UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

# Functional characterization of tumour-derived exosomes in the zebrafish xenograft model

Mafalda Antunes Ferreira

**Mestrado em Biologia Evolutiva e do Desenvolvimento**

Dissertação orientada por:
Doutor Bruno Costa-Silva, PhD
Professora Doutora Gabriela Rodrigues, PhD

2017

# Aknowledgements

Quero agradecer em primeiro lugar ao Bruno e à Rita por me terem escolhido para abraçar este projeto e transpô-lo para a bancada e pelos orientadores excepcionais que foram em todas as fases deste trabalho. Muito obrigada por tudo o que me ensinaram e sinto-me uma privilegiada por poder trabalhar diretamente com cientistas e pessoas como vocês.
Obrigada à Professora Gabriela pela leitura e correções do manuscrito final desta tese e pelo apoio dado.

Ao grupo Systems Oncology, um obrigada a todo o exo gang: Joana, Inês, Bruna (+Ben), Carol, Sergio e Nuno. Pela companhia, motivação e feedback (mesmo sendo a *outsider* do grupo que trabalha com peixinhos). Um obrigada especial às exo girls, sem vocês isto não teria sido a mesma coisa: Joana pelo apoio científico e amizade *incríbel*, Inês por toda a amizade dentro e fora do lab, Bruna (+Ben) por todo o apoio. And Stina, thank you for all the fun at late nights (in and out of the lab).
Ao laboratório Cancer and Telomeres: Vanda, Raquel, Mariana, Magda, Ana e Bruna. Obrigada por todo o apoio ao longo deste ano, o entusiasmo pelos peixes-zebra e ritmo de trabalho contagiante! Em especial, à Vanda por todo o método de trabalho e energia, à Magda pela companhia nesta aventura que é fazer uma tese e conciliar uma vida pessoal e à Mariana um brinde (recíproco) por tudo!
Agradeço também à Fundação Champalimaud e a todo o pessoal do Vivarium pelo suporte no desenvolvimento deste projecto. À Andreia, Sara, Bernardo, Catarina Rato, Ana Vieira, Beatriz e todas as pessoas fantásticas que conheci durante este ano e que me ajudaram a sentir sempre em casa e pertencente a esta grande família da Champalimaud Research.

À minha Família, em especial à Sofia, Carlitos e Raquelinha por, mesmo estando longe, estarem sempre comigo nesta e noutras aventuras ao longo da vida e também à Raquel, Matilde e Kiko (que ainda nasceu antes desta tese). À minha familiazita: Manuel Vicente, Cats, Vera, Fred, Gala, Gil e Manuel Melo, por serem sempre mais do que família.
Ao Mica, obrigada por toda a paciência e apoio ao longo deste ano, por tudo o que significas para mim, *you are my blood bank*, a minha força. (E obrigada pela ilustração fantástica da larva de peixe-zebra nesta tese!). E por último, mas mais importante, à minha mãe: obrigada por me manteres alimentada e feliz durante as fases mais complicadas. Um obrigada gigante pela mulher incrível que és e por todo o amor incondicional, sem o teu apoio isto não seria possível!

# Resumo

Os exossomas, são as vesículas extracelulares mais pequenas que se conhece, com diâmetros entre 30 e 150 nm, compostos por uma bicamada fosfolipídica que pode conter ácidos nucleicos, proteínas, enzimas, lípidos, citoquinas, entre outras moléculas, podendo portanto, representar em parte o genótipo e fenótipo das suas células de origem.

Uma das principais funções dos exossomas é o seu papel como intermediários na comunicação célula-a-célula, suportando a progressão do cancro através da reprogramação do microambiente tumoral. Para tal, os exossomas desempenham diferentes papéis, incluindo modulação de permeabilidade vascular, educação de células estromais, assim como a 'educação' e recrutamento de células derivadas da medula óssea. Sabe-se que estas vesículas preparam um microambiente favorável em futuros locais metastáticos, antes da chegada das células tumorais, os chamados 'nichos pré-metastáticos'. Além disso, estudos recentes demonstram que o padrão de biodistribuição de exossomas tumorais em modelos murinos está diretamente correlacionado com o local da formação de microambientes pró-metastáticos e, consequentemente, com o perfil de disseminação metastática dos tumores em questão. Sabe-se ainda que este padrão de biodistribuição, ou organotropismo, dos exossomas derivados de tumores pode ser explicado, em parte, pelo seu perfil de composição de integrinas membranares.

Desta forma, argumenta-se que a caracterização dos exossomas em circulação pode servir de ferramenta para prever a propensão para doença metastática, assim como, determinar para que órgão a doença poderá metastizar no futuro. Por ser possível obter estes exossomas a partir de fluidos corporais, como o plasma sanguíneo, os exossomas têm potencial como fonte não invasiva para a obtenção de biomarcadores de cancro.

Além de estudos de caracterização de conteúdo molecular, tendo em vista a grande complexidade das possíveis interações de exossomas tumorais com o organismo do hospedeiro, faz-se indispensável a aplicação de estudos *in vivo*, tais como os envolvendo roedores. Apesar de muito informativos, estes modelos requerem uma quantidade significativa de exossomas, dificultando e/ou impedindo a realização de ensaios *in vivo* a partir de fontes limitadas de exossomas, tal como plasma sanguíneo de doentes oncológicos. Tendo em conta a correlação direta entre a biodistribuição exossomal e o organotropismo tumoral, o desenvolvimento de ensaios *in vivo* que permitam identificar a biosdistribuição exossomal, são de grande importância para a caracterização do perfil das doenças oncológicas.

Para tal, neste projeto propusemo-nos a testar a aplicabilidade de modelos de pequena escala, tal como o de peixe-zebra (*Danio rerio*), para estudos *in vivo* da atividade biológica de exossomas derivados de tumores. O peixe-zebra é um modelo já muito utilizado em investigação biomédica, apresentando uma grande semelhança genética aos humanos (70% de todos os genes associados a doenças humanas têm homólogos funcionais em peixe-zebra), sendo vários os sistemas e órgãos notavelmente semelhantes aos dos humanos. Além disso, a vasculatura embriónária e os genes e cascatas de sinalização moleculares que controlam a hematopoiese são altamente conservados entre peixes e mamíferos. A semelhança da hematopoiese entre peixe-zebra e mamíferos vai para além da conservação de genes, partilhando também todos os tipos de células sanguíneas que são geradas de células estaminais de linhagens em comum.

Para além disto, o peixe-zebra apresenta vantagens técnicas e funcionais muito relevantes frente a modelos de roedores, tendo menor custo de manutenção do que os modelos murinos e permitindo um maior número de indivíduos por experiência. Além disso, a transparência dos peixes-zebra em fase larval permite a visualização não intrusiva de órgãos e processos biológicos *in vivo*, ao contrário dos modelos murinos adultos, nos quais tais aspectos só podem ser observados a partir de ensaios *ex vivo*.

Para ser possível efetuar qualquer estudo com exossomas neste novo modelo, foi necessária a estandardização da dose de exossomas a injetar nas larvas de peixe-zebra. Neste sentido, isolámos e testámos diferentes doses de exossomas de algumas linhas de células tumorais murinas: cancro do pâncreas, parental e metastática (nomeadamente, PAN02 e PAN02-H3) e também de melanoma (B16-F10). Em ratinho, PAN02 e PAN02-H3 são altamente metastáticas para fígado e B16-F10 para os pulmões. Como controlo não tumoral, isolámos exossomas a partir da cultura de pâncreas de ratinhos saudáveis. O padrão é injetar 5 a 10 ug de proteína exossomal em ratinhos, os quais têm cerca de 25 g de massa corporal. Sendo a larva de peixe-zebra um modelo muito menor, com aproximadamente 1 mg de massa corporal, tentámos reduzir esta dose de injeção em pelo menos 1000 vezes, de microgramas para nanogramas. Testámos injetar doses num intervalo entre 0.5 a 50 ng, em larvas de peixe-zebra às 48 horas pós fertilização. Concluímos que a dose recomendável para ser possível visualizar sinal exossomal, sem este estar saturado, seria a dose de 5 ng por larva. Observámos que o sinal mais significativo de exossomas apresentou-se sempre na zona caudal das larvas, transversalmente a todos os tipos de exossomas injetados. Esta região é denominada por tecido hematopoiético caudal (*caudal hematopoietic tissue*, CHT), local transitório onde ocorre a hematopoiese na larva e o órgão análogo ao fígado fetal dos mamíferos. Por isso, neste trabalho, focámo-nos principalmente na análise do CHT, subdividindo esta região em dois quadrantes (Q), Q7 e Q8. No Q7 foi possível observar diferenças significativas no número de células que incorporaram exossomas, sendo este número maior nos exossomas PAN02 do que nos B16-F10.

De forma a tentar estudar melhor o organotropismo destes exossomas, testámos também exossomas com biodistribuição semelhante a PAN02, derivados de cancro colorectal (CT26-FL3), altamente metastático para fígado. Além disso, testámos exossomas derivados de Lewis Lung Carcinoma (LLC), para comparar estes com os resultados dos B16-F10, ambos tumores altamente metastáticos para o pulmão. Nestas últimas experiências os resultados obtidos não apresentaram diferenças significativas na zona do CHT entre os diferentes tipos de exossomas tumorais injetados. No entanto, observámos, consistentemente, que a afinidade dos exossomas derivados de pâncreas saudáveis (Normal Pan) com os tecidos na região do CHT era mais reduzida do que todos os exossomas tumorais testados. Este resultado permitiu-nos concluir que a retenção dos exossomas naquele local não era exclusivamente mecânica e que existe algum tipo de interação entre os exossomas tumorais e o tecido hematopoiético caudal das larvas de peixe-zebra. Este padrão de biodistribuição relaciona-se com o que foi observado em ratinho, onde os exossomas têm um papel na comunicação entre as células tumorais e as células derivadas da medula óssea (*bone marrow derived cells*, BMDCs), as quais desempenham um papel preponderante para o desenvolvimento de fenótipos pró-metastáticos. Além disso, medimos no modelo de peixe-zebra a incorporação de exossomas por células mielóides, frequentemente envolvidas nos efeitos *in vivo* de exossomas tumorais, como os macrófagos. Através de resultados preliminares, pudemos observar que uma parte da população de células, cerca de 5-15%, que incorporaram exossomas eram células mielóides.

Neste trabalho, testámos também diferentes doses de exossomas isolados a partir de plasma de pacientes de cancro de pâncreas, no modelo de peixe-zebra. A dose determinada anteriormente para exossomas isolados a partir de culturas celulares, 5 ng, não foi suficiente para obter um sinal exossomal. Desta forma, a dose teve de ser aumentada para 30 ng por individuo. Apesar da necessidade do aumento da dose em 6 vezes, esta ainda é mais de 166 vezes menor do que a utilizada em modelos murinos. Nos dados preliminares obtidos, não foi possível observar diferenças significativas entre exossomas do plasma do controlo saudável e do paciente estudado. Além do limitado número de indivíduos aqui avaliados, a elevada variabilidade da origem dos exossomas presentes no plasma, dificulta a análise dos exossomas

derivados do tumor do paciente, já que cerca de 80% dos exossomas destas amostras são de origem não-tumoral.
A nossa hipótese é que a utilização do modelo animal de peixe-zebra, possa possibilitar a medição da atividade biológica de exossomas tumorais e auxiliar na localização e detecção precoce do risco de doença metastática em pacientes oncológicos. Além disso, acreditamos que este novo modelo experimental possa servir de complemento aos estudos feitos em modelos murinos, permitindo que se façam estudos utilizando uma quantidade de amostra exossomal significativamente menor por indivíduo.

**Palavras-chave**
exossomas, peixe-zebra, biodistribuição, cancro

# Abstract

It has been recently shown that the pattern of biodistribution of tumour-derived exosomes in murine models is directly related to the metastatic distribution profile of the same exosome-producing tumour cells. Although very informative, this model requires significant amounts of exosomes for complete *in vivo* experiments, which is an important limiting factor due to the small quantity of exosomes obtainable from each patient sample. In this work, we tested whether it is possible to use the zebrafish model for *in vivo* studies of the biological activity of tumour-derived exosomes, through the analysis of their biodistribution.

We tested exosomes derived from several tumour cell lines, with different organotropisms and found that these exosomes have a special affinity for the caudal hematopoietic tissue (CHT) of the larvae, which parallels the mammalian bone marrow. Importantly, in mammal animal models, bone marrow cells play a major role in pro-metastatic phenotypes. This large accumulation in the CHT was not detected using exosomes from non-tumour cells, suggesting that this CHT affinity is not based on a mechanical phenomenon.

Furthermore, we tested in the zebrafish model whether exosomes are taken up by myeloid cells, like the macrophages, which are frequently involved in the *in vivo* effects of exosomes and observed that ~15% of the cells that take-up exosomes are from this lineage. Finally, we were able to standardize the injection dose of exosomes derived from tumour cells and plasma of pancreatic cancer patients for *in vivo* zebrafish biodistribution assays. Our hypothesis is that the use of small scale models, like zebrafish, to perform *in vivo* studies, can enable the measurement of the biological activity of tumour-derived exosomes and help to localize and detect the risk of metastatic disease in oncologic patients early.



**Key words**

exosomes, zebrafish, biodistribution, cancer

# Table of Contents

# Table of Figures

# List of abbreviations

| | |
|---|---|
| **AGM** | Aorta-Gonad-Mesonephros |
| **ATCC** | American Type Culture Collection |
| **BMDC** | Bone Marrow-Derived Cell |
| **CHT** | Caudal Hematopoietic Tissue |
| **CTC** | Circulating tumour cells |
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **DMEM** | Dulbecco's Modified Eagle Medium |
| **DMSO** | Dimethyl Sulfoxide |
| **dpf** | Days Post Fertilization |
| **dpi** | Days Post Injection |
| **ECM** | Extracellular matrix |
| **ELISA** | Enzyme-Linked Immunosorbent Assay |
| **EV** | Extracellular Vesicle |
| **FBS** | Fetal Bovine Serum |
| **GFP** | Green Fluorescent Protein |
| **HSC** | Hematopoietic Stem Cell |
| **ICM** | Intermediate Cell Mass |
| **LLC** | Lewis Lung Carcinoma |
| **MIF** | Macrophage Migration Inhibitory Factor |
| **MVB** | Multivesicular Body |
| **MVE** | Multivesicular Endosomes |
| **ns** | Not Significant (Statistically) |
| **PBI** | Posterior Blood Island |
| **PBI** | Posterior Blood Island |
| **PBS** | Phosphate-Buffered Saline |
| **PDAC** | Pancreatic Ductal Adenocarcinoma |
| **PFA** | Paraformaldeyde |
| **PMN** | Pre-Metatatic Niche |
| **PVS** | Periviteline Space |
| **Q** | Quadrant |
| **RPMI** | Roswell Park Memorial Institute medium |
| **TME** | Tumour Microenvironment |

**wpf** Weeks Post Fertilization

# 1. Introduction

## 1.1 Exosomes

Exosomes are the smallest type of extracellular vesicle (EV), ranging from 30 to 150 nm[1]. EVs can be classified as apoptotic bodies, microvesicles or exosomes, depending on their size and also characteristics such as mechanism of secretion, molecular content and function[2, 3]. Exosomes have a specific process of biogenesis, originating from multivesicular endosomes (MVE)[1, 4]. First, endosomes are originated by invagination of the cell membrane. The receptors that are present in the cell surface will be maintained internally in the vesicle. The endosome matures and become a multivesicular body (MVB) that contains numerous intraluminal vesicles, originated by several invaginations of the endosomes membrane. These intraluminal bodies are decorated with molecules all over their surface, which were present originally on the cell surface. When these MVBs fuse with the cell membrane, they release these intraluminal bodies, the exosomes, to the extracellular space[5] (Fig. 1.1)

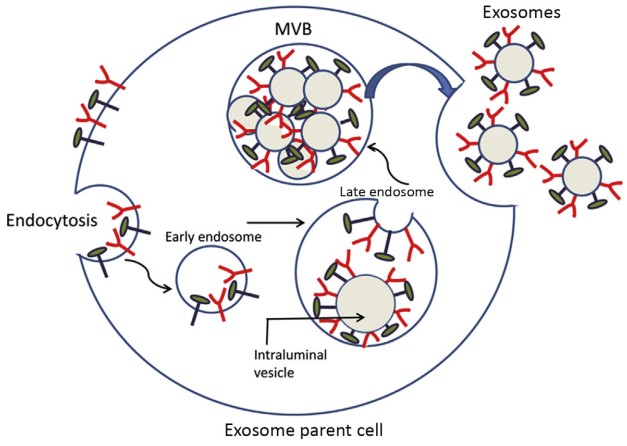


**Figure 1.1 Exosome biogenesis and their release from a parent cell.** The surface membrane undergoes endocytosis enclosing surface-residing molecules into an early endosome. Invagination of the endosomal membrane in late endosomes leads to the formation of intraluminal vesicles, each decorated with molecules originally present on the cell surface. Endosomes form a multivesicular body (MVB) containing pools of intraluminal vesicles. Fusion of the MVB with the cell surface membrane leads to release of free exosomes into the extracellular space. Note that components of the parent cell surface are now present on the membrane surrounding each exosome (Whiteside, 2016).

Exosomes are produced by all types of cells. Also, vesicles with hallmarks of exosomes have been isolated from diverse body fluids, including semen[6–8], blood[9], urine[10], saliva[11], breast milk[12], amniotic fluid[13], ascites fluid[14], cerebrospinal fluid[15], and bile[16].

Exosomes have imprints of their parent cell, containing nucleic acids, proteins, enzymes, lipids, cytokines, among other molecules[2, 3], in the lumen and membrane of these phospholipid bilayer-enclosed vesicles. Although in some cases the content of exosomes does not reproduce exactly all the composition of their parent cells[17], in general it can still give a representation of the parent cell phenotype and genotype. Exosomes are also known to promote the horizontal transfer of molecules to recipient cells[18–20].

Many discussions surround the methods for EV isolation and analysis, which justifies the

importance to improve and standardize protocols in order to have solid proof of the biological material that is being analyzed[21]. The most frequently used procedures are based on the isolation of the EVs released in cell culture by tumour-cells grown in media with foetal calf serum depleted of EVs. A series of differential ultracentrifugation is performed, allowing a purification based on the mass of the particles. Next, exosomes can be efficiently purified based on their density, from non-membranous particles, such as protein aggregates, using Sucrose/Deuterium Oxide (D2O) Density Cushions[22–25].

In the context of cancer, tumour cells secrete much more exosomes than healthy cells. This can be observed in the higher levels of exosomes in plasma and other body fluids of the patients with cancer[26,27]. The mechanisms to regulate exosomes secretion by the tumour cells are still unknown but some insights are emerging. P53, that is usually involved in cancer, can have a role in regulating the production and release of exosomes by tumour cells[28]. Rab proteins, when knocked down, decrease the level of exosomes produced by the tumour cells[29,30]. Heparanase, overexpressed in several tumour cell lines, was shown to also have a part in regulating exosome production[31]. Tumour-derived exosomes have a molecular signature depending on the type of tumour cell of origin. Many studies are showing the role of tumour-derived exosomes in promoting tumour progression and metastasis, altering immune and stromal cell behaviour, enhancing angiogenesis and promoting the survival of tumour cells[5] (Fig. 1.2).

### 1.2 Exosomes as messengers

A major function of exosomes is to serve as an intermediary for cell-cell communication in the body. Tumour-derived exosomes are able to circulate from the primary tumour to distant tissues and organs. They are secreted by the millions by tumour cells and act like their messengers, supporting cancer spreading by reprograming the tumour microenvironment (TME). Strikingly, it has been shown that tumour-derived exosomes prepare a favourable microenvironment at future metastatic sites, even before tumour cell arrival, called the 'pre-metastatic niches' (PMNs)[32].

### 1.3 Exosomes educate the metastatic niche

Tumour-derived exosomes have and important role promoting metastatic niches by educating bone marrow-derived cells (BMDCs) through upregulation of the MET oncoprotein. MET is upregulated in bone marrow progenitor cells by tumour-derived exosomes, promoting the education, mobilization and pro-metastatic behavior of BMDCs[33]. Mice injected with Melanoma (B16-F10) exosomes, which preferentially bind to pulmonary and medullar cells, had a greater metastatic burden in the lungs and developed metastasis with a greater tissue distribution, which included bone and brain, suggesting that qualitative differences in exosome content can mediate metastatic potential and target. Education of bone marrow cells with B16-F10 exosomes was shown to increase primary tumour growth and metastatic burden in less metastatic models such as Lewis lung carcinoma (LLC). Overall it was shown that tumour-derived exosomes have a crucial role for bone marrow cell education by regulating tumour metastasis[33].

By preferentially binding to liver myeloid cells, pancreatic ductal adenocarcinoma (PDAC)-derived exosomes were shown to create hepatic pre-metastatic niches, enhancing PDAC metastasis in the liver. The fibrotic microenvironment formed in this pre-metastatic niche promotes the recruitment of bone marrow-derived macrophages to the liver, which constitutes a

pro-metastatic microenvironment supportive of hepatic metastasis. It was also shown that the targeting of exosomal macrophage migration inhibitory factor (MIF) prevented the formation of this phenomena, suggesting that exosomal MIF primes the liver for metastasis and may be a potential biomarker for predicting PDAC liver metastasis in patients[34].

The organotropism of the tumour-derived exosomes, meaning the preferential binding to some tissues or organs on the body, can be explained in part by exosomal integrin expression. This expression is distinct among tumour cells, which dictates exosome adhesion to specific cell types and extracellular matrix (ECM) molecules in particular organs. It has been shown that treatment with exosomes from lung-tropic models can redirect the metastasis of bone-tropic tumour cells, demonstrating, the importance of these integrin patterns in dictating the organotropism of these vesicles[35]. Exosomes can then, trigger signaling pathways and inflammatory responses in target cells, resulting in the education of that organ. Exosomes can perform distinct roles during each of the sequential steps (that is, vascular leakiness, stromal cell education at organotropic sites, bone-marrow-derived cell education and recruitment) necessary to complete the PMN. Therefore, circulating tumour-derived exosomes may be useful not only to predict metastatic propensity, but also to determine preferential organ sites of future metastasis[35].

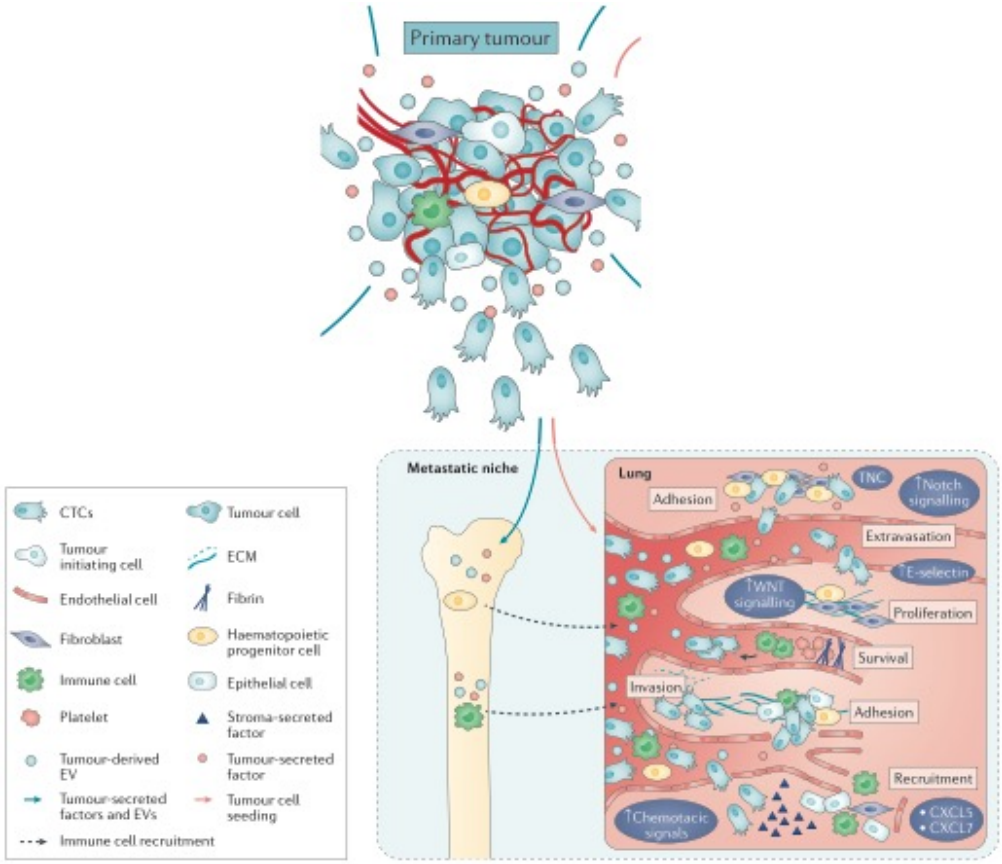


**Figure 1.2 The role of tumour-derived exosomes on the recruitment of bone marrow-derived cells (BMDCs) and the establishment of the metastatic niche** (adapted from Peinado, Zhang, Matei *et al*. 2017). Circulating tumour cells (CTCs), extracellular matrix (ECM), extracellular vesicles (EVs).

### 1.4 Exosomes as biomarkers

The potential of exosomes as a non-invasive source of cancer biomarkers is gaining attention[36,37]. Exosomes are present in all the body fluids and can maintain the parental cell content, even over long distances inside the body. This content reflects the biologically active content present in the parent cells, and the use of these tumour-derived exosomes in *in vivo* models may indicate more about the phenotypic characteristics of the parent cells. However, further research will be needed to standardize the use of these vesicles to measure disease presence, progression and treatment.

### 1.5 Zebrafish model

The *in vivo* study of tumour-derived exosomes is essential to obtain information of the behavior of these vesicles in a complex biological system. Most of the studies involving tumour-derived exosomes have been performed in mouse models. However, alternative rising models such as zebrafish may be advantageous for *in vivo* studies in this area. For instance, zebrafish larvae are very small and translucid, facilitating a non-intrusive visualization of organs and biological processes *in vivo*. In addition, zebrafish has a short generation interval of 2–3 months, producing numerous offspring in a short period of time.

Zebrafish share a high genetic similarity to humans (approximately 70% of all human disease genes have functional homologs in zebrafish) and several zebrafish organ systems are remarkably similar to those in humans. The embryonic vasculature and also the genes and molecular signaling pathways controlling hematopoiesis are highly conserved between fish and mammals [38]. Hematopoiesis in zebrafish and mammals is not only limited to the conservation of genes, it also shares all major blood cell types, that are generated from common lineages of hematopoietic stem cells[39].

As in all vertebrates, there are two waves of hematopoiesis, the primitive and the definitive wave. The primitive hematopoiesis in the anterior lateral mesoderme (ALM) and intermediate cell mass (ICM) lasts for less than 24 hours and is followed by another transient wave of hematopoiesis in the posterior blood island (PBI), which is also known as the caudal hematopoietic tissue (CHT). After this, there is the definitive wave of hematopoiesis, where multipotent hematopoietic stem cells (HSCs) are capable of self-renewal and generate all the different blood lineages. In mammals, the HSCs are first produced in the aorta-gonad-mesonephros (AGM), the ventral wall of the dorsal aorta, after in the fetal liver, and finally in the bone marrow. When comparing to zebrafish, HSCs are first detected in the equivalent to the AGM region, referred to as the ‘‘DP joint’’, a thin mesenchyme between the dorsal aorta and the posterior cardinal vein[40]. Next HSCs are found in the CHT, the intermediate site of hematopoiesis that is equivalent to the mammalian fetal liver. A subset of HSCs from the AGM also migrates directly to the thymus for lymphopoiesis[41]. In the definitive wave, HSCs migrate from the AGM and CHT to the kidney marrow, the equivalent to mammalian bone marrow, and the site of larval and adult HSC production[40,42,43] (Fig. 1.3).

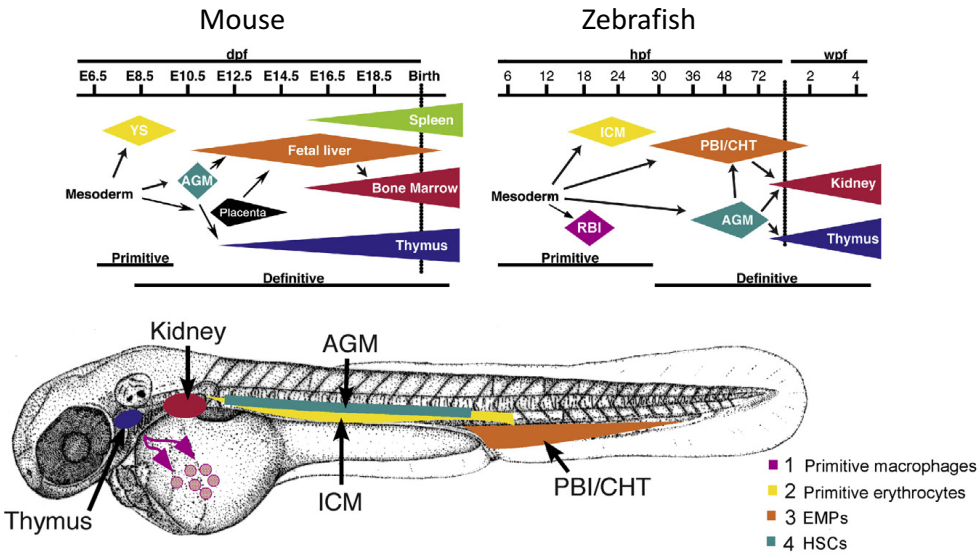


**Figure 1.3 Timing and location of mouse and zebrafish hematopoietic development and the parallel between the two models** (Adapted from Stachura and Traverx, 2016). Rostral blood island (RBI), hematopoietic stem cells (HSCs), yolk sac (YS), erythromyeloid progenitors (EMPs), aorta-gonad-mesonephros (AGM), intermediate cell mass (ICM), posterior blood island (PBI) later in development, named caudal hematopoietic tissue (CHT).

## 1.6 Zebrafish as a model in cancer studies

Regarding the use of zebrafish in the area of precision medicine, a new study promises to bridge the gap between genotype and phenotype in *in vivo* settings. Using zebrafish xenografts, it was possible to test in a quick assay the tumours' chemosensitivity in an *in vivo* setting. Using zebrafish instead of mice it was possible, with a much larger number of xenografts per assay, to obtain faster results, and with fewer patient-derived cells injected per larvae[44]. In addition, when compared to mice, zebrafish present lower cost of maintenance, permitting much larger number of individuals per assay, represents a faster animal model to obtain results, and also facilitates *in vivo* imaging of tumour cells interacting with the tumour microenvironment (TME). Several studies showed evidence of bidirectional communication between the xenografts and zebrafish cells[45–47]. For all the above reasons, zebrafish have a great potential to be used as a complement or alternative to the existent mouse models in *in vivo* studies, also for this new area of medical research, involving EVs.

The goal of this work is to explore the potential use of the zebrafish as a model for *in vivo* assays involving EVs. In zebrafish, there is a great potential to minimize the amount of sample necessary for *in vivo* assays, especially those involving limited patient-derived samples and study exosome effects in target cells by live-imaging in a non-invasive manner. For that, we here standardized the doses of injection of these tumour-derived exosomes in zebrafish to map their biodistribution. Specifically, we used exosomes isolated from murine cell lines that were previously characterized in murine models in terms of biodistribution to different organs, namely hepatic and medullary accumulation in pancreatic cancer (PAN02 and PAN02-H3) and colorectal cancer (CT26-FL3), and medullary and pulmonary accumulation in melanoma (B16-F10) and lung carcinoma (LLC). We observed a hot-spot of exosome distribution in a zebrafish larvae hematopoietic

compartment– the CHT, which parallels the mammalian bone marrow at this stage of development. Furthermore, we tested in the zebrafish model whether exosomes are taken up by myeloid cells, like the macrophages, which are frequently involved in the *in vivo* effects of tumour-derived exosomes. Finally, we were able to standardize the injection dose in zebrafish of exosomes derived from tumour cells and plasma of pancreatic cancer patients. Therefore, we envision the possibility to complement the mouse models for studies involving the biodistribution evaluation of exosomes *in vivo* by those in zebrafish here described.

# 2. Materials and methods

### 2.1 Cell lines and culture

The C57Bl/6 murine pancreatic adenocarcinoma cell line PAN02 was purchased originally from the DTP, DCTD Tumor Repository, NIH. PAN02-H3, derived from PAN02, was obtained by *in vivo* selection involving three hepatic metastatic passages. CT26-FL3 was donated by Marj Pena group. B16-F10 were purchased originally from ATCC (CRL-6475). LLC were purchased originally from ATCC.
All cells lines were tested for mycoplasma before exosomes isolation. PAN02, PAN02-H3 and CT26-FL3 were cultured in RPMI (Biowest). B16F10 and LLC were cultured in DMEM (Biowest). All cell lines in culture were supplemented with 10% fetal bovine serum (FBS - Biochrom) and 1% Penicillin-Streptomycin 10,000 Units/ml in a humidified atmosphere containing 5% $CO_2$ at 37$^{o}$C. All cell lines when thawed, were previously frozen in passages 2-3 and maintained in culture for experiments until a maximum of 15 passages.

### 2.2 Exosome isolation, characterization and labelling

Cells were cultured in RPMI or DMEM, supplemented with 10% exosome-depleted FBS and 1% penicillin–streptomycin. Cells were maintained in a humid incubator with 5% CO2 at 37$^{o}$C. FBS is depleted of bovine exosomes by ultracentrifugation at 100,000*g* for 70 min. The Normal Pancreas exosomes were obtained by culturing pancreata isolated from healthy 4–6-week-old mice in 3 ml of FBS-free RPMI for 12 hours, with 5% $CO_2$ at 37$^{o}$C.
Patient-derived exosomes were obtained from human plasma samples. Normal Samples were collected from 3 healthy subjects and Tumour Samples were collected from 3 pancreatic cancer patients at different stages of the disease.
Isolation of exosomes for all the experiments were done by ultracentrifugation. For cell culture-derived exosomes, supernatant fractions are collected from 72 h cell cultures or cultured organs. As with patient-derived exosomes this is done initially with the blood sample. The samples are pelleted by centrifugation at 500 *g* for 10 min. The supernatant is centrifuged at 3,000 *g* for 20 min, at 12,000 *g* for 20 min and then at 100,000*g* for 70 min. The exosome pellet is fluorescently labelled using PKH26 Red membrane dye and in phosphate-buffered saline (PBS), in a total of 15 mL. A purification based on exosomes density is performed, using 4 mL of Sucrose/Deuterium Oxide (D2O) Density Cushion. Another centrifugation is done at 100,000 *g* for 70 min and the phase with exosomes is obtain with a syringe and mixed with PBS. Exosomes are then collected by an overnight ultracentrifugation at 100,000 *g* and resuspended in PBS. Protein quantification of the samples is performed after isolation (BCA protein assay kit) and exosomes are maintained in -80$^{o}$C. Exosome size and particle number were analyzed using NanoSight NS300. Samples were infused into the NanoSight NS300 using a syringe pump at a rate of 10 (arbitrary units). Data for each sample was collected for 60 seconds and analyzed using NanoSight NTA 2.3 software.

### 2.3 Zebrafish maintenance

The zebrafish larvae for the injections derive from two transgenic lines: Tg(fli1:EGFP), express EGFP under the control of the fli1 promoter[48], allowing *in vivo* observations of both forming and present vessels; and Tg(zpu.1:EGFP), express EGFP under the control of the zebrafish pu.1 promoter, driving GFP expression in myeloid cells[49]. Larvae are maintained in a maximum of 100 individuals per petri dish and dead embryos are discarded. At 48 hours pos-fertilization (hpf), and before the injections, embryos are placed in water with pronase to degrade the chorion. All the procedure is done according with European animal welfare regulations.

### 2.4 Zebrafish injections

Zebrafish larvae injections were performed in a Fluorescent Scope (Zeiss) with a microinjector and larvae aligned in a plate of agarose (2%), anesthetized in tricain (1x)[44]. 14 nL of fluorescently labelled exosomes were injected in PBS and phenol red, to facilitate the observation of the injected suspension. All the needles used were calibrated before the injection. After injected, larvae are maintained at 34$^{o}$C until the end of the experiments. Throughout this work, larvae were analysed *in vivo* at 18, 24 hours pos injection (hpi) and at 4 dpi. All the injections were performed in the periviteline space (PVS) of 48 hpf zebrafish larvae.

### 2.5 Zebrafish larvae processing and immunofluorescence

Zebrafish larvae were fixed in 4% formaldehyde and stored in methanol at -20$^{o}$C. No acetone was used during this protocol in order to avoid fluorescence signal reduction. Larvae were incubated over night at 4$^{o}$C for nuclei counterstaining with DAPI (Molecular probes). The exosomes injected, were always fluorescently labelled using PKH26 Red membrane dye, at the time of the isolation, as mentioned before. Zebrafish larvae were then mounted with fluorescence mounting medium (Dako) and stored at 4$^{o}$C.

### 2.6 Screening and image analyses

For whole larvae screening, a Fluorescent Scope (Zeiss) was used. Fluorescent images are obtained using upright and confocal microscopy (Zeiss710). Exosomes quantification are done using ImageJ Software (NIH) by determining the ratio between the number of cells and/or area with positive signal for exosomes, myeloid cells and DAPI staining, expressed in arbitrary units (a.u.) or percentage. All statistical analysis was carried out with GraphPad Prism 5.0a software (2007). When comparing two groups, unpaired two-tailed *t*-tests. *P* values of less than 0.05 were deemed statistically significant with ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

# 3. Results and Discussion

### 3.1 Standardized dose and analysis of the biodistribution pattern of tumour-derived exosomes in zebrafish larvae model

In order to characterize exosomes in a new model as the zebrafish, the first goal was to define which dose should be used to allow signal detection and avoid signal saturation. In the mouse model, 5 to 10 µg of exosomes are injected per individual with about 25 g body weight. Zebrafish larvae are much smaller than mice, with approximately 1 mg of body weight (wet weight at 6 dpf)[50]. This difference of about 25000 times, suggests a reduction of scale from µg to ng of exosome sample needed for each injection/individual.

We started by testing injection of doses ranging from 0.5 to 50 ng of tumour-derived exosomes per larvae, with exosomes isolated from three types of tumour cell lines: metastatic melanoma and pancreatic cancer (localized and metastatic sublineages). For this, we injected 48 hours post fertilization (hpf) transgenic Tg(Fli1:EGFP) larvae[48], which express eGFP in blood and lymphatic vessels, facilitating anatomical analysis of biodistribution of the exosome signal.

We observed that in some cell lines a high dose of exosomes (50 ng) seem to be lethal for the larvae, which was the case of melanoma B16-F10 tumour-derived exosomes. This is probably due to the high pro-coagulation capability of the exosomes, as already described in previous studies[51]. In the case of pancreatic cancer PAN02-H3 tumour-derived exosomes, the 50 ng dosage was not lethal but the signal was extremely saturated (Fig. 3.1D). With 10 ng, the signal was not saturated (Fig. 3.1C) but, as the main goal is to minimize the amount of exosomes sample and simultaneously be able to obtain the biodistribution information, we reduced the dose to 5 ng (Fig. 3.1B). We found that this dose was optimal, as doses below 5ng resulted in little or no measurable signal to perform this kind of biodistribution assays. Therefore, we found that the lowest exosomes dose able to provide a measurable signal in this biodistribution assay should be in a range between 5 to 10 ng.

At 24 hpi, we analyzed the biodistribution of the different types of tumour-derived exosomes, and observed that the most significant signal was detected in the caudal region of the zebrafish larvae (Fig. 3.1E). This region, posterior to the yolk extension and the urogenital tube, was hereby identified as Quadrant (Q)7 and Q8. These two quadrants correspond in part to the location of the Caudal Hematopoietic Tissue (CHT)[40], being Q7 more anterior in the larvae than the Q8. Subsequently, we focused in further characterizing where the exosome signal was anatomically localized in the larvae. To achieve this, we analyzed injected larvae by high resolution confocal microscopy. At the CHT region, it was possible to observe that exosomes were in the perinuclear area of cells, compatible with previous findings and circulating inside the vessels of the caudal vein plexus (Fig. 3.2).

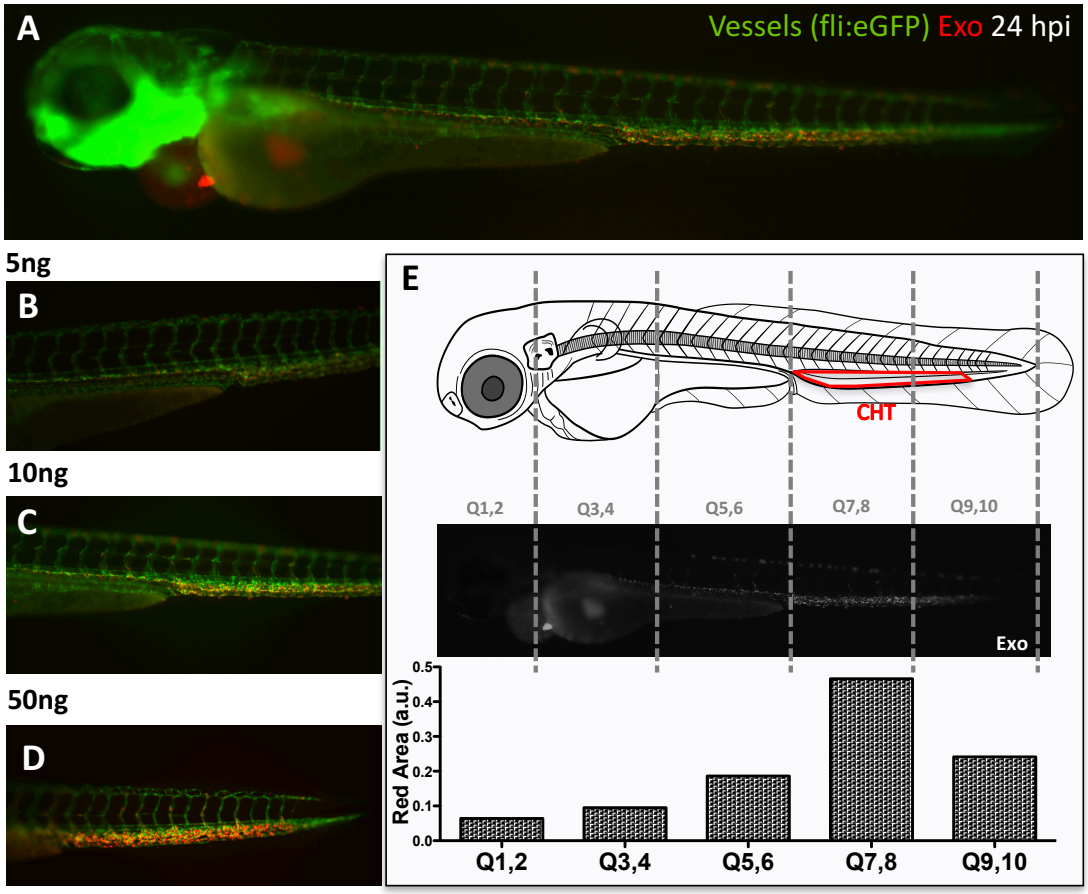


**Figure 3.1 Zebrafish larvae injected at 48hpf with different doses of tumour-derived exosomes isolated from PAN02-H3 cells and screened at 24 hpi.** Tg (Fli:GFP) larvae injected with 10ng (A), 5ng (B), 10ng (C) and 50ng (D) of exosomes. (E) Quantification of the area with exosomes signal, thru the larvae body (region Q1 to Q10, anterior to posterior), larvae injected with 10 ng of PAN02-H3 exosomes. Vessels are stained in green and exosomes in red. (n analysed >50)

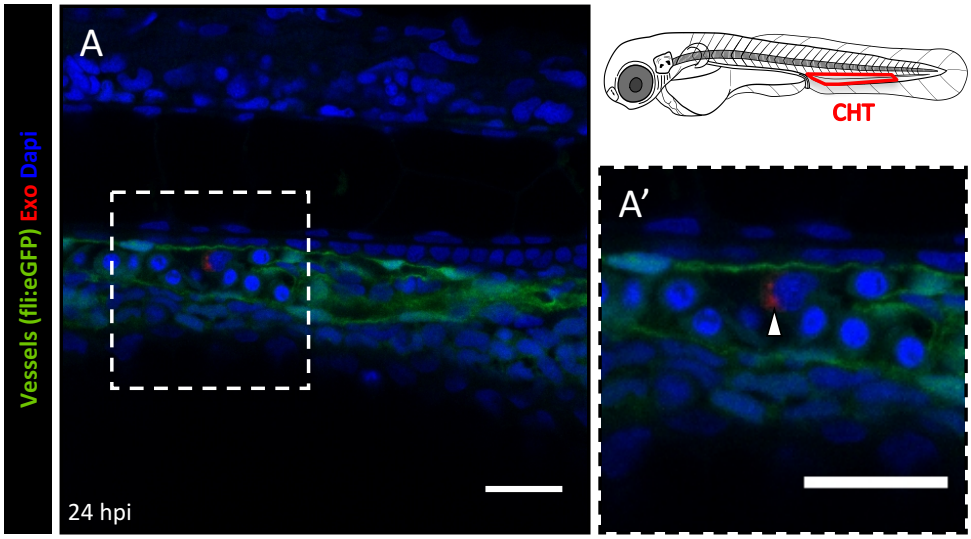


**Figure 3.2 Exosomes-positive cells circulating inside vessels in the CHT region of the zebrafish larvae.** Larvae injected with 5 ng of PAN02-H3 exosomes at 48 hpf and screened 24 hpi. Vessels are stained in green and exosomes in red. Scale bars correspond to 20 µm. Arrowhead is pointing exosomes aggregates. (n analysed >20)

### 3.2 Biodistribution of tumour- versus non-tumour cells-derived exosomes in the zebrafish larvae model

Focusing the analysis on the CHT region, more exactly at the regions Q7 and Q8 (Fig. 3.3A-H), it was possible to observe the accumulation of exosomes already at 18 hpi in the CHT. So further on, all the larvae were fixed at 18hpi, because one of the goals of the models is to be able to have a fast procedure and when compared with larvae 24 hpi or even 4 days after injection, the accumulation of exosomes were at the same place, the CHT.

As a control for pancreatic tumour-derived exosomes, exosomes were isolated from the conditioned media of primary cultures of healthy control pancreas, designated as Normal Pancreas exosomes[34].

In the Q7, when compared to B16-F10 melanoma-derived exosomes, PAN02 primary pancreatic tumour-derived exosomes were taken up by more cells (Fig. 3.3I). In Q8, we can also observe this tendency, although the differences were not statistically significant. Interestingly, we found that Normal Pancreas exosomes had a significantly lower intake by the cells in these regions. This supports the results obtained in mice, where the normal pancreas exosomes were not taken up as efficiently as tumour-derived exosomes[34].

Our results show that the Q7 region was the most appropriate location to measure differences between exosomes derived from distinct tumour models, since it allowed a better comparison amongst exosomes with marked differences in biodistribution (Fig. 3.3I). Based on these results, one can hypothesize that this quadrant would be a good site to compare *in vivo* the biodistribution of different kinds of tumour-derived exosomes, providing an insight to the establishment of a functional assay *in vivo* using the zebrafish model.

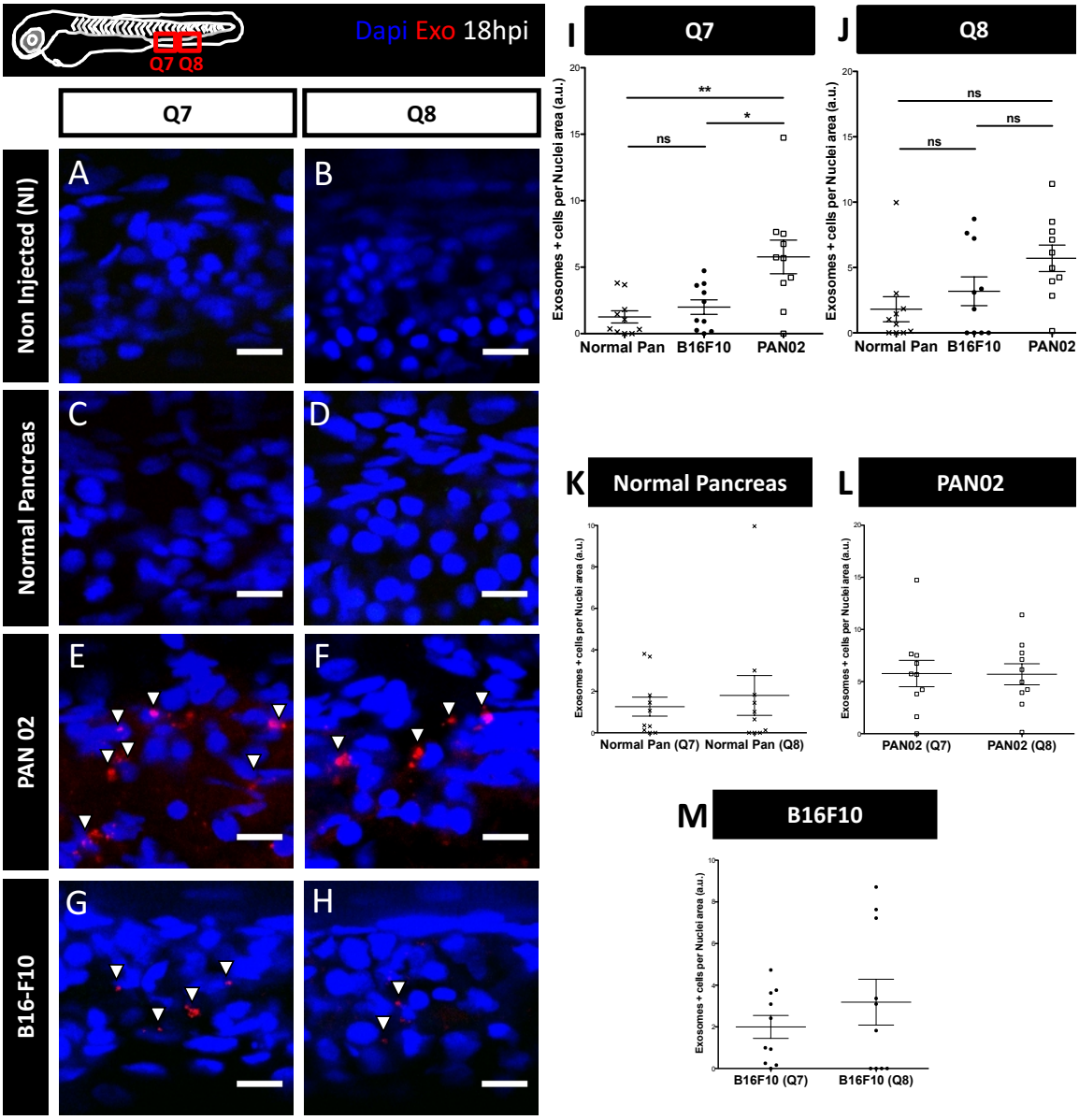


**Figure 3.3 Exosomes from Normal Pancreas, PAN02 and B16-F10 injected in zebrafish larvae at 48 hpf and screened at 18 hpi in the Q7 and Q8 regions.** A-B Non-injected larvae; C-D Larvae injected with exosomes isolated from normal pancreas; E-F Exosomes isolated from the pancreatic cancer cell line PAN02; G-H Exosomes isolated from the melanoma cell line B16-F10; I-M Number of cells taking up exosomes per dapi area in Q7 (I) and in Q8 (J) regions, of larvae injected with Normal Pancreas (K), PAN02 (L) and B16-F10 (M).
Scale bars correspond to 10 µm. Arrowheads are pointing exosomes aggregates.
N analysed= 10 per condition; N injected and treated= 107 (PAN02), 62 (B16-F10), 102 (NORMAL PAN).
***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

### 3.3 Myeloid cells and their role in exosomes intake in the CHT

Exosomes in the CHT were associated to cells circulating inside blood vessels. To understand which cells can have a biological part in the intake of these exosomes, we injected zebrafish transgenic Tg(pu.1:EGFP) larvae[49], that expresses EGFP under the control of the zebrafish pu.1 promoter, driving GFP expression in myeloid cells. The pu.1 myeloid progenitor cells can mature into macrophages and granulocytes[52]. We used this transgenic tool due to previous studies in mice, which showed the important role of cells from the myeloid line in the intake of exosomes and their biological relevance for metastatic niche development, like macrophages[34].

We were able to show that myeloid cells are responsible, in part, for exosome intake in the Q7 and Q8 regions, (Fig. 3.4A, B). Specifically, this preliminary result, shows that about 5-15% of the cells that intake exosomes derived from PAN02 and B16-F10, in this region, are myeloid cells (Fig. 3.4C). These cells can be either macrophages or granulocytes. More detailed screenings need to be done, using more larvae and testing specific cell populations, such as macrophages (Tg(mpeg:GFP)[53] and neutrophils (Tg(mpx:GFP)[54].

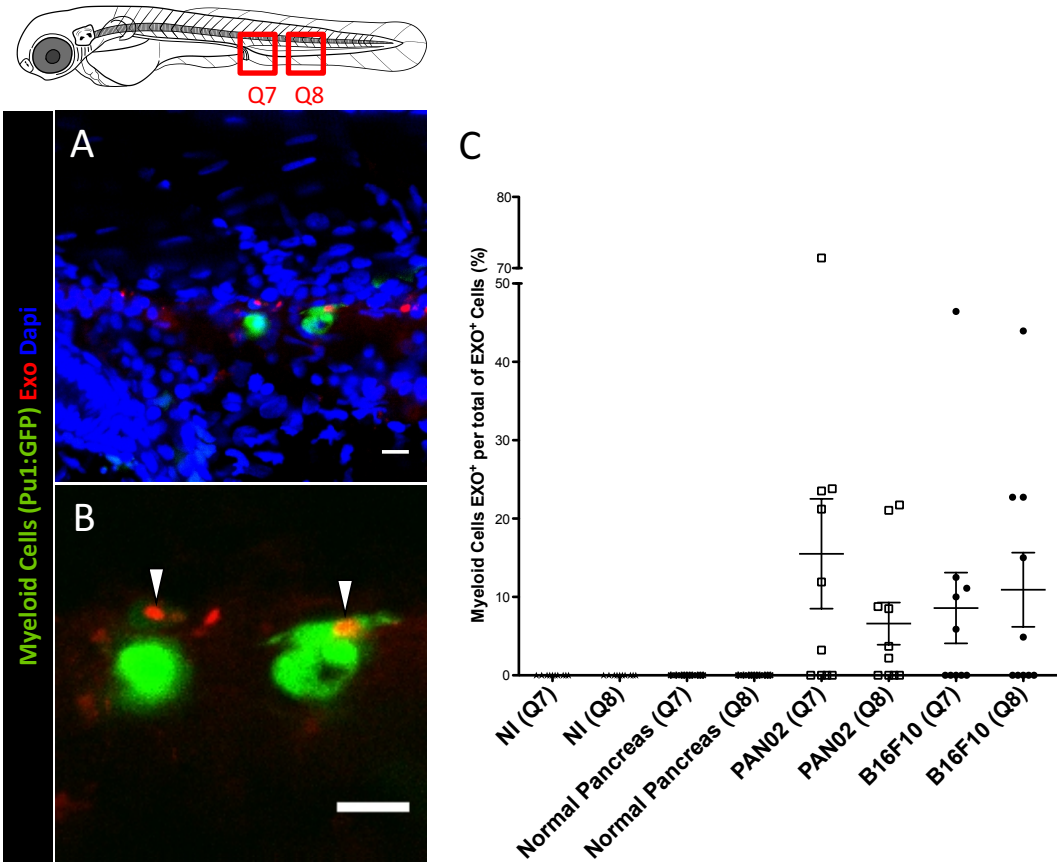


**Figure 3.4 Myeloid cells taking up exosomes in region Q7 and Q8. (**A-B) Zebrafish fish injected with 5 ng of PAN02 exosomes. (C) Quantification of the percentage of cells with exosomes that are myeloid cells.
Scale bars correspond to 10 µm. Arrowheads are pointing exosomes aggregates inside myeloid cells.
N analysed= 10 per condition; N injected and treated= 107 (PAN02), 62 (B16-F10), 102 (NORMAL PAN).

### 3.4 Biodistribution in the zebrafish larvae model of tumour-derived exosomes with different organotropism

In the mouse model, tumour-derived exosomes display organotropisms compatible with the metastatic organ distribution of its tumour of origin[35]. Therefore, the biodistribution analysis of the exosomes gives us important information about their target location, where they can promote a 'pre-metastatic niche' (PMN). PMN are sites more suitable for receiving the metastatic cancer cells, by promoting their survival and growth. To tackle this organotropism, as shown before, we analyze the biodistribution of one type of exosomes with target to the liver, PAN02 and another to the lung, B16-F10. In addition, trying to make a parallel with the results obtained in the previous experiment (Fig. 3.3), we tested more models with different types of organotropic tumours. For that, we used exosomes derived from the highly liver metastatic derivative line of PAN02, PAN02-H3 (Fig. 3.5C,D), and from another liver metastatic cell line, the colorectal cancer CT26-FL3 (Fig. 3.5E, F). In addition, to compare with the results obtained with the lung-tropic melanoma B16-F10 tumour-derived exosomes, we utilized exosomes from the lung-tropic Lewis lung carcinoma (LLC)[32] (Fig. 3.5I, J).

In this experiment the biodistribution in the CHT, regions Q7 and Q8, was not significantly different among the tumour models analyzed, neither between the two regions. Overall, it is possible to observe a tendency for a higher quantity of exosomes in the cells of Q8, especially in the larvae injected with B16-F10 exosomes (Fig. 3.5P). Once again, exosomes from non-tumour pancreatic cells presented a consistently significant lower binding in the regions examined than all the rest of the tumour-derived exosomes. These results are also in accordance with the first experiment (Fig. 3.3), reinforcing the idea that the retention of exosomes is not due to some type of mechanic phenomena in the CHT region of the zebrafish larvae. Due to the small number of analyzed individuals per experimental group, additional repetitions of these experiments are currently in preparation.

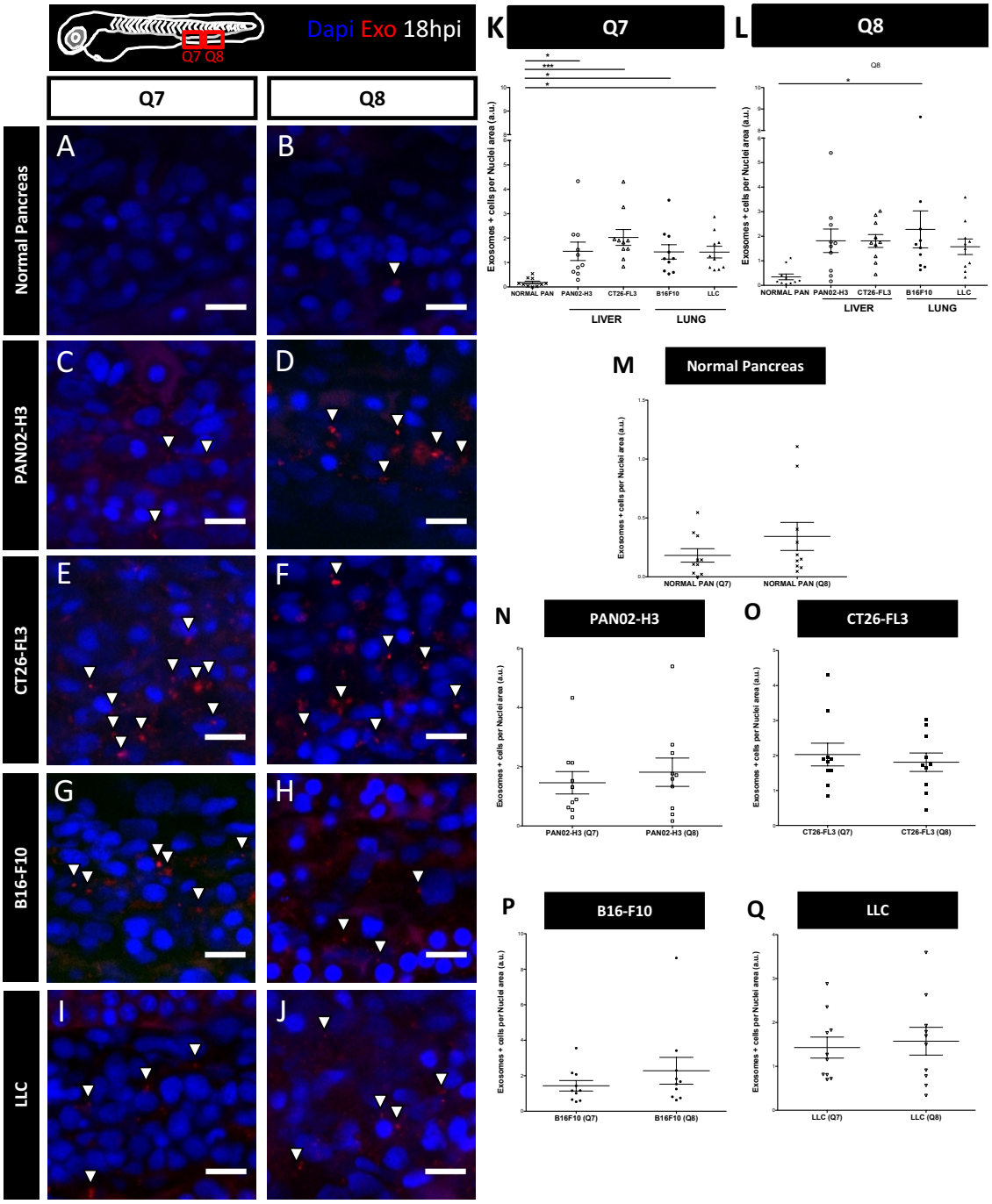


**Figure 3.5 Exosomes from Normal Pancreas, PAN02-H3, CT26-FL3 B16-F10 and LLC injected in zebrafish larvae at 48 hpf and screened at 18 hpi in the Q7 and Q8 regions.** A-B Larvae injected with exosomes isolated from normal pancreas; C-D Exosomes isolated from highly liver metastatic pancreatic cancer cell line PAN02-H3; E-F Exosomes isolated from the highly liver metastatic colorectal cancer cell line CT26-FL3; G-H Exosomes isolated from the highly lung metastatic melanoma cell line B16-F10; I-J Exosomes isolated from the highly lung metastatic lung carcinoma cell line LLC. Number of cells doing intake of these exosomes per dapi area in Q7 (K) and in Q8 (L) regions. (M-Q) Number of cells doing intake of Normal Pancreas (M), PAN02-H3 (N), CT26-FL3 (O), B16-F10 (P) and LLC (Q) exosomes per dapi area.

Scale bars correspond to 10 µm. Arrowheads are pointing exosomes aggregates.

N analysed=10 per condition; N injected and treated= 81 (PAN02-H3), 110 (B16-F10), 83 (NORMAL PAN), 117 (LLC), 99 (CT26-FL3).

***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

Although the hotspot of the tumour-derived exosomes injected was consistently the CHT, it was possible to also detect exosomes from pancreatic cancer PAN02-H3 (Fig. 3.6A-D) and melanoma B16-F10 (Fig. 3.6E-H) in additional locations in the injected zebrafish. We detected exosomes in several regions of the body but always in a lower amount than in the CHT. We observed that these exosomes, were present in different organs such as the brain, gills, liver and the CHT. Although in a very early stage of development, the tissue biology and location of this organs are already present in the larvae at 48 hpf. And, with a conservative analysis, some correspondences to the respective organ in mammalians can be done[55]. For example, although zebrafish do not have lungs, their gills serve the same functions and have a similar tissue structure. With a single epithelial layer scattered with immune cells and a layer of smooth muscle at the base of the lamella, it can be hypothesized that zebrafish gills could be used as a model to study mechanisms of the mammal's lung[56]. These are some of the organs where the exosomes PAN02-H3 and B16-F10 preferentially bind in the mouse model, so it was interesting to analyze the biodistribution in these organs of the zebrafish larvae, for the presence of exosomes.

Specifically, we found that both exosomes types could be found in the brain, which is in agreement with studies showing exosomes uptake by brain cells in zebrafish models[57]. In addition, we found that PAN02-H3 exosomes could be found in the gills and the liver (Fig. 3.6B, C). As shown in other studies, the affinity of the PAN02 exosomes is more expected in the liver, because the exosomes are highly metastatic to that site[34].

Surprisingly, we found that B16-F10 exosomes were only detected in the liver of the larvae (Fig. 3.6G) and not on the gills (Fig. 3.6F), in contrast to what was expected by the B16-F10 exosome high affinity to the lungs in murine models[33]. In spite of this apparent discrepancy of the results between zebrafish and mouse, additional experimental repetitions should be performed to further verify these findings.

Although tumour-derived exosomes did not display evident organotropism in the zebrafish larvae, at least not at this stage of development (66 hpf), and in part with an unrefined analysis in the stereoscope, our results indicate that these exosomes present a strong tropism towards the CHT in the zebrafish, which is the main hematopoietic organ at this stage of development, equivalent to the fetal liver in mice. In both these organs, at this developmental stage, the definitive wave of hematopoiesis is occurring: in the zebrafish, first in the CTH and then in the kidney and thymus (1 wpf) as in mice, first in the fetal liver and later in the bone marrow (after birth)[58]. Interestingly, findings in mouse models show that exosomes can play a key role in the crosstalk between tumour cells and bone marrow derived cells (BMDCs) and their education towards a pro-metastatic phenotype in the bone marrow niche, where these BMDCs are activated and prepared to be recruited to the PMNs[33,34]. Our results, showing a strong preference of these exosomes for the CHT, may suggest some parallel with this process of educating the cells that differentiate and expand at this transitory hematopoietic site, in the zebrafish model.

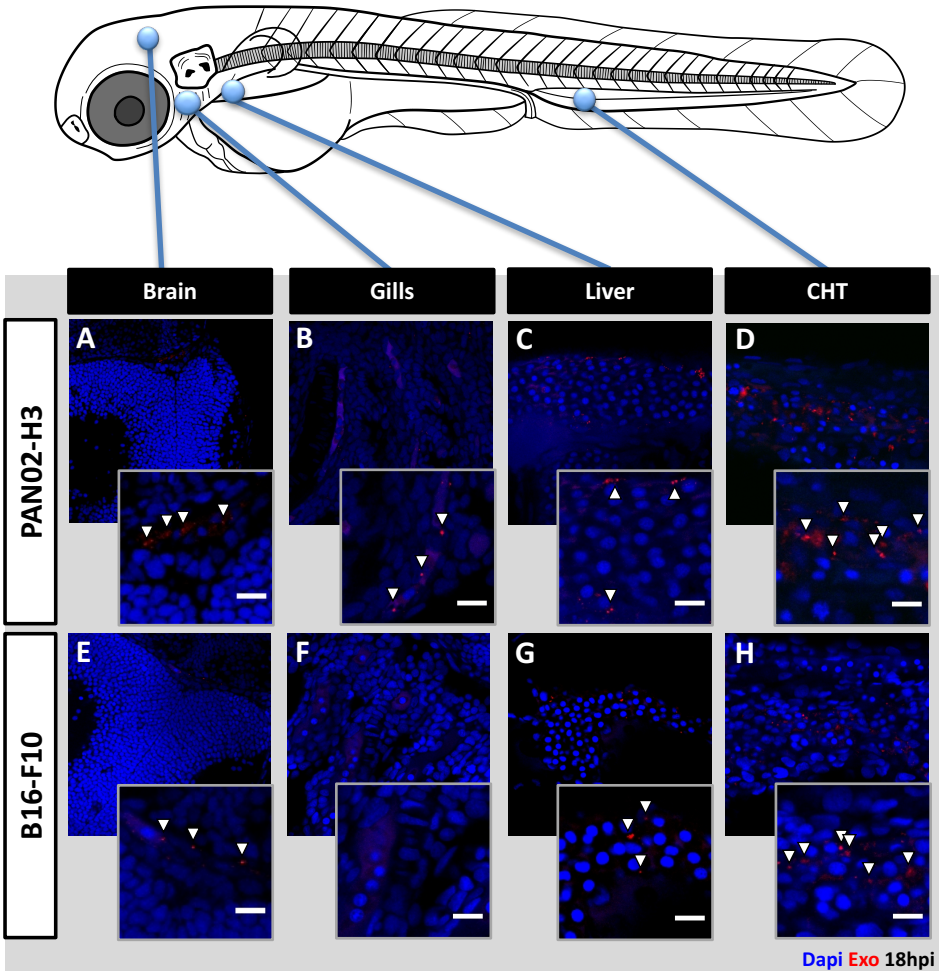


**Figure 3.6 PAN02-H3 and B16-F10 exosomes are taken up by organs other than the CHT**. A-D Zebrafish larvae injected with PAN02-H3 exosomes, brain (A), gills (B), liver (C) and CHT (D); E-H Zebrafish larvae injected with B16-F10 exosomes, brain (E), gills (F), liver (G) and CHT (H). Scale bars correspond to 10 µm. Arrowheads are pointing exosomes aggregates. N analysed>10 per condition; N injected and treated= 81 (PAN02-H3), 110 (B16-F10).

### 3.5 Standardization of doses of exosomes from patients' plasma in zebrafish larvae

Exosomes have a high potential as a non-invasive source of biomarkers for cancer. The possibility of doing tumour biopsies by utilizing exosomes from the patient's plasma, isolated from a blood sample, is gaining attention[36,37]. And the zebrafish could be a potential model for *in vivo* studies in this area of liquid biopsies, especially due to the low amount of sample required. Zebrafish larvae offer the possibility of performing *in vivo* tests faster, cheaper, with lower amount of sample, larger number of individuals and not compromising complex information such as metastatic and angiogenic potential in a vertebrate model, with organs and systems. Differently from the tests done *in vitro*, where the complexity of the system in not considered. Furthermore, the mice models can give us precious information on the interaction of tumour-derived exosomes with the host, but the amount of sample required compromises the use of this model for functional *in vivo* assays, such as those of exosomes biodistribution analysis.

To test the applicability of this model for patient-derived exosomes functional assays, we isolated

exosomes from the plasma of 3 patients with pancreatic cancer and from 3 healthy subjects. The first injections of this plasma-derived exosomes were done with 5 ng, because it was the dose previously standardized using tumour-derived exosomes from cell culture. We found that the signal with 5 ng of plasma-derived exosomes was too faint when compared with the one obtained with tumour-derived exosomes isolated from cell lines (data not shown). Therefore, we proceeded to a quantification just using one of these patients and one of the control samples, testing three different doses- 5, 10 and 30 ng (Fig. 3.7A). Our observations demonstrated that 10 ng with these plasma-derived exosomes were enough to have some small signal, while 30 ng provided a closer signal to the results obtained with exosomes obtained with 5ng of exosomes from tumour cell cultures. Considering that most of the exosomes present in the patient plasma samples are non-tumoural (about 80%)[5], it is expected that the majority of these exosomes will behave similarly to normal cells-derived exosomes, such as those from normal pancreas, and not present a high affinity to any particular tissue. Therefore, we hypothesize that although we injected a higher dose of exosomes, 30 instead of 5 ng, this sample derived from plasma contains exosomes derived from several cell types in the body other than just from tumour cells.

The site with the highest amount of signal of plasma-derived exosomes was once again, the caudal region of the zebrafish larvae, posterior to the yolk and ventrally. Therefore, we proceeded to a quantification of the exosomes' signal in this area (Fig. 3.7A). In order to perform a more detailed analysis, we proceeded with the 30 ng injected larvae and focused again in the caudal area, regions Q7 and Q8, where the signal of exosomes was higher than the rest of the larvae body. Overall the number of cells taking up exosomes was much lower than the observed with cell culture exosomes (Fig. 3.7D). Possibly due to the limited number of samples of plasma-derived exosomes here analyzed, in this preliminary data, we were not able to see significant differences between region Q7 and Q8, neither in control and patient samples (Fig. 3.7D, E). However, interestingly, we could observe that, in contrast to cell-culture- and healthy patient-derived exosomes, the majority of cells with patient-derived exosomes, appeared to have the cytoplasm full of exosomes aggregates (Fig. 3.7B-C) in the perinuclear region of the cells (Fig. 3.5).

One of the causes for this differential distribution in the cell can be due to the usage of a higher dose of exosomes than the previous experiments. Other possibility is the binding of the non-tumour cells-derived exosomes that can have a different affinity to the cells present in the zebrafish, altering their intake by these cells, their degradation and/or target inside the recipient cell. These plasma-derived exosomes appear to be present all over the cytoplasm, which can indicate not exactly a higher amount of exosomes aggregates in the cell. But possibly some differences in the distribution and processing of these exosomes, after entering the cell. In contrast with the observed in the cell culture-derived exosomes before, that appeared to accumulate in a specific compartment of the cell, in the perinuclear region. Being these last ones, possible associated with the endoplasmatic reticulum in the perinuclear region, where the aggregates were observed. Although these data are very preliminary, we were able to find the appropriate exosomes dose plus the preferential zebrafish anatomic region of their biodistribution and therefore set the ground to analyze additional patient-derived exosomes samples.

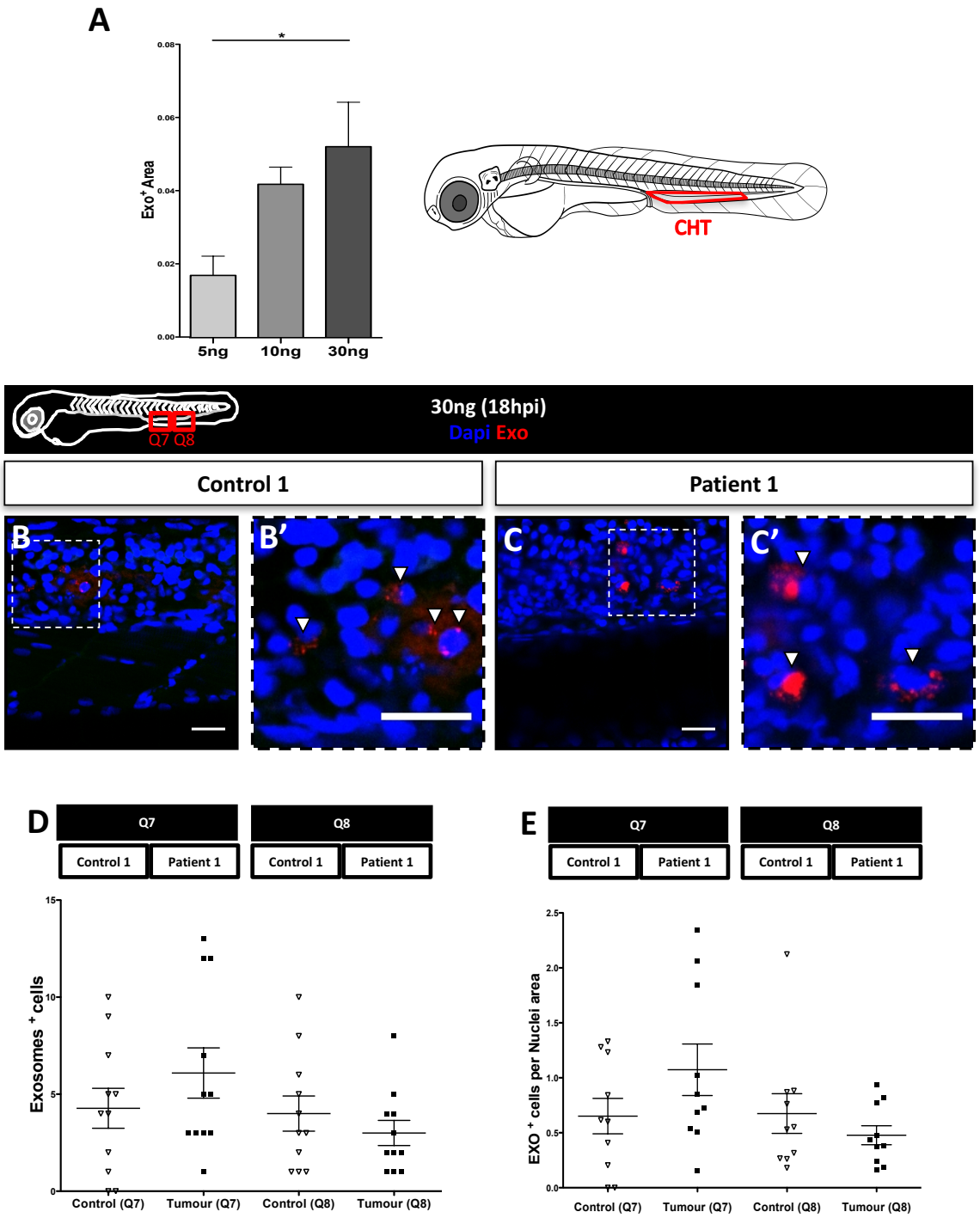


**Figure 3.7 Standardization of doses and biodistribution of exosomes isolated from plasma samples of a pancreatic cancer patient and a healthy control donor.** (A) Exosomes signal in the caudal region, with 3 different doses- 5, 10 and 30 ng from one pancreatic cancer patient plasma sample; (B-C) Cells doing intake of exosomes in the Q7 and Q8 regions of larvae injected with 30 ng of exosomes from the control (B) and the patient (C); (D) Quantification of the number of cells with exosome intake in region Q7 and Q8; (E) Proportion of cells doing intake of exosomes, per total number of nuclei (DAPI). Scale bars correspond to 20 µm. Arrowheads are pointing exosomes aggregates. N analysed=10 per condition; N injected and treated= 49 (Control), 38 (Tumour).
***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

# 4. Conclusions and future directions

Here we show that the zebrafish larvae present a promising model to perform *in vivo* assays of exosomes biodistribution. By isolating tumour-derived exosomes from tumour cell cultures, mouse pancreas in culture and patients' plasma samples, we standardized the doses of exosome injection in zebrafish. We concluded that we can dramatically reduce the amount of exosomes utilized per experiment and at the same time increase the number of individuals per assay, when compared to murine models, which demand approximately 1000 times more exosomes per injection. This is especially true for plasma-derived exosomes, where we found that 30 ng of exosomes are sufficient for our biodistribution assay. In addition, using less amount of sample *in vivo* means that we can still store exosome samples for additional analysis (such as ELISA or proteomics) or to repeat the experiments.

Overall, our more consistent results injecting exosomes in zebrafish larvae, show that the hotspot of biodistribution for these exosomes is the caudal hematopoietic tissue (CHT) of the zebrafish at 66 hpf. This does not occur when we inject non-tumour exosomes, that do not have specific affinity to any tissue of the zebrafish larvae, suggesting that the retention of tumour-derived exosomes in that region is not a mechanical process. This affinity of the exosomes to the CHT of the zebrafish may be compared to what was demonstrated in mice, where exosomes perform a pivotal role in the education and recruitment of hematopoietic progenitor cells and immune cells to support the formation of pre-metastatic niches (PMNs), thus, transforming the PMN into a more suitable environment for the development of tumours, increasing the metastatic potential to those sites.

In the near future, we will need strategies to overcome the variability of exosomes in the plasma samples. For instance, by characterizing exosomes produced from cells in the tumour, peritumoural and healthy tissue, obtained through surgeries, it would be possible to then distinguish tumour from stromal exosome in the plasma samples. Another potential alternative could be the separation of specific exosomes populations with antibody-conjugated beads or other filtration process. However, all of these technics are still in development.

More needs to be done to use the zebrafish as a model for exosomes studies, such as testing more tumour models, increasing the number of tested zebrafish individuals and understanding more about the interaction of these exosomes with the zebrafish larvae biology.

Importantly, quantifying the exosomes in the different organs, or possibly increasing the dose to analyze the organs would be interesting to do in future experiments. Moreover, a zebrafish-based *in vivo* assay to test the effect of exosomes in tumour cells could be an interesting alternative to murine models to study the biological activity of these exosomes in complex biological system.

Overall the results obtained throughout this work open the possibility to use the zebrafish model to study exosome biology in a more rapid and efficient way. Especially, if we can further develop the model to use patient–derived samples, it may represent a highly informative and applicable *in vivo* model for the study of exosome biology in the clinical setting.

# 5. References


1. Brinton, L. T., Sloane, H. S., Kester, M. & Kelly, K. A. Formation and role of exosomes in cancer. *Cell. Mol. Life Sci.* **72,** 659–671 (2015).

2. Raposo, G. & Stoorvogel, W. Extracellular vesicles: Exosomes, microvesicles, and friends. *Journal of Cell Biology* **200,** 373–383 (2013).

3. van der Pol, E., Boing, A. N., Harrison, P., Sturk, A. & Nieuwland, R. Classification, Functions, and Clinical Relevance of Extracellular Vesicles. *Pharmacol. Rev.* **64,** 676–705 (2012).

4. Cocucci, E. & Meldolesi, J. Ectosomes and exosomes: Shedding the confusion between extracellular vesicles. *Trends Cell Biol.* **25,** 364–372 (2015).

5. Whiteside, T. L. Tumor-Derived Exosomes and Their Role in Cancer Progression. *Advances in Clinical Chemistry* **4,** 103–141 (2016).

6. Ronquist, G. & Brody, I. The prostasome: its secretion and function in man. *Biochim. Biophys. Acta* **822,** 203–218 (1985).

7. Park, K.-H. *et al.* Ca2+ Signaling Tools Acquired from Prostasomes Are Required for Progesterone-Induced Sperm Motility. *Sci. Signal.* **4,** 1– 10(2011).

8. Aalberts, M. *et al.* Identification of Distinct Populations of Prostasomes That Differentially Express Prostate Stem Cell Antigen, Annexin A1, and GLIPR2 in Humans1. *Biol. Reprod.* **86,** 1–8 (2012).

9. Caby, M. P., Lankar, D., Vincendeau-Scherrer, C., Raposo, G. & Bonnerot, C. Exosomal-like vesicles are present in human blood plasma. *Int. Immunol.* **17,** 879–887 (2005).

10. Pisitkun, T., Shen, R.-F. & Knepper, M. A. Identification and proteomic profiling of exosomes in human urine. *Proc. Natl. Acad. Sci. USA* **101,** 13368–13373 (2004).

11. Ogawa, Y. *et al.* Proteomic analysis of two types of exosomes in human whole saliva. *Biol Pharm Bull* **34,** 13–23 (2011).

12. Admyre, C. *et al.* Exosomes with Immune Modulatory Features Are Present in Human Breast Milk. *J. Immunol.* **179,** 1969–1978 (2007).

13. Asea, A. *et al.* Heat shock protein-containing exosomes in mid-trimester amniotic fluids. *J. Reprod. Immunol.* **79,** 12–17 (2008).

14. Andre, F. *et al.* Malignant effusions and immunogenic tumour-derived exosomes. *Lancet* **360,** 295–305 (2002).

15. Vella, L. J. Packaging of prions into exosomes is associated with a novel pathway of PrP processing. *J. Pathol.* **211,** 582–590 (2007).

16. Masyuk, A. I. *et al.* Biliary exosomes influence cholangiocyte regulatory mechanisms and proliferation through interaction with primary cilia. *AJP Gastrointest. Liver Physiol.* **299,** 990–999 (2010).

17. Cocucci, E., Racchetti, G. & Meldolesi, J. Shedding microvesicles: artefacts no more. *Trends Cell Biol.* **19,** 43–51 (2009).

18. Ratajczak, J., Wysoczynski, M., Hayek, F., Janowska-Wieczorek, A. & Ratajczak, M. Z. Membrane-derived microvesicles: Important and underappreciated mediators of cell-to-cell communication. *Leukemia* **20,** 1487–1495 (2006).

19. Al-Nedawi, K. *et al.* Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat. Cell Biol.* **10,** 619–624 (2008).

20. Balaj, L. *et al.* Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nat. Commun.* **2,** 180–189 (2011).

21. Théry, C., Clayton, A., Amigorena, S. & Raposo, G. Isolation and Characterization of Exosomes from Cell Culture Supernatants. *Curr. Protoc. cell Biol.* **Chapter 3,** Unit 3.22.1–3.22.29 (2006).

22. Raposo, G. B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* **183,** 1161–1172 (1996).

23. Escola, J. M. *et al.* Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J. Biol. Chem.* **273,** 20121–20127 (1998).

24. Van Niel, G. Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice. *Gut* **52,** 1690–1697 (2003).

25. Wubbolts, R. *et al.* Proteomic and biochemical analyses of human B cell-derived exosomes: Potential implications for their function and multivesicular body formation. *J. Biol. Chem.* **278,** 10963–10972 (2003).

26. Szczepanski, M. J., Szajnik, M., Welsh, A., Whiteside, T. L. & Boyiadzis, M. Blast-derived microvesicles in sera from patients with acute myeloid leukemia suppress natural killer cell function via membrane-associated transforming growth factor-β1. *Haematologica* **96,** 1302–1309 (2011).

27. Kharaziha, P., Ceder, S., Li, Q. & Panaretakis, T. Tumor cell-derived exosomes: A message in a bottle. *Biochim. Biophys. Acta - Rev. Cancer* **1826,** 103–111 (2012).

28. Liu, C. *et al.* Murine Mammary Carcinoma Exosomes Promote Tumor Growth by Suppression of NK Cell Function. *J. Immunol.* **176,** 1375–1385 (2006).

29. Ostrowski, M. *et al.* Rab27a and Rab27b control different steps of the exosome secretion

pathway. *Nat. Cell Biol.* **12,** 19–30 (2010).

30. Bobrie, A. & Thery, C. Unraveling the physiological functions of exosome secretion by tumors. *Oncoimmunology* **2,** e22565 (2013).

31. Atai, N. A. *et al.* Heparin blocks transfer of extracellular vesicles between donor and recipient cells. *J. Neurooncol.* **115,** 343–351 (2013).

32. Kaplan, R. N., Riba, R. D. & Zacharoulis, S. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. **438,** 820–827 (2005).

33. Peinado, H. *et al.* Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat. Med.* **18,** 883–91 (2012).

34. Costa-Silva, B. *et al.* Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat. Cell Biol.* **17,** 816– 826 (2015).

35. Hoshino, A., Costa-Silva, B., Shen T. *et al.* Tumour exosome integrins determine organotropic metastasis. *Nature* **527,** 1–19 (2015).

36. Rolfo, C. *et al.* Liquid biopsies in lung cancer: The new ambrosia of researchers. *Biochim. Biophys. Acta - Rev. Cancer* **1846,** 539–546 (2014).

37. Santiago-Dieppa, D. R. *et al.* Extracellular vesicles as a platform for "liquid biopsy" in glioblastoma patients. *Expert Rev. Mol. Diagn.* **14,** 819–825 (2014).

38. Zhong, T. P. Zebrafish Genetics and Formation of Embryonic Vasculature. *Current Topics in Developmental Biology* **71,** 53–81 (2005).

39. Traver, D. *et al.* Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. *Nat. Immunol.* **4,** 1238–1246 (2003).

40. Murayama, E. *et al.* Tracing Hematopoietic Precursor Migration to Successive Hematopoietic Organs during Zebrafish Development. *Immunity* **25,** 963–975 (2006).

41. Kissa, K. *et al.* Live imaging of emerging hematopoietic stem cells and early thymus colonization. *Blood* **111,** 1147–1157 (2008).

42. Bertrand, J. Y., Kim, A. D., Teng, S. & Traver, D. CD41+ cmyb+ precursors colonize the zebrafish pronephros by a novel migration route to initiate adult hematopoiesis. *Development* **135,** 1853–1862 (2008).

43. Chen, A. T. & Zon, L. I. Zebrafish Blood Stem Cells. *Journal of Cellular Biochemistry* **42,** 35–42 (2009).

44. Fior, R. *et al.* Single-cell functional and chemosensitive profiling of combinatorial colorectal therapy in zebrafish xenografts. *Proc. Natl. Acad Sci. U. S. A.* **114,** E8234–E8243 (2017).

45. Nicoli, S., Ribatti, D., Cotelli, F. & Presta, M. Mammalian tumor xenografts induce neovascularization in zebrafish embryos. *Cancer Res.* **67,** 2927–2931 (2007).

46. Lee, S. L. C. *et al.* Hypoxia-induced pathological angiogenesis mediates tumor cell dissemination, invasion, and metastasis in a zebrafish tumor model. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 19485–19490 (2009).

47. Zhao, C. *et al.* A novel xenograft model in zebrafish for high-resolution investigating dynamics of neovascularization in tumors. *PLoS One* **6,** 1–9 (2011).

48. Lawson, N. D. & Weinstein, B. M. Arteries and veins: Making a difference with zebrafish. *Nat. Rev. Genet.* **3,** 674–682 (2002).

49. Hsu, K. *et al.* The pu.1 promoter drives myeloid gene expression in zebrafish. *Blood* **104,** 1291–1297 (2004).

50. Avella, M. A. *et al.* Lactobacillus rhamnosus Accelerates Zebrafish Backbone Calcification and Gonadal Differentiation through Effects on the GnRH and IGF Systems. *PLoS One* **7,** 1–10 (2012).

51. Yu, J. *et al.* Contribution of host-derived tissue factor to tumor neovascularization. *Arterioscler. Thromb. Vasc. Biol.* **28,** 1975–1981 (2008).

52. Bennett, C. M. *et al.* Myelopoiesis in the zebrafish , Danio rerio. *Blood* **98,** 643–652 (2001).

53. Ellett, F., Pase, L., Hayman, J. W., Andrianopoulos, A. & Lieschke, G. J. Mpeg1 Promoter Transgenes Direct Macrophage-Lineage Expression in Zebrafish. *Blood* **117,** 49–57 (2011).

54. Renshaw, S. & Loynes, C. A transgenic zebrafish model of neutrophilic inflammation. *Blood* **108,** 3976–3978 (2006).

55. Santoriello, C. & Zon, L. I. Hooked! Modeling human disease in zebrafish. *The Journal of Clinical Investigation* **122,** 2337–2343 (2012).

56. Progatzky, F., Cook, H. T., Lamb, J. R., Bugeon, L. & Dallman, M. J. Mucosal inflammation at the respiratory interface: a zebrafish model. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* **310,** L551–L561 (2015).

57. Yang, T. *et al.* Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in Danio rerio. *Pharm. Res.* **32,** 2003–2014 (2015).

58. Stachura, D. L. & Traver, D. Cellular dissection of zebrafish hematopoiesis. *Methods in Cell Biology* **101,** 75–110 (2016).