29a-3p and miR-21-5p that, when up taken by T-cells, induce an imbalance in the T-cell population skewed towards immune suppressive T-cell phenotypes by inhibiting STAT3 activity which led to elevated ovarian cancer proliferation and metastatic potential[67].

Meanwhile, the activation of protumoral immune functions that abet the efforts of malignancies to cultivate candidate PMNs further also helps evoke PMN formation. For example, TD-EVs isolated from mice used as a multiple melanoma murine model were observed to promote expansion of MDSCs in tumour free mice through excitation of the STAT3 pathway[68]. Likewise, gastric cancer secreted EV uptake had a similar effect on MDSCs through DICER inhibition[69]. As with the EVs that oppose antitumorigenic function, EVs that actuate protumoral mechanisms may also do so through surface molecules. For instance, epidermal growth factor receptor (EGFR, a growth factor receptor) has been demonstrated to condition DCs towards phenotypes, which in turn bring forth subpopulations of immune suppressive T-cells[70]. Finally, and in parallel to the inhibition of antitumoral immune factor, immune cells may also secrete EVs whose cargo activate certain protumoral factors. This is exemplified by a mammary tumor mouse model study that indicated the role of mesenchymal stromal cells (pluripotent stromal stem cells with immunomodulatory properties) in adjusting myeloid progenitor cells differentiation towards M2-like macrophages by shedding EVs containing proteins that engage tolerogenic pathways in myeloid cells[71].

In light of the above-mentioned aspects of tumour-immune system interactions, one might suspect the immune system of mostly being a collaborator of cancer progression. However, it is important to remember that the immune system is mandated through evolution to clear out defective cells and both its failure to do so as well as its active participation towards PMN formation are directly ushered by malignant processes. An obvious demonstration of this antitumoral potential of EVs with regard to

the immune system is the fact that NK-cells and cytotoxic T-cells directly lyse cancer cells through the EV mediated deliverance of perforin and granzyme which then induce apoptosis in the targeted cell[19,72,73]. Interestingly, EVs stimulate antigen presentation. EVs carrying TAA and damage associated molecular patterns, such as those released upon irradiation, can be up taken by DCs which in turn leads to antigen presentation[74]. DCs themselves also further antigen presentation by having other DCs and helper T-cells uptake their secreted EVs with TAA bound to MHC-II which in turn leads to them presenting these antigens themselves, a process known as "antigen cross-dressing"[75–77]. As a final example, NK-related EVs are known to further augment a potentially antitumoral response under some conditions. NK-cells treated with the EVs of NK-cells previously exposed to neuroblastoma cells were demonstrated to kill cancer cells more diligently compared to the control group[78]. NK-related EVs were also shown to furthermore facilitate T-cell and monocyte stimulation as well in vitro[79].

Polarization and immune cell phenotype, can be viewed as an emergent property where the culmination of all these immune related pathways dictate cell phenotype. The sum of these collections of activated and suppressed factors determines the specific comportment of a cell within the setting of the local tissue (Figure 2). Thus, one should take into account how said emergent property is affected by both the functional inhibitory and agonistic aspects of TD- and ID-EVs when making an estimation as to how permissive tissue has become to metastatic colonization. The degree of functional inhibition and activation, and the manner in which they correspond with anti- and protumoral mechanisms, is pivotal to PMN development.
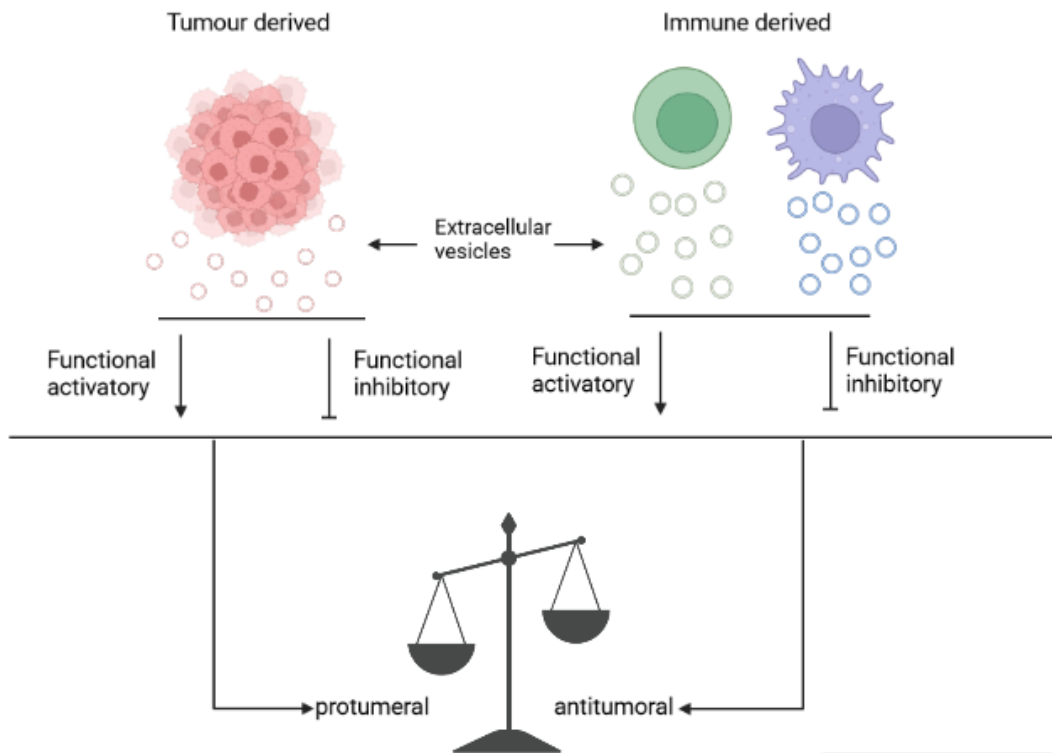
Figure 2 The effect of tumour derived and immune derived EVs on PMN formation Both tumour and immune derived EVs can affect PMN formation by either functionally activating or inhibiting certain immune related pathways related to PMN formation. Inhibiting protumoral pathways PMN formation whilst activating said pathways exacerbates this. The opposite, namely inhibiting antitumoral processes will enhance PMN formation whilst promoting them inhibits it. The culmination of said functional activatory and inhibitory EV cargo dictates PME trajectory. Image created with BioRender.com

The role of EVs in the local vascular and ECM remodeling

In the founder tumour, hypoxia may lead to a surge in EV shedding from cancer cells and stromal cells alike as part of hypoxia-related response[80–82]. Through that consequential surge in EVs, processes such as the reorganization of angiogenesis and ECM modification may be elicited. These released EVs are transported to other tissues where they may potentially participate in PMN formation [7,11,80,83,84].

Angiogenesis is defined as the creation of vasculature and is a process aimed at supplementing the local tissue with more nutrients and oxygen, and is therefore a vital process in cancer progression. Angiogenesis also makes tissues more permissible for dissemination, and tumors have been observed to induce it from a distance through EV transmittance[7,11,80,83,84]. An example of this is a study conducted by Yang et al., where epiregulin (an EGFR ligand) loaded EVs elicited upregulation of proangiogenic genes (such as VEGFA and IL-8). These epiregulin loaded EVs resulted in increased capillary formation of the lungs of mice and was subsequently positively correlated with the dissemination of Salivary Adenoid Cystic Carcinoma metastasis in that in vivo model[85]. An independent yet similar experiment produced concurring results with EV associated annexinA2, which by binding to tissue plasminogen activator and plasminogen to form plasmin effectuated angiogenesis. This in turn increased lung and metastasis in a breast cancer mouse[86]. Vascular permeability tends to magnify as well in these types of settings. This was again seen in Yang et al.'s study, where vascular integrity was shown to decrease both in vitro and in vivo[85]. Such a degradation of the vascular tissue integrity is often instigated through elimination of tight junction molecules like occluding and zonula occludens-1. For example, through downregulation of the aforementioned proteins, something Zhou et al. demonstrated for exosomes transferring the miRNA miR-105 which was able to silence tight junction protein ZO-1 and caused increased vascular permeability in distant organs [7,11,87].

The role of ECM components in PMN formation mostly revolves around enhancing circulating cancer cell and auxiliary immune cell extravasation, as well as stiffening tissue thereby providing cellular survival and proliferation stimuli. Several instances have been documented where EV-related ECM remodifications positively affected metastatic spread[6,7,11,88]. The way this can be achieved is through either depositing ECM materials or molecularly affecting preexisting ECM[88]. For instance, pancreatic cancer EVs containing a marker called macrophage migration inhibitory (MIF) promoted fibronectin production in hepatic stellar cells by eliciting hepatic Kupffer cells to secrete TGFβ. Again, the aforementioned EVs were associated with a higher incidence of metastasis in in vivo models, a phenomenon not observed when these EVs were deprived of their MIF[89]. As a final example, Shinde at al. demonstrated that the ECM related compound fibrolin is crosslinked on EVs shed by breast cancer cells because of the fibronectin crosslinking enzyme Transglutaminase-2 (TG2) being present in them. The presence of TG2 was furthermore determined to aid in education of fibroblasts in the host tissue, which in turn was positively correlated with metastatic activity[90]. Interestingly, EV integrins are demonstrated to specifically bind to ECM components like fibronectin[91]. This suggests it is possible for a positive feedback loop to occur. where TD-EVs remodel the ECM in order to facilitate more TD-EV uptake hence accelerating the transformation of host tissue to PMN.

Future perspectives: utilizing EV cargo to determine metastatic potential

As mentioned in the previous sections, EVs are shed by most cells and as a result EVs have been found in tissues and fluids like urine, blood and saliva[92]. Consequently, one of the advantages of EV analysis is that they can be acquired through isolation from a variety of patient samples. In some instances, EV isolation may therefore occur through relatively non-invasive avenues such as urine, blood and spinal fluid sampling. Once the sample has been obtained, several EV isolation protocols exist. Ultracentrifugation, size-based isolation (such as size exclusion chromatography), immunocapturing (capture based on certain markers) and precipitation are all examples of isolation methods contemporarily used to isolate EVs[92–94]. For diagnostic purposes isolation methodologies should be cost effective, deliver replicable results and easy to use; aspects lacking in most of the afore mentioned isolation methods in one way or another. A promising approach to that end is the use of microfluidic technologies, a method where fluids are led through small areas for separation purposes. Microfluidic technologies are cost effective, provide high yield EV samples, can be adapted to isolate EVs based on either surface markers or size, and have fast recovery times. Although microfluid technologies are to date complicated to use once these hurdles are overcome microfluidic technologies could be upscaled to become useful EV isolation tools within a greater diagnostic workflow. Besides that, an added benefit to microfluid technology is the possibility of synthesizing therapeutic EVs that could be used to counteract PMN formation[95].

Isolating EVs is only the first segment in a conveyor aimed at analyzing EV content and surface markers. Although clinical use of omics data is limited at the moment, as costs cost efficiency and ease of use of omics technology decrease and increase respectively using omics data for clinical purposes may become a viable option. Proteomic, genomic and transcriptomic data gathered EV content derived from diverse EV populations. These data will constitute a big data dataset, as the size of the dataset will require clinicians and scientist make use of informatics. Big data analysis comes with a set of challenges and issues that have to be resolved before clinical implementation, mainly when it comes to (statistical) data interpretation and reproducibility. Although beyond the scope of this thesis, research is being conducted to help set up protocols for big data analysis that limit both of the previously mentioned hurdles. Given the data and relevance of EV content with relation to PMN formation, EV-omics data should yield practical clinical data that can be used to the patient's benefit[96,97].

In the future, when our knowledge with regard to PMN formation has been expanded sufficiently, clinicians may end up using both EV related markers as other markers present in certain samples to gauge metastatic risk and help provide personalized medicine treatment regimens. To that end, a score system may be set up that can tell the physician and patient something about the likelihood of eventual metastatic progression based on the footprint of measured PMN related markers. By utilizing machine learning on marker footprints of cancer patients and comparing those that are struck with metastasis within a certain timeframe with those that stay metastasis free, a patient's marker footprint could be referenced against that. Patient footprints could thereafter be categorized in a metastatic potential staging system in order to inform a physician as to how likely metastasis is for a certain patient and subsequently take precautionary action in the form of personalized treatment. A conceptional framework I created with regard to metastatic potential staging is demonstrated in Figure 3.

A
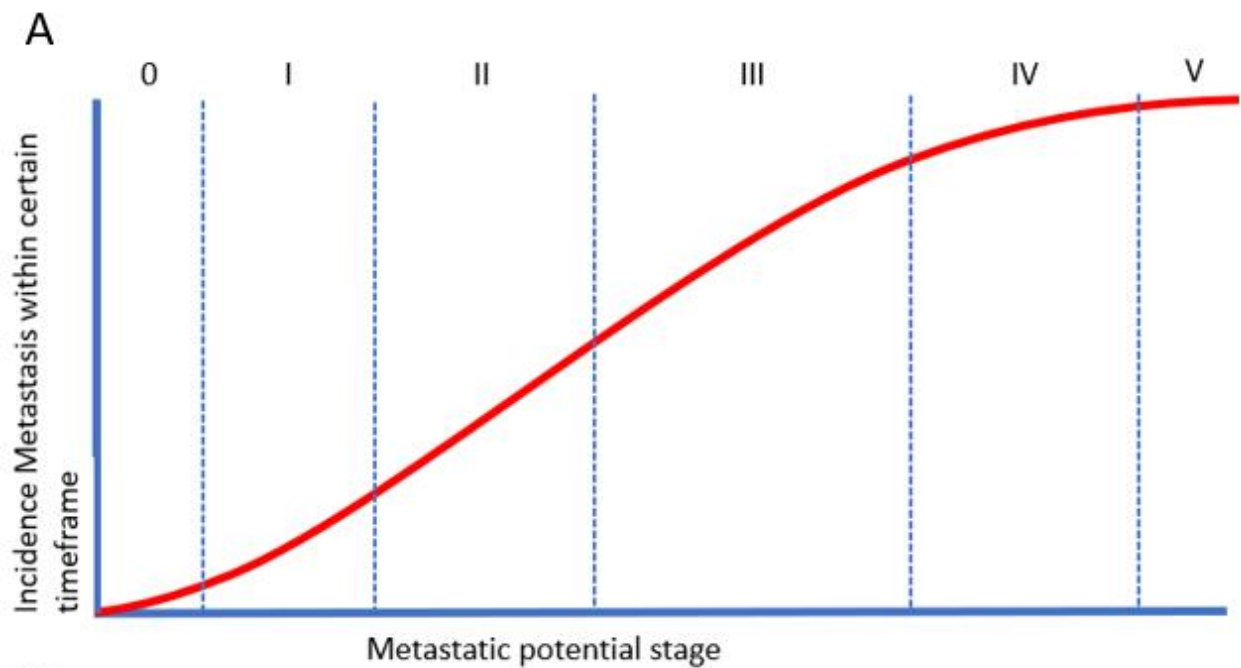
B

stage	Description
0	Baseline. "Healthy individual", perhaps with some slight and vague markers for e.g. chronic inflammation. Metastasis not expected within specified timeframe.
I	Some markers hinting at presence of cancer, perhaps with primary tumour already detected. Markers do not hint at PME presence, but some risk to dissamination exists. Further monitering should be considered.
II	Above mentioned but markers suggest initial PME formation, perhaps hinting at immune suppression at ECM remodification. Substantial percentage of patients with similar MPSS score will go on to develop metastasis within specified timeframe. Metastasis intervention should be considered.
III	Above mentioned but markers suggest advanced PME formation. Without intervention, metastasis will occur in the majority of afflicted patients within specified timeframe.
IV	Metastasis, according to markers now very imminent. Suspected micrometastasis. Circulating cancer cells present. Without intervention, virtually all afflicted patients will go on to develop metastasis within specified timeframe.
V	Metastatic dissemination physically detected.

Figure 3 My conceptual framework of the Metastatic Potential Staging System (MPSS) The Metastatic Potential Staging System (MPSS) is a conceptual framework proposed as part of this report as a future means of arranging patients/patient data into stages of metastatic risk based on (a combination of) certain biomarkers. Staging will occur through specific cut-offs based on incidence of metastasis within a specific timeframe for patients with a similar biomarker profile (A). Based on a specific stage treating physicians could judge the severity of disease, provide useful consultancy/prognoses and apply treatment aimed at metastasis negation (B).

Conclusion

This report was written with the intention to gain insight in the role of EVs in PMN formation, to determine how and to what extent this knowledge may aid in assessing metastatic risks as well as how this knowledge may potentially negate metastatic risk. In recapitulation, this report illuminated how EVs both reflect and transmit their host cell phenotype [14–16,18,25–27]. Thereafter, this report sought to summarize the consecutive steps needed to achieve metastatic formation: priming, licensing, initiation and progression.[7]. In an attempt to link the two previous subjects, this report then went on to describe different relevant facets of this PMN formation process involving EVs, including: the relevance of EV surface markers, EV immune regulation and EV tissue remodification. Overall, there appears to be an abundance of potential when it comes to data with regard to EVs and their role in tissue and immune remodification in relation to metastatic progression. This EV related tissue and immune remodification appear to affect multiple facets of all the different steps of PMN development and the EV content. As such, EV content should provide adequate insight pertaining to the formation of PMNs elsewhere in the body away from the primary tumour.

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