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Extracellular Vesicles Molecular Cargo as Cancer Biomarkers: Insights into EV-DNA Preparation and EV Protein Interactions

Julia Karolina Elzanowska

**Dissertation presented to obtain the Ph.D. degree in Molecular Biosciences,
Biology**

Oeiras, August, 2023

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA ANTÓNIO XAVIER | UNIVERSIDADE NOVA DE LISBOA

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List of Publications

The work presented in Chapter I and Chapter II of this thesis is published in peer-reviewed journals, as listed below:

Chapter I

Elzanowska, J., Berrocal, L., García-Peláez, B., Vives-Usano, M., Sebo, B. P., Maia, J., Batista, S., Teppo, J., Varjosalo, M., Moraes, M. C. S., Molina-Vila, M. Á., & Costa-Silva, B. (2022). Defining Optimal Conditions for Tumor Extracellular Vesicle DNA Extraction for Mutation Profiling. *Cancers*, *14*(13), 3258.

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J.E. designed and performed the experiments. L.B., B.G.-P. and M.V.-U. performed TaqMan and NGS analyses. B.P.S., J.M. and S.B. contributed with EV sample preparation. J.T. and M.V. performed protein mass-spectrometry processing and analysis. M.C.S.M., M.Á.M.-V. and B.C.-S. conceived the project. All authors have read and agreed to the published version of the manuscript.

Chapter II

Couto, N.*, **Elzanowska, J.***, Maia, J., Batista, S., Pereira, C. E., Beck, H. C., Carvalho, A. S., Strano Moraes, M. C., Carvalho, C., Oliveira, M., Matthiesen, R., & Costa-Silva, B. (2022). IgG⁺ Extracellular Vesicles Measure Therapeutic Response in Advanced Pancreatic Cancer. *Cells*, *11*(18), 2800.

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N.C. recruited the patients and designed and performed the experiments; J.E. designed and performed the experiments; J.M., S.B., and C.E.P. contributed to the EV sample preparation and vesicle flow cytometry analysis; H.C.B. and A.S.C. performed protein mass spectrometry processing and analysis; M.O. performed statistical analyses; M.C.S.M. supported results interpretation and presentation; C.C. co-supervised the experimental design and results interpretation; B.C.-S. and R.M. conceived and supervised the project. All authors have read and agreed to the published version of the manuscript.

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List of Abbreviations

Alix	apoptosis-linked gene 2-interacting protein X
BMDCs	bone marrow-derived cells
CAFs	cancer associated fibroblasts
CA19.9	Carbohydrate antigen 19.9
CCM	cell conditioned media
CML	chronic myeloid leukemia
cfDNA	circulating free DNA
CTCs	circulating tumor cells
CTLA-4	cytotoxic-lymphocyte antigen-4
dsDNA	double-stranded DNA
EEs	early endosomes
ESCRT	endosome sorting complexes required for transport
EGFR	epidermal growth factor receptor
ECM	extracellular matrix
EVs	extracellular vesicles
EV-DNA	DNA associated with EVs
gDNA	genomic DNA
GPC-1	glypican-1
Hsp	heat shock protein
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
HER2	human epidermal growth factor receptor-2
ICIs	immune checkpoint inhibitors
IFNγ	interferon gamma- γ
IFNγR	interferon- γ receptor (IFN γ R)
ILVs	intraluminal vesicles
JAKs	Janus kinases
LEs	late endosomes

MHC	major histocompatibility complex
MAGE B1	Melanoma associated antigen B1
mRNA	messenger RNA
miRNA	micro RNA
mtDNA	mitochondrial DNA
MVBs	multivesicular bodies
MDSCs	myeloid-derived suppressor cells
NK-92	natural killer-92
NGFR	nerve growth factor receptor
NLR	neutrophil-to-lymphocyte ratio
NGS	next-generation sequencing
NSCLC	non-small cell lung cancer
PTX	paclitaxel
PDAC	pancreatic ductal adenocarcinoma
P-gp	P-glycoprotein
PD-1	programmed death 1
PD-L1	programmed death-ligand 1
PSA	prostate specific antigen
RECIST	Response Evaluation Criteria in Solid Tumors
rRNAs	ribosomal RNAs
RBP s	RNA-binding proteins
STAT1	signal transducer and activator of transcription 1
STAM1	signal transducing adapter molecule 1
ssDNA	single-stranded DNA
SNARE	soluble N-ethylmaleimide-sensitive fusion attachment protein receptor
STING	stimulator of interferon genes
SEER	Surveillance, Epidemiology, and End Results Program
tRNAs	transfer RNAs
TNFα	tumor necrosis factor α

TNFαR	tumor necrosis factor α receptor
TSG101	tumor susceptibility gene 101
TEPs	tumor-educated platelets
UPR	unfolded protein response
Vps4	vacuolar protein sorting-associated protein 4
VEGF	vascular epidermal growth factor

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Abstract

Extracellular vesicles (EVs) are lipid bilayer-enclosed particles, released by virtually all cells, that have emerged as crucial players in both physiological and pathological processes, including cancer. EVs ability to act as mediators of intercellular communication is strongly determined by the molecular cargo they carry, which includes a variety of biomolecules like proteins, lipids, and nucleic acids, such as double-stranded DNA (dsDNA). Recent studies show that the complex molecular composition of EVs holds great potential as a source of cancer biomarkers; however, multiple barriers exist between these discoveries and the application of EVs as liquid biopsy modalities in clinical practice. This thesis aims to contribute to overcoming these limitations, focusing on the exploration of DNA and proteins in EVs and their roles as biomarkers in cancer detection, progression, and assessment of the response to treatment. The first part of the thesis explores EV-associated DNA (EV-DNA) as a future cancer biomarker for the analysis of clinically relevant mutations. We performed a comprehensive comparison of EV-DNA extraction methods based on DNA quantity, quality, and suitability for quantitative PCR and next-generation sequencing, resulting in the establishment of a workflow for EV-DNA preparation for mutation detection in cancer patients' plasma. Our findings underscore the importance of standardized protocols for EV-DNA extraction and support its potential as a promising cancer biomarker source. In the second part of this thesis, the interest shifts to proteins in EVs and their potential role as treatment response indicators in pancreatic ductal adenocarcinoma (PDAC). The protein profiles of plasma EVs from PDAC patients were characterized at diagnosis and throughout treatment. Notably, we found that the presence of IgG linked with EVs is associated with diagnosis and treatment response and can potentially serve as an additional tool for measuring therapy response in metastatic PDAC patients. We observed that IgG binds to the EV surface via a tumor neoantigen, MAGE B1, suggesting a

protein-protein interaction between EVs and circulating proteins. This discovery prompted an investigation of EV post-secretion interactions, their pathophysiological consequences and potential as cancer biomarkers. Specifically, we investigated the interaction of interferon- γ (IFN γ) with melanoma EVs carrying interferon- γ receptor 1 (IFN γ R1⁺ EVs), and its role in the modulation of IFN γ anti-tumoral signaling and cancer progression. We demonstrated that melanoma EVs bind to IFN γ via IFN γ R1 and decoy it, diminishing its antitumoral activity *in vitro*. Remarkably, the binding of IFN γ to EVs resulted in vesicular accumulation in the lungs and liver, preferential sites of melanoma metastases, suggesting their potential in establishing an immunosuppressive environment in these organs. Our results show that post-secretion protein interactions of EVs can modulate anti-tumor immune response and potentially can have a role in resistance to immunotherapy, unraveling new mechanisms of EVs activity. Overall, this thesis contributes to the understanding and future application of EV-DNA and EV proteins as liquid biopsy biomarkers and provides insights into the use of EVs as a valuable source of molecular biomarkers for cancer diagnosis, treatment response evaluation, immune response modulation, and progression. Furthermore, we highlight the importance of investigating EVs post-secretion interactions and their importance in oncological settings.

Resumo

As vesículas extracelulares (EVs) são partículas lipídicas membranares fechadas, libertadas por quase todas as células, que emergiram como mediadores cruciais em variados processos fisiológicos e patológicos, incluindo o cancro. A capacidade das EVs de agir como mediadores da comunicação intercelular é fortemente determinada pela carga molecular que carregam, que inclui uma variedade de biomoléculas como proteínas, lipídios e ácidos nucleicos, como o DNA de cadeia dupla. Estudos recentes mostram que a complexa composição molecular das EVs tem grande potencial como fonte de biomarcadores do cancro. No entanto, existem múltiplas barreiras entre essas descobertas e a aplicação das EVs como modalidades de biópsia líquida na prática clínica. Esta tese visa contribuir para superar estas limitações e concentra-se na exploração de DNA e proteínas em EVs e no seu papel como biomarcadores na deteção, progressão e monitorização da resposta ao tratamento do cancro. A primeira parte da tese explora o DNA associado a EVs (EV-DNA) como um futuro biomarcador do cancro, para análise de mutações clinicamente relevantes. Foi realizada uma comparação abrangente de métodos de extração de EV-DNA, com base na quantidade, qualidade e adequação do DNA para PCR quantitativo e next-generation sequencing (NGS), resultando no estabelecimento de um fluxo de trabalho para a preparação do EV-DNA com a finalidade de deteção de mutações no plasma de pacientes com cancro. Os nossos resultados enfatizam a importância de protocolos padronizados para a extração de EV-DNA e apoiam seu potencial como uma promissora fonte de biomarcadores do cancro. Na segunda parte desta tese, o interesse foca-se nas proteínas em EVs e no seu potencial papel como indicadores de resposta ao tratamento em adenocarcinoma ductal pancreático (PDAC). Os perfis proteicos das EVs presentes no plasma de pacientes com PDAC foram caracterizados ao diagnóstico e durante todo o tratamento. Notavelmente, os resultados

mostram que a presença de IgG ligada a EVs está associada ao diagnóstico e resposta ao tratamento e que pode potencialmente servir como uma ferramenta adicional para a previsão de resposta terapêutica em pacientes com PDAC metastático. Observamos que o IgG se liga à superfície das EVs através de um neoantígeno tumoral, MAGE B1, sugerindo uma interação proteína-proteína entre as EVs e as proteínas circulantes. Esta descoberta levou a uma investigação sobre as interações pós-secreção de EVs, as suas consequências patofisiológicas, e o seu potencial como biomarcadores do cancro. Especificamente, investigamos o papel da interação do recetor 1 do interferão- γ de EVs de melanoma (IFN γ R1+ EVs) com o interferão- γ (IFN γ) na modulação da sinalização anti-tumoral do IFN γ e na progressão do cancro. Mostramos que as EVs do melanoma se ligam a IFN γ através do IFN γ R1, diminuindo a sua atividade antitumoral nas células tumorais *in vitro*. Notavelmente, a ligação de IFN γ a EVs resultou na acumulação de EVs nos pulmões e fígado, locais preferenciais de metástases de melanoma, sugerindo o seu potencial no estabelecimento de um ambiente imunossupressor nesses órgãos. Os nossos resultados mostram que as interações proteicas pós-secreção de EVs podem modular a resposta imunológica anti-tumoral e potencialmente ter um papel na resistência à imunoterapia, desvendando novos mecanismos de atividade de EVs. No geral, esta tese contribui para a compreensão e aplicação futura de DNA e proteínas de EVs como biomarcadores de biópsia líquida para diagnóstico de cancro, avaliação de resposta ao tratamento, modulação de respostas imunes e progressão. Além disso, destacamos a importância de investigar as interações de EVs pós-secreção e a sua importância em oncologia.

General Introduction

1. Brief history of Extracellular Vesicles

Communication between cells is fundamental for multicellular organisms to ensure cell growth and function, as well as maintenance of homeostasis. Apart from direct cell interactions, cells can communicate via release of molecular messengers, including extracellular vesicles (EVs). EVs have come a long way since their discovery and are now recognized as crucial mediators of cell-cell communication in both physiological and pathological conditions, like cancer¹⁻³. The history of EVs begins in 1940s with studies on coagulation, when Chargaff and West reported the presence of platelet-free fraction with coagulatory activity after high-speed centrifugation of human plasma, which they referred to as "a variety of minute breakdown products of the blood corpuscles"³. Following this observation, twenty years later, Peter Wolf discovered that this sedimentable fraction is indeed a lipid-rich material which he described as "platelet dust", suggesting that these particles can be released from platelets into circulation, even in the absence of coagulation⁴. Later on, while studying the maturation of reticulocytes into erythrocytes, Johnstone and her group reported the formation of multivesicular structures which released those vesicles from the cell upon the fusion with plasma membrane, conceiving the term "exosomes"⁵. This process was described as a mean for the reticulocytes to expel unwanted molecules to mature into erythrocytes. Thus, at that time EVs were considered a waste disposal pathway for removing obsolete cellular components and it wasn't until 1990s that an immunological function of EV secretion was proposed. Raposo et al. showed for the first time that vesicles produced by antigen presenting cells were able to stimulate proliferation of T cells⁶. Later on, EVs secreted by dendritic cells were demonstrated to induce an antitumoral immune response *in vivo*, showing that EVs can serve as a communication tool between cells of the immune system⁷. In the following decades, multiple studies reported the shedding of vesicular structures from a variety of tissues, including tumor cells⁸⁻¹¹. Those results

sparked a growing interest in the field that persists until the present day, demonstrating that EVs act as crucial signaling vehicles able to convey a broad range of information in both physiological and pathological processes such as cell proliferation, cellular homeostasis, apoptosis, cell signaling, angiogenesis, neuronal function, pregnancy, immune response modulation, thrombosis, diabetes, autoimmune, cardiovascular and neurodegenerative diseases and cancer^{1,12-16}.

2. Biogenesis, subtypes, and nomenclature of EVs

EVs are defined as lipid bilayer-enclosed nanoparticles secreted by virtually all cells into the extracellular space¹. EVs are extremely heterogeneous and can be differentiated based upon their biogenesis, size, content, and function^{17,18}. Classification and nomenclature are constantly evolving, but generally they can be categorized into two major subtypes: exosomes and ectosomes (microvesicles and apoptotic bodies), based on their mechanism of biogenesis and size¹⁹⁻²¹. Even though multiple pathways involved in EV biogenesis have been discovered and will be briefly discussed in the following chapter, it is a highly complex and regulated process whose mechanistic details remain to be elucidated (**Fig. 1**)²².

2.1 Exosomes

Exosomes are small, 30-150 nm in diameter vesicles that originate via the endosomal pathway, which involves double invagination of the plasma membrane. The first invagination results in the formation of structures containing endocytosed molecules, which subsequently fuse with each other, forming early endosomes (EEs), which then mature into late endosomes (LEs). The inward budding of early endosomal membrane encapsulates sorted proteins, lipids, and nucleic acids generating intraluminal vesicles (ILVs), resulting in the transformation of LE into multivesicular bodies (MVBs)

containing multiple ILVs. The fate of MVBs depends on multiple factors, including their surface composition, and can be either directed for degradation via fusion with lysosomes or can fuse with the plasma membrane of cells, shedding ILVs contained within MVBs into the extracellular space as exosomes^{21,23}. The formation of ILVs in MVBs and molecular sorting is largely regulated by endosome sorting complexes required for transport (ESCRT), a multi-molecular machinery constituted of four ESCRT complexes (ESCRT-0, ESCRT-1, ESCRT-2, ESCRT-3) and associated accessory proteins that modulate its activity, including vacuolar protein sorting-associated protein 4 (Vps4) and apoptosis-linked gene 2-interacting protein X (Alix)²⁴. ESCRT complexes act sequentially in sorting and packaging of cargo and subsequent formation of ILVs²⁵. Briefly, the process starts with ESCRT-0, which recognizes and captures ubiquitinated cargo on the early endosomal membrane²⁶. This is followed by recruitment and assembly of ESCRT-1 and ESCRT-2, which initiate the invagination of the membrane and subsequent recruitment of ESCRT-3 via Alix. ESCRT-3 starts the membrane scission and formation of ILVs within MVBs. It has been shown that the depletion of specific proteins of ESCRT complexes, including hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), tumor susceptibility gene 101 (TSG101) or signal transducing adapter molecule 1 (STAM1) results in a decrease of exosome secretion and altered molecular composition of EVs, confirming that besides ILV formation, ESCRT is also involved in cargo sorting^{27,28}. More recently, a variation of ESCRT pathway - the syndecan-syntenin-Alix pathway - has been identified²⁹. However, the formation of ILVs and MVBs is more complex than originally thought and can occur in ESCRT-independent manner, which was demonstrated by Stuffers et al., where depletion of all four ESCRT complexes in cells did not abolish formation of MVBs³⁰. Examples of ESCRT-machinery independent pathways involved in exosomes biogenesis and endosomal sorting include syntenin-, ceramide- and tetraspanin-mediated mechanisms^{22,31}. Once MVBs are formed, their primary pathway involves fusion with lysosomes and degradation³².

However, MVBs have an ability to avoid degradation and instead undergo fusion with the plasma membrane, although the mechanisms which favor both pathways are still not understood^{32,33}. MVBs trafficking, docking, and plasma membrane fusion are regulated by several molecular machineries and require, among others, cytoskeletal and molecular motor proteins, Rab GTPases, and fusion machinery including the soluble N-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) and tethering factors^{34,35}. Rab GTPases, a family of small GTPases, regulate vesicle transport intracellularly, mediating the transport of MVBs and ultimately their fusion with the plasma membrane and release of exosomes³⁶. Although their precise actions in these processes remain unknown, there is a list of specific Rab proteins that were shown to play roles in different steps of exosome generation, and that includes Rab2b, Rab4, Rab5a, Rab7, Rab9a, Rab11, Rab27a, Rab27b, and Rab35. Interestingly, even though many different Rab proteins were demonstrated to play a role in exosome secretion, their involvement in this process seems to be cell type-specific^{37,38}. Once MVBs are already transported and docked at the plasma membrane, they interact with SNARE proteins and tethering factors that facilitate membrane fusion, ultimately leading to the shedding of ILVs contained in the lumen of MVBs as exosomes³⁹.

2.2 Ectosomes: microvesicles and apoptotic bodies

Differently from exosomes, larger EVs like microvesicles (100-1000 nm) and apoptotic bodies (>1 μ m), arise directly as a result of outward budding of the plasma membrane^{21,23}. Apoptotic bodies are generated by the plasma membrane blebbing of cells during the late stages of apoptosis⁴⁰. On the contrary, microvesicles are secreted from live cell and their generation involves several mechanistic elements that overlap with the exosome biogenesis pathway, including ESCRT machinery and fusion machinery, like SNAREs and tethering factors^{41,42}. However, in comparison to exosomes, microvesicles biogenesis is less well studied. What is known is that the formation of

microvesicles involves phospholipid redistribution and the fluidity of plasma membrane, molecular cargo sequestration at specific microdomains at the inner surface of plasma membrane and outward budding and fission of the membrane²¹. Also, more recently, larger macrovesicles (up to 10 μm) released by cancer cells were described and called oncosomes⁴³.

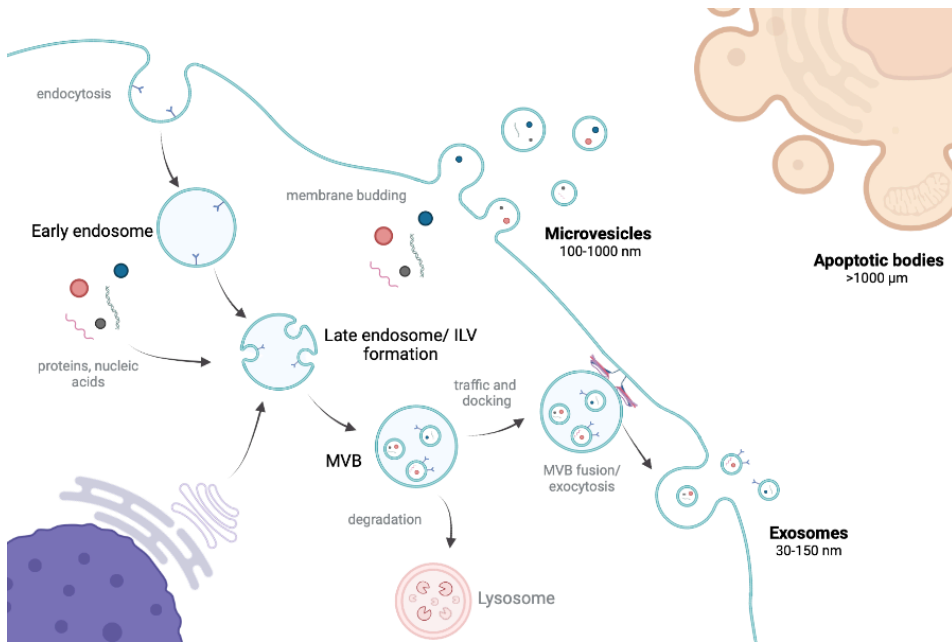


Figure 1. Schematic representation of EV biogenesis and secretion. EVs are produced via a variety of different processes. Exosomes biogenesis starts with endocytosis to form early endosomes, which undergo membrane invagination and generation of intraluminal vesicles (ILVs), resulting in multivesicular bodies (MVBs) formation. MVBs dock at the inner side of the plasma membrane, fuse with it and release ILVs as exosomes into the extracellular space. Microvesicles are generated via direct budding of plasma membrane, while apoptotic bodies are a result of blebbing of cells undergoing apoptosis. Figure created with BioRender.com and adapted from Raposo et al ²³.

2.3 Nomenclature and guidelines

The nomenclature of EVs is constantly evolving due to their high heterogeneity and several limitations regarding their isolation and characterization methods. Over the years, there were different attempts to classify EVs based on their size or markers of their origin. However, despite distinct mechanisms of biogenesis of EVs, the similarities in the overlapping range sizes, and variable molecular compositions of exosomes and microvesicles made it very challenging. Taking together with the fact that the majority of current protocols for EV isolation do not discriminate them based on their origin and cannot provide pure preparations of specific subtypes of EVs, the current guidelines recommend using "EVs" as a generic term for all lipid-bilayer enclosed particles naturally released by cells^{20,44}.

3. Molecular composition of EVs and cargo selection

EVs role as molecular messengers in regulating cell-cell communication lies in their capacity to carry and transfer heterogeneous molecular cargo. Depending on the cell of origin and its metabolic and disease state, the sort and abundance of EVs molecular signature can vary and can be constituted of different biomolecules, including proteins, nucleic acids, and lipids (**Fig. 2**)^{1,13,45–47}. As virtually all cells release EVs, once in circulation these particles together with their diverse constituents can convey signaling locally, at the site of secretion, and also travel and affect distant tissues. The complex cargo of EVs directly affects the fate and biological response they elicit in recipient cells; thus, cargo nature and mechanism of loading into vesicles has been a huge area of interest in recent years. The molecular constituents of EVs resemble that of their cells of origin, as their composition is mostly determined at the stage of their biogenesis via selective cargo sorting⁴⁷. Briefly, sorting of EV cargo starts with an increase in specific cargo concentration at the sites of exosomes and microvesicles formation, which are limiting membrane of MVB

and plasma membrane, respectively²¹. Subsequently, EV cargo is clustered on these membranes which in turn undergo budding, resulting in the release of vesicles. This pathway was reported to be involved in capturing both transmembrane and cytosolic cargoes, however in the latter case the mechanism is less understood²⁶. Cargo can also be loaded into EVs in a passive manner as a result of nonselective sampling, although the extent to which this takes place and how it occurs is not well defined²⁷.

3.1 Proteins

The first ever discovered EV cargo was transferrin receptor, and until now the protein composition of EVs have been studied the most extensively, yielding an extensive list of EV-associated proteins, including plasma membrane, cytosolic, heat shock, cytoskeletal, and nuclear proteins⁴⁸⁻⁵⁰. A considerable proportion of proteins in EVs are so-called pan-EV markers, common for the majority of vesicles regardless of their cell of origin. These include proteins involved in their biogenesis and cargo sorting, such as tetraspanins, Rab GTPases, major histocompatibility complex (MHC) molecules, stress proteins, ESCRT machinery proteins and their accessory proteins, like Alix or TSG101⁵¹. Tetraspanins (such as CD9, CD63 and CD81) are a family of integral membrane surface proteins, which play roles in not only EV formation but also sorting of other proteins into EVs. Initially, those tetraspanins were considered as markers of exosomes, however further studies showed that they can also be incorporated into microvesicles⁵². Other proteins commonly identified in EVs include heat shock proteins (Hsp) 70 and 90 (Hsp90 and Hsp70) and proteins that associate with lipid rafts (flotillin)⁵¹. Besides those, EVs contain subsets of proteins specific for the cell type that secretes them, including proteins related to the pathological state of the cell. As previously mentioned, sorting of transmembrane cargo into EVs is mostly associated with endosomal sorting machineries during their biogenesis, including ESCRT-mediated sorting of ubiquitinated proteins into ILVs^{46,47,53}. However other mechanisms can exist

that allow clustering and packaging of various proteins to vesicles, including post-translational modifications or tetraspanin-enriched microdomains⁵⁴⁻⁵⁶.

3.2 RNA

In the late 2000s a major breakthrough in the EV field was made, when Valadi et al. reported that EVs can carry nucleic acids, namely messenger RNA (mRNA) and micro RNA (miRNA)⁵⁷. This work demonstrated that not only do EVs contain RNAs, but that this transferred RNA can be translated to proteins in the recipient cell, including tumor cells^{2,58,59}. Along with mRNAs and miRNAs, a variety of other RNA species contained in EVs has been identified, including ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), long noncoding RNAs and circular RNAs, but their functions have not been as thoroughly explored⁶⁰. Interestingly, when looking at RNAs in EVs, many of them were found to be considerably enriched in the vesicles relative to the cell of origin, suggesting selective incorporation of RNAs into EVs^{2,46,57,59}. Indeed, several reports showed that mRNAs and miRNAs form complexes with RNA-binding proteins (RBPs) which serve as a mechanism for selective sorting of specific RNAs into EVs, based on the presence of a specific RNA motif that RBPs bind to⁶¹⁻⁶³. Once released, lipid bilayer of EVs protects the RNA present within its lumen from degradation, allowing its delivery to the recipient cell. However, the contribution of both active and passive packaging of RNAs into EVs remains largely unknown^{64,65}.

3.3 DNA

Apart from RNA, different species of DNA, including single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), mitochondrial DNA (mtDNA) and even viral DNA, have been identified to be associated with EVs⁶⁶⁻⁷³. Moreover, Thakur et al. demonstrated the presence of genomic DNA in cancer cell-derived EVs⁷². Interestingly, DNA seems to be present both on the surface as well as

within the lumen of EVs^{72,74–76}. At first, the presence of EV-DNA was assumed to be the result of apoptotic bodies secretion, but in the last years multiple studies showed its presence in exosomes and microvesicles, supporting the idea of DNA secretion in EVs as an active process. Additionally, Lazaro-Ibanez et al. showed that different subtypes of EVs carry different genomic DNA (gDNA) content, suggesting selective DNA packing⁷⁷. The detailed mechanisms of how DNA localizes in the cytoplasm at the sites of EVs biogenesis in order to be packaged into vesicles remain to be revealed, however some explanations behind this phenomenon are emerging. Takahashi et al. reported that DNA secretion in EVs could be a mechanism for clearing of cytosolic DNA to maintain cellular homeostasis by excreting damaged DNA in EVs⁷⁸. Additionally, EV release was inversely correlated with apoptosis, supporting the fact that DNA packaging into EVs is not just a result of cell death. Another proposed mechanism of DNA loading into EVs involves formation of micronuclei, small nuclei-like structures that bud of the nuclear membrane as a consequence of DNA missegregation or DNA damage⁷⁹. In this way, nuclear DNA fragments are released into cytoplasm where they were shown to colocalize with CD63-positive ILVs within MVBs. Reis-Sobreiro et al. showed that tumor cells with induced nuclear shape instability show an increased secretion of EVs containing genomic material⁸⁰. Those few reports correlate DNA nuclear instability with increased DNA packaging into EVs, which may explain why the majority of reports describe EV-DNA in tumor-derived EVs. A more detailed information on possible mechanisms of DNA loading into EVs, and its biological and clinical significance in physiology and pathologies was covered in our recently published review⁸¹.

3.4 Lipids

Other molecules than proteins and nucleic acids are commonly present in EVs, including lipids. Their composition in EVs has been less studied, even though it also impacts the bioactivity of EVs. Vesicles were shown to be enriched in a

variety of different lipids, including cholesterol, phosphatidylserine, glycosphingolipids, and ceramides, which account for the structure and stability of the vesicular membrane^{82,83}. Additionally, lipolytic enzymes that break down triglycerides into lower bioactive lipids have been reported in EVs. Regarding the lipid sorting into EVs, not much has been described, even though a specific lipid loading mechanism has been suggested^{84,85}.

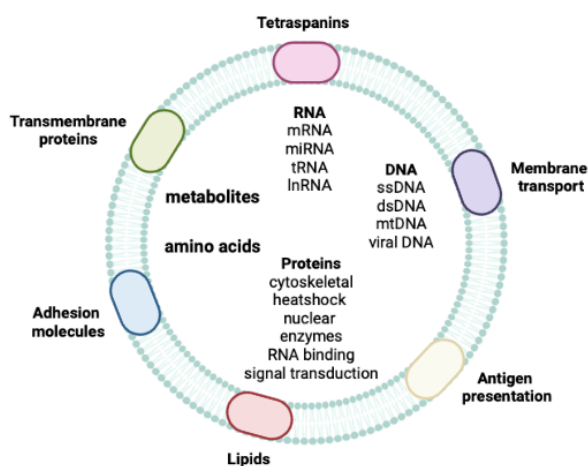


Figure 2. Schematic representation of EV molecular composition. EVs carry a variety of biomolecules, including proteins, lipids, RNA, DNA, amino acids, and metabolites. Figure created with BioRender.com and adapted from Kalluri et al⁴.

4. Mechanisms of EV action and signal delivery

Following their secretion, in order for EVs to act as mediators of intercellular communication and transfer information, they need to interact with recipient cells and deliver their molecular contents to evoke functional responses. Although not fully elucidated, the mechanisms behind EVs targeting, interactions and internalization are briefly covered in the next paragraphs. Those interactions depend on origins and molecular composition of EVs as well as identity of recipient cell⁸⁵. Moreover, more recently it was shown that EVs

function also by interacting with soluble molecules in the extracellular milieu (Fig. 3).

4.1 Interactions of EVs with recipient cells

Once released, EVs can act on the secreting cell itself, on adjacent cells, or they can travel to distant sites in various biofluids. The mechanism of EV recognition and uptake is yet to be fully understood. Some reports describe it as unspecific and stochastic process, while others present evidence that EVs can be selectively targeted to specific cells based on their surface composition and its interaction with different molecules at the plasma membrane of recipient cells^{86–89}. The mechanism behind target cell specificity is still not fully elucidated, but several players involved in it have been identified, including molecules like surface receptors, tetraspanins, integrins, lipids, proteoglycans, and other extracellular matrix (ECM) components^{87,90}. One of the examples of targeted specificity was shown between EVs and target cells of the same origin, where the cellular signature shared between both allowed for the uptake of EVs^{91,92}. Interaction between integrins and ECM proteins was shown to have an important impact on binding of different subtypes of EVs to the recipient cells^{93,94}. In terms of EVs released by tumor cells, Hoshino et al. discovered that the presence of specific integrin patterns on the surface of EVs mediate their tropism to either lungs or liver and thus promote pre-metastatic niche formation and determine organotrophic metastasis^{21,95,96}. Once EVs reach the recipient cell, in order to mediate the intercellular signaling, EVs can either remain at the plasma membrane and interact with it or undergo internalization via different pathways. The fate of EVs after they bind to the target cells is influenced by their molecular composition and presence of specific proteins in the plasma membrane^{21,96}. During the direct interaction, surface vesicular molecules interact with the extracellular receptors of target cells and trigger downstream signaling without the discharge of their cargo. In case of EV internalization, one of the mechanisms is based on membrane fusion between

EVs and plasma membrane, which results in the direct release of EV cargo into the cytosol of the target cells. Other internalization mechanisms include clathrin-mediated endocytosis, macropinocytosis, phagocytosis, or internalization via lipid rafts and caveolae⁹⁷⁻¹⁰⁰. All these pathways target the internalized EVs into endosomal pathway, where they either fuse with the endosomal membrane or end up in lysosomes targeted for degradation. Importantly, all those described mechanisms of EV internalization can coexist and are dependent on various factors, including the protein composition of EVs and their size, as well as the state of the recipient cell^{85,101}.

4.2 Signals delivered by EVs to recipient cells

Once EVs reach the target cells, their molecular content is delivered, which in turn elicits a functional response, ultimately modulating the status of the cell. As described in the previous paragraphs, their signaling capacity depends on the origin and complex molecular architecture of EVs, as well as the mode of vesicle interaction with the cell and its intracellular fate. Different mechanisms of interaction and uptake by the recipient cell also affect the outcome of those interactions. A simple interaction between surface molecules of EVs and proteins of plasma membrane may trigger downstream signaling, subsequently having a functional impact on the target cell. This mechanism of EV action is very common in immune modulation and was first shown in early discoveries of EV roles in intercellular communication, where EVs derived from dendritic and B cells activated T cells via antigen presentation^{6,7,102}. On the other hand, EVs can serve as vehicles for intercellular transfer of molecules. The fusion of EVs with plasma membrane, or with endosomal membrane after their internalization, releases vesicular intraluminal content into the cytoplasm, which can result in inhibition or activation of various processes. For example, protein cargo of EVs can be processed as antigens and used in antigen presentation to stimulate immune response¹⁰³. This mechanism is also key for delivery of genetic material, including miRNAs and mRNAs, that in

turn can regulate gene expression in the recipient cell^{57,59}. Other molecules, including signaling molecules such as cytokines, chemokines and functional receptors, can be delivered to the recipient cell, resulting in the regulation of cellular signaling pathways, including modulation of immune response and cancer growth and metastasis^{16,43,104,105}.

4.3 Interactions of EVs with secreted molecules

Surface composition of EVs is extremely important for their binding and uptake by the recipient cells, and the biological effect they exert. However, before that occurs, secreted EVs, with their highly interactive surface, are present in the circulation. Thus, they can interact with soluble molecules in extracellular milieu, representing a different and less investigated mechanism of EV action. Numerous molecules have been described to associate with EV surface, including immunoglobulins, albumins, extracellular matrix proteins, complement proteins, coagulation factors, chemokines and cytokines, DNA molecules and various enzymes^{13,106}. Those interactions between EVs and soluble molecules can have an effect in the activity and distribution of those EV-bound molecules^{107–109}. Post-secretion interactions of EVs with proteins of the extracellular milieu have been shown to modulate their immune recognition, mobility, uptake and signaling capabilities^{106–112}. For instance, EVs were reported to carry a full-length tumor necrosis factor α receptor (TNF α R) on their surface that can bind tumor necrosis factor α (TNF α). This interaction in Schwann cell-derived EVs carrying TNF α receptor was shown to decrease the availability of TNF α modulating its activity, thus regulating neuroinflammation^{108,113}. However, the details and functions of those interactions remain to be elucidated.

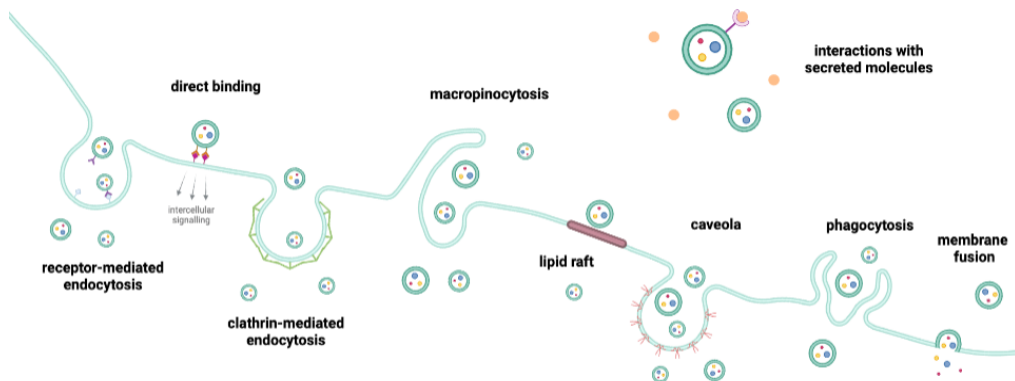


Figure 3. Mechanisms of EV action. Once EVs reach the target cells, they can stay at the plasma membrane and trigger intercellular signaling by a simple interaction between surface molecules of EVs and proteins of plasma membrane. EVs can also fuse with plasma membrane and discharge their cargo to the cytosol or be internalized via various mechanisms including receptor-mediated endocytosis, clathrin-mediated endocytosis, macropinocytosis, phagocytosis or via lipid rafts or cavolae. Figure created with BioRender.com and adapted from Kalluri et al ⁴.

5. Biological functions of EVs

The nature and abundance of EVs molecular cargo determines their biological roles and the effect they exert on the recipient cell. As crucial mediators of intercellular communication, EVs play diverse roles in physiological and pathological processes (**Fig. 4**). While the majority of research has focused on the roles of EVs in pathologies, there are multiple reports demonstrating the functions that EVs play in a range of physiological processes, including cellular homeostasis, innate and acquired immunity, reproductive biology, development, and tissue repair^{114–116}. EVs derived from both immune and non-immune cells were shown to mediate both stimulation and suppression of immune response¹¹⁷. As EVs can carry both surface MHC class I (MHC I) and MHC class II (MHC II) molecules, they can act in antigen presentation, directly stimulating both CD8+ and CD4+ T cells^{117,118}. Another way in which EVs can activate immunity is by transfer of signals, including cytokines, which promote

the acceptor cells into immunogenic. On the other hand, EVs can also display immunosuppressive abilities that support homeostasis, for example by preventing autoimmunity^{119,120}. Interestingly, also in the subject of cellular homeostasis, DNA packaging into EVs was suggested to contribute to this process. Despite little being known about EV-DNA functions, Takahashi et al. suggested that cells may accumulate damaged DNA in EVs in order to avoid an aberrant activation of the immune system, thus maintaining homeostasis^{78,114,115}. Whilst there are still many unknowns about EVs functions in the healthy body, the majority of current understanding about EVs comes from exploring their signaling in disease states. EVs have been shown to be involved in a wide range of pathologies, including autoimmune, cardiovascular, metabolic, and neurodegenerative diseases, as well cancer growth, progression and metastasis^{114,115}.

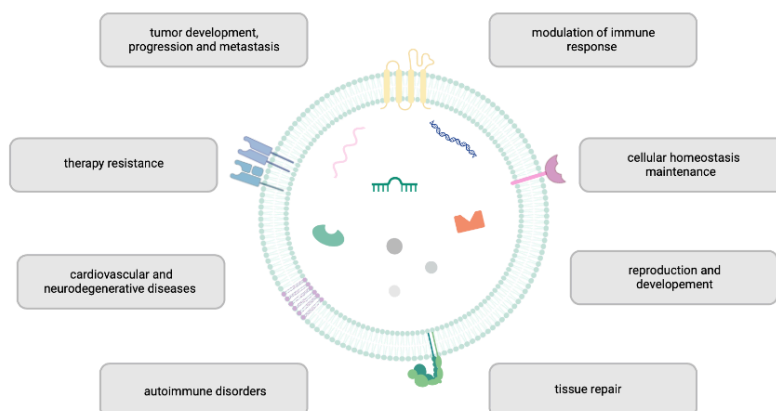


Figure 4. Biological functions of EVs. EVs and the complex molecular cargo they carry play crucial roles in various physiological and pathological processes. Figure created with BioRender.com.

5.1 EV roles in cancer

Cancer is far more than an isolated entity; it represents a complex systemic network involving constant bidirectional cell-cell communication among tumor cells and a variety of non-cancerous cells, including cells of the tumor microenvironment, like epithelial cells, fibroblasts, endothelial cells, and immune cells¹²¹. Over the past years, multiple studies have proven that EVs are crucial players in the intercellular communication network of tumor development, progression, and metastasis^{14-16,122,123}. Generally, cancer cells show an increased secretion of EVs in relation to healthy cells, and the secretion of EVs itself was linked to the enhanced survival of the tumor cells where they originated ¹²⁴. Additionally, biomolecular composition of EVs have also been shown to be altered in cancer. In recent years, other EV roles in the promotion of tumor growth have been explored, such as the transfer of oncogenic molecules, the modulation of the immune system, the regulation of tumor-stroma crosstalk, the promotion of angiogenesis, and the conditioning of future metastatic sites.

5.1.1 Modulation of cell phenotype by transfer of oncogenic molecules

One of the ways in which EVs are implicated in those processes is their ability to transfer oncogenic molecules between cancer and non-malignant cells^{59,125-127}. There is a significant number of studies demonstrating that tumor-derived EVs can serve as vehicles for the transfer of oncogenic proteins, miRNAs, fatty acids or growth factors, which results in the alteration of recipient cells towards an aggressive phenotype, promoting tumor growth and progression. While studying how a highly oncogenic variant of epidermal growth factor receptor (EGFR), EGFRvIII, can promote brain tumor progression, Al-Nedawi showed that glioma cells actively release EGFRvIII-enriched EVs that merge with cancer cells lacking the receptor, leading to the horizontal transfer of EGFRvIII and its transforming phenotype⁴³. Similarly, another group showed that colon cancer

cells derived EVs enriched in Δ Np73 mRNA can transfer the mRNA of this oncoprotein to cancer cells that do not express it, resulting in induced proliferation and drug resistance in the acceptor cells¹²⁸. Moreover, Δ Np73 containing EVs were also shown to be taken up by stromal cells, leading to increased proliferation and reduced apoptosis. Besides mRNA, breast cancer and prostate cancer cell-derived EVs support neoplasia via miRNA transfer^{129,130}. Furthermore, Antonyak et al. demonstrated that breast cancer and glioma-derived EVs induce transformation of normal fibroblasts and epithelial cells by transferring tissue transglutaminase and fibronectin¹³¹. More recently, Hinzman et al. showed that pancreatic cancer-derived EVs enriched in molecules promoting endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) induced these stress response pathways in normal pancreatic epithelial cells upon EV internalization, potentially playing a role in disseminating proliferative and invasive characteristics¹³². Interesting observations were also reported by Cai et al, where they showed that tumor-derived EVs can transfer genomic DNA with pathophysiological significance⁶⁶. EV-mediated transfer of BCR/ABL hybrid gene, involved in the pathogenesis of chronic myeloid leukemia (CML), resulted in the development of CML in immunodeficient NOD-SCID mice^{67,133}. Together with other published data, these examples highlight the importance of EVs in promoting tumor growth of various types of cancer.

5.1.2 Regulation of tumor-stroma crosstalk

EVs are also key in regulating interactions between tumor and their neighboring stromal cells, a mechanism responsible for the alteration of tumor microenvironment, driving cancer progression¹³³. Multiple groups showed that cancer-derived EVs can transform normal fibroblasts into cancer associated fibroblasts (CAFs) and promote tumor growth and invasion¹³⁴⁻¹³⁸. A study on lung metastasis of liver cancer showed that high metastatic hepatocellular carcinoma cells secrete EVs that target and convert fibroblasts into CAFs¹³⁹.

These transformed fibroblasts then showed increased production of pro-inflammatory cytokines, further promoting tumor progression. Naito et al. also showed an increased potential of highly metastatic cancer cells to transform normal fibroblasts into CAFs via EVs, and that those EVs stimulate secretion of chemokines¹⁴⁰. Interestingly, activation of those chemokines correlated with poorer survival of gastric cancer patients. Another key aspect of the implication of EVs in cancer progression is their role in communication with endothelial cells to induce angiogenesis and vascular permeability, both crucial for the continuous tumor growth and metastasis development^{141,142}. For instance, Al Nedawi et al. showed that cancer-derived EVs transfer EGFR to recipient cells, where it stimulates production of vascular epidermal growth factor (VEGF), inducer of angiogenesis and lymphangiogenesis, and subsequent blood vessel formation¹⁴³. In breast cancer, EVs were shown to activate VEGF receptors and thus stimulate angiogenesis via a unique form of VEGF¹⁴⁴.

5.1.3 Pre-metastatic niche formation

In terms of the regulation of tumor microenvironment, EVs have been shown to modulate the sites of future metastasis by supporting the formation of the pre-metastatic niche, defined as an environment conducive to engraftment and growth of metastatic cells¹⁴⁵⁻¹⁴⁷. Peinado et al. showed that melanoma-derived EVs can reprogram bone marrow-derived cells (BMDCs) toward a pro-vasculogenic and pro-metastatic phenotype via upregulation of the MET oncoprotein, thus promoting pre-metastatic niche formation¹⁴². In another case, melanoma EVs were shown to condition sentinel lymph nodes towards formation of a niche facilitating lymphatic metastasis formation¹⁴⁸. More recently, melanoma EVs enriched in nerve growth factor receptor (NGFR) were shown to be taken up by lymphatic endothelial cells where they induced lymphangiogenesis, reinforcing lymph node pre-metastatic niche formation¹⁰⁴. Interestingly, histological analysis of human melanoma samples showed an increased NGFR expression in metastatic lymph nodes correlated with lower

survival of the patients. In pancreatic cancer, Costa-Silva et al. reported a detailed description of liver pre-metastatic niche formation by pancreatic cancer-derived EVs, where those EVs are selectively taken up by Kupffer cells of the liver, which results in fibronectin accumulation and subsequent macrophage recruitment, leading to the establishment of a pre-metastatic environment in the liver¹⁴⁷. As well as inducing angiogenesis, EVs are also capable of destroying vascular endothelial barriers, the natural barriers against metastasis^{149,150}. Tominaga et al. showed that brain metastatic cancer cell-derived EVs can trigger the breakdown of the blood-brain barrier, and that systemic injections of these EVs promoted formation of breast cancer metastasis in the brain¹⁵⁰. In addition, EVs have been proposed as mediators of organotropic metastasis. Hoshino et al. demonstrated that expression of distinct sets of integrins on the surface of tumor-derived EVs leads to their accumulation at specific sites of future metastasis and to the preparation of pre-metastatic niche⁹⁵. To prove that, the group used two clones of an aggressive breast cancer cell line (MDA-MB-231) which had different preferable metastasis sites, namely lung and bones. Mice treatment with EVs from lung-tropic cells followed by injection of bone-tropic tumor cells was enough to redirect metastasis of the originally bone-tropic tumor to the lung. Proteomic analysis of those EVs led to the discovery that specific EV integrins are responsible for organotropic metastasis. Specifically, the expression of ITG $\alpha_v\beta_5$ dictates binding of cancer EVs to Kupffer cells in the liver, while ITG $\alpha_6\beta_4$ and ITG $\alpha_6\beta_1$ mediate lung tropism by binding to lung fibroblasts and epithelial cells.

5.1.4 Modulation of immune response

Another way in which EVs can support tumor growth, pre-metastatic niche development and future metastasis is through the suppression of the immune system. One of the ways for EVs to mediate suppression of the immune response includes inhibition of T cell response via inhibiting T cell and NK-cell

activation or promoting Tregs expansion and myeloid-derived suppressor cells (MDSCs) generation¹⁵¹⁻¹⁵³. Tumor cells have a way of evading immune response by expressing programmed death-ligand 1 (PD-L1), which binds to its receptor programmed death 1 (PD-1) on T cells and results in their deactivation. An alternative mechanism was proposed when the presence of PD-L1 was detected on the surface of EVs. Chen et al. showed that metastatic melanoma-derived EVs carrying PD-L1 suppress the activity of effector T cells, facilitating tumor growth¹¹². This mechanism of immune evasion via direct EV-mediated T cell inhibition was also shown in other cancers, including glioblastoma^{154,155}. As mentioned above, EVs can also suppress the immune response indirectly, by promoting expansion of Tregs and induction of MDSCs, both known for their ability to inhibit activation of T cells^{156,157}. More recently, it has been shown that EVs can exert their immunosuppressive activity through post-secretion interactions with soluble molecules, namely with tumor-reactive antibodies^{110,111}. Tumor-derived EVs were shown to efficiently bind and decoy tumor-reactive antibodies, reducing their binding to tumor cells and subsequently diminishing their cytotoxic activity. Capello et al. showed that PDAC-derived EVs can bind to IgGs in patients' plasma and that those EVs have tumor-associated antigens on their surface that are targets of autoantibodies¹¹⁰. Moreover, those EVs displayed a decoy function by sequestering these antibodies, resulting in the attenuation of complement-mediated tumor cell cytotoxicity. Taken together, these results present a new mechanism by which tumor EVs can modulate immune response, attenuating cytotoxicity against cancer cells and aiding tumor cells to escape from immune surveillance. Even though tumor EVs have been shown to have a predominantly immunosuppressive role, under specific circumstances EVs can display immunostimulatory functions, including activation of T cells by transfer of MHCs to DCs¹⁵⁸. Likewise, Kitai et al. showed the immunostimulatory effect of EV-DNA¹⁵⁹. Treatment of breast cancer cells with an anti-tumor agent resulted in the release of EVs loaded with DNA, which activated dendritic cells

via stimulator of interferon genes (STING) pathway, a cytosolic DNA-sensing pathway, reinforcing anti-tumor immunity. Furthermore, activated T cells were shown to secrete DNA-containing EVs.

5.1.5 Cancer therapy resistance

EVs have also been linked with chemoresistance and other cancer therapies resistance via various mechanisms, including direct drug efflux and inactivation, drug sequestration, and delivery of functional proteins and nucleic acids that promote resistance¹⁶⁰⁻¹⁶³. In breast cancer, EVs have been shown to contribute to resistance to all types of available treatments: chemotherapy, hormonal therapy, targeted therapy, as well as immunotherapy¹⁶⁴. For example, treatment of an aggressive breast cancer cell line MDA-MB-231 with paclitaxel (PTX), a commonly used chemotherapeutic agent, results in increased secretion of EVs that are highly enriched in survivin, promoting enhanced cell survival. Survivin is a cell survival protein linked with poor prognosis, as well as resistance to chemotherapy, in cancer^{165,166}. Treatment of both fibroblasts and breast cancer cells with survivin-enriched EVs derived from PTX-treated breast cancer cells resulted in significant promotion of survival and offered a protective effect from PTX, promoting chemoresistance¹⁶¹. Another study in breast cancer showed that CAF-derived EVs can transfer mtDNA to hormonal therapy-induced dormant cells, which results in their escape from metabolic quiescence and endocrine therapy resistance¹⁶⁷. As mentioned in the previous paragraph, the EV-mediated transfer of immunosuppressive molecules, including PD-L1, can directly inhibit the T-cell response, mediating the resistance to immune checkpoints immunotherapy^{168,169}. Chen et al. showed that PD-L1 expressing EVs decoy anti-PD-L1 antibody (α PD-L1) and that EV-bound α PD-L1 is rapidly cleared by macrophages, resulting in a decreased blockade of tumor PD-L1 and subsequent resistance to α PD-L1¹⁶⁸. Similarly, in the case of acute myeloid leukemia (AML), plasma-derived EVs were shown to mediate resistance to

natural killer-92 (NK-92) cellular immunotherapy¹⁷⁰. AML-derived EVs interfered with NK-92 anti-leukemia functions by delivering inhibitory signals and subsequently reprogramming NK-92 cells, resulting in the suppression of treatment. EVs were also explored in the anti-VEGF therapy resistance, where Ma et al. showed that this treatment results in the increased packaging of VEGF into EVs by ovarian cancer cells, a process mediated by CD63 tetraspanin¹⁶³. Moreover, EV-associated VEGF was not accessible to the anti-VEGF antibodies and resulted in increased angiogenesis and tumor growth.

6. EVs in liquid biopsy

Standard solid biopsy is still considered a gold standard for disease diagnosis and monitoring, regardless of its several important limitations, including invasiveness or inability to capture disease heterogeneity. In recent years, the concept of liquid biopsy has emerged, with a potential to overcome these limitations, offering minimally invasive methods for early disease detection and monitoring based on a variety of different biosources found in body fluids. While circulating tumor cells (CTCs) and circulating free DNA (cfDNA) remain the most researched molecules as liquid biopsies for cancer, other biosources have been gaining a lot of attention in this field, including tumor-educated platelets (TEPs) and EVs. Over the past years, EVs have been found to be present in a variety of body fluids, including blood, saliva, urine, cerebrospinal fluid, breast milk, semen, pleural effusion, amniotic fluid and ascites fluid^{1,171}. Their ubiquitous production by virtually all cells, abundance in body fluids and ability to carry heterogeneous molecular cargo mirroring the state of the cell of origin makes them very promising candidates for liquid biopsy biomarkers. Moreover, the lipid bilayer of EVs provides high stability for encapsulated biomolecules, facilitating their extraction and analysis. Over the recent years, EVs role as a liquid biopsy source has been extensively explored in cancer, but

also in other pathologies, including pregnancy disorders or cardiovascular diseases^{172,173}.

6.1 Cancer liquid biopsy

As described, EVs are now recognized as crucial players in numerous processes involved in tumor growth, progression, and metastasis, thus holding a great potential as future markers for cancer diagnosis, prognosis and assessment of treatment regimens efficacy^{95,122,142,147}. EVs provide valuable information on the presence and molecular characteristics of the tumor, which could facilitate the early diagnosis and characterization of the disease. There has been a significant progress on how the molecular cargo of EVs, namely proteins, RNA and DNA, could be potentially used for cancer detection and monitoring of patients^{59,122,174,175}. While EV-associated miRNAs gained the majority of interest in the recent years and remain one of the best explored EV biomarkers, other EV cargo, including proteins and DNA, offers unique insights into vital information on cancer detection and monitoring. Thus, the next paragraphs will focus on discoveries supporting the potential roles of EV proteins and EV-DNA as emerging liquid biopsy biomarkers¹⁷⁶⁻¹⁷⁸.

6.1.1 Proteins

As it is widely accepted that tumor-derived EV proteins have crucial functional roles in cancer development and progression, there are multiple studies investigating their roles as biomarkers in cancer diagnosis, prognosis and therapy response¹⁷⁹. The presence of particular proteins in EVs, which are commonly associated with specific cancer types, can be used as a biomarker for cancer diagnosis. Melo et al. showed that levels of glypican-1 (GPC-1)-positive EVs in serum of PDAC patients allowed for distinguishing healthy individuals and patients with benign pancreatic diseases from early- and late-stage cancer patients¹⁸⁰. Similarly, in the case of colorectal cancer, elevated

plasma levels of GPC-1 EVs in patient samples allowed for identification of early-stage patients¹⁸¹. Indeed, there are several studies suggesting that the combination of GPC-1 EVs together with C19.9 (pancreatic cancer biomarker) could be potentially used as a biomarker for pancreatic cancer diagnosis^{182,183}. In another example, the levels of plasma EV-associated survivin were significantly increased in plasma of breast cancer and prostate cancer patients versus plasma of healthy controls^{184,185}. Moreover, a multimarker study on more than 400 plasma samples of lung cancer patients and 150 controls, using an EV array equipped with 49 antibodies to capture the vesicles, revealed that three markers CD151, CD171, and tetraspanin 8 were the most significant separators of patients with cancer of all histological subtypes versus cancer-free patients¹⁸⁶. Another group showed that the analysis of the proteomic profile of EVs from more than 400 human samples, including tissue and plasma, revealed a panel of tumor type specific EV proteins which allows for the classification of tumors of unknown primary origin^{142,187}. Interestingly, besides discovering novel EV biomarkers, the expression of clinically validated markers, like prostate specific antigen (PSA) in prostate cancer, has been detected in EVs and suggested to have a diagnostic value¹⁸⁸. Apart from their potential application in cancer diagnosis, EV proteins can also be used for predicting prognosis, as well as for monitoring cancer progression and treatment response. For instance, in the above-mentioned study of Melo et al., the levels of GPC-1 EVs were shown to correlate with tumor burden and the overall survival (OS) of PDAC patients. EVs have also been proven useful in the prediction of metastasis. Costa-Silva et al. showed that PDAC-derived EVs highly enriched in migration inhibitory factor (MIF) prime the liver for metastasis via the establishment of a pre-metastatic niche¹⁴⁷. Moreover, the levels of MIF in EVs from PDAC patients who later developed metastasis were significantly higher than of those who did not progress. Similarly, EVs from a highly metastatic melanoma cell line were shown to promote PMN formation by educating bone marrow (BM) progenitor cells toward a pro-metastatic and

pro-vasculogenic phenotype via transfer of the oncoprotein MET, and BM progenitors from individuals with metastatic melanoma express high MET receptor levels¹⁴². Additionally, the authors identified a signature of EV proteins, including MET from patients with advanced melanoma, which could have a potential use in assessment of metastasis. Hoshino et al. identified specific integrin expression patterns on EV surface that are associated with organ specific metastasis to livers and lungs, suggesting that EV integrins could be used for organ-specific metastasis prediction⁹⁵. Also, several studies in different cancer types showed that PD-L1 EVs were associated with tumor progression and could predict negative prognosis¹⁸⁹⁻¹⁹¹. One of the mechanisms of developing drug resistance by cancer cells is EV-mediated transfer of therapy resistant proteins, and these EV proteins can be used as predictive markers of therapy resistance. Loading of P-glycoprotein (P-gp) into EVs by chemotherapy resistant cells and its transfer to sensitive cells was shown to conduce the development of chemoresistance. This effect was demonstrated in a variety of different cancer types, suggesting the potential of P-gp-positive EVs as a predictive marker of chemotherapy response¹⁹²⁻¹⁹⁵. Moreover, as mentioned in previous paragraphs, EVs with their highly interactive surface can interact with other cells and soluble proteins present in the circulation, and these EV protein interactions can be used to assess and monitor the response to cancer treatment. In breast cancer, human epidermal growth factor receptor-2 (HER2)-overexpressing breast cancer-derived EVs were shown to interfere with the anti-proliferative activity of Trastuzumab by efficiently binding to this anti-tumor antibody and decoying it¹⁹⁶. Moreover, when comparing HER2 EVs from plasma of early and advanced stages of breast cancer, the latter demonstrated higher Trastuzumab-binding capabilities, potentially contributing to therapy resistance and explaining why metastatic patients do not respond to Trastuzumab treatment. Apart from their role as prognostic markers, PD-L1 carrying EVs have gained important interest as potential predictors of anti PD-1/PD-L1 immune checkpoint inhibitor (ICI)

therapy resistance. Chen et al. showed that interferon gamma- γ (IFN γ) upregulates PD-L1-positive EV secretion, which in turn reduces the effectiveness of anti-PD-L1 immunotherapy in melanoma patients¹⁶⁹. Furthermore, EV-PD-L1 levels allowed for stratification of patients into those responding and non-responding to anti-PD-L1 treatment, supporting the application of EV-PD-L1 as a predictor of immunotherapy response. Additionally, when compared to PD-L1 expression in tumor biopsies, EV-PD-L1 levels were presented to be more reliable when predicting ICI response and clinical outcome in melanoma patients. All of those reports present a great potential of EV proteins as cancer biomarkers, although their applicability is still limited by the discovery and validation of accurate biomarkers¹⁹⁷.

6.1.2 EV-DNA

Several groups reported that EVs can contain gDNA and that this DNA reflects the entire genome of parental cells, including their mutational state^{70,72,76,104,198,199}. Thakur et al. was one of the first ones to demonstrate that cancer cell line derived EVs carry dsDNA and that this DNA reflects the mutations present in parental cells, including *EGFR* and *BRAF* mutations⁷². In parallel, another group validated the presence of dsDNA genomic DNA in EVs from cell lines, as well as plasma of PDAC patients^{70,200}. Kahlert et al. saw that those plasma derived EVs can be used for detection of *KRAS* and *p53* mutations, genes most frequently mutated in PDAC²⁰⁰. Following the discovery that EVs can carry cancer-specific mutations, there has been an increase in the number of reports supporting clinical significance of EV-DNA as a tool for cancer diagnosis and prognosis, as well as for detecting the emergence of therapy resistance mutations²⁰¹⁻²⁰³. For example, in PDAC, which is usually detected already in advanced stages due to diagnostic difficulties, a high frequency of *KRAS* mutation was found in EVs from early-stage pancreatic cancer patients²⁰¹. Yang et al. showed that the analysis of EV-DNA allows for identification of *KRAS* and *TP53* mutations and that it could potentially

differentiate PDAC patients from healthy controls²⁰². Another group reported that a longitudinal analysis of plasma EV-DNA of PDAC patients and the dynamics of KRAS mutation detection can be used for monitoring disease progression and that a mutation frequency >5% was related with poorer clinical outcomes²⁰⁴. Moreover, longitudinal analysis of KRAS mutation status in EVs allowed for prediction of outcome in patients with metastatic colorectal cancer²⁰⁵. Similarly, in lung cancer, analysis of plasma EVs allowed for detection of EGFR T790M mutation²⁰⁶. In a follow up study, Castellanos-Rizaldos et al. further confirmed that EV-DNA analysis in plasma of non-small cell lung cancer (NSCLC) allows for the detection of both activating and resistance EGFR mutations, potentially aiding prediction of EGFR inhibitor-based therapy response²⁰⁷. Moreover, in the case of melanoma, the analysis of EV-DNA from lymphatic drainage allowed for *BRAF*^{V600E} detection, correlating with melanoma progression²⁰⁸. Importantly, before the discovery of EV-DNA, cfDNA emerged as a promising nucleic acid source for liquid biopsy approaches. However, EV-DNA proposes several advantages over cfDNA. EVs (apart from apoptotic bodies) are released by living cells, thus EV-DNA is more representative of a viable tumor than cfDNA, which is released by cells already undergoing apoptosis or necrosis. Remarkably, EVs can carry dsDNA fragments of >10kbp, while cfDNA is highly fragmented and has a fragment size range of 60-100bp²⁰⁹. Moreover, the mutation detection sensitivity and specificity are higher for EV-DNA than cfDNA^{201,210,211}. Interestingly, one study that has yet to be validated demonstrated that a large proportion of circulating DNA is associated with EVs⁶⁹. However, the future implementation of EV-DNA into an everyday use in the clinics faces several challenges. Although collectively these results strongly support the potential use of EV-DNA as a liquid biopsy biomarker, additional studies with larger cohorts together with standardized protocols for EV-DNA extraction and quality control are essential.

Outline of the Thesis

Apart from their established roles as intercellular communication mediators, EVs and their complex molecular constituents are emerging as very promising candidates for liquid biopsy biomarkers in cancer diagnosis, prognosis, and assessment of therapy response. Despite a significant number of studies supporting the translational value of EV-DNA and EV protein, several hurdles still exist between those discoveries and the implementation of EVs as a liquid biopsy modality in clinical practice, including lack of standardization, as well as lack of biomarker discovery, and validation. This thesis aimed to further explore the capacity of EVs molecular cargo, namely EV-DNA and EV proteins, as potential liquid biopsy biomarkers and provide new insights into their significance in cancer detection, progression and assessment of therapy response. This thesis is organized in three chapters that cover research aims as follows:

- **Chapter I** addresses EV-DNA and its potential role as a future cancer biomarker source. As the study of EV-DNA is still in its infancy, there are several challenges for its future implementation in the clinical practice. This includes lack of standardization of DNA preparation for mutational analyses, with different reports applying different detection and extraction methods, both having a huge impact on their suitability for downstream analyses and making interpretation and comparison of the findings challenging. In this chapter, we aimed to contribute to overcoming some of those challenges by performing a comprehensive comparison of EV-DNA extraction methods and defining strategic points to consider for EV-DNA preparation for mutational analyses, including detection of clinically relevant mutations in cancer patients.

- In **Chapter II**, the potential of EV proteins as a liquid biopsy source to determine treatment response in PDAC patients is explored. PDAC diagnosis

usually occurs at very advanced disease stages, with very limited therapeutic options, thus it is essential to have improved treatment response indicators. However, the majority of EV protein studies in PDAC focus on their putative diagnostic and prognostic roles and are based on single point analyses of EV cargo, failing to observe their molecular changes during the course of the disease and its treatment. Thus, in this chapter, by characterizing EVs from PDAC patient samples throughout their treatment, we investigated the role of plasma EV proteins as an instrumental tool for therapy response evaluation in metastatic PDAC patients.

- In **Chapter III** we explored the potential of tumor EVs post-secretion protein interactions in cancer progression. Although the majority of EV research focuses on their role as a tool for horizontal transfer of molecules, recent studies show that EVs can serve as a scaffold for interactions with a variety of molecules with both pro- and anti-tumorigenic roles. However, the pathophysiological consequences of those interactions' reactions remain unclear. To contribute to the understanding of this unappreciated mechanism of EVs signaling, we investigated the role of melanoma EVs interaction with IFN γ in the modulation of IFN γ signaling and cancer progression in melanoma.

CHAPTER I

Defining optimal conditions to tumor Extracellular Vesicle DNA extraction for mutation profiling

The content of the present chapter consists of a peer-reviewed article published in the context of this thesis:

Elzanowska, J., Berrocal, L., García-Peláez, B., Vives-Usano, M., Sebo, B. P., Maia, J., Batista, S., Teppo, J., Varjosalo, M., Moraes, M. C. S., Molina-Vila, M. Á., & Costa-Silva, B. (2022). Defining Optimal Conditions for Tumor Extracellular Vesicle DNA Extraction for Mutation Profiling. *Cancers*, 14(13), 3258.

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Author contributions:

J.E. designed and performed the experiments. L.B., B.G.-P. and M.V.-U. performed TaqMan and NGS analyses. B.P.S., J.M. and S.B. contributed with EV sample preparation. J.T. and M.V. performed protein mass-spectrometry processing and analysis. M.C.S.M., M.Á.M.-V. and B.C.-S. conceived the project. All authors have read and agreed to the published version of the manuscript.

Simple Summary: We here performed a comprehensive comparison of commonly used approaches for EV-DNA extraction, by assessing DNA quantity, its quality, and its suitability for downstream analyses. We found that the tested methods resulted in different DNA yields and fragment sizes. As a consequence, we found that different EV-DNA extraction methods had an impact on the detection of specific mutations by qPCR and on the suitability of next-generation sequencing (NGS), which could impact downstream clinical applications.

Abstract: (1) Background: Extracellular vesicles (EVs) have emerged as crucial players in the communication between cells in both physiological and pathological scenarios. The functions of EVs are strongly determined by their molecular content, which includes all bioactive molecules, such as proteins, lipids, RNA, and, as more recently described, double-stranded DNA. It has been shown that in oncological settings DNA associated with EVs (EV-DNA) is representative of the genome of parental cells and that it reflects the mutational status of the tumor, gaining much attention as a promising source of biomarker mutant DNA. However, one of the challenges in studies of EV-DNA is the lack of standardization of protocols for the DNA extraction from EVs, as well as ways to assess quality control, which hinders its future implementation in clinics. (2) Methods: We performed a comprehensive comparison of commonly used approaches for EV-DNA extraction by assessing DNA quantity, quality, and suitability for downstream analyses. (3) Results: We here established strategic points to consider for EV-DNA preparation for mutational analyses, including qPCR and NGS. (4) Conclusions: We put in place a workflow that can be applied for the detection of clinically relevant mutations in the EV-DNA of cancer patients.

Keywords: extracellular vesicles (EVs), DNA, mutation profiling, liquid biopsy

1. Introduction

Extracellular vesicles (EVs) are lipid-bilayer-enclosed particles released in the extracellular milieu by virtually all cells [1]. In recent years, EVs have emerged as crucial mediators of cell–cell communication, acting as messengers in both physiological and pathological conditions [2,3]. The functional roles of EVs are strictly related to their molecular composition, which has been shown to be highly heterogeneous and to consist of a variety of biomolecules, including proteins, lipids, metabolites, and nucleic acids [3,4]. The discovery of DNA associated with EVs (EV-DNA) sparked an interest in its biological and clinical functions, and in recent years, multiple roles of EV-DNA have been explored in terms of intercellular communication, immune response modulation, and maintenance of cellular homeostasis [5,6,7,8,9,10]. Moreover, the ubiquitous presence of EVs in different body fluids makes them strong candidates for nucleic-acid biomarkers in liquid biopsies. Indeed, EVs have lately attracted attention as a potential source of biomarker DNA in various pathophysiological scenarios, especially in oncological contexts [11,12]. Several studies have shown promising results, supporting the idea that EV-DNA represents the entire genome of parental cells and that its analysis allows specific gene mutations, including clinically relevant alterations, to be detected [11,12,13,14]. These studies have indicated that EV-DNA is suitable for liquid biopsies, both for early diagnosis and for disease prognosis.

To date, various studies have addressed the standardization of RNA extraction from EVs (EV-RNA) and have provided key steps to be considered for the EV-RNA preparation for downstream analyses such as RNA-Seq [15,16]. These studies have shown that different RNA purification methods can indeed result in differences in RNA yield and quality. However, comprehensive comparisons of EV-DNA preparation for downstream approaches are still missing. In fact, the implementation of EV-DNA as a clinical biomarker is still halted by the lack

of comprehensive studies on the technical aspects of different strategies for EV-DNA isolation, characterization, and quality control. The literature describes multiple EV-DNA extraction approaches that have been applied (**Table 1**). As DNA mutational analyses can be directly affected by DNA quantity and quality, in order to assure reproducibility as well as high confidence in the results, it is crucial that a standardized workflow for EV-DNA extraction and quality control is developed. Yet, thus far, multiple non-standardized EV-DNA extraction approaches have been employed (**Table 1**).

Table 1. Summary of methods used for EV-DNA extraction in the literature.

Extraction Method	Manufacturer	Number of Times Used in the Literature	Reference
DNeasy Blood & Tissue Kit	Qiagen	3	[7,10,11]
QIAamp DNA Micro Kit	Qiagen	2	[14,17]
Phenol–Chloroform	N/A	2	[5,6]
QIAamp Circulating Nucleic Acid Kit	Qiagen	1	[18]
Qiagen Allprep DNA/RNA Mini Kit	Qiagen	1	[19]
QIAamp DNA Mini Kit	Qiagen	1	[12]
SeleCTEV Low Input DNA Enrichment Kit	Exosomics	1	[20]
TIANamp Genomic DNA Kit	Tiangen Biotech Co. Ltd.	1	[21]
Quick-gDNA Miniprep Kit	Zymo Research	1	[22]
Genomic DNA Mini Kit	Geneaid	1	[23]
XCF Exosomal DNA Isolation Kit	System Biosciences	1	[24]
Isopropyl alcohol precipitation	N/A	1	[25]
Proteinase K	Wako	1	[8]

In this study, we established strategic points for EV-DNA preparation for mutational analyses. This included DNA extraction from EVs, its quantification and quality analysis, and the assessment of its suitability for downstream applications, including qPCR and next-generation sequencing (NGS). By using four different commercial kits, as well as a phenol–chloroform-based protocol, DNA was extracted from EVs isolated from the cell-conditioned media (CCMs) of a panel of cancer cell lines. Extracted EV-DNA was subjected to a comprehensive comparison of the methods, with a specific focus on their suitability for biomarker DNA analyses. Moreover, we present evidence about the utility of the established protocol to detect clinically relevant mutations in

plasma samples of cancer patients. To our knowledge, this is the first study to compare the impact of the EV-DNA isolation protocol on sample quantity, quality, and suitability for mutational analyses.

2. Materials and Methods

2.1 Preparation of conditioned cell culture media – cell lines and cell culture

All cell lines were obtained from the American Type Culture Collection. Human lung cancer cell lines H1975, H1650, and HCC827 and human pancreatic cancer cell line PANC10.05 were maintained in RPMI 1640 (Corning). Human breast cancer cell line MDA-MB-231 (purchased from Memorial Sloan Kettering Cancer Center) and human colorectal cancer cell line SW480 were grown in DMEM (Corning). LoVo, a human colorectal cancer cell line, was cultured in F12-K (Corning), and human pancreatic cancer cell line CFPAC-1 was cultured in IMDM (Gibco). All the above-mentioned media were supplemented with 1% penicillin–streptomycin (Gibco 15-140-122; Waltham, MA, United States) and 10% fetal bovine serum (FBS) (Biowest S181BH-500; Nuaille, France), except for PANC10.05, which was grown in medium supplemented with 15% FBS (Biowest S181BH-500; Nuaille, France) and 10 U/mL insulin (Gibco). Cells were maintained at 37 °C at 5% CO₂ levels. For conditioning, cells were cultured in the designated media supplemented with 1% penicillin–streptomycin and 10% EV-depleted FBS [26]. For the preparation of conditioned culture media (CCMs), cells were seeded in 150 mm culture dishes containing 20 mL of EV-depleted medium and grown for 72 h until they reached a confluency of ~90%. The number of cells seeded for each cell line at the initiation of culture is presented in **Supplementary Table 1**. Non-conditioned medium containing 10% EV-depleted FBS was used as a control. After conditioning, CCMs and the non-conditioned medium were immediately submitted to two centrifugation steps (500× g for 10 min and 3000× g for 20

min, both at 10 °C) to remove dead cells and large debris; then, they were either directly used for EV isolation or stored at –80 °C until use. The cell lines used as positive and negative controls for qPCR are presented in **Supplementary Table 2**.

2.2 Plasma samples and ethics statement

To obtain plasma, blood samples were collected from patients during daytime in sterile Vacutainer tubes (BD) containing EDTA. Blood was directly subjected to 2 consecutive centrifugation steps (500 g, 10 min) and visually inspected for obvious signs of hemolysis. Plasma samples were then aliquoted and frozen at –80 °C. The clinical characteristics of patients and technical aspects of sample collection are summarized in **Supplementary Tables 3 and 4**, respectively.

The study was carried out in accordance with the principles of the Declaration of Helsinki under an approved protocol of Dexeus Hospital Ethical Committee (52/2018 from 22 November 2018). This research study was approved by Research Ethics Committee of Quiron Salud Hospital Group in Barcelona on 25/10/2018 (ethic code: 51/2018). Informed consent was obtained from all patients; samples were deidentified for patient confidentiality.

2.3 Extracellular vesicle isolation and characterization

2.3.1 EV isolation by ultracentrifugation

All EV samples were isolated by differential ultracentrifugation combined with a sucrose cushion, as previously described [27]. For each analyzed cancer cell line, at least 3 independent batches of EVs from separately grown cultures were isolated. First, the CCM was centrifuged at 12,000× g for 20 min to remove larger EVs, including apoptotic bodies. The supernatant (CCM and plasma) was then centrifuged at 100,000× g for 2 h 20 min. The pellet was

resuspended in 16 mL of filtered phosphate-buffered saline (PBS; Corning 15313581; New York, NY, USA) and added to the top of a 4 mL sucrose solution (D2O containing 1.2 g of protease-free sucrose and 96 mg of Tris base adjusted to pH 7.4). Next, centrifugation at 100,000× g for 1 h 10 min was performed; then, a 4 mL fraction was collected using a 18 G needle and mixed with 16 mL of filtered PBS. After overnight centrifugation at 100,000× g, the pellet of purified EVs was resuspended in filtered PBS. All used solutions were filtered using 0.22 µm filters. All centrifugation steps were performed at 10 °C using 45Ti or 70Ti rotors (Beckman-Coulter, Brea, CA, USA).

2.3.2 Nanoparticle Tracking Analysis (NTA)

To determine particle concentration and size distribution, all EV samples were analyzed by NTA using NanoSight NS300 (Malvern Instruments, Malvern, UK) with a red laser (638 nm). Samples were diluted with filtered PBS to obtain concentrations within the optimal range for NTA analyses. The videos were recorded using a camera level of 16 and a threshold of 5-7 and further analyzed with NTA software v3.4 (Malvern Instruments, Malvern, UK).

2.3.3 Protein Quantification

Protein concentration in EV samples was assessed using BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.3.4 Western blotting

Western blotting was used to assess the presence of EV and non-EV protein markers. Equal protein amounts of EV samples were mixed with 4X Laemmli buffer (Bio-Rad), denatured for 5 min at 95 °C, and loaded onto 4–20% Mini-PROTEAN TGX Stain-Free Protein Gels (Bio-Rad). SDS-PAGE was run for 1.5 h at 90 V; then, proteins were transferred to nitrocellulose membranes (Cytiva)

at 100 V for 1 h. Membranes were blocked with LI-COR Intercept Blocking Buffer (LI-COR Biosciences, Bad Homburg, Germany) for 1 h at RT. Blocked membranes were incubated overnight at 4 °C with primary antibodies diluted in LI-COR blocking buffer with 0.1% Tween-20. Membranes were washed with TBS-T (TBS with 0.1% Tween-20) three times for 5 min and then incubated with secondary antibodies for 1 h at RT. Incubation was followed by three additional washes with TBS-T of 5 min each. Blots were imaged using the Odyssey Infrared Imaging System (LI-COR Biosciences). The detailed list of primary and secondary antibodies used is provided in **Supplementary Table 5**.

2.3.5 Transmission Electron Microscopy

The samples were analyzed by negative staining. In brief, the samples were fixed with 2% Formaldehyde in Phosphate Buffer for 5 min. Fixed samples were incubated for 5 min on glow-discharged, formvar-coated, carbon-coated 200-mesh copper grids prior to contrasting with 2% aqueous Uranyl Acetate for 5 min. Imaging was performed on a Tecnai G2 Spirit BioTWIN transmission electron microscope equipped with an Olympus-SIS Veleta CCD camera.

2.4 Mass spectrometry

Liquid chromatography–mass spectrometry (LC-MS) was used to assess the presence of EV and non-EV protein markers. To extract and solubilize the proteins, 9 mol/L urea (Sigma-Aldrich) was added to the final concentration of 8 mol/L, and the samples were sonicated for 15 min. Insoluble debris was removed by two 15 min rounds of centrifugation at 21,460× g at RT. To dilute the samples' urea concentration to 1.5 mol/L, 50 mmol/L ammonium bicarbonate (Fluka) was added. Proteins were reduced and carbamidomethylated with dithiothreitol (Sigma-Aldrich; 5 mmol/L final concentration, 60 min at 37 °C) and iodoacetamide (Sigma-Aldrich; 15 mmol/L final concentration, 30 min at RT in the dark), respectively, followed

by overnight digestion using 1 µg of sequencing-grade modified trypsin (Promega). For C18 purification, 10% trifluoroacetic acid (VWR) was added to the samples to a final concentration of 0.5% and acetonitrile (Merck) to the final concentration of 1%. C18 purification was performed with MicroSpin columns (The Nest Group, Inc.) as previously described [28]. Finally, the samples were vacuum-centrifuged to dryness and stored at -80 °C until LC-MS analyses.

LC-MS analyses were carried out with EASY-nLC 1000 coupled to an Orbitrap Elite mass spectrometer (both from Thermo) using LC-MS-grade solvents (water (VWR); acetonitrile (Merck)) and acids (VWR). The peptides were resolubilized in eluent A (1% acetonitrile + 0.1% formic acid + 0.01% trifluoroacetic acid in water), and 4 µg of total digested protein was injected. Thermo Acclaim PepMap C18 columns were used for separation at a flow rate of 300 nL/min (trap column: PepMap 100, 75 µm × 2 cm, 3 µm, 100; analytical column: PepMap RSLC, 75 µm × 15 cm, 2 µm, 100 Å). The run time was 140 min, 120 min of which were spent gradienting from 5% to 35% eluent B (98% acetonitrile + 0.1% formic acid + 0.01% trifluoroacetic acid in water). Nanospray voltage was 2.9 kV. MS detection was performed with Top20 data-dependent acquisition, where the 20 most intense ions from each MS1 full scan were isolated, fragmented, and analyzed in the ion trap. Then, 30 s dynamic exclusion was applied. The LC-MS parameters have been previously described in detail [28]. MaxQuant/Andromeda version 1.6.1.0 with standard settings was used for protein identification and label-free quantification. The database was a UniProt/Swiss-Prot reviewed human proteome. A false discovery rate of 1% was used on peptide and protein levels. Decoy hits, potential contaminants, and proteins identified only by modified peptides were removed, and LFQ intensities were used for estimating protein abundance.

2.5 DNA extraction

Total DNA associated with EVs was extracted from previously isolated EVs (differential ultracentrifugation) using different DNA isolation methods: QIAamp DNA Mini Kit (Qiagen), QIAamp DNA Micro Kit (Qiagen), SeleCTEV Exosomal DNA Kit (Exosomics), XCF Exosomal DNA Isolation Kit (SBI), and phenol–chloroform extraction. DNeasy Blood & Tissue Kit (Qiagen), which is the most commonly applied approach for EV-DNA extraction in the literature (**Table 1**), was not included in this comparison as it is based on the same technology and used for the same purposes as the QIAamp DNA mini kit (Qiagen), which is our in-house DNA extraction protocol. In order to extract DNA using the SeleCTEV Exosomal DNA kit (Exosomics), we used only the part of the protocol dedicated to DNA purification. For DNA extraction using all methods, each independent EV batch was split, and equal protein amounts of EVs were used for each extraction method (**Fig. 5**). For commercial kits, extraction was performed as per the manufacturer's instructions. For phenol–chloroform extraction, EVs were first mixed with 400 μ L of lysis buffer (0.05 M EDTA, 0.01 M Tris-HCl (pH 8.0), 0.1 M NaCl, 10% SDS, and 200 μ g/mL proteinase K) and incubated for 1 h at 56 °C. Secondly, deproteinization was performed by the addition of an equal volume of phenol–chloroform–isoamyl alcohol mixture (25:24:1), followed by vortexing and centrifugation at 16,000 $\times$ g for 5 min at RT. Next, the upper aqueous phase was separated from the organic phase, mixed with an equal volume of chloroform, and then mixed and centrifuged again at 16,000 $\times$ g for 5 min at RT. The upper phase containing DNA was carefully collected and mixed with one-tenth volume of 3 M sodium acetate (pH 5.2), glycogen (to a final concentration of 0.1 μ g/mL), and 2.5 volumes of cold absolute ethanol. The mixture was incubated overnight at –20 °C and then centrifuged at 16,000 $\times$ g for 30 min at 4 °C. Next, the supernatant was discarded, and the pellet was rinsed with 70% ethanol and centrifuged at 16,000 $\times$ g for 15 min at 4 °C. Finally, the supernatant was removed, and the pellet was left to air dry for 5 min and then

resuspended in 50 μ L of Mili-Q water. For the extraction of genomic DNA from cell lines, either the QIAamp DNA mini kit (Qiagen) or the QIASymphony DSP Virus/Pathogen midi kit (Qiagen) was used, and the extraction was performed as per the manufacturer's instructions. For the extraction of DNA from plasma-derived EVs, the SeleCTEV Exosomal DNA kit (Exosomics) was used according to the manufacturer's instructions. All EV-DNA samples were stored at -20°C .

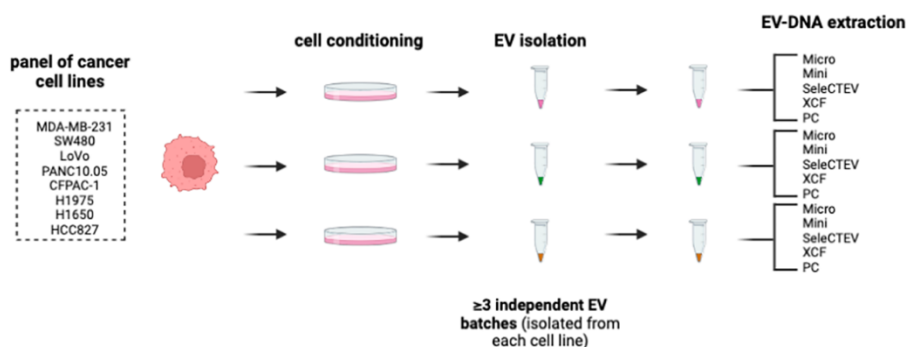


Figure 5. Graphical Abstract. Each cancer cell line (MDA-MB-231, SW480, LoVo, PANC10.05, CFPAC-1, H1975, H1650 and HCC827) was seeded at least 3 times for preparation of CCM. For each CCM, there was a batch of isolated EVs, thus adding up to at least 3 independent batches of EVs. Each batch of EVs was split and equal amounts of EVs were used for EV-DNA extraction using 5 methods: QIAamp DNA Micro Kit (Micro), QIAamp DNA Mini Kit (Mini), SeleCTEV Exosomal DNA Kit (SeleCTEV), XCF Exosomal DNA Kit (XCF) and phenol-chloroform (PC).

2.6 DNA quantification and quality analysis

2.6.1 Qubit fluorometer 3.0

The Qubit quantification of DNA was performed using a Qubit fluorometer (3.0; Invitrogen) and Qubit dsDNA HS Assay Kit (Invitrogen) according to the manufacturer's instructions. A volume of 2 μ L of each EV-DNA sample was diluted in 198 μ L of Qubit working solution.

2.6.2 Fragment Analyzer

The quantification and sizing of DNA was performed by capillary electrophoresis on a fragment analyzer (Agilent) using the High Sensitivity NGS Fragment kit (Agilent). The analyses were performed as per the instructions provided by the manufacturer.

2.7 DNA mutational analysis

2.7.1 PNA-Q-PCR (TaqMan) for mutation testing

EV-DNA and gDNA samples were analyzed for the presence of EGFR (exons 19, 21 and p.T790M), KRAS (exons 12, 13) and BRAF (exon 15) mutations using TaqMan. The assay is based on quantitative real-time PCR (qPCR) in the presence of a PNA clamp (Eurogentec, Seraing, Belgium) designed to inhibit the amplification of the wild-type alleles [29,30,31]. The assay has been fully validated, has an ISO15189 accreditation, and allows the absolute and relative abundances of mutant alleles to be estimated in positive samples. Amplification was performed in a final volume of 12.5 μ L, using 6.25 μ L of Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA), 0.96 pmol of each primer, 1.2 pmol of probes, and 6.25 pmol (for exons 21 and p.T790M) or 62.4 pmol (for exon 19) of PNA. Purified EV-DNA and gDNA were used in the following amounts: 3 μ L (4.5 ng) for BRAF and exon 21 analysis, 1 μ L (1.5 ng) for exon 19 and all exons in KRAS, and 1 μ L (1 ng) for p.T790M analysis. Using QuantStudio™ 6 Real-Time PCR System (Applied Biosystems/Thermo Fisher Scientific), samples were subjected to 50 cycles of 15 s at 92 °C and 1.5 min at 60 °C. The sequences of the primers and probes used are listed in **Supplementary Table 6**. Analyses were carried out in duplicates using one sample of purified EV-DNA or gDNA. In addition, all samples were analyzed in the absence of PNA to confirm the presence of purified EV-DNA and gDNA (WT). Genomic DNAs from cell lines at 1.5 ng/ μ L were used as positive and negative controls (**Supplementary Table 2**). A sample was considered positive if the

same mutant allele was amplified in the two duplicates in the presence of PNA. If amplification was only detected in one duplicate, samples were reanalyzed and considered positive if at least one of the duplicates was positive for the same mutated allele.

2.7.2 NGS for mutation testing

EV-DNA from the H1975 cell line was analyzed by NGS performed with GeneRead® QIAact Pangaea Solid Tumor Custom Panel on plasma (Qiagen), which targets 20 genes frequently altered in cancer (**Supplementary Table 7**). DNA (40 ng) purified from EV-DNA samples was used as a template; clonal amplification was performed on 625 pg of pooled libraries, and following bead enrichment, the GeneReader (Qiagen) instrument was used for sequencing. Qiagen Clinical Insight Analyze (QCI-A) software was employed to align read data and call sequence variants, which were imported into the Qiagen Clinical Insight Interpret (QCI-I) web interface for data interpretation and the generation of the final custom report. Gene copy numbers were determined by QCI-A and QCI-I software according to an “in-house” algorithm.

2.8 Statistical analysis

One-way ANOVA was applied to calculate the *p*-value. *p*-values of <0.05 were considered statistically significant. Statistical analyses and calculations of coefficients of variation were performed using GraphPad Prism software (GraphPad software).

3. Results

3.1 Characterization of EVs isolated from conditioned media

Isolated EVs were characterized by several approaches. First, NTA was used to determine the concentration and size distribution of nanoparticles in isolated

EV samples. Representative histograms of the frequency of particle size in EV samples derived from two representative cell lines, PANC10.05 and CFPAC-1, are shown in **Fig. 6A** and **Fig. 6B**, respectively. To confirm the expression of EV markers (tetraspanins CD9 and CD81, Alix) as well as the absence of non-EV markers (Calnexin and GM130), Western blotting was performed (**Fig. 6C**). Transmission electron microscopy (TEM) revealed the presence of negative-stained EVs, seen as cup-shaped structures (**Fig. 6D**). Additionally, the purity of our isolated EVs was supported by the LC-MS analyses of a panel of proteins differentially present in or absent from small EVs (**Fig. 6E**).

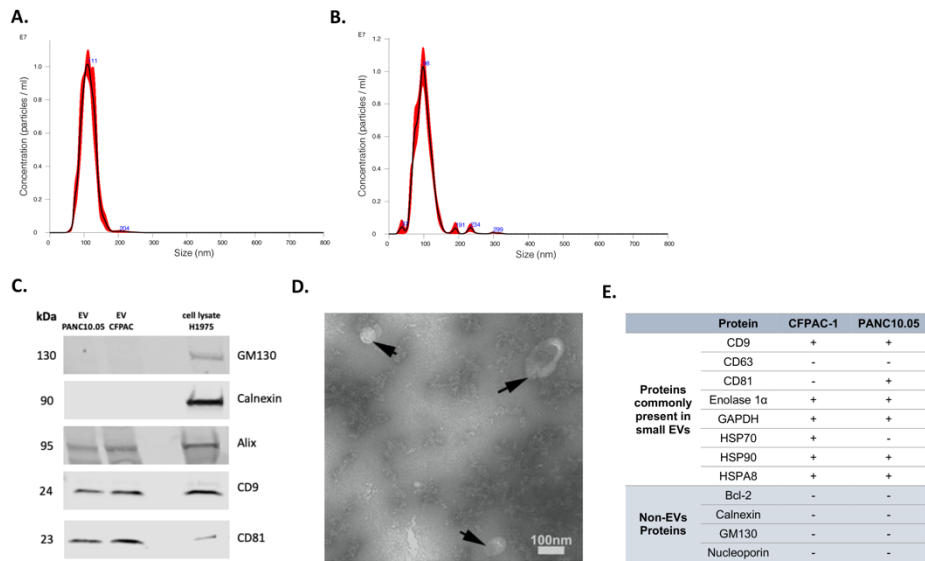


Figure 6. Characterization of EVs isolated from CCM. Representative NTA histograms of EV samples isolated from (A) PANC10.05 and (B) CFPAC-1 CCM. (C) Representative Western blots of EV markers CD9, CD81, and Alix, and of non-EV markers Calnexin and GM130 for EV samples isolated from CCMs derived from CFPAC-1 and PANC10.05 cell lines (H1975 cell lysate was used as a control). (D) Representative TEM image of CCM-derived EV sample. (E) MS analysis of EV-positive and -negative markers of CFPAC-1 and PANC10.05-derived EVs.

Although tetraspanins CD63 and CD81 are commonly enriched in EVs, CD63 was not identified in the LC-MS analyses of our EV samples. Similarly, CD81 was not detected in any of the analyzed EV samples, despite being identified in the Western blot analysis of CFPAC-1 EVs. CD63 and CD81 are membrane proteins and are both hydrophobic (difficult to solubilize) and low-abundant, which makes them difficult to detect with our LC-MS method. Moreover, the scan speed of any mass spectrometer is not sufficient to detect all peptides in a sample, so some peptides cannot be detected even if they are present in sufficient amounts. If the peptides from these proteins elute from LC and enter MS at the same time with many other peptides that are present in higher concentrations, these higher-abundant peptides are prioritized in MS identification, and the lower-abundant ones are missed. Interestingly, a comprehensive analysis of EV protein markers across more than 100 different human tumor and non-tumor cell lines showed that only 40% of them were positive for CD63 [32].

3.2 Preparation of EV-DNA for mutational analyses

3.2.1 Comparison of EV-DNA extraction methods

Previous reports have shown that EVs can carry DNA both in their lumen and on their surface [5,10,12,23]. However, as there is no consensus on which compartment contains DNA of diagnostic interest, we here define DNA associated with EVs as EV-DNA, independently of its location.

For the extraction of EV-DNA, five different methods frequently used in the literature were utilized (QIAamp DNA Mini Kit, QIAamp DNA Micro Kit, SeleCTEV, XCF Exosomal DNA Isolation Kit, and phenol-chloroform). These methods do not discriminate between luminal DNA and surface DNA, and as aforementioned, we considered that both were extracted. As such, these methods can be used in future studies focusing on either fraction. As outlined in the Graphical Abstract (**Fig. 5**), at least three independent batches of EVs

were isolated from each cell line; then, each batch was split into equal amounts, which were used to extract DNA with the five above-referenced methods. The amount of extracted dsDNA was quantified by Qubit, and this assessment showed that the use of different extraction methods resulted in different concentrations of DNA. Specifically, when normalized to samples purified with phenol–chloroform (the least efficient extraction method tested by us), EV-DNA samples extracted using the SeleCTEV Exosomal DNA kit had a significantly higher DNA yield than other methods (**Fig. 7**). This includes the concentration of DNA in ng per μL (**Supplementary Fig. 1**) and in ng per particle (**Supplementary Fig. 2**) in samples with similar EV protein concentrations. Controls involving EV-DNA extraction (SeleCTEV) from corresponding volumes of non-conditioned media revealed that less than 7% of DNA isolated from CCMs may derive from components of the media (**Supplementary Table 8**). These results show that within the tested strategies, the SeleCTEV Exosomal DNA Kit is the method that provides the highest DNA abundance. DNA concentration variability was also assessed for EV-DNA samples extracted through the different methodologies (**Supplementary Table 9**). We found that when all isolation methods and cells were simultaneously evaluated, SeleCTEV displayed the lowest coefficient of variation amongst all cells, reinforcing the application of this method for the isolation of EV-DNA.

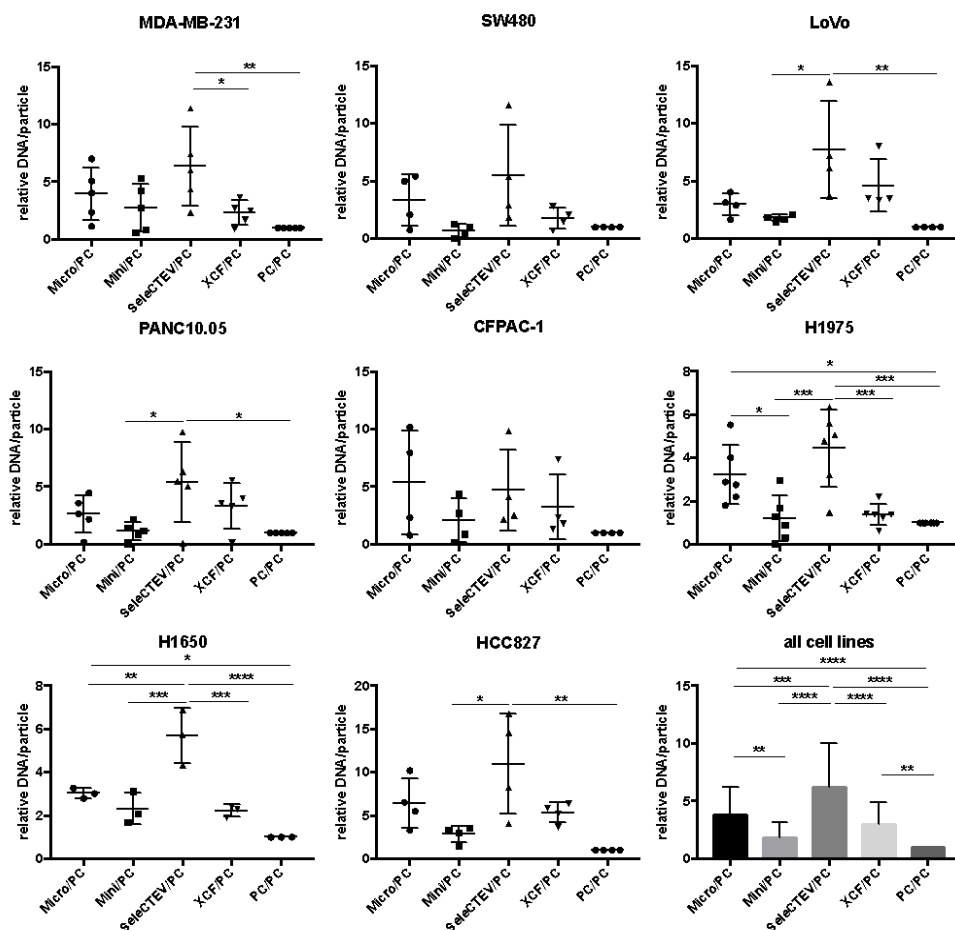


Figure 7. Comparison of EV-DNA abundance among different DNA extraction methods. Qubit DNA quantifications of EV-DNA samples extracted from a panel of cancer cell lines (MDA-MB-231, SW480, LoVo, PANC10.05, CFPAC-1, H1975, H1650, and HCC827) using QIAamp DNA Micro Kit (Micro), QIAamp DNA Mini Kit (Mini), SeleCtEV Exosomal DNA Kit (SeleCtEV), XCF Exosomal DNA Kit (XCF), and phenol–chloroform (PC). At least 3 independent batches of EVs were isolated from each cancer cell line. Next, each EV batch was split in equal amounts and used for DNA extraction with the indicated different methods. EV-DNA abundance is presented as ng of DNA/particle normalized to values obtained using phenol–chloroform. All the data are shown as the mean values $\pm$ SD from independent measurements. p-values were calculated using one-way ANOVA with Tukey's post hoc test; **** p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05.

DNA fragment size is another parameter that plays a key role in the downstream analysis of DNA sequences [33]. Based on that, we compared the size distribution of DNA fragments in EV-DNA using a fragment analyzer (Agilent). A representative electrophoretic histogram of EV-DNA extracted from MDA-MB-231 CCM is presented in **Fig. 8A**. A comparison of the methods showed that the average size of DNA fragments varied among the samples extracted with different DNA extraction protocols, with the highest average sizes having been found in the EV-DNA samples extracted with the SeleCTEV Exosomal DNA kit and phenol–chloroform. However, in the case of phenol–chloroform, only 10 out of 27 extracted EV-DNA samples were successfully analyzed using the fragment analyzer due to the very low DNA concentrations in these samples (**Fig. 8B**).

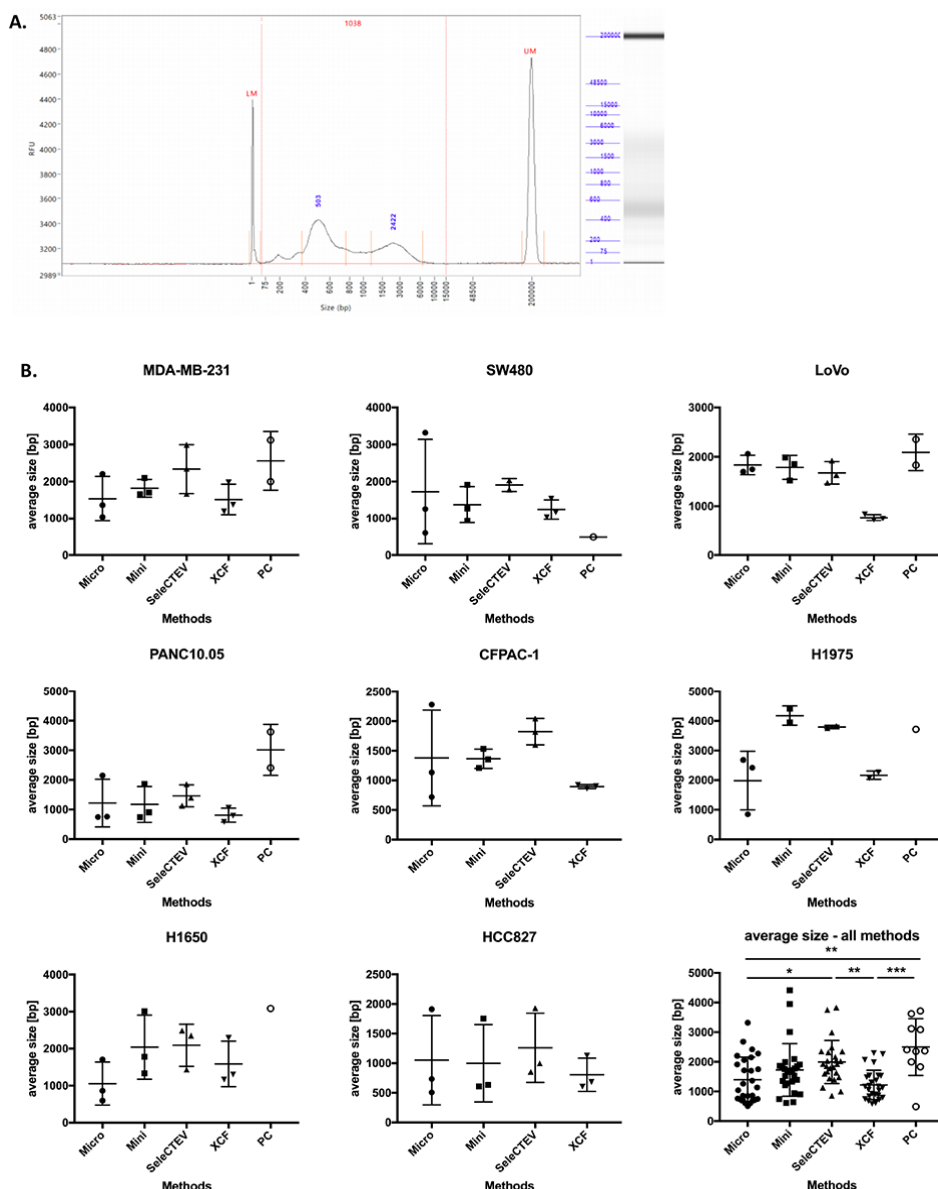


Figure 8. Comparison of the average size of EV-DNA fragments between different DNA extraction methods. (A) Fragment Analyzer analysis of EV-DNA extracted from MDA-MB-231 CCM, showing size distribution [bp] and relative fluorescence intensity (RFU). The peaks at 1bp and 20,000bp correspond to 2 internal size markers. (B) Average size [bp] of EV-DNA fragments extracted with the following kits: QIAamp DNA Micro Kit (Micro), QIAamp DNA Mini Kit (Mini), SeleCTEV Exosomal DNA Kit (SeleCTEV), XCF Exosomal DNA Kit (XCF), and phenol-chloroform (PC). The data is presented as mean values $\pm$ SD from three independent specimens. P-values were calculated using one-

way ANOVA with Tukey's post-test; **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Note: Some samples extracted using phenol-chloroform could not be successfully analyzed using the Fragment Analyzer due to very low DNA concentrations.

3.2.2 Assessment of KRAS and EGFR mutations in EV-DNA and gDNA by TaqMan

To understand the effect of EV-DNA extraction on its suitability for mutational analyses, qPCR (TaqMan) studies were performed. EV-DNA was analyzed to evaluate the mutation status of two genes known to be mutated in the tested cell lines from which EV-DNA samples were isolated, KRAS (MDA-MB-231, LoVo, SW480, CFPAC-1, and PAN10.05) and EGFR (H1975, H1650, and HCC827). For each cell line, three independent EV-DNA samples, previously isolated and analyzed for the comparison of EV-DNA extraction methods (**Fig. 5**), were chosen based on DNA concentration and used for TaqMan (a total of 24 EV-DNA samples per each method). As shown in **Table 2**, the investigated mutations were detected in 21/24 (87.5%), 22/24 (91.7%), 23/24 (95.8%), 22/24 (91.7%), and 14/24 (58.3%) EV-DNA samples extracted with the QIAamp DNA mini kit, QIAamp DNA micro kit, SeleCTEV Exosomal DNA kit, XCF Exosomal DNA Isolation kit, and phenol-chloroform, respectively. This demonstrates that the EV-DNA extraction method affected the suitability of DNA for mutation analysis by qPCR and that the use of the SeleCTEV Exosomal DNA kit resulted in the highest percentage of mutation detection among all methods. Extraction with the QIAamp DNA micro kit and XCF Exosomal DNA Isolation kit also resulted in high mutation detection in more than 90% of the analyzed samples. On the other hand, the mutations were undetectable in over 40% of samples extracted with phenol-chloroform. Moreover, the quantity of mutated DNA in EV-DNA versus gDNA of the corresponding cell lines was comparable regardless of the applied extraction method (**Fig. 9**).

Table 2. Detection of EGFR and KRAS mutations in cancer cells' EV-DNA that were extracted by QIAamp DNA Micro Kit (Micro), QIAamp DNA Mini Kit (Mini), SeleCtEV, Exosomal DNA Kit (SeleCtEV), XCF Exosomal DNA Kit (XCF) and phenol-chloroform (PC).

		EV-DNA Purification Method				
Gene	Cell-Line-Derived EVs	Micro	Mini	SeleCtEV	XCF	PC
KRAS	MDA-MB-231	2/3	3/3	3/3	3/3	1/3
	SW480	3/3	2/3	2/3	1/3	1/3
	LoVo	3/3	3/3	3/3	3/3	3/3
	PANC10.05	3/3	3/3	3/3	3/3	2/3
	CFPAC-1	3/3	2/3	3/3	3/3	2/3
EGFR	H1975	3/3	3/3	3/3	3/3	3/3
	H1650	2/3	3/3	3/3	3/3	1/3
	HCC827	2/3	3/3	3/3	3/3	1/3
Samples with detected mutations		21/24 (87.5%)	22/24 (91.7%)	23/24 (95.8%)	22/24 (91.7%)	14/24 (58.3%)

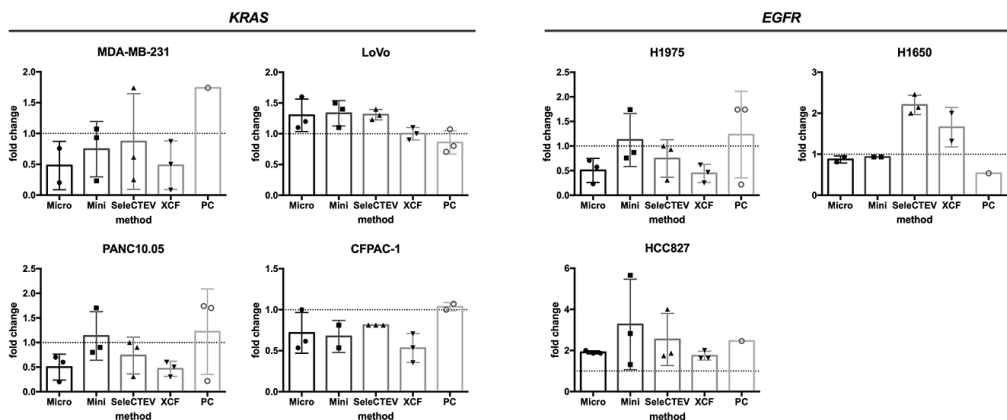


Figure 9. Mutational analysis of EGFR and KRAS genes in CCM-derived EV-DNA by TaqMan. Comparison of the quantity of detected mutated DNA in EV-DNA versus gDNA samples by TaqMan. The $2^{-\Delta\Delta CT}$ method was used as a quantification strategy to assess the fold difference in mutated DNA in EV-DNA in relation to gDNA. This analysis could not be applied to SW480 EV-DNA as the WT KRAS gene was not amplifiable.

3.2.3 Mutation detection in EV-DNA by NGS

To further assess EV-DNA suitability for mutational analyses, we performed NGS using H1975-derived EV-DNA. EV-DNA extracted with all five DNA purification methods was analyzed using a customized panel covering 20 genes. Out of the five analyzed samples, only the one extracted with phenol-chloroform had insufficient material for NGS, resulting in an unsuccessful analysis. In contrast, the mutations present in the gDNA of the cell line from which the EVs were derived were successfully detected in all the EV-DNA samples extracted with the four commercial kits used. The highly similar quality parameters and the almost identical mutant allelic fraction values, representing the percentage of sequence reads carrying a mutant allele, both suggest that those four DNA purification methods allowed us to extract DNA of sufficient quantity and quality for comparable mutation detection by NGS (Table 3 and Table 4).

Table 3. Mutation detection in H1975 EV-DNA by NGS.

Method			Mini	Micro	SeleCTEV	XCF	PC
Gene	Exon	Mutation	Allelic fraction				
<i>EGFR</i>	21	c.2573T > G	66%	65%	67%	62%	N/A
	20	c.2369C > T	64%	64%	65%	69%	N/A
<i>TP53</i>	8	c.818G > A	98%	97%	97%	96%	N/A

Table 4. Summarized quality parameters of H1975 EV-DNA NGS analysis.

Method	Reads	Nucleotides	Average Read Length	Reads with Average Quality > 25	Reads Mapped	Reads in Target Regions	Base Positions in Regions of Interest with UMI Coverage > 100x	Base Positions in Regions of Interest with UMI Coverage > 60x
Mini	4023974	505992583	125.74	95.15%	3980643 (98.2%)	3557632 (89.37%)	97.68%	98.54%
Micro	4034733	512374900	126.99	94.89%	4023363 (99.72%)	3332780 (82.84%)	97.04%	97.55%
SeleCTEV	4194485	523511719	124.81	94.59%	4183430 (99.74%)	2398744 (57.34%)	96.75%	97.31%
XCF	4875693	127.70	94.93	94.93%	4868494 (99.85%)	4568724 (93.84%)	97.60%	98.28%
PC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

3.3 Plasma EV-DNA mutation detection by TaqMan

As stated above, DNA extracted using the SeleCTEV Exosomal DNA kit had a significantly higher DNA yield from CCM-derived EVs than any of the other methods analyzed in this study. In order to assess the usefulness of this approach to detect clinically relevant mutations in plasma samples, EVs were isolated from samples collected from cancer patients. Plasma-derived EVs were then characterized using NTA and Western blotting (**Fig. 10**). Due to the limited plasma volume available, TEM was not performed for these samples. Next, DNA was extracted from these EV samples using the SeleCTEV Exosomal DNA kit. All mutations found in tissue biopsies and circulating free DNA were also identified in EV-DNA (**Table 5**).

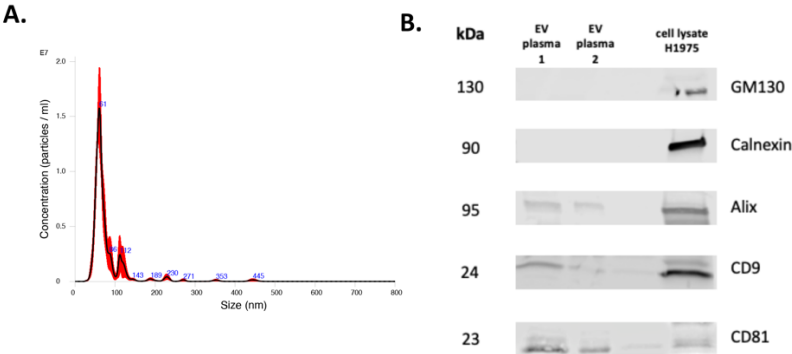


Figure 10. Characterization of EVs isolated from plasma. Representative NTA histogram of EV sample (**A**) and representative Western blots (**B**) of EV markers CD9, CD81, and Alix and of non-EV markers Calnexin and GM130 (H1975 cell lysate was used as a control).

Table 5. Mutational analysis of KRAS and BRAF genes in plasma-derived EV-DNA by TaqMan.

Patient	Tumor Type	Gene	Position	Mutant Allelic Fraction %
#1	Melanoma	BRAF	p.V600E	0.74 $\pm$ 0.04
#2	Colorectal	KRAS	p.G12V	20.33 $\pm$ 1.81
#3	Lung	KRAS	p.G12C	4.65 $\pm$ 0.83
#4	Lung	KRAS	p.G12D	4.84 $\pm$ 1.25
#5	Lung	KRAS	p.G12V	0.39 $\pm$ 0.55
#6	Lung	BRAF	p.V600E	0.84

4. Discussion

Although recent discoveries on EV-DNA and its potential as a biomarker have created excitement, there are still important technical aspects that need to be considered before its future application in clinics. One of the challenges in EV-DNA studies is the lack of the standardization of DNA extraction and quality control, which are crucial factors to be considered for downstream DNA analyses and can vary depending on the chosen DNA extraction method.

Here, we contributed to this standardization by evaluating different methodologies for extracting DNA from EVs. As a first layer of standardization, we fixed the EV isolation method used, so that all DNA would be extracted from comparable isolated EV samples. The chosen method for the isolation of EVs was differential ultracentrifugation coupled with a sucrose cushion, which is one of the most commonly used EV isolation techniques and is considered a gold standard for EV isolation. Other EV isolation methods were not compared, as it was beyond the scope of this paper [34].

In the literature, the characterization of extracted EV-DNA is rather superficial, with DNA quantification being performed using different methods and often presented in various units, making it very difficult to reproduce and compare results. Some of the most commonly applied methods used to assess DNA yield include spectrophotometry-based measurements on NanoDrop spectrophotometers (ThermoScientific), capillary-electrophoresis-based DNA analysis on a fragment analyzer (Agilent) or 2100 Bioanalyzer (Agilent), and methods based on fluorescent dyes, such as Qubit (Life Technologies) or QuantiFluor (Promega). These DNA quantification methods are characterized by different lower and upper limits of detection, as well as different detection size ranges; thus, they need to be considered carefully depending on the analyzed sample type. For instance, NanoDrop has low sensitivity, reaching 1 ng/ μ L, and does not distinguish between single- and double-stranded DNA, while capillary-electrophoresis-based DNA quantification has a very narrow size detection range [35]. Based on previous reports that demonstrate that EVs carry nucleic acids in the order of ng with a broad size range (100 bp→10 kbp), all EV-DNA samples purified in this study were quantified using the HS DNA kit on Qubit, which has a quantitative range of 10 pg/ μ L–100 ng/ μ L [11,12,18,21,36]. According to the results presented by the manufacturer, DNA kits for Qubit are highly selective for dsDNA over RNA and ssDNA, as quantification is based on the fluorescence intensity of the fluorescent dye binding to dsDNA and not on UV absorbance. Thus, we propose that future studies could benefit from using Qubit for EV-DNA quantification.

Most of the data described in this study were obtained from EVs isolated from CCMs, and the conditioning was always performed in media supplemented with EV-depleted FBS. The use of EV-depleted FBS enabled tumor cell growth to be obtained and EVs to be produced in CCMs while avoiding, amongst others, cellular stress, changes in phenotype, and potential alterations in EV cargo packaging that might be caused by the full absence of FBS. Of note, it

was recently proposed that FBS supplementation may result in residues of bovine DNA in CCM preparations [37]. We here found that EV-DNA extraction from volumes of non-conditioned media that corresponded to those utilized for EV isolation from CCMs displayed residual amounts of DNA (less than 7% of the EV-DNA obtained from CCMs). Importantly, as all culture media were prepared with the same lot of EV-depleted FBS, residual bovine DNA would in principle be equally present in all CCM samples that contained the same amount of FBS. Although the culture medium of PANC10.05 contained more than 10% FBS (15%), we did not find any noticeable increment in DNA yield in EVs isolated from PANC10.05 when compared to other cells. Together, these results indicate that in our experimental settings, the traces of bovine DNA in EV-depleted FBS were not sufficient to significantly impact the amount of EV-DNA in CCMs.

When comparing EV-DNA isolation strategies, we observed that there were differences in DNA abundance amongst samples extracted with different methods, agreeable with the studies performed with other nucleic acids, including EV-RNA [15,16]. Our data showed that the highest DNA concentrations were obtained using the SeleCTEV Exosomal DNA kit and the lowest concentrations with phenol–chloroform, suggesting that the SeleCTEV Exosomal DNA kit may be the method of choice if the highest abundance of DNA is considered.

In terms of the size distribution of EV-DNA fragments, we showed that the average sizes of DNA fragments also differed among the analyzed DNA extraction methods, with the average being ~2000 bp. Both the SeleCTEV Exosomal DNA kit and phenol–chloroform extractions resulted in the highest average sizes of DNA fragments. However, despite the high average size of EV-DNA in the case of phenol–chloroform, the efficiency of extraction was lower, leading to lower quantities of DNA. Thus, only a few of the phenol–chloroform

extracted samples amounted to a concentration high enough to be detected by the fragment analyzer for sizing evaluation.

The different quantities and sizes of EV-DNA observed in our studies likely resulted from the potential differences in the extraction methods used. Among the analyzed methods, apart from phenol–chloroform, the remaining four are based on spin-column technology, with two of the kits (SeleCTEV Exosomal DNA Kit and XCF Exosomal DNA Isolation Kit) being specifically dedicated to exosomal DNA extraction. However, the details distinguishing them from regular DNA extraction kits are not included in the descriptions provided by the manufacturers. Both the QIAamp DNA micro kit and the QIAamp DNA mini kit are based on silica-membrane DNA purification. The main difference between these two approaches is that the QIAamp DNA micro kit is dedicated to small-volume samples and includes the addition of carrier RNA, which increases DNA binding to the membrane of the spin column and subsequently enhances the recovery of DNA. Furthermore, we also observed differences in DNA amounts amongst cell lines (**Supplementary Fig. 2B**), which is in line with existing reports suggesting a differential abundance of DNA associated with EVs in various cancer models [12].

Previous studies have shown that DNA in EVs not only covers the entire genome of parental cells, but also allows identical mutations from the cells they are derived from to be detected, including clinically relevant mutations such as KRAS mutations in pancreatic cancer or BRAF in melanoma [11,12,13,14,38]. Thus, in order to compare the suitability of the EV-DNA extracted with all five methods for mutational studies, we analyzed all EV-DNA samples for the presence of mutations known to be present in their parental cells. Mutation detection using TaqMan differed among EV-DNA samples extracted with different methods, with the highest detection rate being observed using the SeleCTEV Exosomal DNA kit (Exosomics) (95.8%). This

result is consistent with the highest DNA concentration and the largest average DNA size of EV-DNA extracted with this method, both of which are factors that can directly affect the TaqMan reaction. In addition, consistently with previous results regarding EV-DNA quantification, respective mutations were detected in less than 60% of samples extracted with phenol–chloroform. qPCR-based amplifications, such as TaqMan, are highly influenced by the quantity, quality, and purity of DNA samples, and the low purity of EV-DNA samples extracted with phenol–chloroform could be a reason for unsuccessful amplification. Phenol–chloroform-based methods use hazardous organic solvents and may result in the presence of PCR inhibitors such as divalent cations or proteins, directly affecting TaqMan analysis. On the contrary, column-based DNA isolation methods are described to yield DNA samples of higher purity, which could explain the differences in mutation detection amongst the compared methods.

Further, we verified the applicability of our strategy for the detection of mutations in EVs derived from the plasma of cancer patients. As pre-analytical steps of plasma processing can affect EV recovery, we recorded a list of parameters describing sample collection and processing. Previous studies have suggested that the presence of lipoprotein and platelet fragments can interfere with molecular studies of plasma EVs [39,40,41]. Therefore, although our plasma EV samples were not controlled for the potential presence of lipoproteins and platelets fragments, we propose that future studies are necessary to address whether these parameters can impact the study of EV-DNA composition. In order to extract DNA from isolated plasma EVs, we used the SeleCTEV Exosomal DNA kit, which had a significant higher DNA yield in CCM-derived EV analysis. It was previously suggested that SeleCTEV's proprietary peptide improves plasma EV isolation for DNA studies when compared to ultracentrifugation [20]. Therefore, future research should consider the potential benefits of using SeleCTEV's proprietary peptide for

improved plasma EV-DNA studies. Due to the limitation of the plasma samples' volume availability, a side-by-side comparison of other DNA extraction methods was not possible in our study. Importantly, the analysis of EV-DNA from the plasma of cancer patients showed that our workflow allowed us to detect clinically relevant mutations in those samples, confirming previous reports on mutation detection in EVs [11,13,14,20].

Along with PCR-based analyses such as TaqMan, one of the most commonly used methods to study mutations in EV-DNA is sequencing. To assess the suitability of EV-DNA samples for sequencing, samples were analyzed with a custom NGS panel. Apart from phenol–chloroform, all the other tested samples were successfully analyzed by NGS, which is in accordance with our previous results demonstrating that the purity of EV-DNA extracted with phenol–chloroform can impede their suitability for mutation detection analyses. In contrast with TaqMan results, which showed that the choice of different DNA isolation methods resulted in differences in mutation detection, NGS analyses indicated that EV-DNA extracted with the four tested commercial kits had very similar results regarding mutation detection and quality parameters. These results show that while SeleCTEV contributes to the improved analysis of EV-DNA by qPCR, no clear advantages amongst the four tested commercial kits were observed in our NGS studies. Recent reports have suggested that this may be due to the high sensitivity of NGS [42].

5. Conclusions

Collectively, our results show that the quantity and quality of EV-DNA can vary depending on the applied method of extraction and highlight the importance of the standardization of EV-DNA preparation for improved reproducibility of results. Our work provides strategic points to consider when preparing EV-DNA samples for downstream applications, including qPCR and NGS (**Table 6**). In agreement with previous reports, we found that EV-DNA reflects the mutational status of parental cells. Therefore, our data support the use of EV-DNA for the detection of tumor mutations [11,12,13,17].

Table 6. Strategic points for EV-DNA extraction and quality control for mutational analysis.

Extraction Method	DNA Yield	DNA Quality (Integrity)	Suitability for TaqMan	Suitability for NGS
QIAamp DNA Mini Kit	++	++	++	+++
QIAamp DNA Micro Kit	++	+++	++	+++
SeleCTEV Exosomal DNA Kit	+++	+++	+++	+++
XCF Exosomal DNA Isolation Kit	++	++	++	+++
Phenol–Chloroform	+	+++	+	-

+++—high; ++—medium; +—low; —not suitable.

Author Contributions: J.E. designed and performed the experiments. L.B., B.G.-P. and M.V.-U. performed TaqMan and NGS analyses. B.P.S., J.M. and S.B. contributed with EV sample preparation. J.T. and M.V. performed protein mass-spectrometry processing and analysis. M.C.S.M., M.Á.M.-V. and B.C.-S. conceived the project. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Quirón Hospitals. Informed consent was obtained from all patients; samples were deidentified for patient confidentiality.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Supplementary Information

Supplementary Table 1. Number of cells seeded at the initiation of culture for conditioning.

Cell line	Number of cells/plate
MDA-MB-231	1.25×10^6
SW480	1.0×10^6
LoVo	1.5×10^6
PANC10.05	2.0×10^6
CFPAC-1	1.25×10^6
H1975	1.0×10^6
H1650	1.5×10^6
HCC827	1×10^6

Supplementary Table 2. Cell lines used as positive and negative controls for TaqMan.

Gene	Positive control	Negative control
<i>EGFR</i>		
Exon 19	PC9	BxPC-3
Exon 21	11-18	BxPC-3
T790M	H1975	BxPC-3
<i>KRAS</i>		
Exon 2	H23, DLD1	BxPC-3
Exon 3	H460	BxPC-3
<i>BRAF</i>		
Exon 15	HT29	BxPC-3

Supplementary Table 3. The clinical characteristics of patients.

Patient characteristics				Mutations	
Patient	Tumor type	Sex	Age	Gene	Position
#1	Melanoma	F	N/A	<i>BRAF</i>	p.V600E
#2	Colorectal cancer	M	84	<i>KRAS</i>	p.G12V
#3	Lung cancer	F	68	<i>KRAS</i>	p.G12C
#4	Lung cancer	M	81	<i>KRAS</i>	p.G12D
#5	Cervical cancer	F	43	<i>KRAS</i>	p.G12V
#6	Lung cancer	M	66	<i>BRAF</i>	p.V600E

Supplementary Table 4. Technical aspects of patients' blood sample collection and processing.

Patient	Tumor type	Time of day of col- Blood collection lection [hour]	First tube volume [mL]	discard	Hemolysis *	Transport and sample pro- cessing
#1	Melanoma	09:57	28	No	No	N/A; samples were kept in an upright position and processed directly after collection
#2	Colorectal cancer	12:29	21	No	No	
#3	Lung cancer	16:32	28	No	No	
#4	Lung cancer	11:36	21	No	No	
#5	Cervical cancer	12:46	21	No	No	
#6	Lung cancer	10:02	28	No	No	

* Plasma samples were visually inspected, and no samples had obvious signs of hemolysis. visual inspection based on color chart for detection of hemolysis.

Supplementary Table 5. List of primary and secondary antibodies used for Western blotting.

Primary Antibodies			
Antibody	Manufacturer	Catalog no	Dilution/concentration
CD81	Santa Cruz Biotechnology	sc-166029	1:50
CD9	Cell Signaling	#13174	1:1000
Alix	Sigma Aldrich	SAB4200476-200UL	1.25 ug/mL
Calnexin	Abcam	ab22595	1:2000
GM130	Abcam	ab52649	1:1000
Secondary Antibodies			
Antibody	Manufacturer	Catalog no	Dilution
Goat anti-rabbit IgG IRDye 800CW	LI-COR Biosciences	926-32211	1:5000
Goat anti-mouse IgG IRDye 800CW	LI-COR Biosciences	926-32210	1:5000

Supplementary Table 6. Primers and probes sequences used in TaqMan.

Gene	Name	Sequence
EGFR	19_F_TAQM	5' TGAGAAAGTTAAAATCCCGTCG 3'
	19_R_TAQM	5' GAAACTCACATCGAGGATTCCTT 3'
	EGFR-T790F	5' CCTCACCTCCACCGTGCA 3'
	EGFR-T790R	5' AGGCAGCCGAAGGGCA 3'
	EGFR-858F	5' AACACCCGAGCATGTCAAGA 3'
	EGFR-861R	5' TGCCTCCTTCTGCATGGTATT 3'
	19_TAQM_WT2	5' VIC-TCAAGGAATTAAGAGAAG-MGB 3'
	19_TAQM_E746-A750del	5' FAM- TCAAAACATCTCCGAAAG -MGB 3'
	19_TAQM_E746-A750*del	5' FAM- TCAAGACATCTCCGAAAG -MGB 3'
	19_TAQM_L747-T751del	5' FAM- TCAAGGAATCTCCGAAAG -MGB 3'
	19_TAQM_E746-T751>A	5' FAM- TCAAGGCATCTCCGAAA -MGB 3'
	19_TAQM_L747-P753>S	5' FAM- AGGAATCGAAAGCCAA -MGB 3'
	19_TAQM_L747-S752del	5' FAM-TCAAGGAACCGAAAGC-MGB 3'
	19_TAQM_E746-S752>V	5' FAM- TCAAGGTTCCGAAAGC-MGB 3'
	19_TAQM_L747-T751>P	5' FAM- AAGGAACCATCTCCGAA-MGB 3'
	19_TAQM_L747-A750>P	5' FAM- CAAGGAACCAACATCT -MGB 3'
	E21_858-FAM	5' FAM-ATTTTGGGCGGGCCAAAC-MGB 3'
	E21_861-FAM	5' FAM-TGGCCAAACAGCTGGGTG-MGB 3'
	E21_858-VIC	5' VIC-ATTTTGGGCTGGCCAAAC-MGB 3'
	E20_T790M-FAM	5' FAM-CTCATCATGCAGCTCATG-MGB 3'
	E20_T790M-VIC	5' VIC- CTCATCACGCAGCTCATG MGB 3'
	PNA_ex19	5' AGATGTGCTTCTCTTA 3'
	PNA_ex21	5' AGTTTGGCCAGCCCA 3'
	PNA_ex20	5' TCATCACGCAGCTC 3'
KRAS	KRAS_TAQM_12F	5' GCCTGCTGAAAATGACTGAA 3'
	KRAS_TAQM_12R	5' TATCGTCAAGGCACTCTTGC 3'
	KRAS_TAQM_61F	5' TGGAGAAACCTGTCTCTTGA 3'
	KRAS_TAQM_61R	5' TCCTCATGTACTGGTCCCTCA 3'
	KRAS_12_WT	5' VIC - ACGCCACCAGCTCCA - MGB 3'

	KRAS_61_WT	5' VIC – CAGGTCAAGAGGAG – MGB 3'
	KRAS_G12C	5' FAM-ACGCCACAAGCTCCA-MGB 3'
	KRAS_G12D	5' FAM-ACGCCATCAGCTCCA-MGB 3'
	KRAS_G12V	5' FAM-ACGCCAACAGCTCCA-MGB 3'
	KRAS_G12A	5' FAM-ACGCCAGCAGCTCCA-MGB 3'
	KRAS_G12S	5' FAM-ACGCCACTAGCTCCA-MGB 3'
	KRAS_G12R	5' FAM-ACGCCACGAGCTCCA-MGB 3'
	KRAS_G13D	5' FAM-ACGTCACCAGCTCCA –MGB 3'
	KRAS_G13C	5' FAM-ACGCAACCAGCTCCA-MGB 3'
	KRAS_Q61H_1	5' FAM-CAGGTCATGAGGAG-MGB 3'
	KRAS_Q61H_2	5' FAM-CAGGTCACGAGGAG-MGB 3'
	KRAS_Q61L	5' FAM-CAGGTCTAGAGGAG-MGB 3'
	PNA_KRAS_EX2R	5' CTACGCCACCAGCTC 3'
	PNA_KRAS_EX3R	5' CTCCTCTTGACCTGC 3'
BRAF	BRAF_TAQ_F	5' CTACTGTTTTCTTTACTTACTACCTCAGA 3'
	BRAF_TAQ_R	5' ATCCAGACAAGTGTCAAAGTATG 3'
	BRAF_F	5' TGCTTGCTCTGATAGAAAATG 3'
	BRAF_R	5' TCAGTGGAAAAATAGCCTCAA 3'
	BRAF_V600E_FAM	5' FAM-TAGCTACAGAGAAATC-MGB 3'
	BRAF_V600E_VIC	5' VIC-CTAGCTACAGTGAATC-MGB 3'
	BRAF_V600K_FAM	5' FAM-TAGCTACAAAGAAATC-MGB 3'

Supplementary Table 7. Customized panel for NGS.

Gene	CNVs analysis	Exons
<i>EGFR</i>	Y	12, 18, 19, 20, 21
<i>BRAF</i>	Y	11, 15
<i>MET</i>	Y	13, 15 (splicing MET14)
<i>ERBB2</i>	Y	8, 17, 19, 20, 21
<i>ALK</i>	N	22, 23, 24, 25
<i>ROS1</i>	N	38
<i>PIK3CA</i>	N	10, 21
<i>KRAS</i>	Y	2, 3, 4
<i>NRAS</i>	N	2, 3, 4
<i>FGFR1</i>	Y	X
<i>KIT</i>	N	8, 9, 11, 13, 14, 17
<i>PDGFRA</i>	N	12, 18
<i>TP53</i>	N	4, 5, 6, 7, 8, 9
<i>CDK4</i>	Y	X
<i>CDK6</i>	Y	X
<i>RCTOR</i>	Y	X
<i>IDH1</i>	N	4
<i>IDH2</i>	N	4
<i>STK11</i>	N	2, 3, 4, 5, 6, 7, 8, 9
<i>ERBB4</i>	N	8, 17, 19, 20, 21

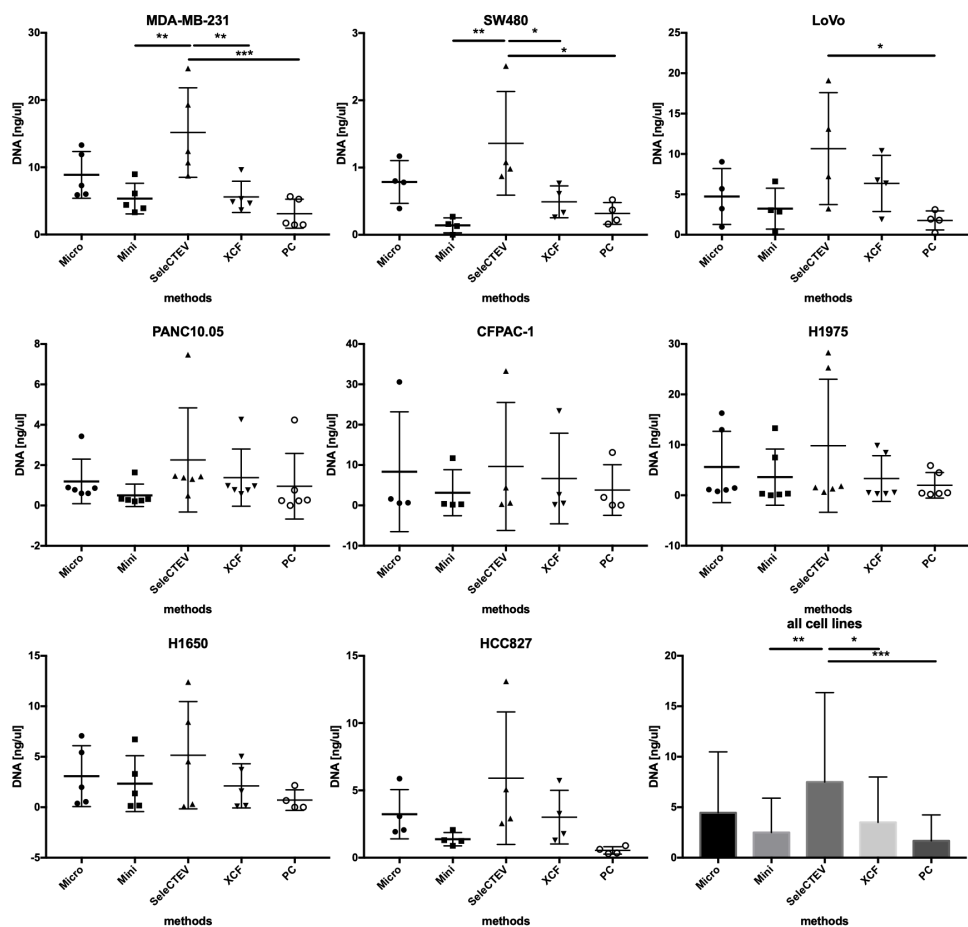
Supplementary Table 8. Comparison of ng of DNA/mL of media in non-conditioned media and cell conditioned.

Sample	Total volume of media [ml]	Total DNA [ng]	ng of DNA/ml of media
nCM1	270	12	0.04
nCM2	135	10	0.07
nCM3	135	4.4	0.03
CCM1	600	262	0.44
CCM2	600	506	0.84
CCM3	520	382	0.74

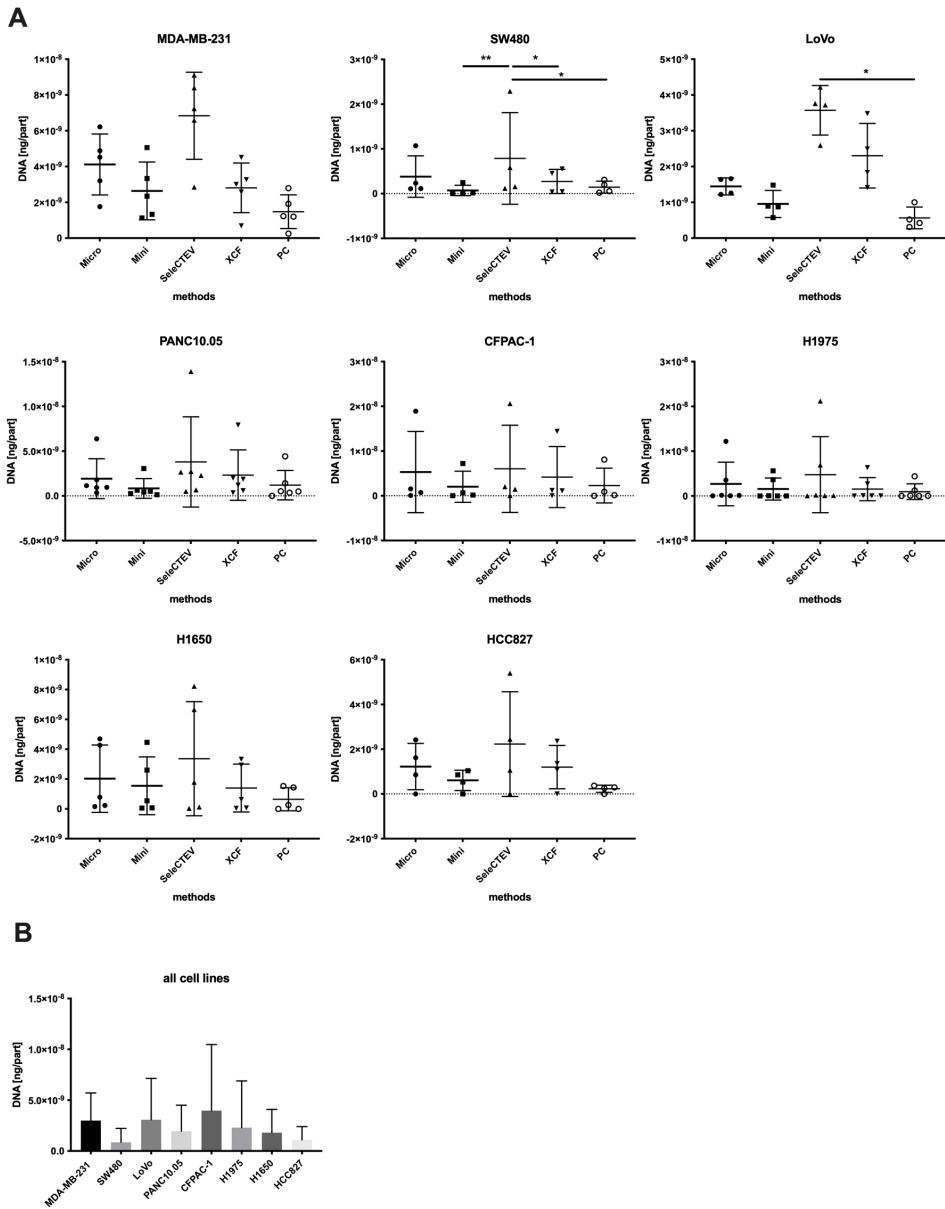
nCM - non-conditioned media; CCM - cell conditioned media.

Supplementary Table 9. Coefficients of variation (CV) calculated for EV-DNA extraction methods.

EV-DNA purification method						
	Cell-line derived EVs	Micro	Mini	SelectEV	XCF	PC
Coefficient of variation (CV)	MDA-MB-231	39.11%	42.69%	43.84%	41.33%	69.52%
	SW480	40.45%	79.33%	56.68%	48.25%	50.90%
	LoVo	73.15%	78.34%	64.98%	54.70%	66.58%
	PANC10.05	92.41%	110.84%	114.26%	102.62%	170.14%
	CFPAC-1	177.88%	181.90%	164.48%	168.28%	164.74%
	H1975	125.77%	154.72%	134.22%	136.49%	127.82%
	H1650	97.93%	118.38%	103.22%	103.38%	144.49%
	HCC827	56.63%	35.90%	83.33%	66.13%	50.89%
	all cell lines	135.67%	136.81%	118.40%	128.51%	153.99%



Supplementary Figure 1. Comparison of EV-DNA abundance between different DNA extraction methods. Qubit DNA quantifications in EV-DNA samples extracted from a panel of cancer cell lines (MDA-MB-231, SW480, LoVo, PANC10.05, CFPAC-1, H1975, H1650 and HCC827) using QIAamp DNA Micro Kit (Micro), QIAamp DNA Mini Kit (Mini), SeleCtEV Exosomal DNA Kit (SeleCtEV), XCF Exosomal DNA Kit (XCF) and phenol chloroform. EV-DNA levels are presented as ng of DNA/μl. All data is shown as the mean values $\pm$ SD from independent measurements. P-values were calculated using one-way ANOVA with Tukey's post-test; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.



Supplementary Figure 2. Comparison of EV-DNA abundance per particle. Qubit DNA quantifications in EV-DNA samples extracted from a panel of cancer cell lines (MDA-MB-231, SW480, LoVo, PANC10.05, CFPAC-1, H1975, H1650 and HCC827) using QIAamp DNA Micro Kit (Micro), QIAamp DNA Mini Kit (Mini), SeleCTEV Exosomal DNA Kit (SeleCTEV), XCF Exosomal DNA Kit (XCF) and phenol chloroform. EV-DNA levels

were compared between different extraction methods (**A**) and analyzed cancer cell lines (**B**). EV-DNA levels are presented as levels of DNA normalized by the number of particles in each EV sample. All data is shown as the mean values $\pm$ SD from independent measurements. P-values were calculated using one-way ANOVA with Tukey's post-test; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

CHAPTER II

IgG⁺ Extracellular Vesicles Measure Therapeutic Response in Advanced Pancreatic Cancer

The content of the present chapter consists of a peer-reviewed article published in the context of this thesis:

Couto, N.*, Elzanowska, J.*, Maia, J., Batista, S., Pereira, C. E., Beck, H. C., Carvalho, A. S., Strano Moraes, M. C., Carvalho, C., Oliveira, M., Matthiesen, R., & Costa-Silva, B. (2022). IgG⁺ Extracellular Vesicles Measure Therapeutic Response in Advanced Pancreatic Cancer. *Cells*, 11(18), 2800. <https://doi.org/>

* These authors contributed equally to this work.

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Author contributions:

N.C. recruited the patients and designed and performed the experiments; J.E. designed and performed the experiments; J.M., S.B., and C.E.P. contributed to the EV sample preparation and vesicle flow cytometry analysis; H.C.B. and A.S.C. performed protein mass spectrometry processing and analysis; M.O. performed statistical analyses; M.C.S.M. supported results interpretation and presentation; C.C. co-supervised the experimental design and results interpretation; B.C.-S. and R.M. conceived and supervised the project. All authors have read and agreed to the published version of the manuscript.

Abstract: (1) Background: Pancreatic ductal adenocarcinoma (PDAC) is expected to be the second-leading cause of cancer deaths by 2030. Imaging techniques are the standard for monitoring the therapy response in PDAC, but these techniques have considerable limits, including delayed disease progression detection and difficulty in distinguishing benign from malignant lesions. Extracellular vesicle (EV) liquid biopsy is an emerging diagnosis modality. Nonetheless, the majority of research for EV-based diagnosis relies on point analyses of EVs at specified times, while longitudinal EV population studies before and during therapeutic interventions remain largely unexplored. (2) Methods: We analyzed plasma EV protein composition at diagnosis and throughout PDAC therapy. (3) Results: We found that IgG is linked with the diagnosis of PDAC and the patient's response to therapy, and that the IgG+ EV population increases with disease progression and reduces with treatment response. Importantly, this covers PDAC patients devoid of the standard PDAC seric marker CA19.9 expression. We also observed that IgG is bound to EVs via the tumor antigen MAGE B1, and that this is independent of the patient's inflammatory condition and IgG seric levels. (4) Conclusions: We here propose that a population analysis of IgG+ EVs in PDAC plasma represents a novel method to supplement the monitoring of the PDAC treatment response.

Keywords: pancreatic cancer, extracellular vesicles, liquid biopsy, biomarker, IgG

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) was the seventh cause of cancer-related fatalities globally in 2020 [1]. As reported by the Surveillance, Epidemiology, and End Results Program (SEER) [2], the incidence of PDAC has increased over the past seven years [3], being projected to be the second-

leading cause of cancer-related deaths by 2030 [4]. The majority of metastatic patients continue to have median survival outcomes of less than one year [5,6], with less than 30% of patients eligible for second-line chemotherapy [7].

Imaging techniques based on the response evaluation criteria in solid tumors (RECIST) criteria [8], which evaluate the number and size of tumor lesions over the course of treatment, remain the gold standards for determining treatment response during chemotherapy. However, these techniques have several drawbacks, including a lack of precision in detecting small tumors and an inability to differentiate between benign inflammatory and malignant lesions. This also includes the necessity to arbitrarily target lesions to evaluate the evolution of the disease in accordance with the treatment, and the usual delay between the imagiological response and the real-time detection of differences in tumor dimensions [9]. In addition, the evaluation solely based on dimensions does not consider changes in tumor attenuation, nor does it discriminate viable cells from non-viable ones, thus complicating the measurement of the treatment response [10].

Serological markers, such as carbohydrate antigen 19.9 (CA19.9), are widely utilized to supplement the ability of RECIST to assess PDAC treatment response [11]. When elevated at the start of treatment, CA19.9 demonstrates dependable outcomes in conjunction with imaging evaluation [12]. However, the CA19.9 evaluation in PDAC patients has limitations, since it is not expressed in 5–20% of patients [12,13]. In addition, false positives are common, especially in patients with obstructive lesions of the biliary tract, which may affect up to 70% of PDAC patients [14].

Due to the aforementioned constraints, it is of the utmost need to develop additional techniques for assessing the therapy response of PDAC. Studies using potential new response indicators, including circulating tumor cells and

circulating tumor DNA, have previously been conducted to uncover new readouts [15]. This also includes our recent findings that plasma extracellular vesicles (EVs) can detect incidence [16], predict progression [17], and locate [18] metastases in PDAC patients. However, the majority of the current research lacks longitudinal information regarding the response to therapy and clinical follow-up.

EVs are nanovesicles that contain all the active biomolecules, including nucleic acids, lipids, and proteins [19]. EVs are critical mediators of cell-to-cell communication under physiological and pathological settings [20]. Studies using EVs as cancer biomarkers usually focus on specific disease timepoints, disregarding potential changes in the molecular composition and population dynamics of EVs during the course of the disease, and investigating the utility of EVs exclusively for prognosis or diagnosis [21]. Moreover, studies of EV biomarkers for follow up of metastatic PDAC patients have been limited by challenges involving their dismal prognosis, which complicates patient recruitment for longer longitudinal studies.

In this study, we characterized EVs obtained from metastatic PDAC patients who were monitored throughout their treatment and the evolution of the disease. We identified a population of plasma EVs bound to IgG (IgG+ EVs) that is connected with the diagnosis of PDAC and the treatment response of PDAC patients (including those without CA19.9 expression). We demonstrated that longitudinal examination of IgG+ plasma EVs may represent a new tool for enhancing the efficacy of chemotherapeutic treatments in patients with PDAC by improving the detection of therapy response and resistance.

2. Materials and Methods

2.1. Patients and Controls

Patients were recruited between May 2016 and October 2019. Patients were eligible to participate in this study if they had a confirmed histological or cytological diagnosis of metastatic PDAC, according to the 8th edition of AJCC Cancer Staging [22], and were able to receive chemotherapy. Samples from healthy individuals were collected from voluntary donors at the Champalimaud Clinical Center and the Champalimaud Research (Champalimaud Foundation, Lisbon, Portugal). Exclusion criteria were previous diagnosis of other tumors or inflammatory diseases. Written informed consent was obtained from both patients and control individuals, and samples were deidentified for confidentiality. All the procedures were conducted in accordance with the Helsinki Declaration and its amendments, with the approval of the Ethical Committees of Nova Medical School and of the Champalimaud Foundation.

Treatment proposals were presented to the multidisciplinary tumor board. Patients were treated with a sequence of chemotherapy in the following approved regimens: association of 5-fluorouracil, irinotecan, and oxaliplatin (FOLFIRINOX); association of gemcitabine and nab-paclitaxel; association of 5-fluorouracil and liposomal irinotecan; or single gemcitabine. The decision regarding the choice of the chemotherapy regimen was at the discretion of the treating oncologist. Demographic and clinical information from the patients, including neutrophil-to-lymphocyte ratio (NLR) and C-reactive protein (CRP) levels, was collected. Treatment response was classified according to the imaging response based on RECIST v1.1 criteria [8]. Timepoints were selected as follows: timepoint I (pretreatment) was collected before the beginning of a new chemotherapy regimen, and timepoint II (posttreatment) at the time of either the best imaging response (if the tumor responded to chemotherapy) or the worst (if the tumor did not respond to chemotherapy). The same patient

can be a responder at one point of treatment and a nonresponder at another. The first samples of one patient that met these criteria were selected for the study until the calculated minimum number of total required samples was obtained.

A total of 5 of the 19 patients who participated of our study were used exclusively for the proteomic analysis of EVs, as there were no samples left for the subsequent experiments. The remaining 14 patients, with a total of 155 plasma samples, were studied by vesicle flow cytometry. **Fig. 11** provides information on the metastatic profile, treatment regimens, and sample collection times. For the control group, we applied the same exclusion criteria (previous oncological diagnosis or previous inflammatory diseases). The group included 21 individuals (ten males and eleven females) with a mean age of 47 years (minimum of 25 years; maximum of 85 years).

2.2. Purification and characterization of EVs from plasma

Blood samples from patients and healthy donors were collected in 9 mL Vacuette NV EDTA K3 tubes and centrifuged twice at 10 °C (500 g for 10 min and 3000 g for 20 min). Prior to analysis, plasma samples were aliquoted and stored at -80 °C. A protocol we previously described involving sequential ultracentrifugation combined with a sucrose cushion was used to purify the EVs [23].

All EV samples were analyzed by nanoparticle tracking analysis (NTA) using a NanoSight NS300, equipped with a red laser (638 nm), to determine particle concentration and size distribution (Malvern Panalytical, Malvern, UK). Samples were pre-diluted in filtered PBS to achieve a concentration within the range for optimal NTA analysis. Video acquisitions were performed at 25 °C using a camera level of 16, and a threshold between 4 and 6. Five videos of

30 s with 10–50 particles per frame were captured per sample. The total protein content of the EV samples was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).



2.3. Characterization of EV protein composition by Mass Spectrometry (MS)

For evaluation by MS, plasma-derived EV samples from PDAC patients were used. Four patients who responded, and four patients who displayed disease progression were selected. For each patient, samples were collected at diagnosis and after treatment response evaluation, totaling 16 samples. In parallel, 5 plasma-derived EV samples from healthy controls were also compared to 8 PDAC patients at diagnosis.

The EV solution containing sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) was loaded onto filtering columns and washed exhaustively with 8 M urea in HEPES buffer [24]. The proteins were reduced with DTT and alkylated with IAA. Protein digestion was performed by overnight digestion with trypsin sequencing grade (Promega).

Peptides samples were analyzed by nano-LC-MSMS (Dionex RSLCnano 3000) coupled to an Exploris 480 Orbitrap mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) virtually, as previously described [25]. Briefly, samples were loaded (flow rate 5 μ L per minute for 6 min) onto a custom-made fused capillary pre-column (2 cm length, 360 μ m OD, 75 μ m ID) packed with ReproSil Pur C18 5.0 μ m resin (Dr. Maish, Ammerbuch-Entringen, Germany), followed by the separation on a custom-made fused capillary column (25 cm length, 360 μ m outer diameter, 75 μ m inner diameter) packed with ReproSil Pur C18 1.9- μ m resin (Dr. Maish, Ammerbuch-Entringen, Germany) using a flow rate of 250 nL per minute. The gradient was from 89% A (0.1% formic acid) to 32% B (0.1% formic acid in 80% acetonitrile) over 56 min. The mass spectra were acquired in positive ion mode by applying an automatic data-dependent switch between one Orbitrap survey MS scan in the mass range of 350–1200 m/z, followed by a higher-energy collision dissociation (HCD) fragmentation and Orbitrap detection of fragment ions, with a cycle time of 2 s between each

master scan. MS and MSMS settings: the maximum injection times were set to “Auto,” the normalized collision energy was 30%, the ion selection threshold for MSMS analysis was 10,000 counts, and the dynamic exclusion of sequenced ions was set to 30 s.

Data obtained from 46 LC-MS runs of 21 subjects and 32 LC-MS runs of IgG bound proteins were searched using VEMS [26,27] and MaxQuant [28]. A standard proteome database from UniProt (3AUP000005640), in which common contaminants were included, was also searched. Trypsin cleavage, allowing a maximum of 4 missed cleavages, was used. Carbamidomethyl cysteine was included as a fixed modification. Methionine oxidation and N-terminal protein acetylation were included as variable modifications; 5 ppm mass accuracy was specified for precursor ions and 0.01 m/z for fragment ions. The FDR for protein identification was set at 1% for peptide and protein identifications. No restriction was applied to the minimal peptide length for the VEMS search. Identified proteins were divided into evidence groups, as defined in [27]. Functional pathway analysis was performed with STRING (string-db.org).

2.4 Western blotting

Western blotting was used to assess the presence of EV and non-EV protein markers. Equal protein amounts of EV samples were mixed with 4X Laemmli buffer (Bio-Rad, Hercules, CA, USA), denatured for 5 min at 95 °C, and loaded onto 4–20% Mini-PROTEAN TGX Stain-Free Protein Gels (Bio-Rad). SDS-PAGE was run for 1.5 h at 90 V, and then proteins were transferred to nitrocellulose membranes (Cytiva) at 100 V for 1 h. Membranes were blocked with LI-COR Intercept Blocking Buffer (LI-COR Biosciences) for 1 h at RT. Blocked membranes were incubated overnight at 4 °C with primary antibodies diluted in LI-COR blocking buffer with 0.1% Tween-20. Membranes were washed with TBS-T (TBS with 0.1% Tween-20) 3 times for 5 min, and then incubated with

secondary antibodies for 1 h at RT. Incubation was followed by 3 additional washes with TBS-T, 5 min each. Blots were imaged using the Odyssey Infrared Imaging System (LI-COR Biosciences). The detailed list of primary and secondary antibodies used is provided in **Supplementary Table 10**.

2.5. Analysis of IgG+ EV population by vesicle flow cytometry

Flow cytometry analysis of plasma EVs was performed as described by our group [29]. A volume of plasma containing 2×10^9 particles was used for staining with 0.5 μ L of anti-IgG (Abbexa abx142503, Houston, TX, USA) in PBS, in a final volume of 40 μ L, and incubated for 1 h at 37 °C. The antibody-stained sample was then incubated with Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE—Thermo Fisher Scientific LTI C34554, Waltham, MA, USA) to a final concentration of 25.6 μ M, for 90 min at 37 °C. For the removal of unbound CFSE and antibody, Size Exclusion Chromatography (SEC) columns (iZON qEV original columns SP1, UK) were used. EV-enriched fractions #7, #8, and #9 were then pooled (total of 1500 μ L) and retrieved for analysis with the flow cytometer Apogee A60-Micro-Plus (Apogee Flow Systems, Hertfordshire, UK), configured as described in **Supplementary Table 11**. For all subsequent analyses, quadrant thresholds were established with unstained and single-stained extracellular vesicles (with CFSE or with anti-IgG) (**Supplementary Fig. 3**).

Internal controls across assays were performed as previously described [29]. The acquired data was exported and analyzed with FlowJo software v10.4.2 (FlowJo LLC, Ashland, OR, USA).

2.6. Total plasma immunoglobulin G quantification

For the quantification of the plasma IgG, samples were processed using the nephelometry method (BNProSpec—Synlab, Lisbon, Portugal). The reference values for healthy controls were 700–1 600 mg/dL.

2.7. Identification of EV surface proteins associated with IgG

To identify which surface proteins of EVs bind to IgG, 200 µg of EVs isolated from 8 PDAC patients and 8 healthy controls were first biotinylated (EZ-Link™ Sulfo-NHS-SS-Biotin, Thermo Scientific, 21331) and then lysed using 0.5% NP-40 lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 0.5% NP-40). Next, the biotinylated surface proteins of EVs were collected using streptavidin magnetic beads [30] (Dynabeads™ MyOne™ Streptavidin C, Invitrogen, 65001), and then detached from the beads (deionized water, 70 °C) [31]. After separation, surface EV proteins that were associated with IgG were co-immunoprecipitated using magnetic beads (Dynabeads™ Protein G for Immunoprecipitation, Invitrogen, 10003D), conjugated with anti-human IgG antibody (Goat anti-Human IgG F(ab')₂ Secondary Antibody, Invitrogen, 31122), then eluted and analyzed by MS.

2.8. Statistical analysis

The sample size was based on previous liquid biopsy studies [32]. Our study involves 155 observations from 30 different situations (15 responders x 15 nonresponders). Experiments were not randomized. The researchers were blinded to allocation during the experiments and the outcome assessment. The response evaluation to the treatment was previously performed by a different researcher. Error bars in graphical data represent means ± standard errors of the means (SEM). Normality and homogeneity of variances from the analyzed variables were tested with the Shapiro–Wilk test and the Bartlett or Levene tests, respectively. If data were parametric, Student's t tests (two populations) were used. If data were not parametric, Wilcoxon or Mann–Whitney tests were performed. For ROC analysis, values were obtained from the division of IgG+ EVs and CA19.9 readings after treatment by the values obtained before treatment in each studied point, as previously described [11]. The statistical packages used was R v.4.0.2. For all evaluations, a p-value

under 0.05 was considered statistically significant, and the null hypothesis was rejected. Graphical design was performed with the GraphPad Prism software (GraphPad software).

3. Results

3.1. Identification of possible EV markers for PDAC diagnosis and therapeutic response

The size distribution and concentration of plasma EVs isolated from patients and healthy controls were characterized. Proteins frequently present or absent in small EVs were measured in our samples (**Supplementary Fig. 4**). We next performed MS analysis of plasma EV samples from 5 healthy controls and 16 samples from 8 PDAC patients, both at the time of diagnosis and after treatment. A total 4 of these 8 patients were considered chemotherapy responders, as tumor shrinkage was observed between the diagnosis and treatment timepoints. In contrast, based on the observed imaging progression of the disease between the 2 time points, the remaining 4 patients corresponded to nonresponders to chemotherapy. For the MS analysis, the same amounts of protein (20 µg) and concentrations (0.5 µg/µL) were utilized.

Protein expression analysis revealed that 102 distinct proteins exhibited statistically significant differences between PDAC patients and healthy controls, 59 of which were upregulated in PDAC patients. Of these, we identified the presence of multiple IgG fragments (**Fig.12A, Supplementary Table 12**). In fact, the functional analysis of proteins significantly upregulated or downregulated in EVs from PDAC patients (responders and nonresponders) and as compared to healthy controls, revealed enrichment in proteins associated with humoral immune response and complement activation, among others (**Fig. 13**).

We were also interested in identifying treatment response indicators. By comparing EVs isolated from PDAC patients who responded to therapy with those who did not, we identified 43 proteins that exhibited statistically significant differences between responders and nonresponders, with 24 of these upregulated in nonresponders (**Fig. 12B, Supplementary Table 13**). We found that 16 of the upregulated proteins in patients who did not respond to chemotherapy were IgG fragments. As a result, we chose to further investigate whether the presence of IgG in populations of plasma EVs is indeed associated with PDAC diagnosis and therapeutic response.

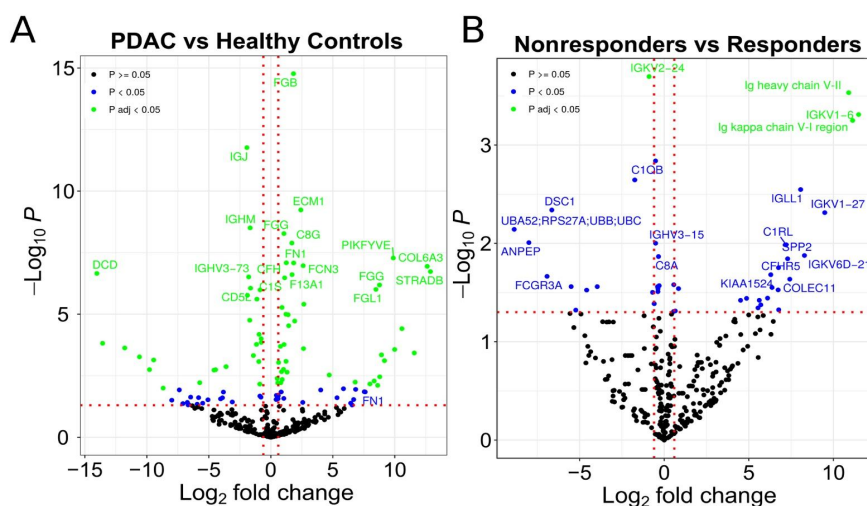


Figure 12. Proteins differentially expressed in EVs. (A) Volcano plot representing the identified proteins in MS (comparison between patients (PDAC) and healthy control samples). (B) Volcano plot representing the identified proteins in mass spectrometry (comparison between patients that are nonresponders and responders to chemotherapy). The green points represent proteins significantly regulated after correction for multiple testing. The blue points represent proteins significantly regulated without correction for multiple testing. Black points represent proteins with insignificant regulation.

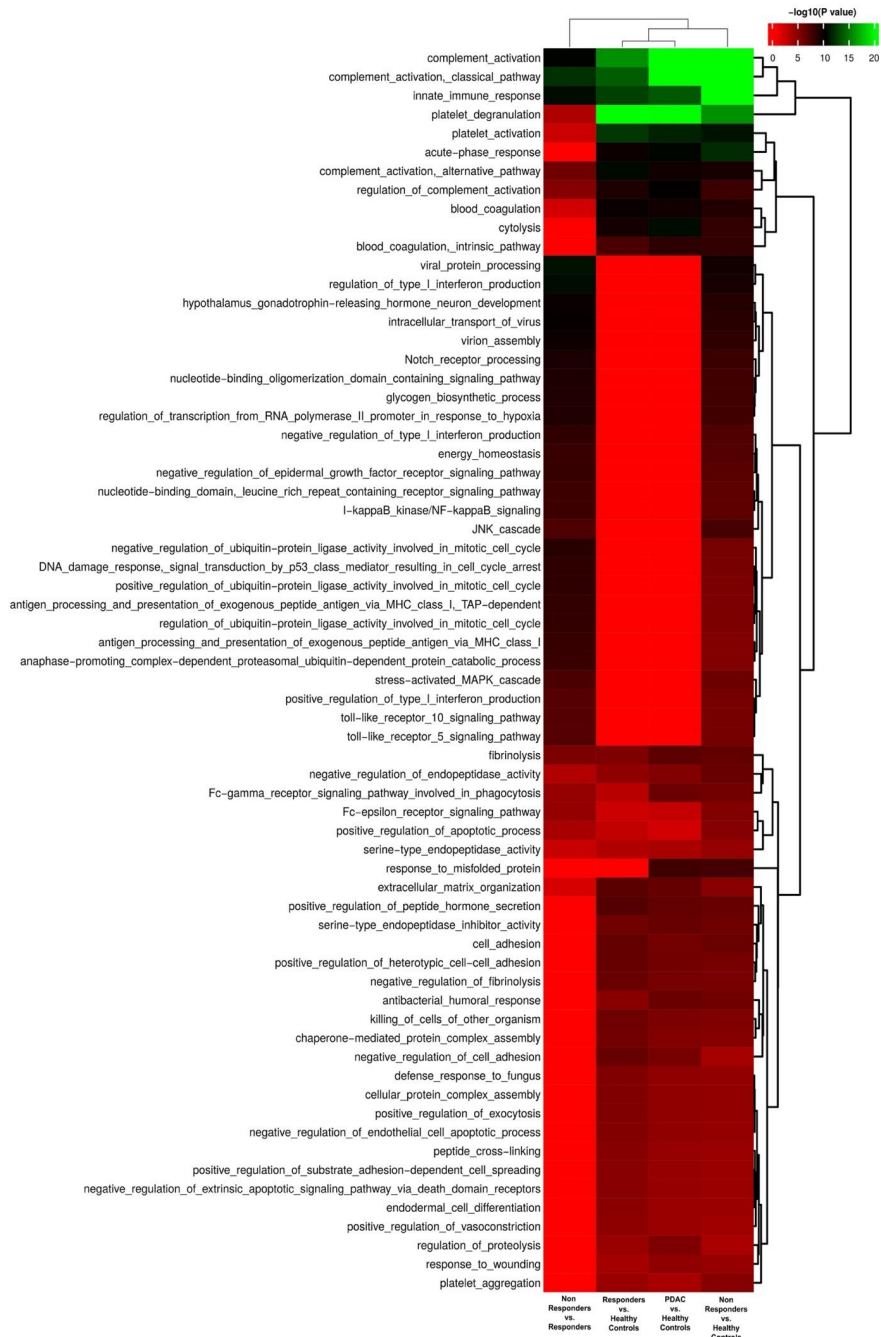


Figure 13. Functional enrichment of significantly regulated EV proteins based on biological processes.

3.2. Evaluation of IgG+ EVs as possible markers of PDAC treatment response

In accordance with the MS findings, vesicle flow cytometry revealed that metastatic PDAC patients have a larger population of IgG+ EVs compared to healthy control donors (**Fig. 14A**), suggesting that IgG+ EVs may be a useful diagnostic marker for advanced PDAC disease. Next, we examined whether this IgG+ EV population could be used to determine whether a patient is responding to chemotherapy or not.

At and after diagnosis, prospective clinical information, CT data, serum levels of CA19.9, hemograms, and serial whole-blood results were collected from patients with stage IV PDAC. To be considered suitable in clinical practice for the care of metastatic PDAC patients, a new marker should: (a) be consistent, independent of the treatment of choice; (b) be able to identify differences in patients with and without CA19.9 expression; and (c) be able to predict treatment response in comparison to the imagiological evaluation, in order to reinforce the maintenance of applied treatment or to suspend futile treatments early [33, 34]. Keeping this in mind, we analyzed the proportion of IgG+ EVs in the plasma of multiple patients who had received at least two lines of chemotherapy (FOLFIRINOX—first line; Gemcitabine/nabpaclitaxel—second line). In contrast to the increase in IgG+ EV populations in the plasma of PDAC patients during tumor progression (nonresponse), the monthly evaluation of IgG+ EV populations in the plasma of PDAC patients (**Fig. 14B**) revealed a decrease in IgG+ EVs upon imagiological response.

To determine whether IgG+ EVs meet the criteria for independence from CA19.9 expression, we performed the same monthly IgG+ EV analysis on two patients without CA19.9 expression (patients 8 and 63). The same pattern was observed as for patients with CA19.9 expression (patients 15, 23, 34 and 49), suggesting that the evaluation of IgG+ EVs as a marker of response to therapy

may be applicable to all metastatic PDAC patients (**Fig. 14B**). In addition, we compared the measurement of IgG+ EVs to that of CA19.9, the gold standard serological marker in PDAC [12]. We observed a significant downregulation of CA19.9 in patients who responded to therapy (**Supplementary Fig. 5A**) while no significant upregulation of CA19.9 was observed in nonresponders (**Supplementary Fig. 5B**).

In addition to the individual longitudinal analysis of patients, we grouped chemotherapy response and nonresponse timepoints to investigate the population of IgG+ EVs in responders and nonresponders. As shown in **Fig. 14C**, we observed a significant decrease in the IgG+ EV population in responders following treatment. In contrast, the proportion of IgG+ EVs significantly increased in patients who did not respond to chemotherapy (**Fig. 14D**). The same patterns of response were observed with CA19.9, as predicted. The evaluation of sensitivity and specificity of IgG+ EV for response evaluation of patients with metastatic PDAC showed an AUC of 0.8311 (95% confidence interval 0.6788 to 0.9834, $p = 0.0020$) (**Fig. 14E**), compared to an AUC of 0.9911 (95% confidence interval 0.9679 to 1.00, $p < 0.001$) for the same evaluation for CA19.9 in our population (**Supplementary Fig. 5C**).

Our findings suggest that levels of IgG+ EVs are associated with imaging evaluations of clinical response to treatment, suggesting that IgG+ EVs could be used as a readout in PDAC clinical settings.

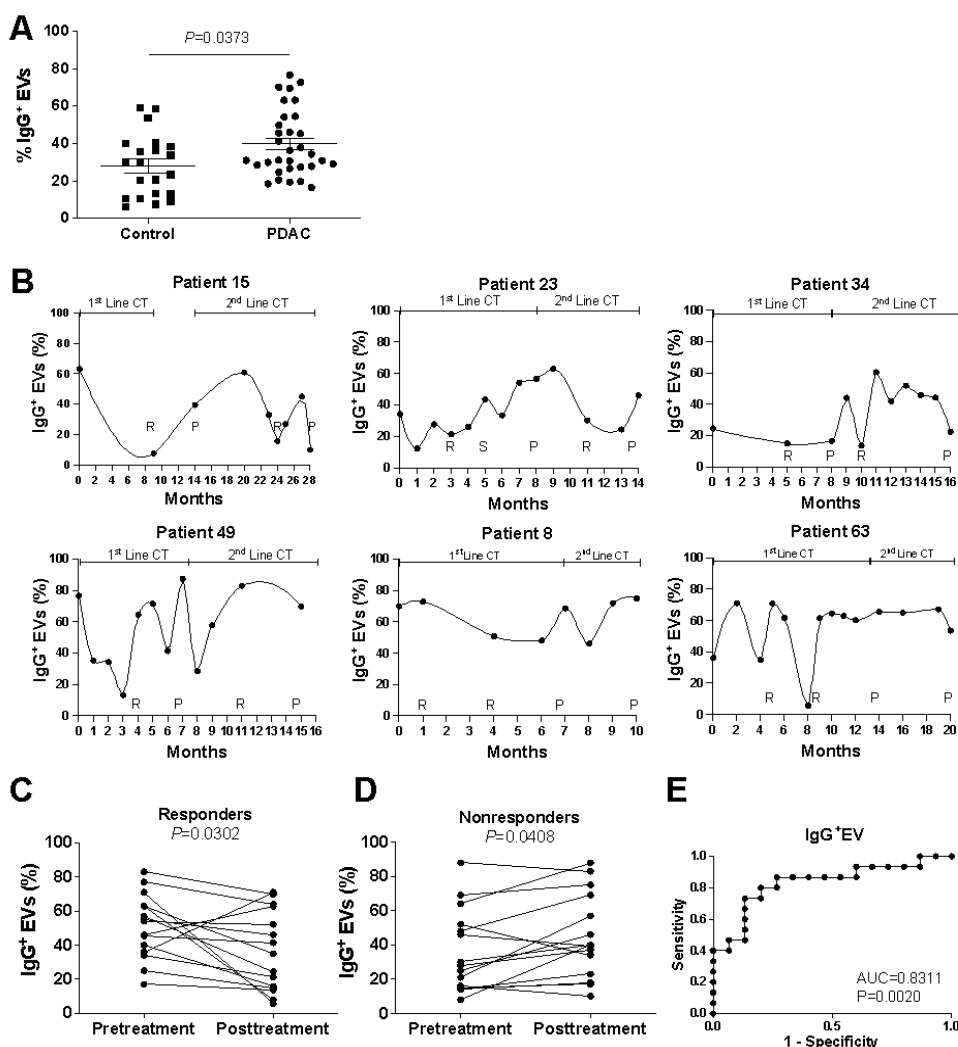


Figure 14. IgG⁺ EVs in healthy controls and PDAC patients. Vesicle flow cytometry data of plasma samples from PDAC patients and healthy controls, as indicated. (A) Comparison of IgG⁺ EVs between PDAC patients and healthy controls, $p = 0.0373$, by Wilcoxon test. (B) Longitudinal evaluation of plasma IgG⁺ EVs in patients followed during at least 2 lines of chemotherapy (mFOLFIRINOX in the 1st line, and Gemcitabine with Nabpaclitaxel in the 2nd line, as indicated), that express CA19.9 (patients 15, 23, 34, and 49); or do not express CA19.9 (patients 8 and 63). The moments of imagiological evaluation are indicated (R for response, S for stabilization, and P for progression to chemotherapy). (C,D) Evolution of IgG⁺ EVs before and after treatment with chemotherapy in 15 situations of response (patients 15, 23, 34, 49, 63, 69, 70,

73, and 94) (C) and no response to treatment (patients 8, 10, 15, 21, 22, 23, 34, 49, 66, and 73) (D). $p = 0.0302$ (C) and $p = 0.0408$ (D), by t-test. (E) ROC curve for the IgG+ EV to discriminate response in patients with metastatic PDAC.

3.3. Evaluation of the potential role of plasmatic IgG levels and inflammation status in IgG+ EV proportion

The fluctuations in the plasmatic proportion of IgG+ EVs could, in principle, be a direct result of variations in plasma IgG [35] levels. To test this hypothesis, the total amount of plasmatic IgG was measured in the same set of patients. We found no significant differences in plasmatic IgG levels during treatment response or disease progression in patients with PDAC (**Supplementary Fig. 6A and 6B**). We found no correlation between the percentage of IgG+ EVs and total plasmatic IgG levels, indicating that the variation in IgG+ EVs is not due to fluctuations in plasmatic IgG levels (**Supplementary Fig. 6C**).

Alternately, IgG+ EVs fluctuations could derive, at least in part, from alterations in the inflammatory status of immune cells during the progression of PDAC. The neutrophil/lymphocyte ratio (NLR) is a clinical marker of inflammation calculated as the quotient of the absolute neutrophil and lymphocyte counts [36]. In light of this, we found no differences between the groups (responders and nonresponders) and no correlation between levels of IgG+ EVs and NLR. In addition, we found no correlation between the levels the inflammatory marker CRP [37] and the levels of IgG+ EVs (**Supplementary Fig. 7**), further suggesting that in the context of our study, the inflammatory status has no effect on IgG+ EV levels.

3.4. Identification of IgG-associated proteins on the surface of PDAC patient EVs

Next, we investigated potential differences in the molecular mechanisms of IgG transport by plasma EVs between healthy controls and PDAC patients. To

test this, we devised a method for characterizing protein interactions at the surface of the EVs. Briefly, biotinylated surface proteins of lysed EVs were isolated by streptavidin binding, followed by co-immunoprecipitation with an antigen-specific antibody. Here, we precipitated IgG from the mixture and searched for EV surface proteins bound to IgG.

Several IgG fragments were detected in both healthy controls and PDAC patients. In addition, soluble proteins (alpha-2-macroglobulin), cell surface receptors (i.e., glutamine receptor), cytoskeletal proteins (i.e., keratin), and cytoplasmic proteins were detected in both groups (e.g., Chloride intracellular channel protein 4, Golgi integral membrane protein 4, Chondroitin sulfate synthase 3, and Protein Argonaute 2). IgG was bound to melanoma associated antigen B1 (MAGE B1) in the EVs of PDAC patients, in addition to IgG fragments and albumin. MAGE B1 is a well-known PDAC antigen [38], suggesting that the population of IgG+ EVs described here is the consequence of an interaction between tumoral antigens on the surface of EVs released by tumor cells and IgG in circulation (**Table 7**).

4. Discussion

Currently, the assessment of treatment response in patients with metastatic PDAC relies mainly on CA19.9 and imaging evaluations, both of which have limitations. A total of 5–20% of PDAC patients lack CA19.9, and false-positive elevations are associated with biliary tree inflammation or infections [12,13]. On the other hand, imaging evaluation cannot detect small lesions and is not an immediate indicator of the tumor's status, as there is a delay between the progression of the disease and its imagiological identification [8,10]. By characterizing a population of plasma extracellular vesicles (IgG+ EVs) that correlates with the treatment response status of metastatic PDAC patients, we

have described a potentially new tool to complement treatment response evaluation.

EVs have the potential to carry biomarkers for liquid biopsies, in both oncologic and non-oncologic diseases [39], due to their abundance in body fluids. EVs produced by tumor cells can populate and alter the composition of biofluids. As a result, the identification of molecular modifications in EVs associated with tumor profile has been utilized to identify putative cancer biomarkers[19,40], including the diagnosis of PDAC [41]. The majority of these studies, however, have focused on the differential expression of specific nucleic acids and proteins in bulk EV samples for diagnosis or prognosis, using single collections from each patient [42]. In cancer patients, longitudinal studies like ours evaluating the response to chemotherapy in a single individual are still scarce [43,44]. To our knowledge, this is the first study to examine the dynamics of circulating EV populations in patients with metastatic PDAC.

The analysis of EV bulks is an effective method for identifying molecules of interest (e.g., proteins, lipids, and RNA) in EV liquid biopsies. In fact, we relied on MS analysis of EVs in bulk to identify IgG as a possible EV marker of therapeutic response in PDAC patients. However, failure to distinguish between EV populations may obscure real differences between experimental groups. Moreover, the implementation of EV biomarkers in clinical practice is hindered by the laborious and time-consuming isolation and analysis protocols commonly employed for EVs. In an effort to characterize populations of extracellular vesicles (EVs) using a method that permits rapid analysis of markers in EV samples, we utilized a vesicle flow cytometry protocol described by our group [29]. By not requiring EV isolation prior to analysis, the processing time is reduced from >24 h to 4 h. Therefore, the use of vesicle flow cytometry has the potential to facilitate the clinical evaluation of IgG+ EVs.

Table 7. IgG associated proteins identified by MS.

Cancer Patients

Immunoglobulin lambda variable 3-21
 Melanoma-associated antigen B1
 Albumin
 Probable non-functional immunoglobulin heavy variable 3-16

Healthy + Cancer

Keratin, type II cytoskeletal 1
 Chloride intracellular channel protein 4
 Alpha-2-macroglobulin
 Glutamate receptor ionotropic, kainate 3
 Keratin, type II cytoskeletal 6B
 HUMAN Ig kappa chain V-I region Lay
 HUMAN Ig kappa chain V-III region NG9 (Fragment)
 HUMAN Ig kappa chain V-III region POM
 HUMAN Ig kappa chain V-III region CLL
 HUMAN Ig kappa chain V-III region VH (Fragment)
 Immunoglobulin kappa variable 3D-7
 Immunoglobulin kappa variable 3/OR2-268 (non-functional)
 Immunoglobulin kappa constant
 Immunoglobulin kappa variable 3-15
 Probable non-functional immunoglobulin kappa variable 3-7
 Ig lambda chain V-II region NIG-84
 Chondroitin sulfate synthase 3
 Ig lambda chain V-II region BUR
 Keratin, type I cytoskeletal 10
 Protein argonaute-2
 Golgi integral membrane protein 4

Healthy Donors

S100A9
 Ig kappa chain V-III region Ti
 Uncharacterized protein (Fragment)

Although the main objective of our work was to identify a novel biomarker to monitor the therapy response of diagnosed metastatic PDAC patients, instead of identifying a new marker to diagnose PDAC, we found elevated levels of IgG+ EVs in PDAC patients compared to healthy controls, reinforcing previous descriptions of the potential application of this marker for PDAC diagnosis [45]. In our study, we found that levels of IgG+ EVs did not correlate with validated clinical markers for inflammation (NLR and CRP), suggesting that this biomarker is not affected by the inflammatory background of the patients. However, as the recruitment criteria for our study excluded individuals with inflammatory conditions, further study will be necessary to address the potential impact of an inflammatory background on the specificity of IgG+ EVs as a diagnostic marker for PDAC.

In our longitudinal studies, we were able to observe the dynamics of IgG+ EV populations within each PDAC patient and their association with the evaluation of chemotherapy response. We also demonstrated that the study of IgG+ EV populations may be utilized in the follow-up of PDAC patients, including those who lack CA19.9 expression. Due to the absence of this established marker, these patients rely solely on imaging evaluations to determine their clinical response to chemotherapy; therefore, a new reliable marker would represent a substantial improvement in their care.

During the characterization of proteins bound to IgG in circulating EVs, a number of proteins were identified in both healthy donors and PDAC patients. This suggests that at least some IgGs bind to EVs via protein-protein interactions that may occur after EVs are secreted by cells. Consequently, circulating IgG and IgG ligands in EVs may interact in the extracellular environment of both cancer patients and healthy individuals [46]. This is further supported by previous studies on autoimmune diseases, showing that immunoglobulins can bind to circulating EVs and form immune complexes that

contribute to disease pathology [47,48,49,50]. In fact, the composition of EVs is not merely a result of their intracellular biogenesis, since their surface is highly interactive with proteins present at the extracellular milieu. The interaction of EVs with secreted proteins has been shown to modulate their immune recognition, mobility, uptake, and signaling capabilities [51]. Remarkably, many proteins that frequently display quantitative changes in cancer patients, such as cytokines/chemokines [52], extracellular matrix proteins [53], coagulation factors [54], complement factors [55], immunoglobulins [56], and albumin [57], can interact with EVs after their release and change their composition [51].

The MS analysis of proteins bound to IgG focused on EV surface proteins, as only these proteins were biotinylated during the immunoprecipitation protocol. Moreover, as vesicle flow cytometry studies were performed with intact EVs, all IgG+ signals originated from IgG bound at the external face of the EV surface. This agrees with the conventional mechanism of IgG binding to the surface of cells, which depends on the binding of IgG to a cell membrane protein on B cells, the Ig α /Ig β heterodimer (CD79 α /CD79 β) [58,59]. Therefore, we consider that, as to CD79 α /CD79 β , at least part of the IgG anchorage on EVs relies on binding to surface proteins of EVs, such as MAGE B1. Although MAGE B1 was identified as an EV surface ligand of IgG in 8 different PDAC patients, further studies which include methods other than MS will be necessary to validate this finding in larger cohorts of PDAC patients. Importantly, although immunoglobulins were traditionally thought to be exclusively produced by B-lineage cells, recent studies have shown that these molecules can also be produced by a large diversity of tumor types [21], including PDAC [35,60,61]. Therefore, although the association with MAGE B1 suggests that IgG binding to circulating EVs may be the result of post-secretion interactions with tumor neoantigens present in tumor EVs, we cannot deny that at least part of the

circulating IgG+EVs associated with metastatic PDAC burden could potentially be packed and secreted at the surface of EVs directly by the PDAC cells.

MAGE B1 is a tumor antigen found in a variety of tumor types, including melanoma and tumors of epithelial origin, such as breast, colorectal carcinoma, lung, and pancreatic [38,62,63,64,65,66] tumors. In addition, MAGE is identified as an antigen normally expressed by the placenta and male germ cells in cancerous testes. It is expressed in 47% of pancreatic tumors [67], offering cells that express it a survival advantage [68] and negatively correlating with prognosis and patient survival [67,69]. MAGE B1 was identified as one of the proteins found exclusively in EVs from PDAC patients. In addition, we found that healthy controls had a significantly smaller IgG+ EV population, with the presence of IgG on the surface of these EVs being unrelated to neo-antigens such as MAGE B1 (**Table 7**). Although the correlation between IgG+ EVs and the response of PDAC patients to chemotherapy is insufficient to conclude that the IgG+ EV population, which varies based on chemotherapy response, is tumor derived, we propose that at least a portion of the IgG+ EVs upregulation during PDAC progression may be due to IgG binding with PDAC EVs expressing MAGE B1. Due to sample limitations, we were not able to further validate this result by additional methods. In spite of that, as the interaction of IgG with MAGE1 and other proteins was verified in all of the 8 different PDAC patients studied in our MS analysis, we are confident that our results are unlikely to be false-positive.

We also found that the proportion of IgG+ EV is independent of the availability of circulating IgG. Alternatively, this may be the result of elevated levels of tumor EV secretion and/or enhanced packaging of tumor antigens (such as MAGE B1) in PDAC EVs. Moreover, it is plausible that this process may result in tumor-directed IgG absorption by tumor EVs and, as a result, may contribute to tumor immune-escape [45] and the chronic inflammatory state observed in

metastatic PDAC patients [70]. The binding of IgGs to EVs could also affect the efficacy of targeted therapies (e.g., immunotherapies). Additional research will be required to fully comprehend the aforementioned implications.

5. Conclusions

We demonstrate that IgG attaches to the surface of EVs in PDAC patients via an interaction with the tumor antigen MAGE B1, and that this process is independent of IgG plasma levels and the inflammatory status of the patient. For the first time, a longitudinal population analysis of plasma EVs revealed that EVs bound to IgG increase during disease progression and decrease when patients react to chemotherapy. Importantly, IgG+ EVs can detect therapy response in a subset of individuals with PDAC who lack the standard PDAC marker, CA19.9. These findings not only have the potential to expand the current monitoring options for PDAC metastasis, but also represent a promising new tool for identifying effective treatments and indicating alternate treatments, in the event of disease progression. In addition, emerging markers of therapy response in PDAC, such as IgG+ EVs, should assist in separating PDAC patients into new groups, hence aiding the development of tailored, more effective treatments.

Author Contributions: N.C. recruited the patients, designed and performed the experiments. J.E. designed and performed the experiments J.M., S.B., C.E.P. contributed with the EV sample preparation and vesicle flow cytometry analysis. H.C.B. and A.S.C. performed protein mass spectrometry processing and analysis. M.O. performed statistical analyses. M.C.S.M. supported results interpretation and presentation. C.C. co-supervised the experimental design and results interpretation. B.C-S. and R.M conceived and supervised the project. All authors wrote and reviewed the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Champalimaud Foundations (project title: Estudo do papel de exosomas na oncopatologia e no diagnostic de tumors malignos). Informed consent was obtained from all patients; samples were deidentified for patient confidentiality.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest

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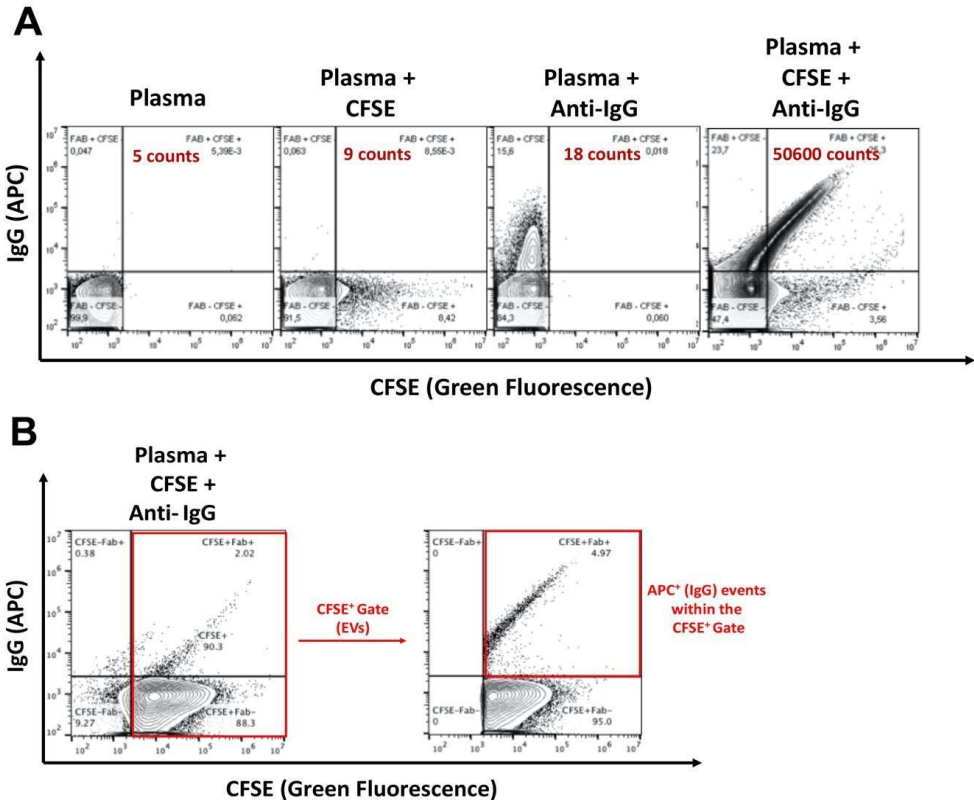
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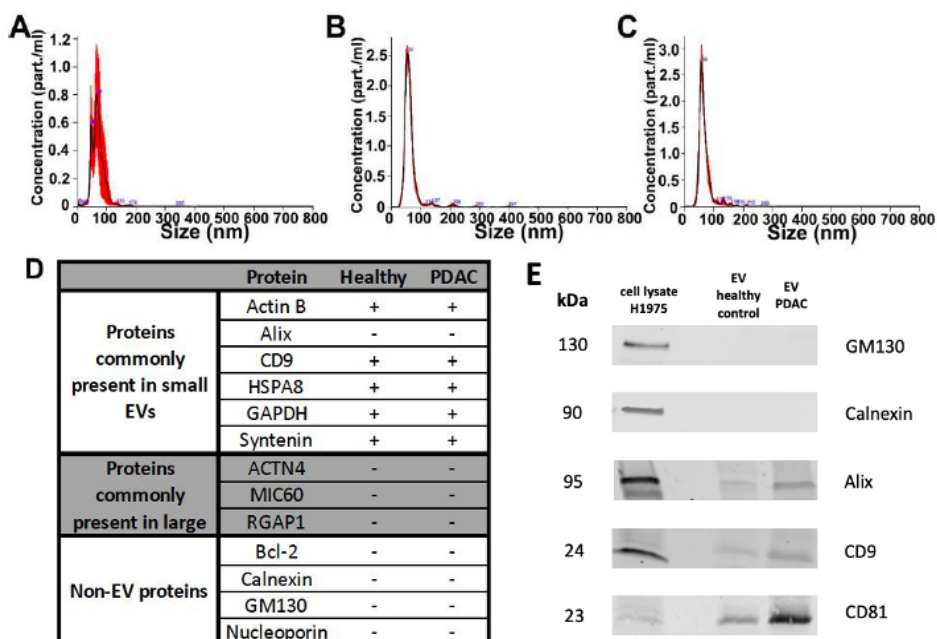
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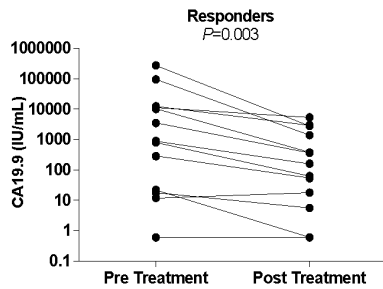
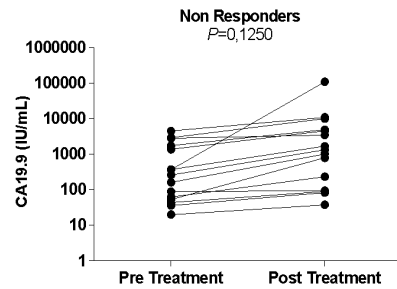
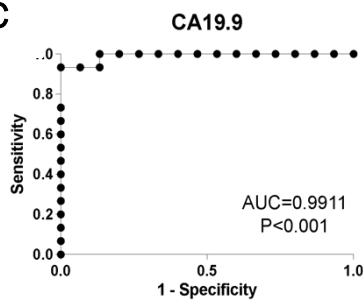
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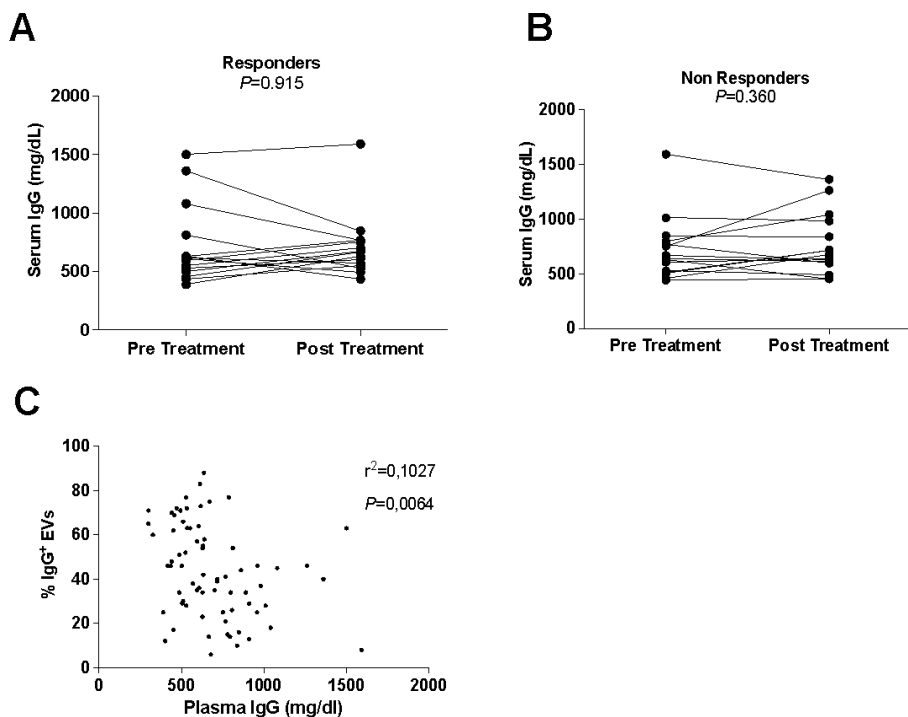
Supplementary Figure 3. Flow cytometry gating strategy. (A) Internal controls: representative plots of unstained plasma, plasma stained with CFSE, plasma stained with anti-IgG (FAB), and plasma stained with both CFSE and anti-IgG; the indicated counts correspond to the events within the upper right quadrant (CFSE⁺IgG⁺). (B) Characterization of EVs in plasma: representative plots of particles labeled with CFSE and anti-IgG (FAB) from plasma; the right panel indicates the IgG⁺ (top right quadrant) and IgG⁻ (lower right quadrant) events within CFSE⁺ particles.



Supplementary Figure 4. Characterization of EVs. Representative size distribution histograms by Nanoparticle Tracking Analysis (NTA) of samples isolated from healthy controls (**A**), and from PDAC patients at diagnosis that did not respond (**B**) or that responded (**C**) to therapy. (**D**) Panel depicting the presence status of the indicated proteins in isolated EVs. **E.** Representative Western blots of EV-markers CD9, CD81, Alix and non-EV markers GM130 and Calnexin for EV samples isolated from healthy control and PDAC plasma (H1975 cell lysate was used as a control).

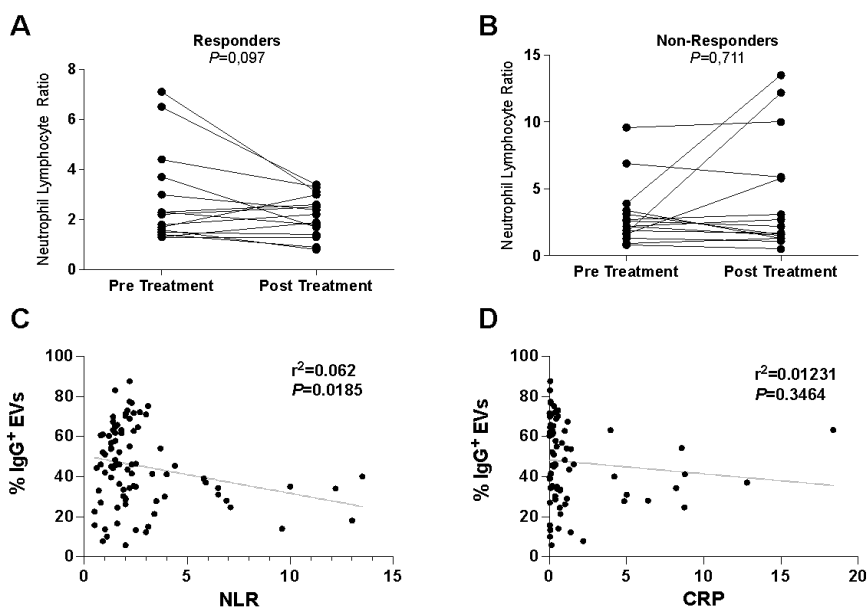
A**B****C**

Supplementary Figure 5. CA19.9 levels in PDAC patients. Evolution of CA19.9 before and after treatment with chemotherapy in responders (**A**) and non-responders (**B**). Depicted are the same patients and timepoints as in Figure 3 D and E. Wilcoxon test was used for statistical analysis. (**C**) ROC curve for the CA19.9 to discriminate response in patients with metastatic PDAC.



Supplementary Figure 6. Plasmatic IgG levels during treatment of PDAC patients.

Comparison between plasmatic levels of IgG before and after treatment with chemotherapy in responders, by Wilcoxon test **(A)** and non-responders, by t-test **(B)**. Depicted are the same patients and timepoints as in Figure 3 D and E. There were no significant differences in both groups. **(C)** Correlation of the same time points of plasmatic IgG (mg/dL) vs percentage of IgG⁺ EVs. Statistical analysis was performed by simple linear regression.



Supplementary Figure 7. Assessment of the inflammatory status of PDAC patients. Evolution of the neutrophil/lymphocyte ratio (NLR) between timepoints pre and post treatment with chemotherapy in responders, by t-test (**A**) and non-responders, by Wilcoxon test (**B**). Depicted are the same patients and timepoints as in Figure 3 D and E. There were no significant differences in both groups. Correlation of the same time points of IgG⁺ EVs vs NLR (**C**) and vs C-Reactive Protein (CRP) (**D**). Statistical analysis was performed by simple linear regression.

Supplementary Table 10. List of primary and secondary antibodies used for Western blotting.

Primary Antibodies					
Antibody			Manufacturer	Catalog no	Dilution/concentration
CD81			Santa Cruz Biotechnology	sc-166029	1:50
CD9			Cell Signaling	#13174	1:1000
Alix			Sigma Aldrich	SAB4200476	1.25 ug/mL
Calnexin			Abcam	ab22595	1:2000
GM130			Abcam	ab52649	1:1000
Secondary Antibodies					
Antibody			Manufacturer	Catalog no	Dilution
Goat	anti-rabbit	IgG	LI-COR Biosciences	926-32211	1:5000
IRDye 800CW					
Goat	anti-mouse	IgG	LI-COR Biosciences	926-32210	1:5000
IRDye 800CW					

Supplementary Table 11. Cytometer configuration and laser power

Channel number	Short Channel Name	Full Channel Name	Optical Filter Name	Laser Wavelength	Laser Power	PMT Voltage
Ch1	405-SALS	Small Angle Light Scatter		405 nm	200 mW	400 V
Ch2	405-LALS	Large Angle Light Scatter		405 nm	200 mW	400 V
Ch3	405-Gm	Green Fluorescence	BP-525/50	405 nm	200 mW	500 V
Ch4	405-Org	Orange Fluorescence	LWP- 590/35	405 nm	200 mW	500 V
Ch5	APC	Red Fluorescence	BP-676/36	638 nm	150 mW	550 V
Ch6	CFSE	Green Fluorescence	BP-525/50	488 nm	200 mW	525 V
Ch7	PE	Orange Fluorescence	BP-575/30	488 nm	200 mW	500 V
Ch8	488-Red	Red Fluorescence	BP-676/36	488 nm	200 mW	500 V
Ch9	488- DRed	Deep Red Fluorescence	LWP-740	488 nm	200 mW	500 V

Supplementary Table 12. EV proteins upregulated in patients with PDAC (vs. healthy controls).

Protein IDs	Gene names	Mean Healthy Controls	Mean PDAC	logFC	P.Value
P02675	FGB	30,80916	32,63287	1,82371 1	1,7E-15
P01591	IGJ	31,04684	29,11787	-1,92898	1,71E-12
Q16610;Q16610-4;Q16610-2;Q16610-3	ECM1	22,3285	24,74706	2,41856 3	5,83E-10
P01871;P01871-2	IGHM	32,80839	31,13976	-1,66863	3,11E-09
P02679	FGG	31,73688	32,79369	1,05681 1	5,31E-09
P07360	C8G	24,25857	25,95475	1,69618	1,28E-08
Q9Y2I7	PIKFYVE	8.610452	18,488365	9,87791 3	5,23E-08
P08603;P08603-2	CFH	25,70408	26,9523	1,24822	8,16E-08
P02751;P02751-8;P02751-3;P02751-15	FN1	29,12809	30,96719	1,83909 1	8,28E-08
O75636;O75636-2	FCN3	27,6463	30,25062	2,60432 2	1,07E-07
P12111-4;P12111-2;P12111-P12111-	COL6A3	0.7664544	13,379808 1	12,6133 5	1,14E-07

3;P12111-5					
Q9C0K7-3;Q9C0K7-2;Q9C0K7	STRADB	6.21537	19,08732	12,87195	1,86E-07
P81605;P81605-2	DCD	15,62981	1.59704	-14,0328	2,21E-07
P00488;P16452-3;P16452;P16452-2	F13A1	23,53023	25,2402	1,709968	2,47E-07
A0A0B4J1V6	IGHV3-73	24,99557	23,20821	-1,78736	3,07E-07
P09871	C1S	22,88309	23,99478	1,111685	3,33E-07
P02679-2	FGG	13,1101	21,87546	8,765356	6,49E-07
O43866	CD5L	29,42484	27,78712	-1,63772	8,76E-07
Q08830	FGL1	13,01631	21,4914	8,475092	9,83E-07
P0D0Y3		32,86217	31,9975	-0,86467	1,03E-06
P01709		27,85825	25,97132	-1,88692	1,71E-06
A0A0B4J1V0	IGHV3-15	25,76245	24,63909	-1,12337	2,45E-06
P00451;P00451-2	F8	18,60007	21,25979	2,659721	3,93E-06
P07358	C8B	23,55035	24,45246	0,902108	5,32E-06
P01011;P01011-2	SERPINA3	24,59445	25,81344	1,218999	1E-05
P01042	KNG1	22,39664	23,79217	1,395532	1,05E-05

014791;O 14791- 2;014791- 3	APOL1	26,07611	24,37361	-1,7025	1,76E-05
P04275;P 04275-2	VWF	27,3174	29,24253	1,92513 8	1,9E-05
P04196	HRG	24,01787	25,45884	1,44096 8	2,94E-05
P12109	COL6A1	5.053083	15,624026	10,5709 4	3,88E-05
P01780		29,81985	28,87501	-0,94484	6,6E-05
A0A087W W87;P016 14	IGKV2-40	28,98482	28,20821	-0,77661	9,96E-05
P06312	IGKV4-1	27,12003	26,34662	-0,77341	0,00014
P04211		23,414648	9.843152	-13,5715	0,00015 2
Q08380	LGALS3BP	27,12358	25,96748	-1,1561	0,00016 8
P13671	C6	22,07315	23,13764	1,06449 5	0,00017 1
P10909- 4;P10909; P10909- 5;P10909- 2;P10909- 3	CLU	26,44996	27,21772	0,76775 8	0,00020 3
A0M8Q6	IGLC7	16,343175	4.559787	-11,7834	0,00023 4
P02792	FTL	23,44187	26,07389	2,63202 1	0,00024 9

Q4LDE5;Q 4LDE5- 4;Q4LDE5- 3;Q4LDE5- 2	SVEP1	4.153104	14,13788	9,98477 7	0,00027 1
Q9HCU4	CELSR2	7.209845	18,779373	11,5695 3	0,00037 6
Q14520- 2;Q14520	HABP2	9.420802	18,345494	8,92469 2	0,00044 9
Q9Y6R7	FCGBP	22,3186	20,91387	-1,40473	0,00047 9
Q9NZT1	CALML5	15,381387	4.785034	-10,5964	0,00054 2
Q08554- 2;Q08554	DSC1	12,67823	3.226449	-9,45178	0,00072 3
Q96IY4;Q9 6IY4-2	CPB2	9.124999	18,288738	9,16373 9	0,00077 2
P01009;P 01009- 2;P01009- 3;P20848	SERPINA1	27,45175	28,67493	1,22317 7	0,00082 3
A0A0C4DH 68	IGKV2-24	27,98671	27,01923	-0,96748	0,00084
P02743	APCS	26,49926	27,12377	0,62451 1	0,00091 8
O43166	SIPA1L1	3,611292	0	-3,61129	0,00135 3
P01859	IGHG2	28,50515	29,53058	1,02543 7	0,00174 8
Q01469	FABP5	13,333104	3.547731	-9,78537	0,00178 5

Q562R1	ACTBL2	4,454414	0	-4,45441	0,00180 4
AOAOAOM RZ9	IGLV5-52	4,580166	0	-4,58017	0,00186 9
P01860	IGHG3	28,57457	29,43901	0,86444	0,00216 6
P02763	ORM1	23,88044	25,18618	1,30574	0,00226 3
P29622	SERPINA4	15,58681	20,29616	4,70935 4	0,00295 9
000189	AP4M1	8.074653	16,858605	8,78395 2	0,00348 9
P07357	C8A	24,73622	25,25004	0,51381 6	0,00406 4
P01594;P 01593		23,88627	24,80415	0,91788	0,00450 6
Q15582	TGFBI	5.345572	13,679386	8,33381 4	0,00517 3
P98160	HSPG2	8.859966	15,662051	6,80208 5	0,00573 6
A2NJV5;A0 A075B6S2 ;AOAOAOM RZ7	IGKV A18;IGKV2 D- 29;IGKV2 D-26	6,2640694	0,5281823	-5,73589	0,00604 3
P00748	F12	21,33945	22,1381	0,79865 8	0,00608 5
P22792	CPN2	22,25886	22,8205	0,56164 2	0,00617 3
P11021	HSPA5	3.720965	11,713255	7,99228 9	0,00682 6

P01876	IGHA1	31,13867	30,24995	-0,88872	0,00691 1
Q92496- 2;Q92496; Q92496-3	CFHR4	2.42436	11,03821	8,61384 6	0,00776 6
AOA075B6 I1	IGLV4-60	13,87167	5.185394	-8,68628	0,01005 1
P01703		17,37782	23,23569	5,85787 2	0,01064 9
P18428	LBP	11,47416	18,31519	6,84103 2	0,01132 3
Q8NI99	ANGPTL6	11,526017	4.144387	-7,38163	0,01190 6
AOA0B4J1 U7	IGHV6-1	22,59996	26,62302	4,02305 2	0,01200 4
Q9BXR6	CFHR5	5.800607	13,338935	7,53832 8	0,01415 4
P02751- 10;P0275 1-13	FN1	9.087818	16,69571	7,60789 2	0,01441
P02790	HPX	25,28332	26,11439	0,83107 6	0,01444 3
P21980- 2;P21980; P21980-3	TGM2	4,2720915	0,4457934	-3,8263	0,01456 5
P02746	C1QB	27,56949	28,01983	0,45033 6	0,01977 3
P02760	AMBP	24,33717	24,93431	0,59714 2	0,02035 5
P01701		27,48918	26,60877	-0,88041	0,02118 3

P01766		24,45966	17,99343	-6,46623	0,02337 4
P01019	AGT	23,4802	22,67968	-0,80053	0,02351 3
AOA0G2JS 06		25,3019	19,47542	-5,82649	0,02455 2
P01704		19,9328	25,27153	5,33873 3	0,02482 7
Q5T749	KPRP	4,4687764	0,5660902	-3,90269	0,02487 2
000187;0 00187-2	MASP2	19,93343	20,97891	1,04548	0,02643 3
Q03591	CFHR1	23,1884	23,76836	0,57996 1	0,02717
P11217- 2;P11217; P06737- 2;P11216; P06737	PYGM	4,6574862	0,6082011	-4,04929	0,02759 3
P07225	PROS1	25,57112	25,99843	0,42731 2	0,02920 7
Q12805- 5;Q12805- 2;Q12805- 4;Q12805- 3;Q12805	EFEMP1	2,296374	8,973493	6,67712	0,02924 5
AOA075B6 H9	IGLV4-69	25,56016	20,4878	-5,07236	0,02992 8
P01715		19,30839	11,32053	-7,98786	0,03114 6
Q02413;Q 02413-2	DSG1	3,6552677	0,5459463	-3,10932	0,03685 8

Q66K66	TMEM198	20,92864	23,50407	2,57543	0,03823
				7	3
P08473	MME	15,310028	8.574814	-6,73521	0,03823
					9
P68133;P	ACTA1;ACT	7.430236	13,875415	6,44518	0,04095
68032;P6	C1;ACTG2;				5
3267;P62	ACTA2				
736;P632					
67-2					
AOA0C4DH	IGKV6-21	24,16357	18,66946	-5,4941	0,04129
24					7
P01817		12,696209	5.607219	-7,08899	0,04282
					7
P15144	ANPEP	20,31834	14,37981	-5,93853	0,04514
					8
P62987;P	UBA52;RP	19,56519	12,92859	-6,63661	0,04616
62979;P0	S27A;UBB;				2
CG47;POC	UBC				
G48					
AOA075B6	IGKV2D-	12,474057	5.802547	-6,67151	0,04853
S6	30				2
P08519;Q	LPA	5.945479	12,500058	6,55457	0,04900
16609				9	1

**Supplementary Table 13. EV proteins upregulated in non-responder PDAC patients
(vs. responders)**

Protein IDs	Gene names	Mean .Non Responders	Mean Responders	logFC	P.Value
A0A0C4DH72; A0A0C4DH73; P01611	IGKV1-6	15,060969	3.574371	-11,4866	0,000489
P04432;P015 97		14,692537	3.558026	-11,1345	0,000562
P01817		10,90292	0.00000	-10,9029	0,000293
A0A0C4DH68	IGKV2-24	26,58469	27,47934	0,89465 7	0,000201
P02746	C1QB	27,77124	28,28304	0,51179 9	0,001447
P01714		25,10489	26,84546	1,74056 3	0,00226
P15814;P158 14-2	IGLL1	8,060513	0	-8,06051	0,002832
Q08554- 2;Q08554	DSC1	0	6,642689	6,64268 9	0,004575
A0A075B6S5; A0A0C4DH69	IGKV1-27	15,14843	5.662179	-9,48625	0,004863
P62987;P629 79;POCG47;PO CG48	UBA52;RPS2 7A;UBB;UBC	8.622959	17,487488	8,86452 9	0,007196
P15144	ANPEP	10,49458	18,49358	7,99899 8	0,009811
A0A0B4J1V0	IGHV3-15	24,3923	24,90039	0,50809 5	0,009961
Q9NZP8	C1RL	9,544	2,343031	-7,20097	0,010317
A0A0A0MT36	IGKV6D-21	10,839119	2.553621	-8,2855	0,013266

P07357	C8A	25,08541	25,42435	0,33894 1	0,013604
Q13103	SPP2	9,603383	2,31008	-7,2933	0,014323
Q9BXR6	CFHR5	16,619122	9.865795	-6,75333	0,017651
Q8TCG1- 2;Q8TCG1	KIAA1524	8,173923	1,876418	-6,2975	0,020795
P08637;O750 15	FCGR3A	11,79168	18,71712	6,92543 9	0,021632
Q9BWP8- 8;Q9BWP8- 7;Q9BWP8- 6;Q9BWP8-5	COLEC11	12,04857	4.62706	-7,42151	0,023107
P01857	IGHG1	32,56523	31,99845	-0,56677	0,02629
P03952;P207 18	KLKB1	23,64847	23,94836	0,29989 5	0,026916
P13164;Q016 29;Q01628	IFITM1;IFITM 2;IFITM3	0	5,501942	5,50194 2	0,027497
P05106- 2;P05106- 3;P05106	ITGB3	0	3,959515	3,95951 5	0,027508
P02745	C1QA	27,34568	27,7409	0,39521 5	0,02757
A0A0C4DH43; P01814		23,72722	17,35704	-6,37018	0,028112
A0A0A0MS15	IGHV3-49	23,7816	22,93867	-0,84293	0,028814
P07225	PROS1	25,81825	26,18921	0,37095 3	0,028895
P04278- 4;P04278- 2;P04278- 3;P04278;P04 278-5	SHBG	13,2751	6.543573	-6,73153	0,029683

P08670;P176 61;Q16352;P0 7197-2	VIM	0	4,58408	4,58408	0,029827
P02747	C1QC	28,01889	28,39234	0,37345 1	0,030901
P23083		25,56806	26,25761	0,68954 6	0,031442
P28074- 3;P28074	PSMB5	8,442728	2,336585	-6,10614	0,03599
P04430		21,55277	16,68508	-4,8677	0,036212
A0A087WSX0	IGLV5-45	5,619149	0	-5,61915	0,037874
P01699		4,512501	0	-4,5125	0,037909
P27169	PON1	23,04364	23,63591	0,59226 8	0,040998
A0A0C4DH29	IGHV1-3	21,04053	15,33251	-5,70801	0,042131
A0A075B6I0	IGLV8-61	25,2758	19,73274	-5,54307	0,04525
A0A075B6Q5	IGHV3-64	18,17808	11,41315	-6,76493	0,047306
Q01469	FABP5	1,009046	6,235751	5,22670 5	0,04767
P01861	IGHG4	27,21168	26,56055	-0,65113	0,048619
P00739;P007 39-2	HPR	26,29785	25,7406	-0,55725	0,049185

CHAPTER III

IFN γ R+ Extracellular Vesicles as regulators of IFN γ antitumor immunotherapy

1. Introduction

IFN γ is a pleiotropic cytokine that plays crucial roles in the regulation of both innate and adaptive immune responses, including antiviral and antitumoral processes^{212,213}. It is mainly produced by T cells and NK cells as a consequence of stimulation by interleukins, tumor or viral antigens, or by IFN γ itself via a positive feedback loop^{214–219}. IFN γ exerts its downstream effect by binding to interferon- γ receptor (IFN γ R), which consists of two subunits: IFN γ R1 and IFN γ R2^{220,221}. The binding of IFN γ results in formation of a complex by the subunits and conformational changes that lead to the recruitment and activation of Janus kinases (JAKs)^{220,222}. Subsequently, JAKs phosphorylate and activate transcription factor signal transducer and activator of transcription 1 (STAT1). STAT1 undergoes dimerization and translocates to the nucleus, where it binds to specific DNA sequences and modulates transcription of IFN γ regulated genes, resulting in immune cell activation^{223,224}. In oncologic settings, due to its cytostatic, pro-apoptotic and anti-proliferative functions, IFN γ was shown to be the key modulator of antitumor immunity by orchestrating a variety of immune responses (**Fig. 15**)^{225–227}. First reports of the antitumoral roles of IFN γ signaling come from the 1990s, where several studies showed that IFN γ enhances the cytotoxic activity of NK cells and CD8⁺ cytotoxic T cells, augmenting their ability to directly kill tumor cells, and that IFN γ production was required for rejection of transplantable tumors and prevention of primary tumor development in mice^{228–230}. Over the next years, multiple mechanisms via which IFN γ performs its antitumoral roles have been demonstrated, including both immunologic and non-immunologic actions. IFN γ can have a direct anti-proliferative function on tumor cells that is dependent on STAT1 action, which activates transcription of inhibitors of cellular progression, including p21 and p27, that cause cell cycle arrest^{226,231}. On the other hand, also via STAT1 signaling pathway, IFN γ was shown to induce caspase-1 and -8 and stimulate apoptosis of tumor cells²²⁵. Moreover, IFN γ

signaling can inhibit angiogenesis in tumor tissue by induction of angiostatic chemokines secretion, as well as contribute to destruction of tumor-associated blood vessels^{232–234}. Furthermore, numerous immunological actions of IFN γ have been described, such as the activation of macrophages. IFN γ was shown to stimulate M1 proinflammatory macrophage polarization, while suppressing M2 phenotype^{235,236}. Another way through which IFN γ contributes to antitumor immunity is by promoting tumor cell recognition by immune cells via stimulation of MHC I expression on tumor cells, which increases their immunogenicity and immune destruction^{227,237}. Despite its established antitumorigenic effects, there are reports demonstrating that, under specific circumstances, IFN γ can play a protumorigenic role, promoting tumor escape from the immune system^{228,238,239}. For instance, IFN γ was shown to be involved in the establishment of an immunosuppressive tumor microenvironment by promoting the expression of immunosuppressive molecules, including checkpoint inhibitors like PD-L1^{240–242}. Interestingly, it is proposed that the effect of IFN γ depends on the duration and magnitude of IFN γ signaling, as well as tumor specificity and other tumor microenvironmental conditions, where IFN γ orchestrates the anti- and pro-tumoral signalling²⁴³. While clinical trials on IFN γ had mixed successes with conflicting outcomes, the effectiveness of the majority of cancer immunotherapies, including immune checkpoint inhibitors (ICIs), depends on the signaling and antitumoral functions of IFN γ ^{239,244–249}. For instance, metastatic melanoma patients that did not respond to an anti-CTLA-4 therapy had tumors with genomic alterations in IFN γ pathway genes²⁵⁰. Mutations in JAKs were associated with a lack of response to IFN γ and acquired resistance to anti-PD-1 immunotherapy²⁵¹. Additionally, IFN γ upregulates secretion of PD-L1-positive EVs, whose high levels were linked with poor clinical outcome and ICI resistance^{169,252}. Although IFN γ is a known player in anti-tumoral immunity, it is still unclear how its signaling can be downregulated in cancer settings, enabling tumor initiation and progression.

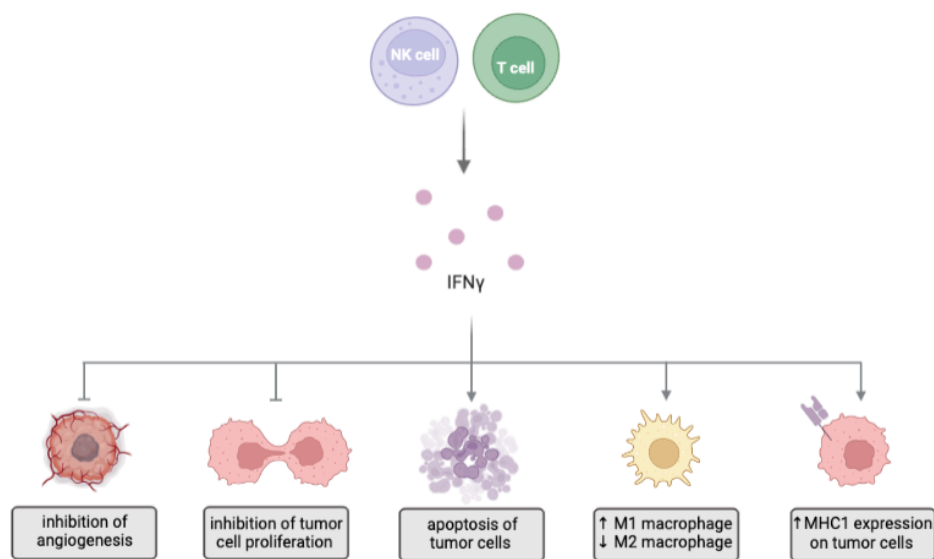


Figure 15. The anti-tumoral roles of IFN γ . IFN γ exerts its antitumoral role via a variety of different processes, including inhibition of angiogenesis in tumor tissue, inhibition of proliferation and promotion of apoptosis of tumor cells, stimulating M1 macrophage polarization and enhancing immunorecognition of tumor cells by enhancing MHC I expression on tumor cells.

EVs are crucial mediators of intercellular communication and modulation of immune response, playing a key role in tumor progression and metastasis¹⁶. In the previous chapter, characterization of EVs obtained from samples of metastatic PDAC patients revealed that an IgG+ EV population can detect response to chemotherapy, representing a promising new tool for evaluating the effectiveness of treatments. The binding of IgG to EV surface proteins, including tumor neoantigen MAGE B1, suggests a protein-protein mediated interaction between secreted EVs and circulating proteins. Indeed, as already mentioned in General Introduction, due to their highly interactive surface, EVs were shown to serve as a scaffold for interactions with a variety of soluble molecules in the extracellular environment¹⁰⁶. Importantly, those EV post-secretion interactions were shown to have functional consequences, potentially modulating intercellular communication in different processes,

including pathologies like cancer. Some of the examples of the effects those interactions exert in the bound proteins include regulation of their immune recognition, delivery, and activity^{107-111,169}. One of the mechanisms behind that is based on the discovery that EVs can act as molecular decoys by scavenging the soluble molecules and preventing their function, phenomena initially demonstrated as a mechanism for protection against MRSA-produced bacterial toxins²⁵³. In oncological settings, tumor derived EVs were shown to interact and decoy cytokines, including TNF α , tumor-reactive and immunotherapy antibodies, modifying the effects they exert on target cells^{108,110,111,169}. EVs can also act as an intercellular delivery tool. For instance, post-secretion interaction between IFN γ R1-positive EVs (IFN γ R1⁺ EVs) and IFN γ was shown to mediate delivery of that cytokine to target cells, where it exerted its downstream signalling¹⁰⁷. As already mentioned, the surface composition of EVs plays crucial roles in their targeting and interaction with the recipient cell, as well as their biological activity. Thus, binding to various proteins in the extracellular milieu can also result in altered EVs surface composition, modulating their biodistribution and uptake²⁵⁴.

The ability of EVs to interact with soluble proteins, including signaling molecules, adds another layer of complexity to the functional roles they play in cell-cell communication. However, despite the growing evidence of the ability of tumor EVs to bind to circulating proteins, including pro- and anti-tumorigenic proteins, it is still not fully understood how these post-secretion interactions can affect the activity and distribution of both molecules and EVs themselves in oncological settings. Therefore, in this chapter we wanted to provide new insights into the relevance of EV post-secretion interaction in modulating immune response and cancer progression. To address that, we decided to investigate whether an interaction between melanoma-derived EVs and IFN γ can potentially regulate IFN γ signaling. We hypothesize that melanoma EVs can

act as a decoy, sequestering IFN γ and preventing its binding to tumor cells, thus exerting its antitumoral functions (**Fig. 16**).

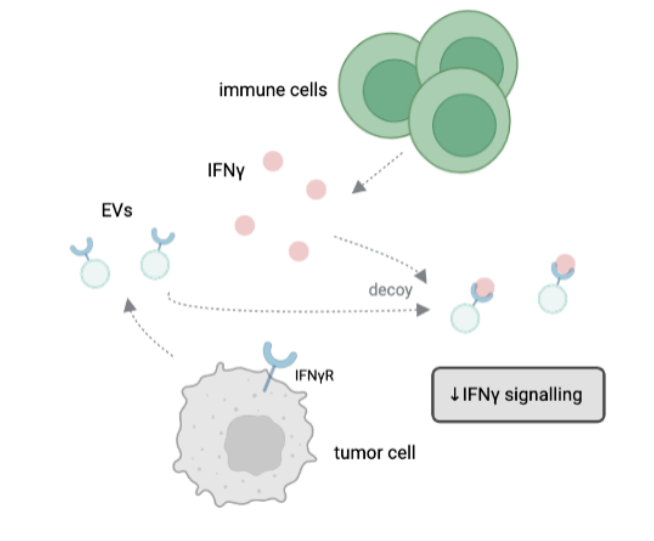


Figure 16. Schematic representation of Chapter III hypothesis. Melanoma cells secrete EVs that bind and decoy circulating IFN γ , which results in a downregulation of IFN γ signaling on tumor cells.

2. Materials and methods

2.1 Cell lines and cell culture

The murine melanoma cell line B16F10 was obtained from the American Type Culture Collection. Cell line was maintained in DMEM (Corning) and supplemented with 1% penicillin-streptomycin (Gibco 15-140-122, United States) and 10% fetal bovine serum (FBS) (Biowest S181BH-500, Nuaillé, France). Cells were maintained at 37 °C with 5% CO₂ levels. For conditioning, cells were cultured in DMEM supplemented with 1% penicillin-streptomycin and 10% EV-depleted FBS. For the preparation of conditioned culture medium (CCM) 1x10⁶ cells were seeded per 150 mm culture dish containing 20 mL of

EV-depleted medium and grown for 72 h until they reached a confluency of ~90%. After conditioning, CCM was immediately submitted to two centrifugation steps (500 g for 10 min and 3,000 g for 20 min, both at 10 °C) to remove dead cells and large debris, and were then either used directly for EV isolation or stored at -80 °C until use. Cells were regularly tested to make sure they were negative for mycoplasma.

2.2 Knockdown of IFN γ R1 gene in melanoma cell lines

Knockdown of IFN γ R1 in B16F10 cells was done using lentiviral particles of mouse shRNA specific to IFN γ R1 gene and control scramble shRNA (Santa Cruz Biotechnology). Transduction of cells was carried out according to the manufacturer's instructions. Briefly, 4 x10⁴ cells were seeded in a 12-well plate to reach 50% confluence, and after 24h cells were transduced with lentiviral particles. Next, cells were expanded and selected with puromycin-containing medium. Western blot was used to confirm the extent of IFN γ R1 knockdown.

2.3 Extracellular vesicle isolation and characterization

2.3.1 EV isolation by differential ultracentrifugation

EV isolation was performed by differential ultracentrifugation coupled with sucrose cushion. CCM was centrifuged at 12,000 g for 20 min to remove larger EVs, including apoptotic bodies, then supernatant was collected and centrifuged at 100,000 g for 2 h 20 min. The pellet was resuspended in 16 mL of filtered phosphate buffered saline (PBS, Corning 15313581, NY, United States) and then carefully added on top of a 4 mL sucrose solution (1.2 g of protease-free sucrose dissolved in D₂O containing and 96 mg of Tris base adjusted to pH 7.4). Next, sample was centrifuged at 100,000 g for 1 h 10 min, and after a 4 mL fraction was collected using an 18G needle. Fraction was then mixed with 16 mL of filtered PBS and centrifuged overnight at 100,000 g. The pellet of purified EVs was collected and resuspended in filtered

PBS. All solutions were filtered using 0.22 μm filters. All centrifugations were performed at 10 °C using rotors 45Ti or 70Ti (Beckman-Coulter, CA, United States).

2.3.2 Nanoparticle Tracking Analysis (NTA)

To determine particle concentration and size distribution, all EV samples were analyzed by NTA using the NanoSight NS300 (Malvern Instruments) equipped with a red laser (638 nm). In order to obtain concentrations within the optimal range for NTA analysis, all samples were diluted in filtered PBS. The videos were recorded at camera level of 16 and a threshold of 5-7. Videos were analyzed using NTA software v3.4.

2.3.3 Protein Quantification

Protein concentrations in cell lysates and EV samples were assessed using the BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

2.4 Mass Spectrometry

EVs were analyzed using liquid chromatography-mass spectrometry (LC-MS), as described previously¹⁴⁷.

2.5 Western blotting

Equal protein amounts of cell lysate or EV samples were mixed with 4X Laemmli buffer (Bio-Rad), denatured for 5 min at 95 °C, and separated by SDS-PAGE for 1.5 h at 90 V, using 4-20% Mini-PROTEAN TGX Stain-Free Protein Gels (Bio-Rad). Next, samples were transferred to a nitrocellulose membrane (Cytiva) at 100 V for 1 h, which was later blocked with LI-COR Intercept Blocking Buffer (LI-COR Biosciences) for 1 h at RT, and incubated overnight at 4 °C with the primary antibody diluted in LI-COR blocking buffer with 0.1% Tween-20.

Membrane was washed with TBS-T (TBS with 0.1% Tween-20) three times for 5 min, and then incubated with the secondary antibody for 1 h at RT. This incubation was followed by three additional washes with TBS-T, 5 min each. Blots were imaged using the Odyssey Infrared Imaging System (LI-COR Biosciences). The detailed list of primary and secondary antibodies used is provided in **Supplementary Table 14**.

2.6 "EV precipitation" assay

To investigate whether IFN γ R1⁺ EVs can bind soluble IFN γ , we developed an assay which we refer to as "EV precipitation" assay. Briefly, EVs were incubated with IFN γ for 30 minutes at RT. As controls, samples of EVs alone or IFN γ alone were maintained in the same conditions. Afterwards, samples were centrifuged at 100,000 x g, ON, 10°C. Next, supernatants containing soluble proteins and pellets containing precipitated EVs were collected, and Western blotting was performed to assess IFN γ signal detection.

2.7 Cell proliferation assay

Cell proliferation after treatment with IFN- γ was assessed using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). Briefly, 4,000 cells were seeded in 96-well plates and treated with IFN γ alone, IFN γ R1⁺ EVs alone, IFN γ R1^{KD} EVs alone, IFN γ R1⁺ EVs + IFN γ or IFN γ R1^{KD} EVs + IFN γ . Non-treated cells served as a control. After 72h incubation, CellTiter 96AQueous One Solution Reagent was added to the media, according to the manufacturer's instructions, and absorbance at 490 nm was measured for 1-3 hours. All experiments were performed in triplicates and repeated at least three times.

2.8 Animal studies

Adult C57Bl/6 female mice (5 to 8 weeks old) were used for all experimental procedures. Animal work was performed in accordance with national animal experimentation guidelines (DGAV), animal protocol 0421/000/000/2018. For the EV incorporation assay, mice were anesthetized using 1.5–3% isoflurane and injected into the retro-orbital venous sinus with 10 µg of EVs in 100 µl filtered PBS, previously labelled with NIR815 (LI-COR). Mice were injected either with EVs alone or EVs mixed with IFN γ (Abcam, ab198567). For that, EVs were incubated with IFN γ for 30 min, RT and then ultracentrifuged overnight, 100,000 x g, 10 °C. Supernatant was then removed and the pellet was resuspended in sterile filtered PBS to a final concentration of 10 µg of EVs per 100 µl PBS. As controls, mice were injected with 100 µl filtered PBS. After 24 hours, mice were sacrificed, and lungs and livers were collected and imaged using Odyssey Infrared Imaging System (LI-COR Biosciences).

3. Results

3.1 Particles isolated from B16F10 murine melanoma cells display small EV features and package IFN γ R

Isolated EVs were characterized by NTA to determine the concentration and size distribution of nanoparticles in samples (**Fig. 17A**). Moreover, the presence of proteins commonly identified in EVs (tetraspanins, heat shock proteins, TSG101) as well as absence of common contaminant proteins (non-EV proteins) were confirmed in our samples by mass spectrometry (**Fig. 17B**). Taken together, these data indicate that B16F10 vesicles in our isolated samples display characteristics of small EVs. Also, preliminary mass spectrometry results from our lab showed that B16F10 melanoma cells pack both IFN γ R1 and IFN γ R2 into EVs (**Fig. 17C**). We then confirmed the presence of IFN γ R1 in B16F10 EVs by Western blot (**Fig. 17D**).

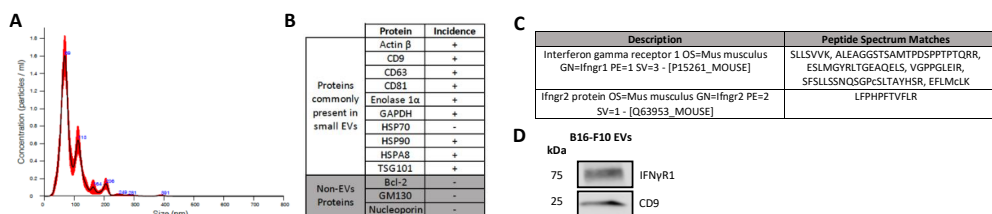


Figure 17. Characterization of B16F10 EVs. (A) Representative NTA histogram of EV sample isolated from B16F10 cell line. (B) Mass Spectrometry analysis of EV-positive and -negative markers of B16F10-derived EVs. (C) Mass Spectrometry identification of IFN γ R1 and IFN γ R2 in B16F10 EVs. (D) Identification of IFN γ R1 in B16F10 EVs by Western blot.

3.2 IFN γ R⁺ melanoma EVs bind soluble IFN γ and this interaction is decreased when IFN γ R1 expression is reduced

To understand if IFN γ R⁺ B16F10 EVs can bind to IFN γ , we developed and performed the "EV-precipitation" assay (Fig. 18A). IFN γ alone, B16F10 EVs alone and B16F10 EVs + IFN γ were incubated, ultracentrifuged, and analyzed by Western blotting for IFN γ detection. We found that, while IFN γ alone or EVs alone do not produce a detectable IFN γ signal in the pellet, the presence of EVs resulted in IFN γ precipitation, supporting the fact that IFN γ R⁺ melanoma EVs can bind soluble IFN γ , potentially decoying it (Fig. 18B). To verify the role of IFN γ R in the binding of IFN γ by IFN γ R⁺ EVs, we generated a B16F10 cell line with IFN γ R1 knockdown (IFN γ R1^{KD}), and obtained IFN γ R1^{KD} EVs (Fig. 18C). "EV-precipitation" assay with WT EVs (IFN γ R1⁺) and IFN γ R1^{KD} EVs showed that ~80% decrease of IFN γ R1 expression results in ~60% reduction of IFN γ binding to EVs (Fig. 18D).

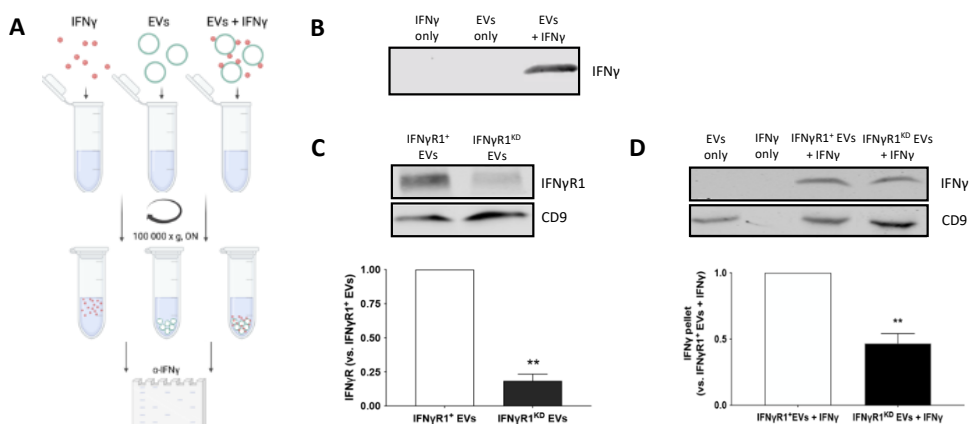


Figure 18. IFN γ interaction with B16F10 EVs. (A) Schematic representation of "EV precipitation" assay. Briefly, IFN γ alone, B16F10 EVs alone or a mix of B16F10 EVs and IFN γ were incubated for 1h at RT and then centrifuged at 100,000xg, overnight. Pellets were analyzed for the expression of IFN γ by Western Blot (B). (C) Validation of IFN γ R1 Knock-Down (by sh-RNA) in B16F10 EVs. Expression normalized by CD9 levels in EVs and in relation to sh-Control EVs (IFN γ R1⁺ EVs), by Western Blot. (D) IFN γ binding to IFN γ R1⁺ EVs and sh-IFN γ R1 B16F10 EVs (IFN γ R1^{KD} EVs). Expression normalized by CD9 levels in EVs and in relation to IFN γ R1⁺ EVs, by Western Blot. All data is shown as the mean values $\pm$ SD from independent measurements. P-values were calculated using t-test; ** P < 0.01.

3.3 Binding of IFN γ R⁺ EVs to IFN γ prevents the antitumoral action of IFN γ in melanoma cells

Based on our results showing that IFN γ R⁺ EVs can bind to soluble IFN γ , we examined whether this binding could decoy this cytokine, and prevent it from exerting its antitumoral effect on B16F10 cells. To that end, B16F10 cells were treated with IFN γ alone, IFN γ R1⁺ EVs alone, IFN γ R1^{KD} EVs alone, IFN γ R1⁺ EVs + IFN γ or IFN γ R1^{KD} EVs + IFN γ . We observed that EVs alone (both IFN γ R1⁺ and IFN γ R1^{KD}) did not affect cell viability (Fig. 19). As expected, treatment with IFN γ alone resulted in decreased viability, which could be prevented when the cells

were co-incubated with IFN γ R1⁺ EVs + IFN γ . On the contrary, incubation with IFN γ R1^{KD} EVs + IFN γ did not rescue cell viability, presenting an effect similar to that of IFN γ alone. Thus, IFN γ R1⁺ EVs can decoy IFN γ and prevent its antitumoral action on target cells, supporting the EV protein decoy function in mediating cellular viability.

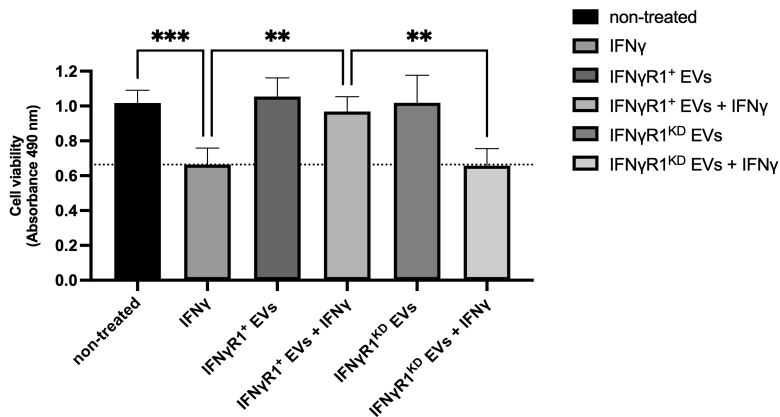


Figure 19. The effect of IFN γ interaction with B16F10 EVs on B16F10 cell viability. Viability of B16F10 cells when non-treated or treated with IFN γ alone, IFN γ R1⁺ EVs alone, IFN γ R1^{KD} EVs alone, IFN γ R1⁺ EVs + IFN γ or IFN γ R1^{KD} EVs + IFN γ . Cell viability was assessed using MTS assay by absorbance measurement at 490 nm. Data is presented as a mean $\pm$ SD of two independent experiments, each with two or three technical replicates. P-values were calculated using one-way ANOVA with Tukey's post-test; *** P < 0.001, ** P < 0.01.

3.4 IFN γ binding to melanoma IFN γ R1⁺ EVs increases vesicular accumulation in lungs and liver

As binding of proteins to EVs can affect not only the protein activity, but also EVs themselves, we decided to investigate whether the interaction of melanoma EVs with IFN γ has an effect on the distribution of vesicles. Labelled EVs, either alone or after incubation with IFN γ , were injected retro-orbitally in mice and their distribution in livers and lungs was assessed (**Fig. 20A**). We

observed that IFN γ binding to EVs results in a significant increased vesicular accumulation in lungs (Fig. 20B) and livers (Fig. 20C).

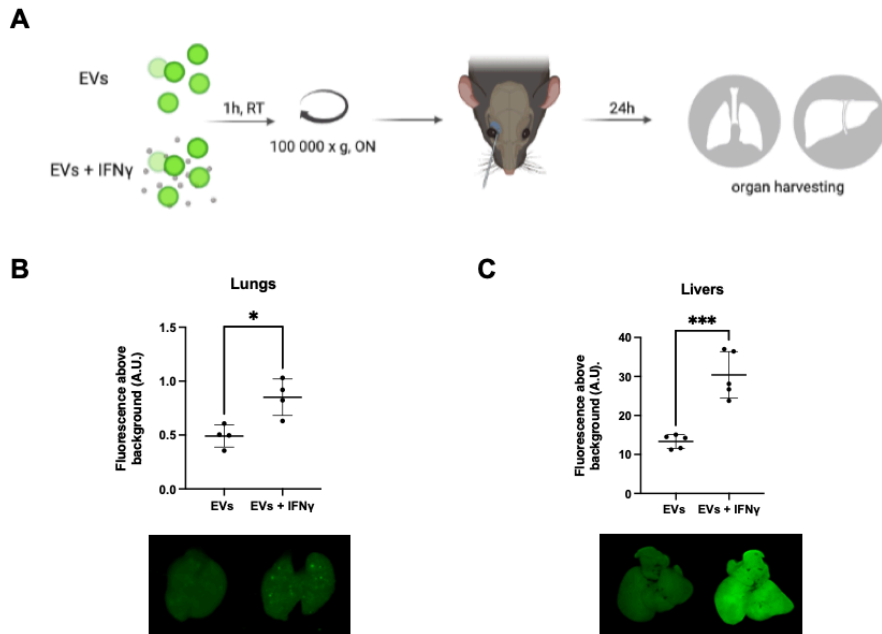


Figure 20. The effect of IFN γ interaction with B16F10 EVs on vesicular biodistribution.

(A) Schematic representation of this EV biodistribution experiment. NIR labelled EVs alone or EVs + IFN γ were incubated for 1h, RT, then centrifuged overnight, 100,000 x g, 10°C and injected retro-orbitally in mice. After 24 hours, mice were sacrificed, and lungs and livers were collected and imaged using Odyssey Infrared Imaging System (LI-COR Biosciences). Biodistribution of EVs alone and EVs + IFN γ in lungs (B) and liver (C). All data is shown as the mean values $\pm$ SD from independent measurements. P-values were calculated using t-test; *** P < 0,001, *P < 0,05.

4. Discussion

Although EVs are established mediators of intercellular communication in cancer, to date the majority of this research falls under the umbrella of their role in the horizontal transfer of molecular cargo. However, after their secretion, EVs are capable of interacting with and regulating the activity of a

variety of proteins in the extracellular milieu, including cytokines with pro- and anti-tumoral functions, like IFN γ ^{106-110,168}. The consequences of those post-secretion interactions in terms of cancer development and progression have yet to be explored.

Our data showed that B16F10 melanoma cells package IFN γ R1 into EVs and that those EVs can bind and interact with soluble IFN γ , potentially decoying it. Reduction of IFN γ R1 levels in EVs resulted in a decreased interaction with IFN γ , demonstrating that the interaction between EVs and IFN γ is mediated by IFN γ R1. Consistent with our hypothesis, we observed that, by binding to IFN γ , IFN γ R1⁺ cancer EVs decoy it and downregulate the anti-tumoral effect of this cytokine on melanoma cells. This presents a new mechanism of IFN γ signaling modulation via protein post-secretion interaction with EVs, and supports the idea that tumor-derived EVs can modulate the activity of antitumoral cytokines, thus helping tumor cells to evade the immune response. Even though EVs are established mediators of the immune system regulation, the concept of the "molecular decoy" function of tumor EVs and its implication in interfering with immune surveillance is still in its infancy^{110,117,152}. For instance, PDAC-derived EVs were shown to carry a large repertoire of tumor associated antigens that bind and decoy circulating autoantibodies, inhibiting complement-dependent cytotoxicity. Importantly, the decoy role of EVs has been implicated in resistance to cancer therapies^{111,168,196,255}. Battke et al. reported that tumor EVs bind and sequester tumor-reactive antibodies, resulting in a significant reduction of their binding to tumor cells and downregulation of antibody-dependent cytotoxicity by immune cells¹¹¹. Breast cancer- derived HER2-overexpressing EVs can bind to Trastuzumab, an anti-HER2 antibody, decoy it and subsequently inhibit its therapeutic efficacy¹⁹⁶.

The breakthrough in cancer treatment in recent years has undoubtedly been the discovery of immunotherapy and the introduction of antibody blockade of

T cell inhibitory pathways, referred to as ICIs^{208,209}. ICIs act via binding and blocking immune checkpoints, present on both immune cells, like T cells, and tumor cells. Under normal circumstances, the binding between an immune checkpoint ligand and its receptor on the surface of a T cell transmits an inhibitory signal suppressing the immune response, which helps prevent a too strong immune response and thus maintains immune homeostasis. However, by expressing immune checkpoint proteins, cancer cells have an ability to exploit this mechanism in order to evade immune surveillance. ICI-mediated binding to immune checkpoints releases that blockage and restores T-cell mediated anti-tumoral response. The most widely used ICI treatments target immune checkpoint pathways, such as CTLA-4, and PD-1 and its ligand PD-L1, and have achieved significant clinical benefits in patients with a variety of tumor types, including melanoma, lung cancer, and bladder cancer^{256,257}. Importantly, ICI immunotherapy revolutionized the treatment of advanced melanoma, being one of the first therapies to show an increased overall survival of metastatic patients^{258,259}. Despite a major clinical success, only a subset of patients benefits from this therapy, while the majority do not show any response^{260,261}. Moreover, the efficacy of ICIs was shown to vary significantly among different tumor types, with tumors like pancreatic cancer showing a minimal response rate²⁶². Thus, in order to predict patients' response to ICIs and increase treatment success, it is essential to understand the molecular processes behind this resistance. While the details behind the lack of ICI response are not understood, several mechanisms involved in it have been proposed, including disruption of IFN γ signalling^{244-249,263-265}.

As IFN γ signaling plays a pivotal role in the effectiveness of many immunotherapy treatments, we hypothesize that tumor-derived EVs regulate ICI resistance by modulating IFN γ signaling. Specifically, we hypothesized that IFN γ interaction with IFN γ R1⁺ EVs can modulate its activity and bioavailability, which could potentially contribute to a lack of response to ICI. Interestingly, EV

decoy function has already been proposed in terms of immunotherapy resistance. In an already mentioned work by Chen et al., tumor EVs interaction with anti-PD-L1 antibody results in its sequestration and resistance to ICI treatment¹⁶⁸. To further investigate whether IFN γ decoy by IFN γ R1⁺ EVs could be one of the molecular mechanisms responsible for ICI resistance, we plan to characterize *in vivo* whether IFN γ R1⁺ EVs regulate tumor growth, metastasis and ICI response. If confirmed, this mechanism of post-secretion interaction between EVs and IFN γ could expand our understanding of the complex interplay between EVs and tumor immune responses, and represent a novel EV liquid biopsy strategy to assess and monitor response to ICIs in cancer patients.

Additionally, our data revealed that IFN γ binding to melanoma IFN γ R1⁺ EVs results in an increased vesicle accumulation in liver and lungs, preferential sites of melanoma metastasis²⁶⁶. As described previously, EVs can act as tools for molecular cargo delivery. For instance, IFN γ delivery by IFN γ R1⁺ EVs was shown to serve as a communication tool between neural stem cells and the host immune system¹⁰⁷. However, the intercellular fate of EVs and biomolecules they carry depends on multiple factors and can result in degradation upon phagocytosis or their incorporation and release in the cytoplasm of recipient cells. Thus, the binding of IFN γ by melanoma EVs may result in either its degradation or delivery to target cells, representing a novel mechanism of IFN γ signalling regulation by cancer EVs. Despite the most studied and established role of IFN γ being its anti-tumoral function, in certain contexts this cytokine can exert tumor promoting effects and contribute to immunosuppression in tumor microenvironment²⁶⁷. For instance, IFN γ can upregulate expression of PD-L1, a crucial player in the suppression of T-cell mediated anti-tumor response, both in tumor cells and in various non-transformed cells, including fibroblasts, macrophages and epithelial cells^{268,269}. Considering that we previously showed that cancer EVs target lung

fibroblasts and endothelial cells and resident macrophages in the liver, melanoma EV-bound IFN γ may be delivered to those cells and stimulate expression of PD-L1. Thus, tumor EVs-mediated delivery of IFN γ could potentially have a role in the formation of an immune-suppressive microenvironment, supporting the future formation of metastasis. However, the fate of EV-bound IFN γ and the potential roles of this interaction in IFN γ signaling needs careful further validation.

Taking all together, understanding the post-secretion EV interactions with soluble proteins can unravel a new layer of a previously unappreciated complexity of EV functions in cancer progression and immunotherapy resistance. Our results regarding the ability of EVs to interact and decoy proteins in the extracellular milieu, resulting in the modulation of the activity and of the distribution of both EVs and proteins, advance the knowledge on cancer EV post-secretion interactions and their pathophysiological consequences. We propose that cancer EV interactions can regulate tumor progression by modulating anti-tumoral cytokine activity, representing a novel potential mechanism of EV-mediated anti-tumoral immunity regulation. As EVs were shown to interact with a variety of proteins with different functions, this EV-mediated process could have applications in other tumor types, as well as other pathologies. However, further studies are needed to identify and elucidate the functional consequences of these interactions.

To summarize, in this study we proposed a new mechanism in which melanoma EVs interaction with IFN γ affects the activity and biodistribution of both EVs and IFN γ , downregulating IFN γ signaling and potentially regulating response to ICI treatment. Moreover, we saw that EV post-secretion interaction with IFN γ affects the biodistribution of EVs and could serve as a IFN γ delivery mechanism with a role in the formation of an immunosuppressive microenvironment. These results are groundbreaking in understanding how

tumor EV protein interactions can influence cancer progression by regulating the activity of anti-tumoral cytokines, the biodistribution of EVs and the delivery of cytokines to future sites of metastasis. Based on the role of IFN γ signaling in the efficacy of ICI treatments, these results could shed a new light on mechanisms behind immunotherapy resistance and potentially allow for the identification of new therapeutic strategies. Furthermore, if confirmed, IFN γ interaction with IFN γ R1⁺ EVs could serve as a new marker of resistance to ICI treatment in melanoma.

Supplementary Material

Supplementary Table 14. List of primary and secondary antibodies used for Western blotting.

Primary Antibodies				
Antibody		Manufacturer	Catalog #	Dilution/concentration
IFN γ		Invitrogen	PA5-95560	1:500
IFN γ R1		Abcam	ab280353	1:1000
CD9		Invitrogen	MA1-10309	1:1000
Secondary Antibodies				
Antibody		Manufacturer	Catalog #	Dilution
Goat anti-rabbit IgG	IRDye 800CW	LI-COR Biosciences	926-32211	1:5000
Goat anti-rat IgG	IRDye 800CW	LI-COR Biosciences	926-32219	1:5000

Final considerations

Although significant progress has been made in recent years in understanding the biology and roles of EVs, further studies are needed to explore the full potential of these particles. The complex molecular composition of EVs, along with their cargo sorting mechanisms, are crucial to their performance as intercellular carriers of biological information. However, besides their undoubted role as regulators of cell-cell communication, compelling evidence shows that EVs and their bioactive cargo, namely proteins and nucleic acids, can serve as potential tools for liquid biopsy applications in cancer. The broad interest in EVs as a liquid biopsy source resulted in numerous studies showing that EVs cargo analysis could serve as early detection biomarkers and provide insights into disease progression and treatment response in various types of cancers. However, future clinical implementation of EVs and their biomolecular cargo as a liquid biopsy source faces several challenges. These include limitations related to the heterogeneity of EVs themselves, as there is a lack of consensus regarding the standardization of methods for vesicles' purification. Moreover, the field of EV-based liquid biopsy lacks protocols' standardization for extraction and analysis of EV biomolecules, limiting the discovery and validation of new biomarker candidates. In this PhD Thesis, we aimed at addressing some of those challenges by further exploring the potential of EV molecular cargo, namely DNA and proteins, as cancer biomarkers. Specifically, we focused on the standardization of EV-DNA preparation for mutational analyses, as well as the investigation of the roles of EV protein interactions as biomarkers in treatment response assessment and as mediators of immunotherapy resistance.

In **Chapter I**, we addressed one of the challenges that hamper the implementation of EV-DNA as a liquid biopsy biomarker in clinical practice. We performed a comprehensive comparison of commonly applied methods for DNA extraction from EVs based on their quantity, size distribution and applicability for downstream analyses. Based on that, we established a

workflow for EV-DNA preparation for mutation detection, including TaqMan and NGS. Our findings showed that the choice of extraction method affects both DNA abundance and average size of DNA fragments, important factors that can directly affect mutational analyses. When examining tumor EVs for mutations known to be present in their parental cells, we observed that TaqMan mutation detection varies significantly among samples extracted with different DNA purification methods. On the other hand, NGS analysis did not show significant differences in detection of mutations among the methods, probably due to the high sensitivity of NGS. Importantly, by analyzing EV-DNA from plasma of cancer patients, we demonstrated the applicability of our workflow for the detection of clinically significant mutations, which is in line with previous reports supporting the use of EV-DNA as nucleic acid biomarker for liquid biopsy. Collectively, our results show that the DNA extraction method affects the quantity and quality of EV-DNA and highlights the importance of the development of standardized protocols for EV-DNA preparation to assure reproducibility, as well as high confidence, of the results. Of note, it should be noted that some reports show increased performance of mutation detection when combining EV-DNA with more established nucleic acid biosources, like cfDNA, representing another approach of how EV-DNA could be applied in liquid biopsy assays ^{206,207}. While our results support the potential use of EV-DNA as a cancer biomarker, more work is required to ensure its clinical validation and future application in clinical settings. Besides its clinical application, the standardization of protocols for EV-DNA preparation will be key to further understand the biological functions of EV-DNA in physiological and pathological scenarios.

In **Chapter II**, we explored the potential of EV proteins as a complementary indicator of treatment response in metastatic PDAC patients. By analyzing plasma EV protein composition at diagnosis and throughout therapy, we saw that the levels of IgG presence in EVs are linked with PDAC diagnosis and

response to chemotherapy. By assessing the dynamics of IgG⁺ EV population over the course of treatment, we observed that it increases with disease progression and decreases when patients respond to therapy, demonstrating that analysis of IgG⁺ EVs can represent an additional tool for monitoring PDAC patients. Furthermore, while investigating the mechanism behind IgG association with EVs, we obtained a list of EV surface proteins, suggesting that IgG presence in EVs may be a result of post-secretion interaction. Indeed, as described in the General Introduction, due to their highly interactive surface, EVs can bind to circulating proteins. Interestingly, this is in line with previous reports where binding between IgGs and EVs have been already shown and suggested to serve as a potential biomarker in different diseases, including PDAC¹¹⁰. Among the identified EV surface proteins, MAGE B1, an PDAC antigen, was present exclusively in EVs from PDAC patients, suggesting that tumor EVs can interact with circulating IgG via a tumor antigen and that at least partially, the increase in IgG⁺ EVs in PDAC progression could be caused by IgG binding to MAGE B1 on EVs. As this observation is based on MS results, further experiments are required to validate this interaction. Importantly, post-secretion interactions between EVs and circulating proteins can have functional consequences on both the bound proteins and EVs themselves. For instance, Capello et al. showed that PDAC EVs can bind and decoy IgGs, inhibiting complement-dependent cytotoxicity towards cancer cells. Collectively, in this chapter we demonstrated that IgG binds to the surface of PDAC EVs, at least in part through an interaction with tumor antigen MAGE B1, and that analysis of IgG⁺ EVs can detect chemotherapy response, representing a novel approach in improving PDAC treatment response evaluation. These findings may have important implications for understanding the role of EV proteins and EV-mediated post-secretion protein interactions as biomarkers of therapy response and their potential significance in personalized treatment strategies in PDAC.

With this in mind, we decided to further investigate the consequences of tumor EV post-secretion interactions with secreted proteins in **Chapter III**. We saw that B16F10 melanoma-derived EVs package IFN γ R and that those EVs can bind IFN γ . Furthermore, this post-secretion interaction results in the downregulation of IFN γ antitumoral activity *in vitro*, demonstrating that tumor EVs act as molecular decoys and sequester IFN γ , representing a new aspect on how tumor cells can use EVs to evade the immune system. As IFN γ signalling is crucial for the efficacy of many immunotherapies, we suggest that, by decoying IFN γ , melanoma EVs may downregulate response to immunotherapy, resulting in resistance. To understand the importance of this phenomena, future *in vivo* studies are essential, assessing the impact of this interaction on tumor growth, metastasis, and ICI response. Additionally, it is important to verify the interaction and EV decoy effect in other melanoma cell lines. It would also be interesting to learn whether EV post-secretion interactions influence ICI responsiveness in tumors that respond to it only moderately (like breast cancer) or not at all (pancreatic cancer). As previously mentioned, EV post-secretion interactions can have an impact not only on the bound protein, but also on the EVs themselves. In line with this, we saw that IFN γ binding to melanoma EVs affects their distribution and results in increased accumulation of vesicles in organs that melanoma metastasizes to, namely lungs and liver. As IFN γ is known to induce expression of PD-L1, its interaction and delivery by EVs could have a role in the formation of an immunosuppressive environment in the sites of future metastasis. Collectively, we showed that tumor EV protein interactions can regulate the activity of anti-tumoral cytokines, EVs distribution, as well as the delivery of cytokines to future sites of metastasis. Our findings open the door to understanding the roles of tumor EV post-secretion interactions in terms of immune modulation and cancer progression, proposing a new mechanism of resistance to ICI and a potential new marker for immunotherapy resistance prediction. As the EV molecular composition is far more complex than initially assumed and can be influenced by interactions

that occur after their release, our findings may have important implications in unravelling the yet-to-be appreciated mechanism of EV activity.

Taking all together, this thesis highlights the potential of EV molecular cargo as a valuable source of biomarkers in oncological settings, but also emphasizes the need for standardization and development of protocols for EVs extraction and analysis. We provide evidence that tumor EVs can be used as DNA source for mutational detection as well as a tool for the assessment of therapy response in PDAC. Moreover, we not only shed a light on the biological importance of tumor EV post-secretion protein interactions in terms of cancer progression, but also on their implications as potential biomarkers for assessing treatment response and predicting immunotherapy resistance.

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Extracellular Vesicles Molecular Cargo as Cancer Biomarkers: Insights into EV-DNA Preparation and EV Protein Interactions

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