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ROLE AND PREDICTIVE VALUE OF RED BLOOD CELLS IN BREAST CANCER METASTASIS

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“El amor es tan importante como la comida, pero no alimenta”

G.García Márquez

“Mi diagnostico es sencillo, sé que no tengo remedio”

Julio Cortazar

A Miriam,

Por la suerte que he tenido de compartir estos cuatro años contigo,

y por todo lo que queda por venir

INDEX

INDEX

ABBREVIATIONS 11

RESUMO 13

SUMMARY 25

INTRODUCTION 30

1. Breast cancer: 31

1.1 Epidemiology and risk factors 31

1.2 Breast Cancer subtypes 31

1.3 Staging of BC disease 32

2. The metastatic cascade 33

2.1 Angiogenesis, migration and invasion: 33

2.2 Intravasation and circulation in blood 35

2.3 Colonization and formation of metastasis. 36

3. Liquid biopsy 38

3.1 Utility of Liquid Biopsy on clinical practice 38

3.2 Liquid biopsy biomarkers 39

4. Understanding the complexity of RBCs 42

4. 1 Non-oxygen-transport related functions of RBCs 43

4.2 Role of RBCs in blood rheology and coagulation 43

4.3 RBCs immune and angiogenic functions 44

4.4 Role of RBCs in cancer 45

5. In vitro models to study RBCs 46

5.1 RBCs differentiation 46

5.2 CD34+ primary cells and immortalized cell lines derived from it 46

5.3 Differentiation in vitro of primary CD34+ and immortalized cell lines 47

OBJECTIVES 49

MATERIALS AND METHODS 51

1. Breast cancer patient cohorts and samples 52

1.1 Cohort I 52
1.2 Cohort II 53
2. Cell lines and cell cultures 54
3. Isolation and preservation of erythrocytes 55
4. Elisa assays 55
4. Thawing glycerol-frozen RBCs and lysates obtaining 56
5. RBC-tumor cell adhesion 56
6. MTT proliferation assay 56
7. Angiogenesis assay 56
8. Wound healing assay 57
9. Migration Transwell assay 57
10. Zebrafish assays 58
11. Tumor cell adhesion to endothelial cells 58
12. Tumor cells adhesion to collagen I 59
13. Cluster formation assays 59
14. Cytokine and Chemokines detection 60
15. Liposome synthesis 61
16. RNA extraction 61
17. RNA-Sequencing analysis 61
18. qRT-PCR analysis 62
19. PAK4 Inhibition 63
20. Generation of a LAMP2 knockout (KO) on an erythroid cell line 63
21. RNA extraction and qRT-PCR protocol for HUDEP-2 cells 64
22. Immunofluorescence 65
23. Western Blot 65
24. Protein extraction 66
25. HUDEP-2 differentiation protocol 66
26. HUDEP-2 Cytometry analysis 67
27. Vesicle Production analysis 67
28. Statistical analysis 67

RESULTS 68
1. RBCs as breast cancer biomarkers 70
1.1 RBCs from BC patients have altered clinical blood values 70
1.2 LAMP2: Potential biomarker of the presence of metastasis 71
2. Functional characterization of RBCs effects in BC cell lines. 73
2.1 RBC from M1 patients adhere to cancer cells 73
2.2 RBCs presence does not affect the proliferation of cancer cells 75
2.3 Coculture with M1 RBCs alter tumour cell Cytokine profile 75
2.4 RBCs from M1 patients have a proangiogenic effect 76
2.5. Effects of RBCs in migration of BC cell lines. 77
2.6. Tumor-exposed RBCs enhanced tumor cell aggregation *in vitro* 81
2.7 RBCs co-culture affects tumor cell adhesion 82
3. RBCs from M1 patients modify gene expression in MDA-MB-231 83
3.1 RNA-sequencing analysis of MDA-MB-231 cocultured with RBCs 84
3.2 qRT-PCR analysis of genes upregulated by RBCs 87
4. Role of PAK4 in RBCs´ modulation of MDA-MB-231 functions 88
5. Study of LAMP2 involvement in vesicle biogenesis using erythoblast model 90
5.1 Study of LAMP2 role in differentiated HUDEP cells 92
5.2 LAMP2 KO has an altered production of vesicles 94
5.3 LAMP2 play a role in paracrine communication between primed WT HUDEP-2 and MDA-MB-231 94
DISCUSSION ..... **¡Error! Marcador no definido.**
CONCLUSIONS 105
BIBLIOGRAPHY 107
SUPPLEMENTARY MATERIAL 131
AGRADECIMIENTOS 136
ANNEXES 139
Favorable reports from the clinical research ethics comitte of Galicia 140
Favorable report from the clinical research with farmaceutics ethics comitte of Galicia 142
Figures Autorship and list of publications used in this thesis 144

ABBREVIATIONS

Abbreviations

AUC	Area under curve
BC	Breast cancer
BEL-A	Bristol Erythroid Line Adult
CFC	Cancer free control
CTC	Circulating tumour cell
ctDNA	Circulating tumor DNA
CT	Computed tomography
DEG	Differentially expressed genes
DOX	Doxycycline
DARC	Duffy antigen receptor for chemokine
DMEN	Dulbecco's Modified Eagle's Medium
EGF	Epithelial growth factor
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
ECM	Extracellular matrix
EVs	Extracellular vesicles
FBS	Fetal Bovine serum
EMA	European Medicine Agency
FDA	Food and Drug Administration
GO	Gene Ontology
Hb	Haemoglobin
HSPC	hematopoietic stem progenitor cells
HER2	Human Epithelial Growth factor receptor 2
HT	Hormonal therapy
HR	Hormonal receptor
HUDEP-2	Human umbilical cord blood derived erythroid progenitors 2
HIF-1α	Hypoxia-inducible factor 1 alpha
IHC	Immunohistochemistry
IMDM	Iscove's Modified Dulbecco's Medium
LB	Liquid biopsy
LAMP2	Lysosomal-associated membrane protein
MRI	Magnetic resonance imaging
M1	Metastatatic breast cancer
miRNA	Micro RNA
MDSC	Myeloid-derived suppressive cells
M0	Non-metastatic breast cancer
OS	Overall survival
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PET	Positron emission tomography
PFS	Progression free survival
PNP	Purine nucleotide phosphorylase
ROC	Receiver operating characteristic
RBCs	Red blood cells
RT-qPCR	Reverse transcription quantitative PCR
Stemspan	StemSpan™ Serum-Free Expansion Medium (Stemspan)
TN	Triple negative
TLR	Toll-like receptor
TGF-β	Transforming growth factor beta
TME	Tumour microenvironment
TNM	Tumour, node and metastasis
TEP	Tumour-educated platelets

RESUMO

RESUMO

O cancro de mama (CM) é o cancro máis común diagnosticado en mulleres en todo o mundo, con máis de 2,3 millóns de casos novos en 2020. A pesar dos recentes avances nos tratamentos e programas de detección, o CM aínda é responsable do 16 % das mortes relacionadas co cancro, principalmente debido ao desenvolvemento de metástase. O prognóstico do paciente dependerá do seu subtipo de tumor e do estadio da enfermidade. En CM existen 3 subtipos clasificados en base á expresión do Receptor 2 do Factor de Crecemento Epitelial Humano (HER2) e dous receptores hormonais (HR): receptor de estróxenos (ER) e receptor de progesterona (PR). O CM luminal ou HR positivo é o subtipo máis común (máis do 70 % dos casos) e menos agresivo. Este subtipo caracterízase pola expresión de HR (ER e/ou PR) e expresión ocasional de HER2 en doentes con CM Luminal B. O CM HER2 caracterízase pola sobreexpresión de HER2 e a ausencia de HR. O tratamento de estos dous últimos subtipos ha mellorado notablemente grazas ao desarrollo de terapias dirixidas contra os HR e HER2 Finalmente, o subtipo triple-negativo (TN) non expresa HR nin HER2 (principal diana terapéutica). É o subtipo máis agresivo e non ten ningunha terapia dirixida, requirindo quimioterapia ou inmunoterapia como tratamento alternativo. Na maioría dos casos, o CM é diagnosticado nas primeiras fases cando o tumor está localizado, porén, ao redor do 30 % dos pacientes acabarán desenvolvendo metástases durante o seu tratamento ou preséntanas no momento da diagnose (máis do 6 %). A metástase é responsable do 90 % das mortes relacionadas co cancro e converte ao CM nunha enfermidade incurable cunha taxa de supervivencia a cinco anos do 26-30 % tendo en conta todos os subtipos, sendo aínda menor para os subtipos máis agresivos como o TN.

O desenvolvemento da metástase é un proceso complexo que implica varios pasos que conducen ás células cancerosas dende o tumor primario cara a tecidos distantes, onde forman tumores secundarios. Estes pasos xuntos constitúen o que se coñece como metástase: anxioxénese, migración, intravasación, supervivencia no torrente sanguíneo, extravasación e colonización de tecidos distantes. A anxioxénese a miúdo indúcese no tumor primario pola crecente necesidade de osíxeno debido á proliferación incontrolada de células cancerosas. A indución da anxioxénese no tumor leva á formación de novos vasos sanguíneos que presentan ocose unha estrutura defectuosa, o que permite unha extravasación máis sinxela. Durante este proceso, as células tumorais tamén comezan a desprenderse unhas das outras e invaden os tecidos circundantes. Neste punto, as células tumorais comezan a migrar a través do estroma tumoral cara aos vasos sanguíneos. Esta migración pódese realizar individualmente/en pequenos grupos, o que é característico das células que sufriron transición epitelio mesenquimal (EMT), ou en grandes grupos (migración epitelial), xeralmente compostos principalmente por células tumorais cun fenotipo epitelial. Unha vez que estas células chegan aos vasos sanguíneos, necesitan atravesar a barreira endotelial para entrar no sangue. As células tumorais requiren pasar pola EMT para romper a barreira endotelial, non obstante, os vasos linfáticos ou a vasculatura defectuosa recentemente formada ofrecen unha vía máis sinxela para a extravasación. Unha vez que estas células extravasan, coñécense como células tumorais circulantes (CTC). As CTC teñen un tempo de residencia medio no sangue de 1-2,4 horas. A maioría delas morren debido a presión do sangue, ataques inmunes ou anoikis, e só unha porcentaxe moi pequena pode sobrevivir e extravasarse en órganos distantes para formar metástase. Durante a extravasación, as células deben unirse ás paredes dos vasos e cruzar a

barreira endotelial. Unha vez que as CTC extravasan, teñen que sobrevivir no tecido colonizado e, finalmente, proliferar para formar un tumor secundario. En CM, a metástase xeralmente localízase no óso, pulmón e/ou cerebro, porén, cada subtipo presenta patróns de tropismo diferentes. É importante destacar que, como se dixo antes, o CM TN é o subtipo máis agresivo e é máis propenso a presentar estadios avanzados e recaídas temperás. Estudar os mecanismos e as diferentes interaccións que interveñen na metástase é fundamental para mellorar a nosa comprensión deste proceso e o desenvolvemento de novas terapias dirixidas. Neste sentido, nunha publicación previa do noso laboratorio, observáronse glóbulos vermellos (GV) en contacto directo con CTC illados de pacientes con CM metastásico, sendo a súa presenza indicativa dun peor prognóstico. Ademais, tamén se reportou que o perfil proteómico dos GV de pacientes con CM difire dos controis libres de cancro (CLC), o que suxire que a presenza dun tumor pode modificar o seu contido. A pesar de en xeral ser ignorados e considerados uns meros transportadores de osíxeno, publicacións recentes demostraron que os GV participan na regulación de múltiples procesos como a coagulación, a inmunidade e a anxioxénese. Tendo en conta a súa crecente complexidade, a súa abundancia en sangue e a evidencia da súa interacción coas células tumorais, é importante estudar o papel potencial destas células no proceso da metástase.

Ademais do estudo do proceso metastásico, o desenvolvemento de novas tecnoloxías e a busca de novos biomarcadores son fundamentais para mellorar o tratamento e diagnóstico dos pacientes con CM metastásico. A práctica clínica actual baséase na elaboración de perfiles de tumores primarios para estudar tumores sólidos. Non obstante, a biopsia tisular tradicional non representa a heteroxeneidade do tumor, é altamente invasiva e a súa aplicabilidade para estudar metástase é limitada debido ao pequeno tamaño e á mala accesibilidade dos tumores secundarios. Debido a estas limitacións, desenvolveuse unha tecnoloxía alternativa coñecida como biopsia líquida. A biopsia líquida baséase no estudo de material derivado do tumor como CTC, ADN tumoral circulante (ctADN) e vesículas extracelulares (EVs) presentes en diferentes fluídos corporais, principalmente no sangue. Estes biomarcadores reflicten a heteroxeneidade do tumor e estanse a utilizar amplamente para a estudar a recaída da enfermidade, monitorear a resposta á terapia e determinar mecanismos de resistencia ou como obxectivos terapéuticos. Non obstante, estas entidades teñen varios inconvenientes. As CTC e o ctADN están presentes en cantidades moi baixas no sangue, o que limita o seu uso para detectar os estadios iniciais da enfermidade e a metástase. As vesículas extracelulares, aínda que son máis numerosos e están presentes nas primeiras fases da enfermidade, o seu material é difícil de extraer e purificar. Debido a estas limitacións, outros biomarcadores como os GV poderían ser unha alternativa interesante. A pesar de non ser materiais derivados de tumores, demostrouse que os GV están modificados polos tumores e estes cambios reflicten a presenza da enfermidade, como se confirmou noutras enfermidades como o Alzhéimer ou a Diabetes entre outras. Ademais, os GV ofrecen varias vantaxes como cantidades elevadas, fácil illamento e extracción do seu material.

Tendo en conta toda esta información, esta tese pretende estudar o papel dos GV na metástase así como avaliar o seu valor como biomarcadores en CM. Para conseguilo, recolleuse sangue de doentes con CM, tanto non metastásicos (M0) como metastásicos (M1), no momento do diagnóstico e de CLC. Os GV destes pacientes foron illados mediante

centrifugación e categorizados en dúas cohortes distintas para facilitar os enfoques diverxentes neste traballo.

Como primeiro paso, avaliouse o valor dos GV como predictores da presenza de metástase. Extraeuse a proteína dos GV e recompiláronse os datos clínicos das probas de sangue rutineiras. Un estudo da análise de sangue mostrou que algúns parámetros relacionados cos GV estaban alterados, incluíndo o ancho de distribución de glóbulos vermellos (RDW), o hematocrito e a concentración de hemoglobina. Baseándonos nos nosos resultados dun análise proteómico anterior reportado por en Pereira-Veiga *et al.*, utilizouse o ensaio ELISA para examinar a sobreexpresión da proteína 2 de membrana asociada a lisosomas (LAMP2) e a purina nucleósido fosforilase (PNP) nos GV de doentes metastásicos. Debido á expresión variable de PNP dependendo da localización da metástase, só se validou o valor preditivo de LAMP2 para detectar metástase. Os resultados da análise das da curva de eficacia diagnóstica (ROC) mostraron que a concentración de LAMP2 tiña un AUC de 0.71. Non obstante, o AUC aumentou ata 0.89 cando se combinou a concentración de LAMP2 cos valores de RDW e hematocrito, mostrando que os GV teñen un bo valor preditivo para detectar metástase.

Despois de avaliar o valor dos GV como biomarcador, tamén se estudou o papel dos GV na metátase en CM. Baseándose na identificación de GV unidos a CTC illados de doentes con CM, propuxose o estudo *in vitro* da adhesión dos GV ás células cancerosas. Do mesmo xeito que os fenómenos visualizados nas mostras de pacientes, unha maior porcentaxe de pacientes con CM tiñan GV adheridos a células tumorais, principalmente de pacientes M1 en comparación cos controis. Tendo en conta estes resultados iniciais, se evidencia que os GV de CM non só se alteraron en canto ao seu contido senón tamén funcionalmente. Co fin de avaliar os efectos potenciais dos GV na metástase, deseñouse un conxunto de ensaios para estudar o papel destas células en diferentes etapas deste proceso: anxioxénese, migración, formación de clústers e adhesión. Ademais, tamén se estudaron os efectos potenciais dos GV no tumor primario que poden afectar a metástase, principalmente a proliferación e a produción de citocinas. En ambos enfoques, realizouse unha análise funcional co-cultivando lisados de GV de pacientes ou controis con dúas liñas de células tumorais de CM. Non obstante, nalgúns ensaios como a quimiotaxis ou os ensaios de agregación, os GV utilizáronse directamente despois do illamento para garantir que se mantivese a súa integridade.

Os resultados mostraron que os GV, independentemente da súa procedencia, non mostraron ningunha influencia na proliferación das liñas celulares CM TN MDA-MB-231 ou luminais MCF7. Pola contra, os GV dos pacientes M1 modificaron o perfil de citocinas de ambas liñas de células tumorais, provocando a produción de citocinas da familia CCL, de acordo coas observacións de Yin *et al*. Os ensaios de anxioxénese mostraron que os GV M1 teñen un efecto proanxioxénico que non se observa cando se usan GV CLC, o que reflicte a capacidade dos GV para unirse a factores proanxioxénicos probablemente a través de DARC, tal e como describen Karsten *et al*. Os GV tamén tiveron un impacto na migración das MDA-MB-231. Os GV M1 aumentaron a migración tanto *in vivo* como *in vitro*, así como mostraron a capacidade de atraer por quimiotaxis a células tumorais. Ademais, cando os GV M1 foron utilizados como quimio-atraentes, as células migradas presentaban unha morfoloxía alterada, mostrando protuberancias da membrana que se asemellan aos lamelipodios. A continuación, os ensaios de agregación de células tumorais realizados con MDA-MB-231 mostraron un aumento da formación de clústers cando estaban presentes os GV de pacientes M1. Curiosamente, a exposición dos GV de CLC a medios condicionados de MDA-MB-231

alterou o seu comportamento neste ensaio, provocando un aumento da agregación de células tumorais semellante ao observado cando se usan GV M1. Isto último indica que os GV están condicionados debido ao microambiente tumoral. Finalmente, estudouse a adhesión ás células endoteliais e ao coláxeno I, un compoñente principal da membrana basal vascular, como parte do proceso de extravasación. Os resultados destes ensaios mostraron que os GV M1 aumentaron a adhesión de MDA-MB-231 a ambos. É importante destacar que non se observaron cambios na migración, adhesión ou proliferación para a liña celular luminal BC MCF7.

Despois de estudar os diferentes pasos do proceso metastático, probouse que os GV dos pacientes M1 teñen un impacto na capacidade de migración e adhesión das MDA-MB-231, con todo, os mecanismos que median estes efectos seguen sendo descoñecidos. Para determinar o potencial mecanismo, realizouse unha análise de secuenciación de ARN para estudar como afectan os GV á expresión de ARN de MDA-MB-231. Os resultados da análise de ontoloxías xenéticas dos xenes expresados de forma diferencial mostraron que as vías como a adhesión celular ou o esamblaxe de filamentos contráctiles de actina e de fibras de tensión atopábanse alteradas despois do co-cultivo cos GV. Ademais, o análise por qRT-PCR dun conxunto seleccionado de xenes expresados de forma diferencial amosaron que *PAK4*, *HMOX1 e EPHX1* estaban sobreexpresados en MDA-MB-231. Entre estes xenes, *PAK4* destaca como un importante regulador da migración, adhesión, EMT e morfoloxía. Debido á importancia deste xene na EMT, analizouse outro conxunto de xenes asociados a este proceso, mostrando que *PLS3, VIM e STAT1* tamén estan significativamente sobreexpresados en MDA-MB-231 co-cultivadas con GV M1. Tendo en conta estes resultados, estudouse a *PAK4* como un mecanismo potencial para os efectos dos GV M1 debido ao seu papel como regulador da EMT e da migración. O uso do inhibidor de PAK4 LCH-7749944 en células MDA-MB-231 co-cultivadas con GV M1 restableceu a migración e a adhesión aos niveis basais da liña celular antes do co-cultivo, o que suxire a implicación de PAK4 na alteración do fenotipo e o comportamento das células MDA-MB-231.

Estes datos demostraron que os GV dos doentes M1 son diferentes dos procedentes dos CLC e poden afectar a diferentes etapas do metastático. Non obstante, aínda non está claro como se modifican GV durante o seu tempo na circulación dos pacientes con enfermidade metastásica. Publicacións recentes demostraron que os GV maduros teñen compartimentos endosómicos e conservan a capacidade de realizar endocitose así como de xerar exosomas e microvesículas. Ademais, unha investigación realizada por Thorn *et al* proporcionou evidencias da capacidade dos GV para transportar moléculas como os aminoácidos. É importante destacar que os resultados desta tese demostraron que os efectos dos GV poden ser mediados de forma paracrina, implicando potencialmente a produción de vesículas. Esta información suxire que os GV maduros poden captar factores presentes no plasma ou no tumor primario e liberalos. Debido á súa implicación na regulación da produción de vesículas e á súa regulación positiva nos GV M1, LAMP2 podería ser un importante mediador neste proceso. Para estudar o papel de LAMP2, xerouse un modelo LAMP2 KO usando CrisprCas9 en células proxenitoras eritroides derivadas do sangue do cordón umbilical humano 2 (HUDEP-2), unha célula eritroide inmortalizada. LAMP2 está implicado na produción de vesículas nos GV, mostrando que o silenciamiento a LAMP2 reduciu a cantidade e tamaño da liberación de vesículas. Ademais, a ausencia de LAMP2 vese prexudicada coa capacidade do eritroblasto ortocromático para atraer por quimiotaxe as células cancerosas. A pesar dos

interesantes resultados, é necesario realizar máis estudos para determinar a posible implicación de LAMP2 no transporte de vesículas neste contexto.

Este traballo proporciona a primeira evidencia da implicación dos GV en varios pasos da metástase, ademais de demostrar o potencial destas células como un novo biomarcador subrogado para ser usado na biopsia líquida. Ademais, tamén destaca o valor de utilizar liñas celulares CD34+ eritroides inmortalizadas como HUDEP-2 para estudar a función das proteínas nos GV. En conxunto, estes resultados sentan a base dunha nova liña de investigación que requirirá máis traballo para dilucidar o posible mecanismo de acción dos GV no cancro.

RESUMEN

RESUMEN

El cáncer de mama (CM) es el cáncer más común diagnosticado en mujeres en todo el mundo, con más de 2,3 millones de casos nuevos en 2020. A pesar de los recientes avances en tratamientos y programas de detección, el cáncer de mama sigue siendo responsable del 16 % de las muertes relacionadas con el cáncer, principalmente debido al desarrollo de metástasis. El pronóstico del paciente dependerá del subtipo de tumor y estadio de la enfermedad. En CM existen 3 subtipos clasificados por la expresión del Receptor 2 del Factor de Crecimiento Epitelial Humano (HER2) y dos receptores hormonales (HR): receptor de estrógenos (ER) y receptor de progesterona (PR). El CM luminal o HR positivo es el subtipo más común (más del 70 % de los casos) y menos agresivo. Este subtipo se caracteriza por la expresión de HR (ER y/o PR) y la expresión variable de HER2 en pacientes Luminal B. El cáncer HER2 CM se caracteriza por la sobreexpresión de HER2 y la ausencia de HR. El tratamiento de estos dos últimos subtipos ha mejorado notablemente gracias al desarrollo de terapias dirigidas contra los HR y HER2. Finalmente, el subtipo triple negativo (TN) no expresa HR ni HER2 (principales dianas terapéuticas). Es el subtipo más agresivo y no tiene ninguna terapia dirigida requiriendo quimioterapia o inmunoterapia como tratamiento alternativo. En la mayoría de los casos, el CM se diagnostica en las primeras etapas cuando el tumor está localizado, sin embargo, alrededor del 30 % de los pacientes eventualmente desarrollan metástasis durante su tratamiento o la presentan al diagnóstico (alrededor del 6 %). La metástasis es responsable del 90 % de las muertes relacionadas con el cáncer y convierte al CM en una enfermedad incurable con una tasa de supervivencia a cinco años del 26-30 % considerando todos los subtipos, siendo incluso menor para subtipos más agresivos como el TN.

El desarrollo de metástasis es un proceso complejo que implica varios pasos que conducen a las células tumorales desde el tumor primario hacia tejidos distantes, donde forman localizaciones secundarias. El conjunto de estos pasos constituye lo que se conoce como cascada metastásica: angiogénesis, migración, intravasación, supervivencia en el torrente sanguíneo, extravasación y colonización de tejidos distantes. La angiogénesis a menudo se induce en el tumor primario debido a la creciente necesidad de oxígeno debido a la proliferación descontrolada de células cancerosas. La inducción de la angiogénesis en el tumor conduce a la formación de nuevos vasos sanguíneos, generalmente permeables y con una estructura defectuosa, lo que permite una extravasación más fácil. Durante este proceso, las células tumorales también comenzarán a desprenderse unas de otras e invadir los tejidos circundantes. En este punto, las células tumorales comienzan a migrar a través del estroma tumoral hacia los vasos sanguíneos. Esta migración puede realizarse de forma individual/en pequeños grupos, característica de células que han sido sufrido transición epitelio mesénquima (EMT), o en grandes grupos (migración epitelial), generalmente compuestos principalmente por células tumorales con fenotipo epitelial. Una vez que estas células llegan a los vasos sanguíneos, necesitan cruzar la barrera endotelial para ingresar a la sangre. Las células tumorales requieren haber experimentado EMT para atravesar la barrera endotelial y migrar; sin embargo, los vasos linfáticos o la vasculatura defectuosa recién formada ofrecen una ruta más fácil para la extravasación. Una vez que estas células se han extravasado, se las conoce como células tumorales circulantes (CTC). Las CTCs tienen un tiempo de residencia medio de 1 a 2,4 horas. La mayoría de ellas mueren debido a la presión de la sangre, ataques

del sistema inmune o anoikis, y sólo un porcentaje muy pequeño puede sobrevivir y extravasarse en órganos distantes para formar metástasis. Durante la extravasación, las células deben adherirse a las paredes de los vasos y cruzar la barrera endotelial. Una vez que las CTC se han extravasado, tienen que sobrevivir en el tejido colonizado y eventualmente proliferar para formar un tumor secundario. En CM, las metástasis generalmente se localizan en hueso, pulmón y cerebro, sin embargo, cada subtipo presenta patrones de tropismo diferentes. Es importante destacar que, como se indicó anteriormente, el CM TN es el subtipo más agresivo y es más propenso a presentar estadios avanzados y recaídas tempranas. Estudiar los mecanismos y las diferentes interacciones que intervienen en esta cascada metastásica es esencial para aumentar nuestra comprensión de este proceso y el desarrollo de nuevas terapias dirigidas. En este sentido, en una publicación anterior de nuestro laboratorio, se observaron glóbulos rojos (GR) en contacto directo con CTC aisladas de pacientes con CM metastásico, siendo su presencia indicativa de un peor pronóstico. Además, también hemos reportado que el perfil proteómico de los GR de pacientes con CM difiere del de los controles libres de cáncer, lo que sugiere que la presencia de un tumor puede modificar su contenido. A pesar de que generalmente se pasan por alto y se consideran meros transportadores de oxígeno, publicaciones recientes han demostrado que los GR participan en la regulación de múltiples procesos como la coagulación, la inmunidad y la angiogénesis. Teniendo en cuenta su creciente complejidad, su abundancia en la sangre y la evidencia de su interacción con las células tumorales, es importante comprender mejor el papel potencial de estas células en la cascada metastásica.

Además del estudio de la cascada metastásica, el desarrollo de nuevas tecnologías y la búsqueda de nuevos biomarcadores son fundamentales para mejorar el tratamiento y diagnóstico de los pacientes con cáncer de mama metastásico. La práctica clínica actual para el estudio de tumores sólidos se basa en el análisis del tumor primario. Sin embargo, la biopsia de tejido tradicional no representa la heterogeneidad tumoral, es altamente invasiva y su aplicabilidad para estudiar metástasis es limitada debido al pequeño tamaño y mala accesibilidad de los tumores secundarios. Debido a estas limitaciones, se ha desarrollado una tecnología alternativa conocida como biopsia líquida. La biopsia líquida se basa en el estudio de material derivado del tumor como las CTCs, el ADN tumoral circulante (ctADN) y las vesículas extracelulares (VEs) presentes en diferentes fluidos corporales, principalmente la sangre. Estos biomarcadores reflejan la heterogeneidad tumoral y se utilizan ampliamente para la predicción de la recaída de la enfermedad, el seguimiento de la respuesta al tratamiento y la determinación de mecanismos de resistencia y dianas terapéuticas. Sin embargo, estas entidades tienen varios inconvenientes. Las CTCs y el ctADN están presentes en cantidades muy bajas en la sangre, lo que limita su uso para detectar etapas tempranas de la enfermedad y la metástasis. Las EVs, aunque son más numerosas y están presentes en las primeras etapas de la enfermedad, presentan dificultades para la extracción y purificación del material que contienen. Debido a estas limitaciones, otros biomarcadores como los GR podrían ser una alternativa interesante. A pesar de no ser materiales derivados del tumor, se ha demostrado que los GR son modificados por el cáncer y estos cambios reflejan la presencia de la enfermedad como se ha confirmado en otras enfermedades como el Alzheimer o la Diabetes, entre otras. Además, los GR ofrecen varias ventajas, como encontrarse en grandes cantidades en la sangre o un fácil aislamiento y extracción de su material.

Considerando toda esta información, esta tesis tiene como objetivo estudiar el papel de los GR en la cascada metastásica en CM así como evaluar su valor como biomarcadores en CM. Para lograrlo, se recogió sangre de pacientes con CM, tanto no metastásicos (M0) como metastásicos (M1), en el momento del diagnóstico y de controles libres de cáncer (CFC). Los GR de estos pacientes se aislaron mediante centrifugación y se clasificaron en dos cohortes distintas para facilitar los distintos enfoques dentro de este trabajo.

Como primer paso, se evaluó el valor de los GR como predictores de la presencia de metástasis. Se extrajeron proteínas de los GR y se recogieron datos clínicos de análisis de sangre rutinarios. El estudio del análisis de sangre mostró que algunos parámetros relacionados con los GR estaban alterados, incluido el ancho de distribución de los GR (RDW), el hematocrito y la concentración de hemoglobina. Basándonos en nuestros resultados de un análisis proteómico previo, reportado por Pereira-Veiga *et al*., se utilizó el ensayo ELISA para evaluar la sobreexpresión de la proteína lisosomal asociada a membrana 2 (LAMP2) y la purina nucleósido fosforilasa (PNP) en GR M1. Debido a la expresión variable de PNP dependiendo de la ubicación de la metástasis, solo se estudió el valor predictivo de LAMP2 para detectar metástasis. Los resultados del análisis de la curva de eficacia diagnóstica (ROC) mostraron que LAMP2 por sí solo tenía un AUC de 0.71. Sin embargo, el AUC aumentó hasta 0.89 cuando la concentración de LAMP2 se combinó con los valores de RDW y hematocrito, lo que demuestra que los GR tienen un buen valor predictivo para detectar metástasis.

Después de evaluar el valor de los GR como biomarcador, también se estudió el papel de los GR en la cascada metastásica del CM. Basándonos en la identificación de los GR adheridos a las CTC aisladas de pacientes con CM, se estudió *in vitro* la adhesión de los GR a las células tumorales. De manera similar al fenómeno visualizado en las muestras de pacientes, un mayor porcentaje de pacientes con CM presentaba GR con adhesión a células tumorales, principalmente en GR de pacientes M1 en comparación con los CLC. Teniendo en cuenta estos resultados iniciales, quedó claro que los GR de CM no sólo se modificaron en términos de su contenido sino también a nivel funcional. Para evaluar los efectos potenciales de los GR en la cascada metastásica, se diseñó un conjunto de ensayos para estudiar el papel de estas células en diferentes pasos de la cascada metastásica: angiogénesis, migración, formación de grupos de CTCs y adhesión. Complementariamente, también se estudiaron los posibles efectos de los GR en el tumor primario que pueden afectar la cascada metastásica, principalmente la proliferación y producción de citocinas. En ambos enfoques, se realizó un análisis funcional co-cultivando lisados de GR de pacientes y controles con dos líneas de células tumorales de CM. Sin embargo, en algunos ensayos, como la quimiotaxis o los ensayos de agregación, los GR se utilizaron directamente después del aislamiento para garantizar que se mantuviera la integridad de los GR.

Los resultados mostraron que los GR, independientemente de su procedencia, no tenían ninguna influencia en la proliferación de las líneas celulares de CM TN MDA-MB-231 o luminal MCF7. Por el contrario, los GR de pacientes M1 modificaron el perfil de citocinas de ambas líneas celulares tumorales, induciendo la producción de citocinas de la familia CCL, de acuerdo con las observaciones de Yin *et al*. Los ensayos de angiogénesis mostraron que los GR M1 tienen un efecto proangiogénico que no se observa cuando se usan GR con CLC, lo que refleja la capacidad de los GR para unirse a factores proangiogénicos probablemente a través de DARC, como lo describe Karsten *et al*. Los GR también tuvieron un impacto en la migración de MDA-MB-231. Los GR M1 aumentaron la migración tanto *in vivo* como *in*

vitro, y también mostraron la capacidad de quimio-atracción de células tumorales. Además, cuando se utilizaron GR M1 como quimio-atrayentes, las células migradas presentaron una morfología alterada, exhibiendo prolongaciones de la membrana que se asemejan a los lamelipodios. A continuación, los ensayos de agregación de células tumorales realizados con MDA-MB-231 mostraron una mayor formación de grupos cuando estaban presentes GR de pacientes M1. Curiosamente, la exposición de los GR de CLC a medios condicionados de MDA-MB-231 alteró su comportamiento en este ensayo, lo que llevó a un aumento en la agregación de células tumorales similar al observado cuando se usaron GR M1. Esto último indica que los GR están condicionados por el microambiente tumoral. Finalmente, se estudió la adhesión a las células endoteliales y al colágeno I, componente principal de la membrana basal vascular, como parte del proceso de extravasación. Los resultados de estos ensayos mostraron que los GR M1 aumentaron la adhesión de MDA-MB-231 a ambos. Es importante destacar que no se observaron cambios en la migración, adhesión o proliferación para la línea celular luminal BC MCF7.

Después de estudiar los diferentes pasos de la cascada metastásica, quedó claro que los GR de pacientes M1 tenían un impacto en la capacidad de MDA-MB-231 para migrar y adherirse; sin embargo, los mecanismos que median estos efectos siguen siendo desconocidos. Para determinar este mecanismo, se realizó un análisis de secuenciación de ARN para estudiar cómo los GR afectan la expresión de ARN de MDA-MB-231. Los resultados del análisis de ontología genética de los genes expresados diferencialmente mostraron que vías como la adhesión celular, el filamento contráctil de actina y el ensamblaje de fibras de estrés estaban alteradas tras el cocultivo con GR. Además, un análisis posterior por qRT-PCR de un conjunto seleccionado de genes expresados diferencialmente mostró que *PAK4*, *HMOX1* y *EPHX1* estaban sobre-expresados en MDA-MB-231. Entre estos genes, *PAK4* destaca como un importante regulador de migración, adhesión, EMT y morfología celular. Debido a la importancia de este gen en la EMT, se analizó otro conjunto de genes asociados con este proceso, mostrando que *PLS3*, *VIM* y *STAT1* también se sobre-expresaron significativamente en MDA-MB-231 co-cultivado con GR M1. Teniendo en cuenta estos resultados, se estudió la expresión de *PAK4* como un mecanismo potencial mediador de los efectos de los GR M1 debido a su papel como regulador “aguas arriba” de la EMT y la migración. El uso del inhibidor de *PAK4* LCH-7749944 en GR M1 co-cultivados con células MDA-MB-231 restauró la migración y la adhesión a los niveles basales de la línea celular antes del co-cultivo, lo que sugiere la participación de *PAK4* en la alteración del fenotipo y el comportamiento de células MDA-MB-231.

Estos datos demostraron que los GR de los pacientes M1 son diferentes de los que provienen de los CLC y pueden afectar diferentes pasos de la cascada metastásica. Sin embargo, aún no está claro cómo se modifican los GR durante su estancia en la circulación de pacientes con enfermedad metastásica. Publicaciones recientes demostraron que los GR maduros tienen compartimentos endosomáles y conservan la capacidad de realizar endocitosis, así como de generar exosomas y microvesículas. Además, una investigación realizada por Thorn *et al* proporcionó evidencias de la capacidad de los GR para transportar moléculas como los aminoácidos. Es importante destacar que los resultados de esta tesis mostraron que los efectos de los GR pueden estar mediados de forma paracrina, lo que potencialmente implica la producción de vesículas. Esta información sugiere que los GR maduros pueden captar factores presentes en el plasma o en el tumor primario y liberarlos. Debido a su participación en la regulación de la producción de vesículas y su regulación positiva en los GR M1, LAMP2 podría ser un mediador importante en este proceso. Para

estudiar el papel de LAMP2, se generó un modelo LAMP2 KO utilizando CrisprCas9 en células progenitoras eritroides derivadas de la sangre del cordón umbilical humano 2 (HUDEP-2), una línea celular eritroide inmortalizada. La eliminación de LAMP2 redujo la cantidad y el tamaño de las vesículas liberadas, mostrándose la asociación entre LAMP2 y la producción de vesículas. Además, la ausencia de LAMP2 impide que los eritroblastos ortocromáticos actúen como quimio-atrayentes de células cancerosas. A pesar de los interesantes resultados, es necesario realizar más estudios para determinar la posible participación de LAMP2 en el transporte de vesículas en este contexto.

Este trabajo proporciona la primera evidencia de la participación de los GR en varios pasos de la cascada metastásica, además de demostrar el potencial de estas células como un nuevo biomarcador subrogado para ser utilizado en biopsia líquida. Además, también destaca el valor del uso de líneas celulares eritroides CD34+ inmortalizadas, como HUDEP-2, para estudiar la función de las proteínas en los GR. En conjunto, estos resultados sientan las bases de una nueva línea de investigación que requerirá más trabajo para dilucidar el mecanismo de acción de los GR en el cáncer.

SUMMARY

SUMMARY

Breast Cancer (BC) is the most common cancer diagnosed in women around the world, with over 2.3 million new cases in 2020. Despite the recent advances in treatments and screening programs, BC is still responsible for 16 % of cancer-related deaths, mainly due to the development of metastasis. Patient's prognosis will depend on their tumour subtype and stage of the disease. In BC there are 3 subtypes classified by the expression of Human Epithelial Growth Factor receptor 2 (HER2) and two Hormonal receptors (HR): HR estrogen receptor (ER) and progesterone receptor (PR). Luminal or HR-positive BC is the most common (over 70 % of the cases) and less aggressive subtype. This subtype is characterized by the expression of HR (ER and/or PR) and occasional HER2 expression in Luminal B patients. HER2 BC cancer is characterized by the overexpression of HER2 and the absence of HR. The treatment of these subtypes has improved notably due to the development of targeted therapies against HR and HER2. Finally, Triple-negative (TN) subtype does not express HR or HER2 (main therapeutic targets). It's the most aggressive subtype and does not have any targeted therapy, requiring chemotherapy or immunotherapy as an alternative treatment. In most cases, BC is diagnosed in the first stages when the tumour is localized, however, around 30 % of the patients will eventually develop metastasis during their treatment or present it at diagnosis (over 6 %). Metastasis is responsible for 90 % of cancer-related cell deaths and turns BC into an incurable disease with a five-year survival rate of 26-30% considering all subtypes, being even lower for more aggressive subtypes such as TN.

The development of metastasis is a complex process involving several steps that lead cancer cells from the primary tumour towards distant tissues, where they form secondary sites. These steps together constitute what is known as the metastatic cascade: angiogenesis, migration, intravasation, survival in the bloodstream, extravasation and colonization of distant tissues. Angiogenesis is often induced in the primary tumour due to the increasing need for oxygen due to the uncontrolled proliferation of cancer cells. Induction of angiogenesis in the tumour leads to the formation of new blood vessels that are generally leaky and with a defective structure, allowing for easier extravasation. During this process, tumour cells will also start to detach from each other and invade the surrounding tissues. At this point, tumour cells start to migrate through the tumour stroma towards the blood vessels. This migration can be performed individually/in small groups, which is characteristic of cells that have undergone epithelial-mesenchymal transition (EMT), or in large groups (epithelial migration), generally composed mainly of tumour cells with an epithelial phenotype. Once these cells arrive at the blood vessels, they need to cross the endothelial barrier to enter the blood. Tumour cells go through EMT to disrupt the endothelial barrier; however, lymphatic vessels or defective newly formed vasculature offer an easier route for extravasation. Once these cells have extravasated they are known as circulating tumour cells (CTCs). CTCs have a media time of residence of 1-2.4 hours. Most of them die due to shear stress, immune attacks or anoikis, and only a very small percentage can survive and extravasate in distant organs to form metastasis. During the extravasation, cells are required to adhere to the vessel walls and cross the endothelial barrier. Once CTCs have extravasated, they have to survive in the colonized tissue and eventually proliferate to form a secondary tumour. In BC, metastasis is generally localized in bone, lung and brain, however, each subtype presents different tropism patterns. Importantly, as stated before, TNBC is the most aggressive subtype and is more

prone to present advanced stages and early relapses. Studying mechanisms and the different interplay that take part in this metastatic cascade is essential to increase our understanding of this process and the development of new targeted therapies. In this regard, in a previous publication from our laboratory, Red blood cells (RBCs) were observed in direct contact with CTCs isolated from metastatic BC patients, being their presence indicative of a worse prognosis. Besides, we have also reported that the proteome profile of RBCs from BC patients differs from cancer-free controls (CFCs), suggesting that the presence of a tumour can modify their content. Despite being generally overlooked and considered mere oxygen transporters, recent publications have demonstrated that RBCs take part in the regulation of multiple processes such as coagulation, immunity and angiogenesis. Considering their increasing complexity, their abundance in blood and the evidence of their crosstalk with tumour cells, it is important to further understand the potential role of these cells in the metastatic cascade.

Besides the study of the metastatic cascade, the development of new technologies and the search for new biomarkers are essential to improve the treatment and diagnosis of metastatic BC patients. The current clinical practice relies on primary tumour profiling to study solid tumours. However, traditional tissue biopsy does not represent the tumour heterogeneity, is highly invasive and its applicability to study metastasis is limited due to the small size and bad accessibility of secondary tumours. Due to these constraints, an alternative technology known as liquid biopsy has been developed. Liquid biopsy is based on the study of tumour-derived material such as CTCs, circulating tumour DNA (ctDNA) and extracellular vesicles (EVs) present in different body fluids, mainly the blood. These biomarkers reflect tumour heterogeneity and are being extensively used for disease relapse, monitoring therapy response and determining resistance mechanisms and therapeutic targets. However, these entities have several drawbacks. CTCs and ctDNA are present in very low amounts in the blood, limiting their use to detect early stages of the disease and the metastasis. EVs, although more numerous and present in the early stages of the disease, their material is challenging to extract and purify. Due to these limitations, other biomarkers such as RBCs could be an interesting alternative. Despite not being tumour-derived materials, RBCs have been demonstrated to be modified by tumours and these changes reflect the presence of the disease as has been confirmed in other diseases such as Alzheimer's or Diabetes among others. Moreover, RBCs offer several advantages such as high quantities or easy isolation and extraction of their material.

Considering all this information, this thesis aims to study the role of RBCs in the metastatic cascade in BC as well as evaluate their value as biomarkers in BC. To achieve this, blood from BC patients, both non-metastatic (M0) and metastatic (M1), at the moment of diagnosis and from CFC were collected. RBCs from these patients were isolated using centrifugation and categorized into two distinct cohorts to facilitate the divergent approaches within this work.

As a first step, the value of RBCs as predictors of the presence of metastasis was evaluated. Protein from the RBCs was extracted and clinical data from routine blood tests was collected. A study of the blood test showed that some RBC-related parameters were altered, including red cell distribution width (RDW), haematocrit and haemoglobin concentration. Based on our previous proteomic analysis, reported by Pereira-Veiga *et al*., ELISA assay was used to examine the overexpression of lysosomal-associated membrane protein 2 (LAMP2) and purine nucleoside phosphorylase (PNP) in M1 RBCs. Due to the variable expression of PNP depending on the location of the metastasis, only the predictive value of LAMP2 for

detecting metastasis was further validated. Results from the receiver operating characteristic (ROC) analysis showed that LAMP2 alone had an AUC of 0.71. However, the AUC increased up to 0.89 when LAMP2 concentration was combined with the RDW and Haematocrit values, showing that RBCs have a good predictive value for detecting metastasis.

After studying the value of RBCs as a biomarker, the role of RBCs in the BC metastatic cascade was also studied. Based on the identification of RBCs attached to CTCs isolated from BC patients, the adhesion of RBCs to cancer cells was studied *in vitro*. Similarly to the phenomena visualized in patient samples, a greater percentage of BC patients had RBCs with adhesion to cancer cells, mainly from M1 patients compared with CFC. Considering these initial results, it was clear that RBCs from BC were not only altered in terms of their content but also functionally. To assess the potential effects of RBCs on the metastatic cascade, a set of assays was designed to study the role of these cells in different steps of the metastatic cascade: angiogenesis, migration, cluster formation, and adhesion. Complementary, potential effects of the RBCs in the primary tumour that can affect the metastatic cascade were also studied mainly the proliferation and cytokine production. In both approaches, functional analysis was performed by co-culturing RBC lysates from patients and controls with two BC tumour cell lines. However, in some assays such as chemotaxis or aggregation assays, RBCs were used directly after isolation to ensure that the integrity of the RBCs was maintained.

Results showed that RBCs, independently from their provenance, did not show any influence on the proliferation of both TNBC MDA-MB-231 and luminal MCF7 cell lines. In contrast, RBCs from M1 patients modified the cytokine profile of both tumour cell lines, inducing the production of CCL family cytokines, in agreement with the observations of Yin *et al*. Angiogenesis assays showed that M1 RBCs have a pro-angiogenic effect that is not observed when using CFC RBCs, reflecting the ability of RBCs to bind pro-angiogenic factors likely through DARC as described by Karsten *et al*. RBCs also had an impact on the migration of MDA-MB-231. M1 RBCs increased migration both *in vivo* and *in vitro*, as well as exhibited the ability to chemo-attract tumour cells. Moreover, when M1 RBCs were used as chemo-attractants, migrated cells presented an altered morphology, exhibiting membrane protrusions that resemble lamellipodia. Next, tumour cell aggregation assays performed with MDA-MB-231 showed an increased cluster formation when RBCs from M1 patients were present. Interestingly, exposure of RBCs from CFC to conditioned media from MDA-MB-231 altered their behaviour in this assay, leading to an increase in tumour cell aggregation similar to the observed when using M1 RBCs. This latter indicates that RBCs are conditioned due to the tumour microenvironment. Finally, adhesion to endothelial cells and collagen I, a main component of the vascular basement membrane, was studied as part of the extravasation process. Results from these assays showed that M1 RBCs increased the adhesion of MDA-MB-231 to both. Importantly, no changes in migration, adhesion or proliferation were observed for luminal BC cell line MCF7.

After studying the different steps of the metastatic cascade, it was clear that RBCs from M1 patients had an impact on MDA-MB-231´s ability to migrate and adhere; however, the mechanisms that mediate these effects remain unknown. To determine this mechanism, an RNA-sequencing analysis was performed to study how the RBCs impact the RNA expression of MDA-MB-231. Results from gene ontology analysis of the differentially expressed genes showed that pathways such as cell adhesion, contractile actin filament and stress fiber assembly were altered after the co-culture with RBCs. Moreover, further qRT-PCR analysis of a selected set of differentially expressed genes showed that *PAK4, HMOX1* and *EPHX1*

were overexpressed in MDA-MB-231. Among these genes, *PAK4* stands out as an important regulator of migration, adhesion, EMT and cell morphology. Due to the importance of this gene in the EMT, another set of genes associated with this process was analysed, showing that *PLS3, VIM* and *STAT1* were also significantly overexpressed in MDA-MB-231 co-cultured with M1 RBCs. Given these results, *PAK4* was studied as a potential mechanism for the effects of M1 RBCs due to its role as an upstream regulator of EMT and migration. The use of the PAK4 inhibitor LCH-7749944 on MDA-MB-231 cell co-cultured with M1 RBCs restored migration and adhesion to basal levels of the cell line previous to co-culturing, suggesting the involvement of *PAK4* in the phenotype alteration and behaviour of MDA-MB-231 cells.

This data demonstrated that RBCs from M1 patients are different from those coming from CFCs and they can affect different steps of the metastatic cascade. However, it remains unclear how RBCs are modified during their time in the circulation of patients with metastatic disease. Recent publications demonstrated that mature RBCs have endosomal compartments and retain the ability to perform endocytosis as well as to generate exosomes and microvesicles. Moreover, research performed by Thorn *et al* provided evidence of the ability of RBCs to transport molecules such as amino acids. Importantly, results from this thesis showed that the effects of the RBCs can be mediated in a paracrine way, potentially involving vesicle production. This information suggests that mature RBCs can uptake factors present in the plasma or the primary tumour and release them. Due to its involvement in the regulation of vesicle production and its upregulation in M1 RBCs, LAMP2 could be an important mediator in this process. To study the role of LAMP2, a LAMP2 KO model was generated using CrisprCas9 on human umbilical cord blood-derived erythroid progenitor 2 (HUDEP-2) cells, an immortalized erythroid cell line. LAMP2 is involved in the vesicle production in RBCs, showing that knocking out LAMP2 reduced the amount and size of the vesicle release. Moreover, the absence of LAMP2 is impaired with the ability of orthochromatic erythroblast to chemo-attract cancer cells. Despite the interesting results, further studies need to be performed to determine the potential involvement of LAMP2 in vesicle transport in this context.

This work provides the first evidence of the involvement of the RBCs in several steps of the metastatic cascade, as well as demonstrates the potential of these cells as a new surrogate biomarker to be used in liquid biopsy. Moreover, it also highlights the value of using immortalized erythroid CD34+ cell lines such as HUDEP-2 to study the function of proteins in RBCs. Altogether, these results set the basis for a new line of research that will require more work to further elucidate the possible mechanism of action of RBCs in cancer.

INTRODUCTION

INTRODUCTION

1. Breast cancer:

1.1 Epidemiology and risk factors

Breast cancer (BC) is one of the leading health concerns for women around the world. GLOBOCAN data from 2020 reported that BC is the most diagnosed cancer in women around the world with 2.3 million new cases each year (24,5 % of the total) and a mortality rate of 6.9 %[1]. In Spain, the Spanish Society of Medical Oncology (SEOM) estimated that up to 35.001 new cases of BC were diagnosed in Spain during 2023, being the second most diagnosed cancer in our country[2]. The inclusion of screening programs and the development of new treatments have allowed to reduce BC mortality a 40 % between 1980 and 2020[1]. Despite these advances, mortality is still very high, making up to 16 % of cancer-related deaths, mainly due to the development of metastasis[3,4].

In terms of the aetiology of this disease, several factors have been described to be associated with the development of BC. Age is considered to be the most important risk factor in developed countries. Almost 80 % of the patients diagnosed with BC are women over 50 years old and 50 % are aged between 50 and 69 years[5,6]. Other important factors could be divided into reproductive and non-reproductive risk factors. Starting with the first group, BC risk increases with early menarche, late menopause or older age at first childbirth. Non-reproductive factors that can also increase BC risk are obesity, which doubles the risk of BC in postmenopausal women, and alcohol consumption, which is estimated to cause up to 4% of the cases that were diagnosed in 2020. Finally, a reduced percentage of the BC cases, between 5 to 10%, have a genetics aetiology[7]. These cases are mainly associated with the presence of mutations in the *Breast Cancer gene 1/2* (*BRCA1/2*).

1.2 Breast Cancer subtypes

Breast cancer is a heterogeneous disease composed of different subtypes, that are mainly classified based on classical immunochemistry (IHC) markers such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER2)[8]. This classification is generally complemented with clinic-pathological variables that include proliferation index (ki67), tumour size, tumour grade and nodal invasion. Altogether, these parameters and markers provide information about the prognosis of patients and guide treatment selection[8].

In BC we can find 3 different subtypes based on the expression of the aforementioned classical IHC markers (Table 1). Luminal or ER-positive BC is characterized by the expression of Hormonal receptors (PR and/or ER) on the cell surface[9]. This subtype is the most common (over 70 %) and has two sub-classifications, luminal A (low expression of ki67 and absence of HER2) and luminal B (high expression of ki67 and can express HER2). HER2 subtype is characterized by the absence of hormonal receptors (PR and ER) and the overexpression of HER2[10]. Finally, triple negative BC (TNBC) does not express any known markers on its surface.

Most of BC patients are not metastatic at the moment of diagnosis[11]. In the majority of the cases, the patient's tumours are surgically resected before neoadjuvant therapy and, depending on the cases, they can receive postoperative radiation and/or adjuvant therapy[11], systemic therapy to prevent disease recurrence as well as to prolong their life expectancy. In BC patients that have undergone metastasis, systemic therapy is palliative to alleviate the symptoms [11]. Systemic therapies vary greatly depending on the patient subtype. Luminal A BC is the least aggressive cancer and greatly benefits from Hormonal Therapy (HT). Luminal B is more aggressive and generally requires HT combined with chemotherapy[9]. The recent introduction of Cyclin-dependent kinase inhibitors (CDKi) combined with HT has greatly improved the treatment effectiveness in advanced luminal patients[11]. HER2+ tumours are more aggressive and grow faster than luminal ones, but recent advances in targeted drugs against HER2 used in combination with chemotherapy have improved the prognosis of these patients[8,10]. TNBC is the most aggressive subtype, and the patients are treated with chemotherapy or immunotherapy because there is no targeted therapy[12]. Altogether, this causes TNBC to be the subtype with the worse prognosis and shorter survival[12,13].

Table 1. Breast cancer subtypes and classification based on marker expression. Ki67 Low (<20%) and High (>20%)

IHC status	Luminal A	Luminal B	HER2	TNBC
ER	++	+	-	-
PR	++	-/+	-	-
HER2	-	-/+	++	-
Ki67 (%)	Low	High	High	High
Outcome				
	Good	Intermediate	Poor	Poor

1.3 Staging of BC disease

When cancer is diagnosed it is important to study and describe the tumour site, tumour profile (histological, morphological and molecular characteristics that determine the subtype) and the tumour stage or anatomic disease extent. This last feature significantly influences the prognosis, since those patients with extended disease will have worse outcomes than those where the tumour is localized[14,15]. Besides, the therapeutic strategy will be different depending on the stage of the disease. The most used tumour staging system is tumour, node and metastasis (TNM) system first developed by Pierre Denoix and the Union for International Cancer Control (UICC)[16]. This system was further refined in collaboration with the American Joint Committee on Cancer (AJCC), leading to the creation of the AJCC-TNM staging system, which is currently the preferred system for most clinicians[17].

In the AJCC-TNM system classification, the T category corresponds to the extent of the primary tumour in terms of size and invasion of adjacent structures and ranges from T0 to T4 depending on the degree of spread[16]. N category refers to the absence or presence of regional lymph node metastasis and ranges from N0 to N3 depending on the amount and extension of lymph node affectation[16]. Finally, the M category indicates the absence (M0) or presence of

distant metastasis (M1)[16]. This category was expanded in 2010 to include M0 (i+) which refers to patients with no signs or symptoms of metastasis but having circulating tumour cells (CTCs) in the blood and forming micrometastasis in bone marrow or other tissues[18]. Based on this classification, clinicans classify tumours in 4 different stages. Stage I and Stage II include small tumours (T1-2) and larger tumours (T2-4), respectively, which have not spread to nearby lymph nodes or other parts of the body (M0)[16]. Stage III includes tumours from various sizes (T1-4) that have spread to the lymph nodes (N1-3) but not to other parts of the body[16]. Finally, Stage IV or metastatic cancer includes tumours (T1-4) that have spread to the lymph nodes (N1-3) and other areas from the body (M1). Metastasis is responsible for 90% of all cancer-related deaths[19]. Between 5 to 10 % of BC patients will show metastasis at the moment of diagnosis, and around 30 % of all BC patients will eventually develop metastasis during their treatment or even after finishing it[2]. Despite the advances in terms of prevention, diagnosis, and therapies, metastatic BC is still an incurable disease with a very bad prognosis, with a five-year relative survival rate of 26-30%[6,20–22].

Imaging diagnostic tools such as ultrasound, positron emission tomography (PET), computed tomography (CT) or magnetic resonance imaging (MRI) have allowed clinicians to assess tumour size, location, the relationship with the adjacent normal tissues and the presence of nodal and/or distant metastasis[23]. Despite the utility of these technologies, they still have sensitivity limitations for the detection of micrometastasis, which is one of the main clinical issues for advanced cancer diagnosis and monitoring[18].

2. The metastatic cascade

The study of the metastatic process and the different steps that form it is especially relevant to understand the mechanism and interplayers that take part in it. As the disease advances through different stages, tumour cells start to disseminate from the primary tumour to the adjacent tissues and local lymph nodes[16]. Eventually, these cells will reach the nearest blood vessels and intravasate into the blood, becoming CTCs. These CTCs will travel through the blood and will attach to the vasculature and extravasate in distant tissues, forming microscopic metastasis that will grow to form observable metastasis[24,25]. At this stage, patient management is complex, ruling out surgery as a viable choice and leaving palliative treatments as the sole option available.

In the following sections, we will introduce the steps of metastasis, namely, migration, angiogenesis, intravasation, survival in the bloodstream, extravasation, colonization and growth in a distant tissue[25]. All of these steps outlined above represent what is known as the metastatic cascade, the process that leads to the formation of metastasis and the transition from a localized disease to a systemic one. The chances of a cell completing all the steps and forming a metastasis are very low and most of the cells leaving the tumour fail to colonize distant organs[19,25].

2.1 Angiogenesis, migration and invasion:

Malignant tumours are characterized by rapid growth, which eventually leads to oxygen starvation in the region. The absence of oxygen will act as a double edge sword, killing many

tumour cells but also leading to the expression of Hypoxia-inducible factor 1 alpha (HIF-1α), a major regulator of response to acute and intermittent hypoxia in normal tissue[26–29]. Activation of HIF-1a increases cell plasticity and can induce partial or total epithelial-to-mesenchymal transition (EMT), depending on the duration and periodicity of the hypoxia[29,30]. HIF-1α is also a master regulator of angiogenesis at different levels, such as upregulating the production of pro-angiogenic factors, mainly vascular endothelial growth factor (VEGF), recruiting endothelial cells , and regulating the extracellular matrix (ECM) to favour the alignment and assembling of endothelial cells into tubular structures to form new tumour vessels (Figure 1)[31–33]. Moreover, tumour cells can also form tubular structures similar to normal blood vessels in a process known as vasculogenic mimicry[34]. These vessels can connect to other existing blood vessels, further increasing the newly developed vascular network[35]. Tumour neovascularization will foster direct tumour growth by increasing the supply of oxygen, and nutrients and the removal of metabolic waste. Moreover, pro-angiogenic factors such as VEGF are able to modify the microenvironment and promote immunosuppression through the inhibition of dendritic cell maturation and the recruitment of myeloid-derived suppressive cells (MDSC)[36]. Importantly, the imbalance in the production of pro-angiogenic factors leads to the formation of a defective new vasculature[32]. These abnormal tumour vessels have sections without pericytes and basement membrane, smaller diameter and are extremely permeable even to large molecules, facilitating the entrance of cancer cells into the blood[33,37,38]. Moreover, both the distribution of the vessels and blood flow in the tumour are highly irregular, leading to further hypoxia and activation of HIF-1[31].

As the tumour grows and differentiates, cancer cells start to detach from the tumour mass, penetrate the basement membrane and invade the surrounding stroma (Figure 1). Tumour invasion is the first step in the metastatic cascade[25] and is considered the point where pre-cancerous neoplasia advances to malignant cancer. During the invasion, cells can migrate using two main mechanisms: EMT or complex tumour cell migration. Tumour cells that undergo EMT downregulate epithelial markers (such as E-cadherin and ZO-1) and transit to a mesenchymal phenotype, generally exchanging keratin for α-actin or vimentin[39,40]. EMT can be initiated by the activation of transforming growth factor beta (TGF-β), Wnt or Notch pathways among others, that lead to the upregulation of *TWIST1, ZEB1/2* or *SNAIL*[39]. Cells that suffer EMT lose partial or total cell-cell adhesion as well as attachment to the basement membranes. On the other hand, these cells will gain plasticity and invasive properties, that will allow them to move more efficiently through the ECM and even remodel it [41,42]. These cells generally migrate alone or form small groups. Tumour cells can also migrate forming larger groups through complex tumour cell migration, where cells do not undergo EMT or do it partially, receiving also the name of epithelial migration[40,43]. Cells that migrate collectively maintain cell polarity as well as cell-cell adhesion through cadherins (E, N and P) and GAP junctions. These groups are heterogeneous and their migration is generally supported by the cells located on the leading front that have more invasive and metastatic properties, generally acquired through partial EMT. The movement of these groups is based on coordinated actin dynamics and ECM remodelling performed by leading cells[44]. As cells migrate through the stroma, they remodel the extracellular matrix through the use of metalloproteinase and the activation of fibroblast into cancer-associated fibroblast (CAFs), creating a stiffer environment by restructuring the stromal network[45]. This remodelling is essential for directing and making easier the migration of the cells towards lymphatic and blood vessels, especially for those cancer cells with an epithelial phenotype[42,46]. During the migration, cancer cells will interact and communicate with immune cells present in the stroma. Many tumours, including

BC, will induce M2 type differentiation of macrophages present in the tissue, which will provide an immunosuppressive microenvironment by recruiting MDSCs and producing anti-inflammatory cytokines such as IL-10 or TGF-β [26,30,39]. Tumour cells will also protect themselves from cytotoxic T cells or Natural killers through the expression of PD-L1 or arginine accumulation among other mechanisms[47].

Altogether, the previously mentioned processes will allow tumour cells to invade and colonize the stroma, as well as to initiate the migration towards lymphatic and blood vessels present in the tumour, which is the next step of the metastatic cascade, the intravasation.

2.2 Intravasation and circulation in blood

Intravasation is an early step of metastasis that involves the crossing of the endothelial barrier and entrance into the luminal space vessels by cancer cells (Figure 1). Tumour cells can disseminate through blood or lymphatic vessels, making the lymphatic vasculature an easier route due to the lack of thigh inter-endothelial junction and the presence of a discontinuous basement membrane[39,48]. Moreover, lymph nodes are often one of the first sites of metastasis, being considered an important marker in BC for cancer staging and risk of distant metastasis[49]. From the lymph nodes, tumour cells can enter the venous system, finally reaching the blood[50]. To extravasate through blood vessels, especially intact healthy vasculature, tumoural cells generally require undergoing total or partial EMT to be able to invade the basement membrane and cross the endothelial barrier[48,50]. Tumour-associated macrophages (TAM) present in the tissue also help in this process by degrading the basement membrane and producing factors such as epithelial growth factor (EGF) to attract cancer cells[51]. Importantly, as mentioned before, as primary tumour grows and angiogenesis is induced, new abnormal and leaky vasculature is formed, allowing for an easier extravasation[50]. The extent of haematogenous or lymphatic intravasation may also depend on the relative density of blood and lymphatic vessels present in the tumour[50,52]. Despite the importance of lymphatic intravasation and lymph node metastasis, there is no clear evidence that metastasis are generated in that way[53,54]. The most likely mechanism of initiation of distant metastasis is a direct intravasation into the blood in the primary tumour or the lymph nodes[52,54].

Once cells intravasate and enter into circulation they will be referred to as CTCs. Tumour cells can intravasate into blood as single cells or forming clusters of cancer cells that can be accompanied by stromal cells from the tumour microenvironment (TME) [55–57]. CTC cluster can also be generated by aggregation of CTCs in circulation[58,59]. Also, depending on the properties of the ECM in the primary tumour or in the metastasis, cancer cells can form clusters by a mechanism called jamming, which consists in the formation of CTC cluster with mesenchymal phenotype due to increased confinement and density of ECM[60,61].

To reach a distant organ and form secondary metastasis, CTCs will have to face 3 main obstacles: anoikis (detachment-induced apoptosis), immune system attacks and physical damage due to shear stress[55,57]. Due to these reasons, the metastatic process is very inefficient, with only about 0.01% of CTCs reaching secondary sites[52]. Tumour cells rely on different mechanisms to survive in the blood. Thus, CTCs that have undergone EMT avoid more easily anoikis thanks to their mesenchymal phenotype. However, CTCs that retain an epithelial phenotype can still survive to anoikis if they are part of a cluster, which will also benefit

CTCs against shear stress and immune attacks, especially in heterotypic clusters containing non-tumoural cells[56,60,62]. Indeed, having clusters in the blood has been linked with a worse prognosis in BC patients as reported by us and others[63,64]. Importantly, CTCs that expressed receptors for the coagulation factors VIIa and X can also form aggregates with platelets and fibrin clots that will provide them protection against shear stress and immune surveillance, especially against NK cells[65–67].

CTC has a media time of residence in the blood of 1-2.4 hours. During this time, CTCs may interact and adhere to endothelial cells from vessels of reduced diameter and low flow pressure, especially high endothelium venules[24]. CTC clusters can also be trapped on these vessels due to their bigger size. Coagulation is also an important factor in this process since it further reduces the blood flow and allows CTCs a better and more stable contact with the endothelial cells[24,39]. To extravasate, CTCs with a more mesenchymal phenotype perform diapedesis which is similar to the mechanism that white blood cells) use to extravasate[26]. This process is composed of three steps: cell rolling (weak interactions, for example via CD44 or integrin αVβ3), adhesion to endothelial cells (strong interactions, for example, the integrin α5β1) and transmigration (cancer cells actively disrupt endothelial barrier and extravasate)[24,68]. Alternatively, CTCs adhered to the vessel walls can also cross passively the endothelium through a mechanism known as angiopellosis (migration mediated by the activation and remodelling of endothelial cells)[69,70]. Once cancer cells have extravasated into the secondary organs, they will start the colonization and formation of metastasis.

In BC, there are 3 main organs where CTCs tend to extravasate and form metastasis: bone, lung and brain[39]. Bone metastasis is the most frequent, partly due to the presence of sinusoids (irregular vasculature that allows for an easier migration)[71] which facilitate CTC extravasation. The lung is the next most common organ where metastases are formed. In this organ, capillaries are tighter and less accessible than the leaky vasculature in the primary tumour or the sinusoids present in bone. BC cells require different molecules such as angiopoietin-like 4 (ANGPTL4), cyclooxygenase 2 (COX2)/ metalloproteinase 1/2 (MMP1,2)/Epiregulin to disrupt the endothelial barrier in the lung[72,73]. Lastly, the brain is the less common of all 3, especially due to the presence of the blood-brain barrier which hampers tumour cells` extravasation. However, cancer cells have managed ways to overcome it, especially through the expression of COX2, MMP2 or Cathepsin S, promoting transmigration via junctional protein cleavage[74]. Importantly, certain regions of the brain such as supratentorial regions have a low perfusion and flow pressure, which allows an easier extravasation of CTCs [24].

2.3 Colonization and formation of metastasis.

The establishment of metastasis depends on multiple factors, such as the origin of the cancer and subtypes and the microenvironment of the pre-metastatic niche. Two main theories explain how the metastasis are formed and why they are present in certain organs. Firstly, in 1889 Stephen Paget, proposed that some tumour cells ("seed") grow preferentially in the microenvironment of certain organs ("soil"), implying that metastasis only forms in a suitable soil or organ for a certain type of cancer (organotropism)[75]. As mentioned in the extravasation section, BC cells can develop certain mechanisms that allow them to extravasate in their main metastasis organs (bone, lung and brain)[39]. Moreover, many publications also describe how BC tumour cells can specifically modify the microenvironment and the stromal cells present

in the lung, brain and bone. These mechanisms allow cancer cells to survive the initial apoptosis signals and manipulate later on the stromal cells in order to foster their growth[76–80]. Despite the compelling evidence, the Seed and Soil theory was not universally accepted. Alternatively, some experts suggested that the pattern of tumour metastasis relied on the anatomical characteristics of the vascular and lymphatic vessels[75]. This hypothesis argues that CTCs are arrested non-specifically in the first organ that they encounter. This theory has also solid evidence such as the abundant liver metastasis for tumours from the gastrointestinal tract due to the drainage of these organs that takes place through the portal venous system. Nowadays, the consensus is that the reality is something in between these two theories, but further research is needed to better characterize it.

In the context of BC, distinct tropism patterns are evident, exhibiting subtype-dependent characteristics. HER2 and luminal BC tend to form metastasis in the brain and bone[8]. Meanwhile, TNBC generally establishes secondary sites on the brain, lung and distant nodes. Due to its aggressiveness and poorly differentiated histology, TNBC tends to present advanced stages and early relapses[13].

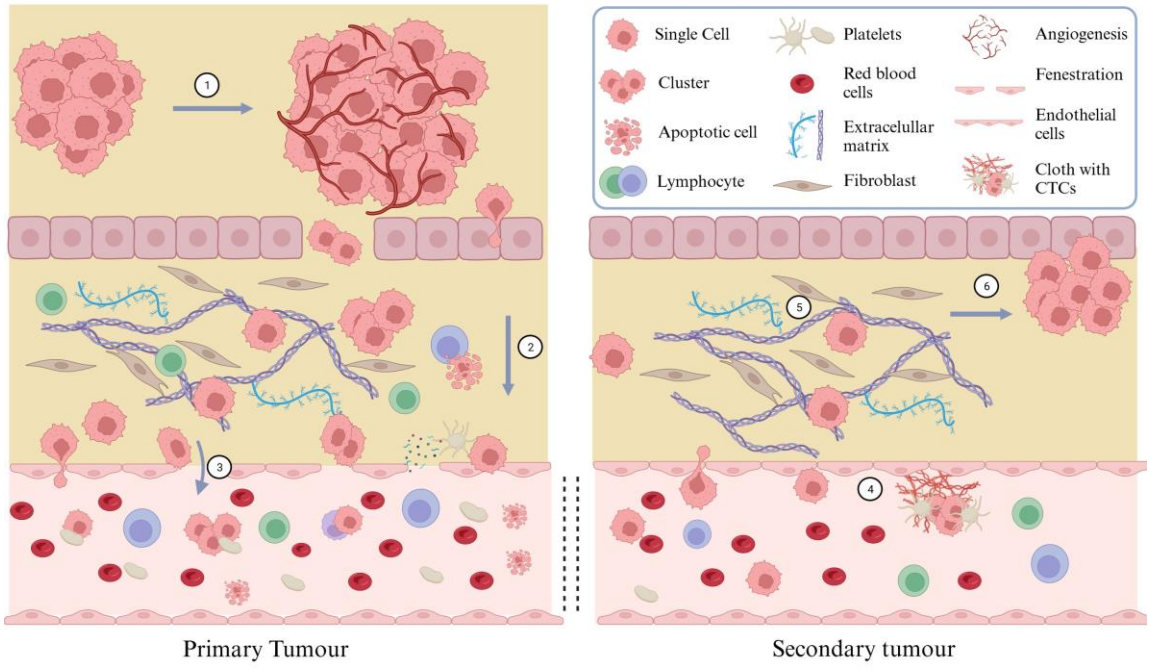


Figure 1. Representative scheme of the different steps composing the metastatic cascade. 1. Proliferation of cancer cells and induction of angiogenesis. 2. Invasion and migration of tumour cells through the stroma. 3. Intravasation of cancer cells into the bloodstream 4. Extravasation of the Circulating tumour cells present in the blood to distant tissues. 5. Anchorage and survival of the extravasated CTCs in the secondary site. 6. Growth of the extravasated cells and formation of a secondary tumour.

3. Liquid biopsy

3.1 Utility of Liquid Biopsy on clinical practice

The current clinical practice relies on primary tumour profiling in order to study solid tumours[81]. Despite many clinicians´ base diagnostic and treatment of their patients on this analysis, it has several drawbacks: samples not being representative of the tumour heterogeneity, incapability to perform serial testing, high cost and the invasiveness of tissue biopsy[81,82]. Additionally, the development of metastasis further limits the effectiveness of traditional biopsy techniques. Metastasis can be very difficult to biopsy due to their small size, number and inaccessible location[83]. Moreover, secondary tumours tend to evolve differently than the primary tumour, increasing even more the heterogeneity[84]. Taking together all of these drawbacks, oncology research has been focusing on an alternative technique called liquid biopsy (LB), based on the analysis of the tumour material that is being released into different biological fluids (Figure 2). LB generally focuses on blood sampling, although saliva, pleural effusions, cerebrospinal fluid or urine are other biological fluids that are currently being studied[82]. This technique allows for real-time, non-invasive and repetitive analysis of tumour derived material such as CTCs, circulating tumour DNA (ctDNA), cell-free RNA and extracellular vesicles (EVs)[83]. More importantly, it reflects better the heterogeneity of the tumour. LB is currently being extensively used for disease relapse, monitoring the therapy response or determining resistance mechanisms and therapeutic targets[83,85]. The increasing importance of the use of LB is reflected in the 638 clinical trials using CTCs, 511 using ctDNA and 79 using exosomes that are listed on the ClinicalTrials.gov database[85,86]. Currently, other novel biomarkers such as Tumour-educated platelets (TEP) are also showing promising results[87,88]. Down below, the advantages, disadvantages and the current state of each biomarker are depicted (Table 2).

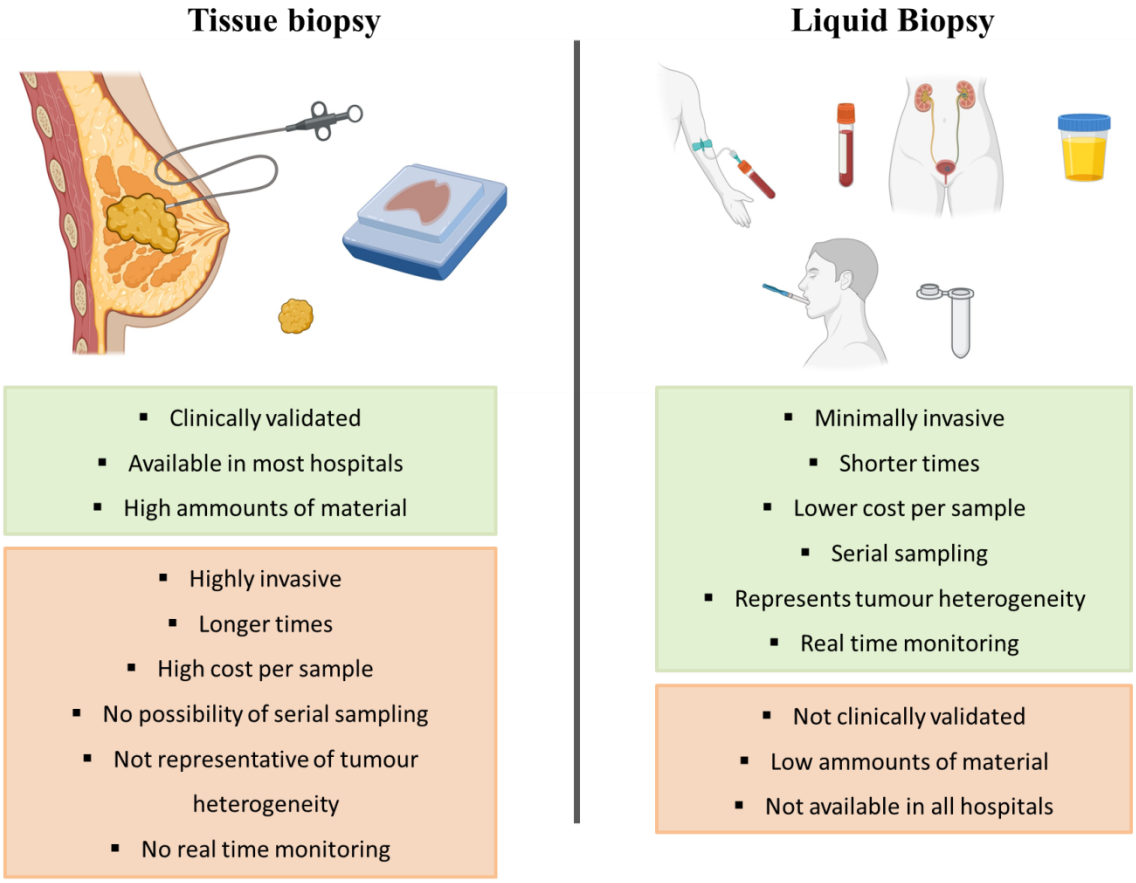


Figure 2. Comparison of the advantages (green) and disadvantages (red) of the liquid biopsy versus the traditional tissue biopsy currently employed in the clinic as the gold standard.

3.2 Liquid biopsy biomarkers

3.2.1 Circulating tumour cells (CTCs)

CTCs are the cancer cells that have extravasated into the blood. This tumoural entity has been validated in the clinical practice. The gold standard method for CTCs isolation and enumeration, known as CellSearch® system, was approved by the FDA in 2004 to predict the outcome of metastatic BC patients[89]. In 2007 and 2008, CellSearch® system use was also approved by the FDA for monitoring metastatic colorectal and prostate cancer[89,90]. Moreover, the use of an alternative isolation method, known as Parsortix® system, also received FDA approval in 2022 for detecting CTCs in metastatic BC patients[91]. Thus, having ≥ 5 CTCs or ≥ 3 CTCs per 7.5 mL of blood is indicative of worse outcome for breast and prostate or colon cancer patients, respectively[84]. CTCs detection can vary depending on the tumour, going from a 70% of the treatment naïve patients in breast cancer to a 30% in colorectal cancer[92,93]. However, this system has several limitations. Firstly, CellSearch® is based on a positive enrichment of cells expressing EpCAM, an epithelial marker on their surface[84]. Due to this technical limitation, all cancer cells that have a mesenchymal phenotype are not recognized by this system[83]. However, many of the breast and prostate cancer cells strongly express EpCAM[84]. Secondly, CTCs are present in a very low concentration even in blood from metastatic cancer patients (< 10 CTCs/mL), being generally below the optimal detection

levels in those patients with early stage disease[84,84,94]. This greatly limits the application of this marker for screening and early cancer detection.

3.2.2 Circulating tumour DNA (ctDNA)

Under physiological conditions, normal cells release DNA into the blood through apoptosis, necrosis and direct secretion[81]. This material receives the name of cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA), which is released by tumoral cells, composing just a very small fraction of it (<1%)[84]. Currently, its application in the clinic is becoming more prominent, with four tests related to ctDNA approved by the FDA. Among them we can count Cobas® *EGFR* mutation test for non-small cell lung cancer, BRACAnalysis CDx test based on the detection of *BRCA1/2* mutations in breast, pancreatic, ovarian and prostate cancer, EpiproColon® test that detect methylation patterns of *SEPT9* in colorectal patients and Therascreen PIK3CA RGQ test that detect mutations in *PI3KCA* gene to guide treatment in BC patients[95–100]. Despite the recent advances, the ctDNA presents similar limitations as the CTCs, mainly due to the low amount of material that can be extracted from the blood of the patients, the limit in sensitivity of detection and the lack of standardized extraction protocols[83,101]. ctDNA can only be found in 75% of the patients with metastatic disease, and the percentages drop even further in earlier stages of the disease, limiting its applicability as a marker for screening and early cancer detection[101,102]. Nonetheless, the device known as CancerSEEK, focused on the analysis of ctDNA and circulating proteins by mass spectrometry to detect early-stage cancers, received FDA approval in 2020, providing new platforms for early detection[103]. Despite this, the efficacy of this system has proven to be limited in BC patients[104].

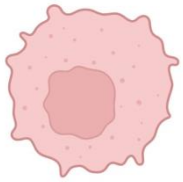
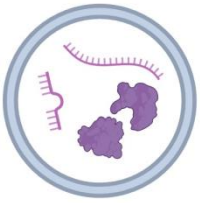
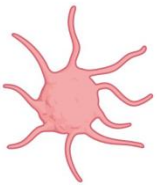
3.2.3 Extracellular vesicles (EVs)

EVs are small membranous particles present in the vast majority of the body fluids, mainly in blood[84]. They play a fundamental role in intercellular communication, both in physiological and pathological conditions[81]. Depending on their biogenesis, these particles contain different variety of proteins, carbohydrates, lipids, metabolites, RNAs and DNA fragments[105]. This content acts as a fingerprint of their cell of origin and it can be used as potential biomarkers of the tumour presence, evolution and response to treatment[84,106]. Although EVs are less explored than CTCs or ctDNA, they have several advantages such as a higher availability and longer half-life of their content thanks to the protection of their membrane[84]. In recent years, important advances have been achieved, putting EVs closer to the clinic with two systems using EVs being granted by the FDA breakthrough device status [107–110]. These devices are EXO-pancreatic ductal carcinoma (PDAC), which has been developed to detect the early stages of this aggressive disease, and ExoDx Prostate IntelliScore, which focuses on the study of microvesicles in urine and can stratify prostate cancer patients in terms of the grade of the disease [107–110]. Importantly, ExoDx has proved to be an independent and more effective biomarker than the currently used prostate-specific antigen (PSA)[110]. Although EVs are a promising biomarker, they present several limitations such as a lack of standardization in their isolation protocols and the contamination of isolated EVs by cfDNA, lipoproteins and cells[105].

3.2.4 Tumour educated platelets (TEP)

Platelets, the smallest of our blood cells, are enucleated cells that primarily work to create a clot at the site of vascular damage, effectively halting blood loss. Platelets are deeply affected by the presence of cancer. Interaction of these cells with tumour cells leads to the formation of TEPs with an altered RNA profile and properties[111]. The education of these platelets is mediated by different mechanisms such as the sequestration of biomolecules from the tumour, tumour-specific splice events produced by external stimuli such as platelet activation (despite not having a nucleus, platelets have fully functional transcriptional machinery) or alteration of their producer cells (megakaryocytes) in the bone marrow via cytokines or extracellular vesicles from the tumour cells[111–113]. These modified platelets support tumour growth and metastasis, aiding tumoural cells to build the stroma, increasing angiogenesis, protecting tumour cells in circulation and promoting invasion[111]. Along with these changes in behaviour, researchers found that TEP content is altered. This, coupled with the fact that platelets are highly abundant and their isolation is very simple, makes TEP an appealing surrogate biomarker in cancer. One of the first studies performed comparing platelets from healthy donors and six different primary cancer types showed that TEP from cancer patients had a differential mRNA expression[88]. This same study developed a predictive algorithm using these differentially expressed RNAs to distinguish healthy donors and cancer patients with 96% accuracy and identification of cancer types with a 71% accuracy[88]. Several articles suggest that TEP RNA content could provide information about the presence and subtypes of different cancers, including breast, lung, colorectal, prostate and glioblastoma tumours[87,88,111,113,114]. Importantly, the increasing relevance of this biomarker is reflected on the 9 clinical trials listed on the ClinicalTrials.gov database[86]. Despite these results, TEP remains understudied, and more knowledge is needed about the splicing mechanism that takes place in these cells and, similarly to EVs and ctDNA, there is a lack of standardized protocols for their isolation[115].

Table 2. Advantages and disadvantages of the different biomarkers that could be used in liquid biopsy.

CTC	Advantages	Provide vast information about the tumour (RNA, DNA, protein, methylation), detects CTCs derived from metastasis and primary tumours, provide rapid information, FDA-approved applications, functional assays permitted.	
	Disadvantages	Low amount in blood, current limitations in their isolation (low/negative EpCAM CTCs not detected), difficult to detect in early stages, short half-life.	
ctDNA	Advantages	Provides information about mutations and methylation patterns present in tumour cells, relatively easy to isolate, high sensitivity, good representation of tumour heterogeneity, FDA-approved applications.	
	Disadvantages	Low amount in blood (mainly in early stages), short half-life, challenging standardization, less common mutations are hard to detect.	
EVs	Advantages	High amount in blood, provide a lot of information about the tumour (RNA, DNA, protein, methylation), can be detected at early-stage, easy to isolate, long half-life	
	Disadvantages	No FDA-approved applications (two breakthrough device status), challenging standardization, difficult purification and quantification of the material contained in EVs.	
TEP	Advantages	High amounts, long half-life, present in early stages of the disease, easy to isolate and purify	
	Disadvantages	No FDA-approved applications, does not provide direct information about the tumours, less studied, lack of standardization	

4. Understanding the complexity of RBCs

During the tumour growth as well as in their time in circulation, cancer cells interact with many different cells such as platelets, neutrophils, macrophages, natural killers, endothelial cells, RBCs or fibroblast[116]. In these last years, different researches have shown that these interactions have an important impact in the development of the disease and can be exploited for developing new biomarkers as well as targeted therapies[116]. Among these cells, RBCs have been generally overlooked and considered as mere bystanders. Despite this, an increasing number of publications are providing evidence of the RBCs´ role in different processes and ability to interact with other cells. In the following sections, this newly

discovered RBCs´ complexity will be reviewed and the importance of studying these cells in cancer will be highlighted.

4. 1 Non-oxygen-transport related functions of RBCs

RBCs are one of the most abundant cells in the blood, with 20 to 30 trillion cells in the average adult[117]. In the past, RBCs were regarded as mere oxygen transporters. This is mainly due to the fact that RBCs do not possess many of the organelles normally present in the rest of the cells such as nuclei, ribosomes or mitochondria[118]. This abnormal composition allows RBCs to enrich themselves in haemoglobin (Hb), an essential protein in the oxygen transport, as well as to reduce their size, become highly deformable and acquire their typical biconcave shape, allowing them to traverse easily through small capillaries[118]. Despite being highly specialized in oxygen transport, these cells take part in many other biological processes[118,119].

4.2 Role of RBCs in blood rheology and coagulation

RBCs play an important role in regulating blood rheology directly by secreting various substances (release of NO, NO metabolites, ATP and other bioactive molecules)[120], especially in tissues with low oxygen tension or by physical activation of mechanoreceptors present in the RBC surface[121]. Through these mechanisms, RBCs are able to regulate endothelial cells activation (further production of vasoactive molecules) and control the vasodilatation of the vessels, maintaining tissue oxygenation and cardiovascular homeostasis[121]. Under pathological conditions, RBCs that retain damage due to oxidative stress, lose partially or totally their ability to produce vasoactive substances[120]. Moreover, cells become stiffer, making difficult RBCs circulation as well as diminishing the sensitivity of their mechanoreceptors[122].

RBCs also participate in the regulation of the coagulation[118,123]. It has been described that RBC's axial flow promotes platelet margination towards vessel walls[123]. Moreover, RBCs can interact with platelets through αIIbβ3-ICAM4 receptors and activate them through the secretion of ATP and ADP under certain conditions such as hypoxia or high shear stress[123]. Notably, activation of platelets promotes the activity of αIIbβ3, further increasing this interaction[124]. RBCs can also promote coagulation by externalization of phosphatidyl serine (PS), a negatively charged phospholipid that provides a scaffold for factor X activation and active thrombin generation[118]. PS externalization is associated with RBC aging, being more present in older RBCs as well as RBCs that have sustained damage[125]. PS is only externalized in this small subset of RBCs in normal conditions, but under pathological conditions (due to oxidative stress produced by the inflammatory state of the patients) expands significantly, being responsible for up to 40% of the thrombin generation in blood[118,126]. In addition, RBCs with PS on their membrane are also more adhesive to endothelial cells, further increasing the probability of generating vascular occlusion[124,127].

During their maturation and aging, RBCs release from their membrane microvesicles (MVs)[125]. The generation of these MVs is generally triggered by the loss of membrane phospholipid asymmetry, with the previously mentioned externalization of PS, and band 3 oxidation and clustering, which leads to membrane blebbing[128,129]. This RBC derived MVs contain several pro-coagulant proteins such as phospholipid scramblase I, complement component C9 precursor or β2-glycoprotein I, allowing them to activate coagulation through

tissue factor and contact pathways[118]. Although these MVs are produced in high amounts, they are also rapidly cleared by macrophages through recognition of PS exposure and Fc region of IgG antibodies that opsonize oxidized band 3[128,129]. During acute and chronic inflammatory states, the oxidative stress that RBCs suffer triggers an increased production of RBC derived MV that exceeds the capacity of macrophage clearance[123,125]. The accumulation of MVs derived from RBC in stored blood leads to decreased clotting times, providing evidence of their procoagulant role[130]. Moreover, as RBCs accumulate damage from oxidative stress, they reduce their size and Hb content (oxidized Hb is also released in these vesicles)[129,131]. This leads to an increase in the RDW that can be appreciated in many diseases, due to the increase in the fraction of old/damaged RBCs that have a smaller size[131]. These RBCs have their deformability and elasticity affected, which hampers them to return to their normal biconcave shape after deformation and makes them to get tightly trapped in the clots, further increasing the risk of thromboembolic events [124,132].

4.3 RBCs immune and angiogenic functions

Besides their role in regulating haemostasis and blood flow, RBCs have an important role in regulating the immune system. Recent studies have shown that a subset of RBCs express nucleic acid toll-like receptor 9 (TLRs) that is involved in scavenging of CpG-containing cell-free DNA from pathogens and host cells, preventing the accumulation of this pro-inflammatory signal[133]. In homeostatic conditions, it has been found that intracellular TRL9 in RBCs mediated the clearance of mitochondrial cfDNA[134]. Interestingly, TLR9 can also be found in the surface of the RBCs and its expression is increased during sepsis[134]. During infection, excessive DNA binding to RBCs TLR9 generally leads to altered RBC function, structure and loss of CD47 in the membrane[117,134]. Loss of CD47 and presentation of CpG cfDNA by TLR9 to macrophages ultimately leads to erythro-phagocytosis and innate immune activation during infection and systemic diseases such as cancer[134–136]. Recent studies have discovered a wide variety of TLRs in the transcriptome of mammalian reticulocytes, indicating that mature RBCs could be capable of scavenging other types of nucleic acids[117,137].

Interestingly, RBCs also modulate immune response by capturing a wide variety of cytokines through Duffy antigen receptor for chemokine (DARC)[117]. It has been demonstrated that RBCs act as a sink for CXCL8, as well as other CXC and CC chemokines, preventing neutrophil signalling, recruitment and activation. Interestingly, binding to DARC does not trigger signal transduction in RBCs[138]. Although it has less affinity, DARC can be also found in microvesicles derived from RBCs[138]. This scavenging role, prevents an overstimulation of the immune system that could lead to tissue damage. Other authors have indicated that the role of DARC is involved in the maintenance of blood chemokine levels, acting more like a reservoir (DARC-Chemokines interaction is reversible)[138,139]. Despite these contradictory functions, it has been hypothesized that RBCs may scavenge chemokines from inflammation sites but eventually release them when the concentration in plasma is low[117].

Finally, RBCs can also regulate and influence the angiogenesis. Amongst DARC targets, we can also find pro-angiogenic factors that attach with high affinity this receptor, allowing RBCs to regulate angiogenesis onset[140]. Moreover, RBCs are also involved in the production of Sphingosine-1-Phosphate (S1P) an important signalling molecule involved in vascular development and maintenance[141]. Although indirectly, RBCs can also regulate the

angiogenesis via the stimulation of erythropoietin, an erythropoiesis activator, in cases of anemia when haematocrit is low[142].

4.4 Role of RBCs in cancer

There is increasing evidence that the presence of cancer affects the RBCs. Firstly, Red cell distribution width (RDW), a morphological parameter that measures the variability in size and volume of RBCs, is increased in patients with breast, lung, prostate and colorectal cancer[143–147]. The increase in the RDW is a reflection of the inflammation produced by the disease, and despite the correlation with the presence of cancer it can be found altered in other systemic diseases such as COVID-19 or cardiovascular diseases[148–151]. Secondly, most of the patients will also develop anemia due to the extensive loss of RBCs and inhibition of erythropoiesis mediated by inflammation[131,152]. Moreover, treatments given after the diagnosis of the disease further accentuate the anemia in oncologic patients[153]. Recent studies from our laboratory have found that RBCs from cancer patients have an altered proteomic profile in breast cancer [154]. Moreover, independent studies have also found similar observations in hepatocellular cancer[149]. Interestingly, Geng *et al.* found altered micro RNA (miRNA) profiles in RBCs derived from lung cancer patients, indicating that these alterations are not limited just to the protein content[155]. Despite the clear evidence of cancer effects on RBCs, how RBCs and cancer cells interact remains an understudied topic. A recent study performed in our laboratory has provided evidence of CTCs and RBCs interaction. The primary objective of this study was to cultivate CTC *in vitro*. For that, CTCs were isolated from metastatic BC patients using a negative enrichment technique. The pre-enriched CTC sample exhibited a clear contamination of RBCs[156]. When those cells were cultured *in vitro* and observed, direct contact between RBCs and CTCs was evident, forming networks that captured these cells in some cases[156]. This phenomenon was not observed performing spiking experiments in healthy blood. Interestingly, the presence of escorting RBCs that accompany CTCs associated both with worse progression-free and overall survival[156]. Supporting this evidence, other publications have shown that the contact between CTCs and RBCs could be mediated directly through GAL-4 or indirectly through RBCs´ attachment to platelets[118,157]. This interaction with CTCs might play a role akin to platelets, providing physical and immune protection to the tumoural cells as well as facilitating metastasis by inducing coagulation and increasing attachment to the vessel walls[67,118,158].

Besides their direct contact and their role inside the circulation, RBCs can also interact with the tumour. It has been demonstrated that RBCs are capable of uptaking cytokines produced by tumour cells through DARC receptors and regulating immune cells after this contact with the tumour[159]. Peripheral blood mononuclear cells (PBMCs) exposed to conditioned RBCs previously exposed to cancer cells led to the production of immuno-suppressive cytokines[159]. However, results from the same experiment showed that after exposure to conditioned RBCs, GATA-3 expression was not reduced in comparison to the control conditions whereas naïve RBCs did have a significant decrease in expression[159]. Despite contradictory results, these experiments show that RBCs exposed to cancer cells are able to modify the immune response. Other studies performed by Yin *et al.* have provided evidence that extravascular RBCs can promote tumour growth as well as mediate tumour resistance to chemotherapy[160]. Leaky and fragile vasculature in tumours can lead to the collapse of the vessels and the release of RBCs (ranging from scattered RBCs to large blood

lakes) in the tumour microenvironment[160–162]. The presence of intratumoral haemorrhages is associated with poor prognosis in cancer patients[162]. This study shows that the presence of RBCs activates NF-kB pathway due to the release of Hb, which acts as an endogenous damage signal[160]. This signalling leads to an increase in macrophage recruitment as well as a polarization towards an immune-suppressive response that fosters tumour growth and resistance[160].

5. In vitro models to study RBCs

5.1 RBCs differentiation

The use of *in vitro* erythroid differentiation systems is an important tool to understand how different factors or genetically alterations can affect erythropoiesis and the mature RBCs derived from it[163]. The process by which mature RBCs are generated from hematopoietic stem cells is known as erythropoiesis[164]. It can be divided into 3 different stages: early erythropoiesis, terminal erythroid differentiation and finally, reticulocyte maturation[165]. During the first step, early erythropoiesis, multi-potential hematopoietic stem cells proliferate and differentiate into committed erythroid progenitors[165]. Next, the process continues towards terminal erythroid differentiation, where recently differentiated proerythroblasts undergo sequential mitoses becoming basophilic, polychromatic and finally orthochromatic erythroblasts that will enucleate to become immature reticulocytes[165]. During this step, RBC precursors will experiment important changes, including a reduction in cell size, an increment in haemoglobin content, chromatin condensation and enucleation[166]. These changes will also be accompanied by modifications in expression, notably affecting membrane proteins[164,166,167]. The last step of erythropoiesis consists of the maturation of multilobular reticulocytes into mature discoid RBCs without organelles[168].

5.2 CD34+ primary cells and immortalized cell lines derived from it

Currently, erythroid differentiation cultures are often initiated from purified CD34+ hematopoietic stem progenitor cells (HSPC) extracted from the PBMC fraction cultured in fully defined serum-free growth media[169,170]. Differentiation of CD34+ HSPC has been used to study the disease mechanism of many haematological disorders such as Diamond-Blackfan anemia, congenital dyserythropoietic anemia type I/II, β-thalassemia and sickle cell disease[171,172]. However, patient-derived CD34+ HSPC are primary cultures with limited expansion potential, meaning that only a limited number of erythroid cells can be generated from each extraction[173]. To overcome this setback, researchers have focused on obtaining immortalized human erythroid cell lines. The most important ones that have been extensively used in these last years are Human umbilical cord blood-derived erythroid progenitor 2 (HUDEP-2), obtained in 2013 and Bristol Erythroid Line Adult (BEL-A) obtained in 2017[174,175]. HUDEP-2 and BEL-A were generated using a doxycycline-inducible HPV16-E6/E7 construct[173]. Progenitor cell lines transformed with this construct were immortalized, and in addition, the expression of HPV16-E6/E7 proteins inhibited the terminal differentiation of these cells[175]. Consequently, the removal of doxycycline (DOX) will allow cells to reach terminal differentiation and enucleate[175].

There are several differences between these cell lines. Firstly, BEL-A is derived from adult bone marrow CD34+, meanwhile, HUDEP-2 cells were generated from cord blood CD34+ cells[173–175]. Secondly, CD34+ progenitors used for BEL-A were cultured in an optimized medium before immortalization whereas CD34+ used for HUDEP-2 were cultured in a non-erythroid specific media[173–175]. Finally, BEL-A cell line differentiation is more efficient than HUDEP-2 cell line. BEL-A can reach higher percentages of enucleation (around 40% at day 12) than the HUDEP-2 (20 % at day 12), being able to recapitulate better adult erythropoiesis[173]. Moreover, HUDEP-2 tends to have higher percentages of cell death due to orthochromatic normoblasts being unable to proceed with the enucleation (Figure 3) [175]. However, both models still have low percentages of enucleation when compared to non-immortalized adult progenitors[173].

5.3 Differentiation in vitro of primary CD34+ and immortalized cell lines

Primary human peripheral-blood-derived CD34+ are RBCs precursors from terminal erythroid differentiation, mainly proerythroblast[173,176]. Similarly, immortalized cell lines HUDEP-2 and BEL-A are constituted of proerythroblast, but basophilic erythroblast can also be found in these cell lines, especially HUDEP-2 (Figure 3)[173,177]. *In vitro* differentiation of both cell lines requires the use of specific media for expansion periods (up to 10 days for primary CD34+ HSPC) and for differentiation[173,176]. Importantly, immortalized CD34+ cell lines require the removal of DOX during the differentiation to allow them to fully progress towards the final steps of the terminal erythroid differentiation[178]. Differentiation is considered finished once most of the orthochromatic erythroblasts have enucleated to become reticulocytes[176].

In vitro erythroid differentiation also needs to be assessed using flow cytometry to analyse changes in surface protein expression[165]. Studying proteins such as CD49d (integrin alpha 4), CD235 (Glycophorin A) and Band 3 allows to assess the stage of the differentiation as well as to verify a correct progression[173,176,178]. Individually, CD235 is a major protein of the RBC membrane that is upregulated steadily along the differentiation[176,179]. Importantly, the HUDEP-2 cell line express high levels of CD235 in the early stages due to being slightly more differentiated than primary CD34+ HSPC[177]. CD49d is an early differentiation marker whose expression declines at later stages[180]. Finally, Band3 is a major protein of the RBC cytoskeleton and its expression increases in the later stages of the differentiation. Flow cytometry analysis can be also complemented with image studies of the morphological change of the cells from early to late stage[173]. Reduction in size, nucleus condensation and enucleation are the primary changes evaluated in these observations[173].

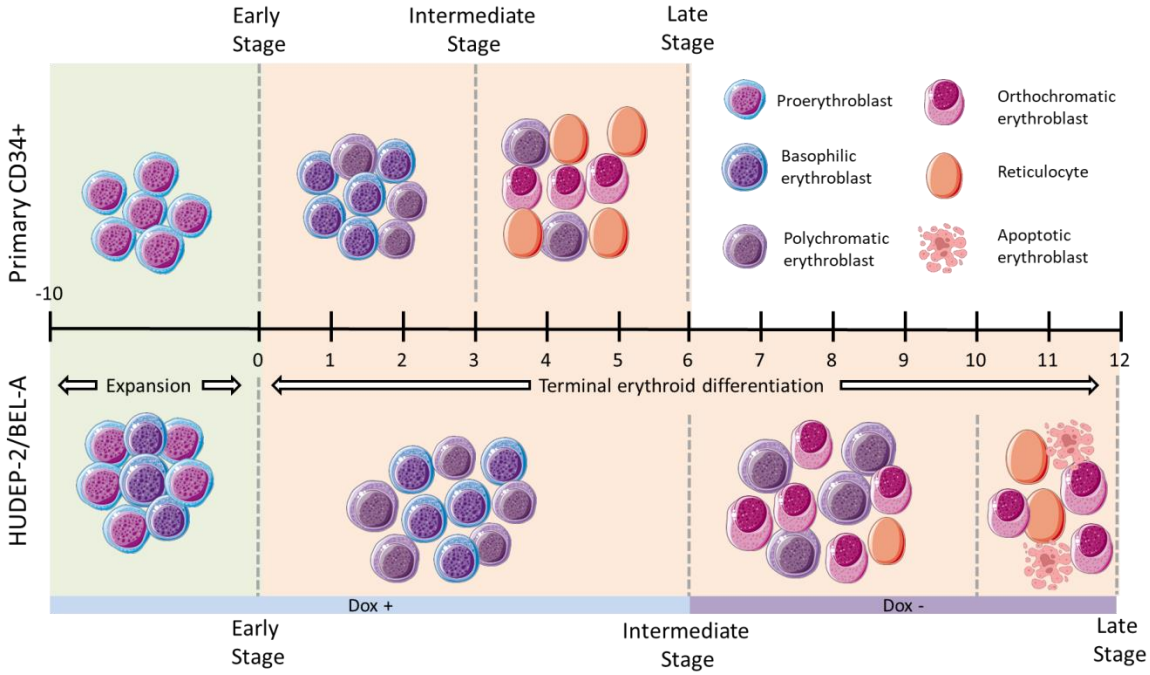


Figure 3. Descriptive diagram of the differentiation process in Primary Human CD34+ cells and immortalized cell lines HUDEP-2 and BEL-A.

OBJECTIVES

OBJECTIVES

This thesis aims to study the role of Red Blood Cells (RBCs) in metastatic breast cancer and its potential use as surrogate biomarkers in liquid biopsy in breast cancer patients. To achieve this, the following specific objectives have been proposed:

1. To identify biomarkers indicative of metastasis in Breast Cancer patients based on RBCs analysis.

2. Define the impact of RBCs from breast cancer patients and controls on the different steps of the metastatic cascade through the performance of functional assays employing Breast Cancer tumour cell lines.

3. Study the capacity of RBCs from breast cancer patients and controls to modulate gene expression of breast cancer tumour cells.

4. Study an immortalized erythroid cell line *in vitro* model for studying RBCs using genome editing.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Breast cancer patient cohorts and samples

Blood samples from breast cancer patients and associated clinical information were obtained after signing the corresponding informed consent at the Oncology Department of the Clinic University Hospital of Santiago de Compostela. This study was approved by the Ethics Committee of Galicia (Reference Number 2015/772). In this study, were included women with breast cancer (BC) from Stages I-IV at the moment of diagnosis previous to therapy initiation. Criteria of inclusion and exclusion are available at Table S1 in the supplementary material. In parallel, blood from donors without cancer and paired age was extracted as Cancer-Free Controls (CFC). For both patients and controls, three EDTA tubes of 7.5 mL of blood were extracted. One of them was used for blood test analysis, processed in the clinical laboratory of the Oncology Department by standard assays. Patient recruitment was performed from November 2019 until September 2023. The follow-up of the patients was performed for 42 months. BC patients and CFC were categorized into two distinct cohorts to facilitate the divergent approaches within this thesis, ensuring a better understanding. Cohort I was employed in the search for BC metastasis biomarkers in Red Blood Cells (RBCs) and Cohort II was used to study the role of RBCs in the BC metastatic cascade.

1.1 Cohort I

Cohort I is composed of 48 patients diagnosed with stage IV BC (M1), 43 patients diagnosed with stage I-III (M0), and 46 cancer-free controls (CFC) (Table 3). Patients from all groups had a similar age, with a mean of 56.80 ± 11.82 years. Luminal subtype patients were the most prevalent (81.2 % for M1 and 72.1 % for M0) meanwhile triple-negative (14.6% for M1 and 20.93% for M0) and HER2 (2.1% for M1 and 4.65% M0) subtypes were less represented. This cohort also includes 2 patients with mixed subtypes (Histological analysis detected both subtypes in the biopsied tissues or different subtypes in each breast). Stage IV patients were further classified depending on the location of their metastasis into 3 groups: Bone, visceral and mixed (when both, bone and visceral metastasis, were detected).

Table 3. Clinic and pathologic characteristics of breast cancer patients and cancer-free control from Cohort I.

Category	M1		M0		CFC	
	Media	SD	Media	SD	Media	SD
Age (years)	59.25	12.13	54.12	11.69	56.74	11.31
Tumour stage	n	%	n	%	n	%
0					46	100
I-III			43	100		
IV	48	100				
Subtype						
Luminal	39	81.2	31	72.1		
Her2	1	2.1	2	4.65		
Triple Negative	7	14.6	9	20.93		
Mixed Subtypes	1	2.1	1	2.33		
Metastasis Location						
Bone	15	31.2				
Visceral	14	29.2				
Bone & Visceral	19	39.6				
Others	1	2.1				

1.2 Cohort II

Cohort II is composed of 33 patients diagnosed with stage IV BC (M1), 29 patients diagnosed with stage I-III (M0) and 29 cancer-free control (CFC)(Table 4). For this Cohort the median age was 58.3 ± 11 years, with a slight bias in the M0 group, being the younger women. Luminal subtype patients were the most prevalent (60.6 % for M1 and 72.5 % for M0) meanwhile triple negative (18.2% for M1 and 10.3% for M0) and HER2 (3% for M1 and 17.2 % M0) subtypes were less represented. This cohort includes 6 patients with mixed subtypes for the M1 group. Stage IV patients were further classified depending on the location of their metastasis as previously described. No significant differences were found in terms of age, subtypes and metastatic localization between cohorts I and II.

Table 4. Clinic and pathologic characteristics of breast cancer patients and cancer-free controls from Cohort II.

Category	M1		M0		CFC	
	Media	SD	Media	SD	Media	SD
Age (years)	62.76	12.22	54.07	9.863	57.55	9.12
Tumour stage	n	%	n	%	n	%
0					29	100
I-III			29	100		
IV	33	100				
Subtype						
Luminal	20	60.6	21	72.5		
Her2	1	3	5	17.2		
Triple Negative	6	18.2	3	10.3		
Mixed subtypes	6	18.2				
Metastasis Location						
Bone	7	39.4				
Visceral	12	21.2				
Bone & Visceral	13	36.4				
Others	1	3				

2. Cell lines and cell cultures

Two BC cell lines were employed: the triple-negative MDA-MB-231 cell line and the luminal A MCF7 cell line were purchased from the American Type Culture Collection (ATCC). MDA-MB-231 Green Fluorescent Protein (GFP) expressing cells were obtained from Tebu-bio (UK).

Human Umbilical Vein Endothelial cells (HUVEC) were obtained from ATCC. Human Umbilical cord blood-derived Erythroid Progenitor-2 (HUDEP-2) was kindly transferred from Higg group from the University of Oxford (UK).

MDA-MB-231 and MCF7 were cultured in DMEN High glucose with L-Glutamine (Biowest, USA) supplemented with 10 % Fetal bovine serum (FBS)(NE Biotech, USA) and 1 % Penicillin Streptomycin (P/S),(Corning, USA). HUVEC cells were cultured with EGM™-2 Endothelial Cell Growth Medium-2 BulletKit™ (Lonza, Spain) and culture plates were pre-

treated with gelatine 0.2 % (Sigma, USA). HUDEP-2 cells were cultured with StemSpan™ Serum-Free Expansion Medium (Stemspan) (Stem Cell Technologies, Canada) supplemented with glutamine 2 mM (Thermo Fisher, USA), 1 % P/S, Human Stem Cell Factor 50 ng/mL (SCF, Peprotech, USA), 3 IU/mL Erythropoietin (EPO, Sandoz, Austria), Dexamethasone 330 µg/L (DEX, Sigma, USA) and 1 µg/mL Doxycycline (DOX, Sigma, USA). Stemspan was aliquoted and used in batches. Stempan supplemented with all factors except DOX (HUDEP Growth media) was prepared and used for a period of two weeks. DOX was added every 48 h due to its instability at 37 ºC. For HUDEP-2 differentiation, base media was prepared with Iscove's Modified Dulbecco's Medium (IMDM, GIBCO, UK) supplemented with 200 µg /mL Holotransferrin (HT, Prospecbio, Israel), 10 µg/mL Human insulin (Sigma, USA), Heparin 3 IU/mL (STEMCELL Technologies, Canada), Heat inactivated AB Plasma 3 % (Sigma-Aldrich, Poole, UK), 2 % FBS and 1 % P/S. Base differentiation media was used for Phase I differentiation media, supplemented with 3 IU/Ml EPO, 1 ng/mL IL3 (Peprotech, UK), 10 ng/mL hSCF and DOX 1 µg/mL. Also, base differentiation media was used for Phase II differentiation media supplemented with 3 IU/mL EPO and 300 µg/mL HT.

All cells were cultured at 37 ºC in an incubator with humidified atmosphere of 5 % CO_2. All adherent cells were cultured until 80-90 % of confluence was reached and suspension cell lines (HUDEPS) were subcultured after cell density was above 1 million cells/mL.

3. Isolation and preservation of erythrocytes

Two EDTA tubes with 7.5 ml of blood were centrifuged (1,700g/10’), plasma and peripheral blood mononuclear cells (PBMCs) were discarded and RBCs were collected from the bottom of the tube. RBCs were stored directly for protein analysis purposes. For functional studies were stored with glycerol freezing buffer, containing glycerol 6.2 M, Lactate 0.14 M, KCl 5 mM and Na_2PO_4 5 mM (Sigma Aldrich, USA). All samples were kept at -80ºC until further use. For subsequent assays, RBCs were either used fresh or preserved with the glycerol buffer.

4. Elisa assays

Elisa assays were performed according to the manufacturer´s protocol. The adequate dilution of the samples was assayed for each Elisa kit individually. The following kits were used: Human Hemoglobin Subunit Delta (HBD) ELISA Kit (Cusabio, USA), Human PECAM-1/CD31 ELISA Kit (Neo Biotech, USA), Human GNAQ ELISA Kit (Antibodies-online, Germany), Human LAMP2 (Lysosomal Associated Membrane Protein 2) Sandwich ELISA Kit (NE-Biotech, USA) and Human Purine Nucleotide Phosphatase ELISA kit (Mybiosource, USA). Concentrations of each protein were relativized to the total protein concentration of each sample in order to normalize the results. Absorbance was measured using BIOTEK EPOCH 2 microplate reader (Agilent technologies, USA) according to manufacturer´s protocol.

4. Thawing glycerol-frozen RBCs and lysates obtaining

Vials containing frozen RBCs were defrosted in a 37ºC bath. Afterwards, RBCs were transferred into a 15 mL tube with 5 mL of a sterile 12 % NaCl solution and centrifuged for 5' at 200 g. The supernatant with lysed RBCs and glycerol was discarded; the pellet was resuspended in a sterile 3.4 % NaCl solution and centrifuged again 5' at 200 g. Finally, the supernatant was discarded, 20 µL of RBC pellet was collected and resuspended in fresh media without FBS. The RBCs were lysed due to osmotic stress and a solution of RBC lysate was obtained for further use.

5. RBC-tumor cell adhesion

20,000 MDA-MB-231 or MCF7 cells were seeded in a P24 multiwell plate low attachment plate in 1 mL of RMPI with 4% plasma from the corresponding patient or control and 100 µL of RBCs dilution (20 µL of fresh RBCs in 10 mL of RPMI). Cells and RBCs were incubated for 24 h. Afterwards, photos of 8-10 random fields were taken using a DMi8 microscope (Leica Microsystems) under the bright field. The presence of RBCs attached to the tumor cells was considered a positive result, if no evidence of adhesion was seen, negative.

6. MTT proliferation assay

Proliferation of BC cell lines were assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays by indirect measurements of metabolic conversion of MTT to formazan. First, 4,000 MDA-MB-231 and MCF7 cells were seeded into 96-well plates in 100 µL of DMEN without serum and allowed to attach overnight. The following day, media was discarded and 160 µL of fresh DMEN with 10 % FBS was added to each well. 40 µL of the RBC lysate dilution was added to the corresponding well, in negative control wells, 40 µL media with FBS was added. Proliferation was measured at 24, 48 and 72 h for MCF-7 and MDA-MB-231. For the measurement, old media was discarded and fresh media with 0.5 mg/mL of MTT was added. After 3h, media was carefully removed, and formazan crystals were dissolved in 200 µL of DMSO. Absorption was measured at 540 nm and 650 nm (reference wavelength) using BIOTEK EPOCH 2 microplate reader (Agilent technologies, USA).

7. Angiogenesis assay

Extracelullar matrix (ECM) 625 *in vitro* angiogenesis assay kit (Sigma, USA) was employed for this assay. Matrix was prepared using precooled tips according to manufacturer protocol. 10 µL of the matrix was added to each well of a u-Plate Angiogenesis P96 (Ibidi, USA) and the plate was incubated for 1h at 37ºC. Once the matrix was already polymerized, 14,000 HUVEC cells were seeded on each well in 51.1 µL of Endothelial Basal Growth

Medium (EBGM). 18.9 µL of RBC lysate solution (approximately ¼ dilution) was added to the corresponding conditions. As a negative control condition, 18.9 µL of DMEN media without FBS was added. All conditions were set in triplicates. Images of the newly formed structures were taken at 6-8 h after seeding. Images of 3-4 FOV were obtained from each triplicate. Angiogenesis was quantified by measuring tube length and number of nodes per field using ImageJ.

8. Wound healing assay

150,000 MDA-MB-231 were plated on a P12 (VWR, USA) containing 2 mL of DMEN with 10 % FBS and incubated overnight. Next day, once cells were attached, 400 µL of the RBC lysate solution (see section 4) was added. After 24 h, media was removed, cells were washed, and a wound was generated scratching the cell monolayer with a p1000 tip. The residual cells were washed and fresh media was added. Different points of the scratch were selected randomly and images were taken at 0, 8 and 24 h using a Leica DMi8 (Leica Microsystems). The area of the scratch was measured using ImageJ. Each condition had at least two replicates.

9. Migration Transwell assay

Tumor cells pre-stimulated with RBCs. 150,000 MDA-MB-231 and MCF7 cells were plated on a P12 (VWR, USA) with 2 mL of DMEN with 10 % FBS and were cultured overnight. Next day, 400 µL of the RBC lysate dilution (see section 4) was added. After 24 or 48 h of pre-stimulation, tumor cells were washed and further cultivated for other 24 h with FCS-free medium (NE Biotech, USA). The transwell migration assay was performed by adding 200 µL FCS-deprived media containing 50,000 cells into cell culture inserts (BD Falcon, DE; 8 µm pores). Media containing 10% FCS as a chemo-attractant was placed in the lower chambers (Figure 4A). After 8 h, the media was removed, and the membrane was washed in PBS and fixed with 4% (w/v) PFA/PBS for 10 min. Cells were permeabilized with methanol for 20' and stained with 0.5% (w/v) crystal violet/H_2O solution for 10'. Excess staining solution was removed by washing in PBS. Non-migrated cells were scraped off from the inside of the inserts with a sterile cotton. The transwell membrane was imaged with a microscope Leica DMi8 (Leica Microsystems) under the bright field. Membrane surface of the transwell was analyzed by counting manually alternative Fields of view (FOV)(Figure 4C).

RBCs as chemo-attractants of tumour cells. The transwell migration assay was performed by adding 200 µL FCS-deprived media containing 10,000 cells into cell culture inserts (BD Falcon, DE; 8 µm pores), while media containing 10% FBS and 440,000 RBCs as a chemo-attractant was placed in the lower chambers (Figure 4B). After 16 h, the media was removed, and the transwell membrane was washed in PBS. Same protocol as previously described was used for fixation, permeabilization, staining of the migrated cells andcounting (while all FOV were measured, which means that all Transwell surface was counted). This protocol was also used for HUDEP-2 cell line. Instead of RBCs in the lower chambers, differentiated HUDEP-2 cells were placed on the lower chamber. These cells were previously conditioned for 4 hours

with plasma from M1 or CFC. Negative control settings of HUDEP-2 without conditioning were included.

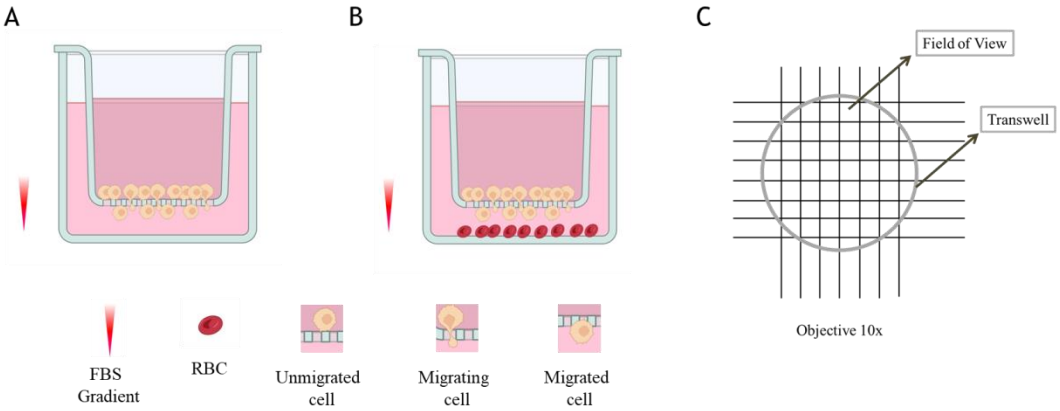


Figure 4. Transwell migratory asssays. A and B. Representative images of the different conditions used for the transwell migration assay: tumor cells pre-estimulated with RBCs (A) and RBCs as chemo-attractants (B). C. Representative image of the transwell counting. The circle in grey represented the Transwell membrane and the black squares represented the different fields of view that were counted.

10. Zebrafish assays

500.000 MDA-MB-231 cells were seeded in T25 flask (Corning, USA) in DMEN 10% FBS and left to attach overnight. Next day, 800 µL of the RBC lysate dilution (see section 4) was added and cells were further cultured for 24 h. In parallel, a CN condition without RBCs was also cultured. Next day, cells were harvested, and 1 million of cells were concentrated in 10 µL with 2% of PVP (Polyvinylpyrrolidone) to avoid cellular aggregation. Cells were injected in the Cuvier Duct of anesthetized 2 days old Zebrafish embryos at a rate of 100-200 cells per injection. Embryos were incubated for 24 h after the injection. Migration to the caudal hematopoietic tissue (CHT) of the Zebrafish embryos was measured using images taken from a fluorescence stereomicroscope (AZ-100, Nikon). These images were analyzed using the Quantifish software to obtain fluorescence intensity (FI) in the CHT. FI values of MDA-MB-231 co-cultured with RBCs were normalized to mean FI value from the CN condition. Injection and analysis of the zebra fish was performed in collaboration with the Genetics department of University of Santiago de Compostela site in Lugo´s campus.

11. Tumor cell adhesion to endothelial cells

80,000 HUVEC were plated on a P12 (VWR, USA) and cultured for 2 days until a monolayer was formed. Before the monolayer of endothelial cells was ready, MDA-MB-231-GFP cells were co-cultured for 24 h with RBCs lysates (see section 4) from CFC or M1 RBCs. A CN condition without RBCs was also added to act as control. Breast cancer cells were cultured in the HUVEC monolayer and allowed to attach for 1 h at 37ºC. Once incubation was finished, media was removed and wells were washed 3 times with PBS. Next,

new media was added and cells were imaged using a microscope Leica DMi8 (Leica Microsystems). Photos of 8 random FOV were taken for each condition. Adhered MDA-MB-231 cells expressing GFP were counted manually.

12. Tumor cells adhesion to collagen I

100,000 MDA-MB-231 or MCF7 cells were plated on a P24 (VWR, USA) and left to attach overnight. The following day, RBC lysate dilutions from CFC, M0 or M1 RBCs (see section 4) were added to their corresponding wells and cells were incubated for further 24 h. CN conditions without RBCs were also added for each assay. After co-culture with RBCs, cells were harvested and counted.100,000 cells from each condition were seeded on a 48 well Cytoselect Collagen I Plate (Cell biolabs, USA) and incubated for 1 h at 37 ºC and 5 % CO_2. After incubation, media was aspirated and wells were washed up 5 times with PBS. After that, adhered cells were incubated with the kit´s staining solution and washed 5 times with distillated water following manufacturer´s protocol. Absorbance was measured at 560nm using BIOTEK EPOCH 2 microplate reader (Agilent technologies, USA).

13. Cluster formation assays

For this assay fresh RBCs from CFC or M1 patients were employed. CFC RBCs were used directly after isolation or after a 24h exposure to MDA-MB-231 conditioned media, to generate conditioned RBCs from CFC (cCFC). Before the assay, an RBC dilution was prepared using 20 µL of RBCs in 10 mL of DMEN. Once RBCs were ready to use, MDA-MB-231 cells were harvested and a single cell suspension of 200,000 cells/mL was obtained. To ensure that all cells are individualized and not forming clusters, the solution was pipetted up and down with a p200 tip and filtered with a 100 µm cell strainer (Corning, USA). Once solution was ready, 200,000 individualized cells were seeded in a low attachment p24 (Corning, USA). Alongside the cells, 200 µL of each RBC dilution was added to their corresponding wells. All conditions had two wells, one that acted as a basal control (t0) and was fixed with PFA 4% when the assays started, and the other that was fixed after 1 h incubation in an orbital shaker (Lan Technics G) at 37 ºC and 150 rpm (t1). Next, single cells from each condition (t0 and t1) were counted using a Neubauer chamber and an optical microscope Leica DMI1 (Leica Microsystems). At least 6 measures were taken from each condition (Figure 5). Quantification was performed following the formula: $1 - \frac{Nº\ of\ single\ cells\ at\ t1}{Nº\ of\ single\ cells\ at\ t0}$ to count the percentage of aggregation indirectly. Cluster were considered when two or more cells were attached. Clusters present in the Neubauer chamber were not counted.

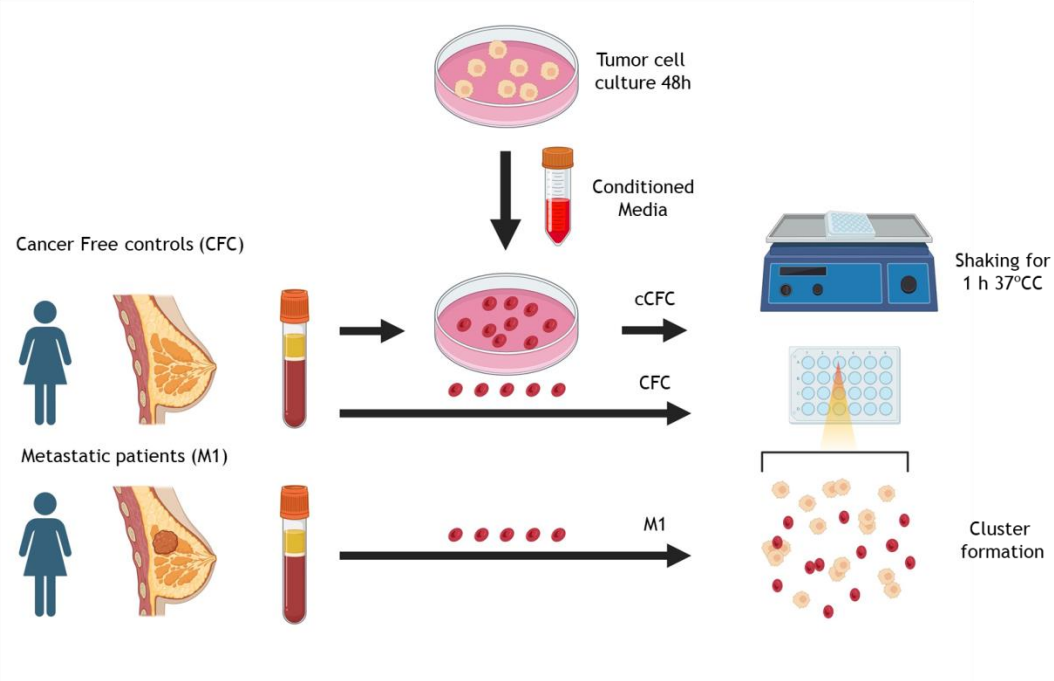


Figure 5. Diagram of the cluster formation protocol. Red blood cells (RBCs) isolated from blood extracted from cancer-free controls (CFC) or metastatic patients (M1) were cocultured in low attachment conditions and agitation with individualized MDA-MB-231. CFC RBCs were also conditioned (cCFC) using media from a 48 h culture of MDA-MB-231.

14. Cytokine and Chemokines detection

BC tumor cells (MDA-MB-231 and MCF7) were co-cultured with RBCs from CFC or M1 patients. Negative controls without RBCs were also included. After 24h, media from the co-culture was transferred to a 15 mL tube and centrifuged at 1000 g for 5' to remove any residue from the culture. The supernatant was taken to a new 15 mL tube and stored in a -80ºC freezer. Samples were used to check the production of chemokines and cytokines by the BC cells using the kit Proteome Profiler™ Human Cytokine Array Kit (R&D SYSTEMS) (Table 5). According to manufacture protocols, samples were processed and let to incubate with the membranes. After secondary antibody incubation, images were acquired using ChemiDoc™ MP Imaging System (Biorad, USA). Signal was obtained through chemoluminiscence reactives provided by the kit. Relative levels of expression from each cytokine were measured employing ImageJ to measure the signal from each spot in comparison to the control spot.

Table 5. Chemokines and Cytokines included in the kit Proteome ProfilerTM Human Cytokine Array Kit (R&D SYSTEMS)

C5a	CD40 Ligand	G-CSF	GM-CSF	CXCL1/GRO alpha	IL-2
IL-4	IL-5	IL-6	IL-8	IL-10	IL-21
IL-27	IL-32 alpha	CXCL10/IP-10	CXCL11/I-TAC	CCL2/MCP-1	TREM-1
CCL1/I-309	ICAM-1	IFN-gamma	IL-1 alpha	IL-1 beta	IL-1ra
IL-12 p70	IL-13	IL-16	IL-17	IL-17E	IL-18
MIF	MIP-1 alpha/MIP-1 beta	CCL5/RANTES	CXCL12/SDF-1	Serpin E1/PAI-1	TNF-alpha

15. Liposome synthesis

The liposomes were synthesized with lipid content similar to the erythrocytes membranes: 60% Phospholipids (30% Sphingomyelin (SM) and 30% phosphatidylcholine (PC)) and 40% Non-esterified cholesterol (CH) as described by Vahedi et al. and Oliveira et al[181,182]. This work was done in collaboration with Ana Belén Dávila PhD (FIDIS).

16. RNA extraction

400,000 MDA-MB-231 cells were seeded in a P6 (VWR, USA), with 3 mL of DMEN with 10 % FBS and cultured overnight. Next, cells were co-cultured with 600 µL of RBC lysates for 24 h. Also, as control conditions, MDA-MB-231 without RBC lysate co-culturing and cells co-cultured with liposomes were included. After the co-culture, cells were washed with PBS (Corning, Manassas, VA, USA), trypsinized and stored in RNA later (Thermofisher Scientific Baltics, Lithuania) at -80°C. RNA was extracted using AllPrep DNA/RNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer´s protocol. Extracted RNA concentration was measured using Nanodrop One (Thermo Fisher, USA).

17. RNA-Sequencing analysis

1 µg of RNA from each sample was sent for an RNA-sequencing analysis to find differentially expressed genes. RNA-sequencing was carried out by the company Macrogen (Seul, South Korea). Total RNA was isolated, treated to remove DNA contaminants, and then prepared for sequencing using the TruSeq Stranded Total RNA LT Sample Prep Kit (Globin). The cDNA libraries were generated, sequenced using the Illumina platform (NovaSeq6000). Reads were mapped to reference genome GRCh37 with HISAT2, transcripts were assembled

with StringTie, and Differential Expression Gene (DEG) analysis was performed using DESeq2 for 4 comparisons pairs. Analysis included 14,470 genes (Genes with at least one read count value of 0 were not included). Statistical analysis was performed using Fold Change, nbinomWaldTest using DESeq2 per comparison pair. Gene-enrichment and functional annotation analyses was performed with the g:Profiler tool (https://biit.cs.ut.ee/gprofiler/). This analysis, based on the Gene Ontology (GO) database (http://geneontology.org/), aimed to identify over-represented biological information within DEGs.

18. qRT-PCR analysis

1 µg RNA was transcribed to cDNA using SuperScript III (Thermofisher Scientific, Schwerte, Germany), according to manufacturer protocol. For quantitative Real Time-Polymerasa Chain Reaction (qRT-PCR), a dilution of 1:20 of the material obtained from the retrotranscription was prepared. Gene expression analysis was performed with probes for the selected genes (see Table 6) and Master mix from TaqMan (Applied Biosystems®, Foster City, CA, USA) on a LightCycler 480 II (Roche Diagnostics, Basel, Switzerland), using the amplification protocol described in Table 7. B2M was used as a reference gene in Δct calculation. Gene expression was normalized to negative control Δct.

Table 6. List of genes analyzed through qRT-PCR.

Gene	Taqman Reference	Function
ALAS1	Hs00963537_m1	Metabolism
B2M	Hs00187842_m1	Reference
CTNNB1	Hs00355049_m1	Cell adhesion/Transcription
CDKN1 (p21)	Hs00355782_m1	Cell cycle
EPHX1	Hs01116806_m1	Metabolism
HMOX1	hs01110250_m1	Metabolism
PAK4	Hs01100061_m1	Cytoskeleton regulation and cell signalling
PI3K	Hs00907957_m1	Cell cycle
PLS3	Hs00543971_m1	Cell adhesion, EMT
STAT1	Hs00244839_m1	Transcription Factor
VIM	Hs00958116_m1	EMT

Table 7. qRT-PCR amplification protocol.

Step		Temperature	Time
Preamplification		95º	10 ’
Amplification x 45 cycles	Denaturation	95º	10 ’’
	Annealing	60º	30 ’’
	Extension	72º	1 ’’
Cooling		40º	30 ’’

19. PAK4 Inhibition

LCH-7749944, a PAK4 inhibitor, was bought from Selleckchem (USA). MDA-MB-231 cell line was exposed for 4 h to different concentrations (ranging from 10 to 60 μM) of this drug in order to study the IC50 of this drug. Proliferation was assessed at 24, 48 and 72 h using MTT assay as previously described (see section 6). A concentration of 20 μM was chosen for the *in vitro* assays. To study the effect of PAK4 in migration, MDA-MB-231 were cocultured for 24 h with M1 RBC, as previously described on Transwell migration assay (see section 9). CN condition without RBCs was also included. After coculture, cells were incubated for 4 h with 20 μM of LCH-7749944 or without the drug. Rest of the assay was performed as previously described.

20. Generation of a LAMP2 knockout (KO) on an erythroid cell line

HUDEP-2 cell line was selected for LAMP2 expression editing following a similar protocol as described by Moir-Meyer et al[183]. Transfection of cells was performed using Amaxa™ 4D Nucleofector Protocol for insulated Human CD34+ cells (Lonza Bioscience, Germany). LAMP2 KO was generated using Crispr associated protein 9 (CrisprCas9) paired with a guide RNA (gRNA) that targets exons 2 and 3 (Figure 6). 1.5 million of HUDEP-2 cells (with at least 90% viability) were mixed with the kit buffer, the gRNA and Cas9. The mix was electroporated using DZ-100 protocol for P3 primary cells with Amaxa™ Nucleofector 2B (Lonza Bioscience, Germany). Cells were transferred in culture in a p24 with 1.5 mL of fresh HUDEP-2 growth media. After 48 h, viable single cells were sorted for clonal selection on to 5 Terasaki plates of 96 wells using FACS ARIA III cell sorter (BD Biosciences, USA). Cells were let to grow until reaching 200/300 cells per well and the clones were further expanded.

Cell pellets were washed and next were incubated overnight with a lysis buffer (50 mM Tris, 1mM EDTA, 0.5% Tween 20) supplemented with 0.01 mg/mL of proteinase K (Thermofisher Scientific, UK) and genomic DNA from the clones was extracted. The presence of the deletion was studied through conventional PCR. Amplification was performed using: 0.5 μL of forward and reverse primers (target the edges of exon 2 and 3, see Figure 6), 1 μL of cell lysate, 200 μM deoxynucleotide triphosphates (dNTPs), a unit of FastStart Taq DNA Polymerase (Roche, Germany) and 1x of the corresponding amplification buffer of the

kit. Amplification reactions were incubated at 95 °C for 3′ and then cycled 35 times at 95 °C for 30″, 58 °C for 30 ″ and 72 °C for 30 ″ before a final incubation at 72 °C for 10 ′. The wild type (WT) cells product was 2 kilo base pairs (kbp) whereas the LAMP2 KO product was 800 base pairs (bp). The presence of the mutation was confirmed observing the different bands generated after agarose gel electrophoresis. Of the 53 clones studied, two KO were obtained (KO 9 and KO 43). KO samples were sent to MRC WIMM Sequencing facility (Oxford, UK) to confirm the presence of the correct deletion between exon 2 and 3.

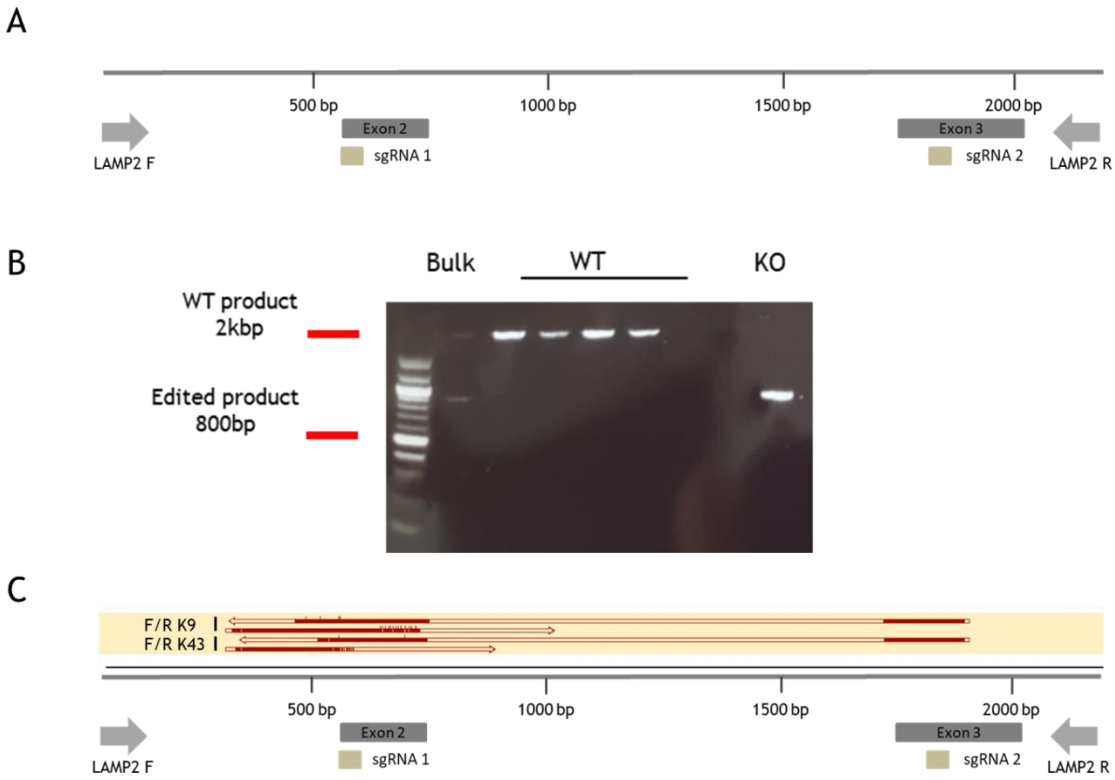


Figure 6. Generation of the knockout for LAMP2. A. Representative image of the LAMP2 region that was removed (exon 2 and 3) using CrisprCas9 and the short guide RNAs (sgRNA) used for this task. B. Exemplary results of a conventional PCR gel where we can see the wild type (WT) bands at 2kb (unedited LAMP2 fragment) and knockout (KO) bands at 800bp (edited LAMP2 fragment). C. Results from the sequencing of gDNA extracted from ko 9 and ko43 showing the deletion of the targeted LAMP2 region.

21. RNA extraction and qRT-PCR protocol for HUDEP-2 cells

RNA was extracted using Direct-Zol RNA Miniprep kit (Zymo Research, Germany) following manufacturer protocol. cDNA was obtained using High-Capacity Reverse Transcription kit (Thermofisher Scientific, USA) and qRT-PCR was performed using SYBR Green qPCR Master Mix (Thermofisher scientific, USA), Nuclease free water and cDNA from the sample. qRT-PCR was run using ThermoFisher QuantStudio 3 system using the protocol described at the Table 8.

Table 8. SYBR Green qRT-PCR conditions.

Step		Temperature	Time
Preamplification		95º	10 ’
Amplification x 45 cycles	Denaturation	95º	15 ’’
	Annealing/Extend	60º	60 ’’
Cooling		40º	30 ’’

22. Immunofluorescence

Slides of HUDEP-2 cells were prepared using Cytospin 4 machine (Thermo fisher, USA) at 400 g during 4’. Slides were allowed to dry for 30’ and then the samples were fixated using PFA 4% for 15’. After fixation, cells were washed with PBS twice and permeabilized with Saponin 0.1 % (Sigma, USA) PBS for 10’. Cells were washed twice with PBS; the primary antibody was added and incubated for 1 h at room temperature (RT). During the last 10’, DAPI (Thermo fisher, USA) was added at a concentration of 1µg/mL. After the incubation, cells were washed twice with PBS and once with deyonized water. Excess liquid was removed and cover slip was placed with mounting media for visualization. Images were obtained using fluorescence microscope Leica. Primary antibody used was LAMP2 (H4B4), Alexa Fluor™ 488 #MA5-18122 (Thermo fisher, USA) 1/150.

23. Western Blot

Protein sample were prepared with a concentration of 1.5 µg/µL of total protein in 1x loading dye (Thermofisher Scientific, USA) and boiled for 5’ at 95ºC. Samples were used directly or stored at -80ºC. 20 µL from each sample were pipetted on each well of a 12 % acrylamide gel 1.5 mm. Electrophoresis was performed at 50 V for 30’ and 120 V for 1 h. Proteins were blotted to a nitrocellulose membrane (Bio-Rad, USA) for 1 h 30’ at 30 V. Membranes were blocked for 1 h in 5% milk (Thermo Fisher, USA) TRIS-buffered saline-Tween 0.1 % (TBS-T) and incubated overnight at 4ºC and in agitation with primary antibody (see below). Next day, membranes were washed with TBS-T, and incubated at RT, protected from the light and in agitation with the secondary antibody.

The following antibodies were used: Histone H4 Antibody #2592, LAMP2 (D5C2P) Rabbit mAb #49067 (Cell signaling, USA) and IRDye 680 RD/800 CW (Li-Cor, Germany). Primary and secondary antibodies were diluted 1/1000 and 1/2500 respectively in TBS-T 5% Milk.

24. Protein extraction

RBCs, stored at -80ºC, were defrosted and protein was extracted using a lysis buffer compound of Hepes 20 mM, Triton x100 1%, NaCl 100 mM, MgCl2 20 mM, EGTA 10 mM, β-Glycerophosphate 40 mM, PMSF 20 µM and 1x Protease inhibitors (Sigma Aldrich, USA). Samples were incubated for 15-20'at 4ºC and centrifuged at 13000 gs 4ºC for 5'. Protein concentration was measured using DC protein Assay (Bio-Rad, USA). Extracted protein was stored at -80ºC until further use.

HUDEP-2 cells were lysed with RIPA buffer supplemented with Protease Inhibitor (PI) cocktail (Sigma Aldrich, USA) 20' in agitation at 4ºC. Then, centrifuged 20'at 10000 gs at 4ºC and the supernatant was transferred to a different tube. In both cases, protein was quantified with Qubit protein assay kit (Thermofisher Scientific, USA) following the manufacturer´s instructions.

25. HUDEP-2 differentiation protocol

HUDEP-2 cells were resuspended in Phase I differentiation media supplemented with doxycycline at a concentration of 500,000 cell/mL. Cells were passed every two days and new media was added with fresh doxycycline. During this time, cells will keep growing. After 6 days, cells were cultured in Phase II differentiation media without doxycycline and cultured for two more days. Cells did not grow after the removal of doxycycline. At different stages of the differentiation (day 0, 6 and 8), a sample of cells was taken to confirm a correct differentiation of the cells (Figure 7). Part of the cells was cytospined and stained with Wright-Giemsa to track the morphological change of the HUDEPs in their transition to RBCs. The other part of the cells was used for flow cytometry analysis to study the changes in expression of the different membrane markers along the erythroid differentiation.

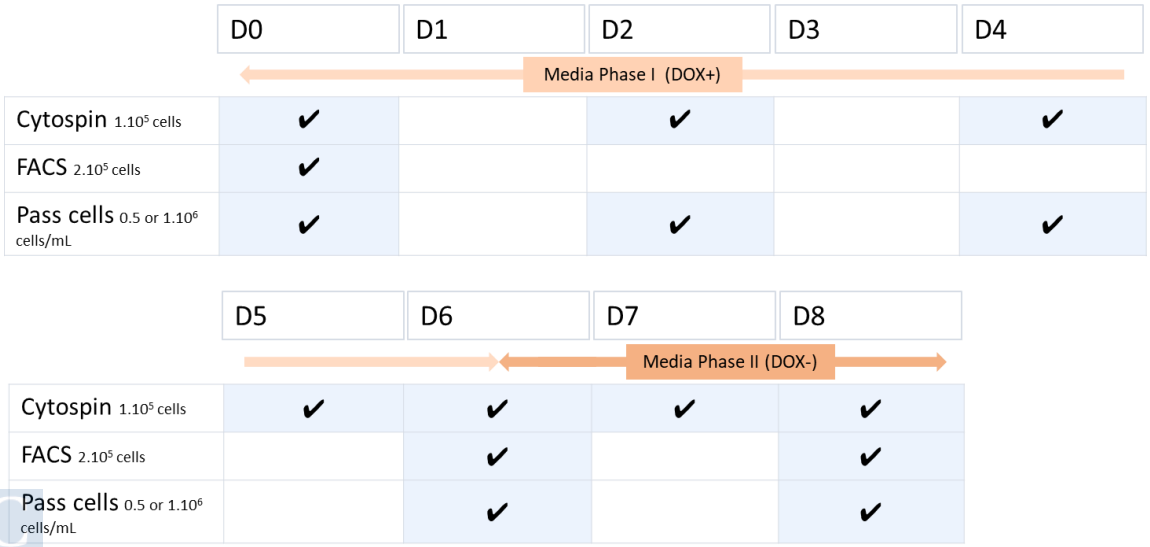


Figure 7. Representative scheme of the differentiation process. Summary table showing the sampling, passage and DOX removal points for HUDEPs cells during the differentiation.

26. HUDEP-2 Cytometry analysis

Flow cytometry analysis was performed using FACS ARIA III cell sorter (BD Biosciences, USA). Study of the changes in expression of the different membrane markers along the erythroid differentiation was performed using the following panel of antibodies:CD235a PE (BD Bioscience, 555570) 1/100, CD49d APC (BD Bioscience, 561892) 1/50, CD233 FITC (IBGRL, 9439FI) 1/100 and eFluor™ eBioscience™ 780 (Thermofisher, USA) 1/1000. Optimization and calibration of the different fluorophores was performed using HUDEP-2 WT cell line at day 0 previous to the start of the differentiation. Data obtained was analysed using flow cytometry analysis software Flowjo version 7.6 (BD biosciences, USA).

27. Vesicle Production analysis

HUDEP-2 cells at day 8 of the differentiation were seeded at 1,500,000 cell/mL and cultured for 2 days in Phase II media. For this media, FBS reduced in exosomes by ultracentrifugation was used. After the culture, cells were removed from the media by a centrifuging 5' at 300 gs. Supernatant was centrifuged at 500 gs for 10' to remove cell debris. The supernatant was transferred to 12.5 mL Open Top SIN 331372 tubes (Beckman Coulter, USA) and ultracentrifuged at 10,000 gs for 20' using SW41Ti rotor (Beckman Coulter, USA) prior to the vesicle isolation. Finally, the resulting supernatant was ultracentrifuged at 100,000 for 16 h at 4ºC. After this last step, media was completely removed and the resulting pellet containing the vesicles was resuspended using 500 µL of PBS. Vesicles were stored at -80ºC until measure in NTA Nanosight NS300 (Malvern panalytical, UK):

28. Statistical analysis

Statistical analysis was performed using R Studio (version 4.3.0) and GraphPad Prism 6.0.1 software (GraphPad Software Inc.). A receiver operating characteristic (ROC) analysis was conducted to assess the predictive capability of LAMP2 and LAMP2/Haematocrit/RDW levels to detect the presence of metastasis, using the pROC package in R. To determine the association between categorical variables a Chi-Squared or Fisher test was performed using R. Mann Whitney and Kruskal-Wallis analysis were performed using GraphPad Prism to determine if there are statistically significant differences between two, three or more independent groups, respectively. Only $p\ value < 0.05$ was consider statistically significant.

RESULTS

The results from part 1, RBCs as breast cancer biomarkers, have been adapted/extracted from our previous published article

Red Blood Cells Protein Profile Is Modified in Breast Cancer Patients

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RESULTS

1. RBCs as breast cancer biomarkers

RBCs can be altered in systemic diseases such as cancer, in terms of their rheological and physical properties as well as their content. To study the effects of breast cancer (BC) on these cells, RBCs from breast cancer patients, including non-metastatic BC (M0) and metastatic BC (M1) patients, and cancer-free controls (CFC) were isolated. All patients included in this study were women of around the same age (Cohort I, see M&M section 1.1, Table 3). Blood from BC patients was collected at the moment of diagnosis (primary tumour or metastasis) before the initiation of treatment to avoid bias due therapy. Isolated RBCs were stored at -80ºC. Protein from the RBCs was extracted, quantified and stored at -80ºC until further use. In parallel, a blood tube was used for blood parameters analysis in the standard clinical laboratory.

1.1 RBCs from BC patients have altered clinical blood values

To check whether BC patients depicted altered blood parameters, data from routine blood analysis from BC patients (M0, n=34; M1, n=44) and CFC (n=30) were checked. Firstly, M1 patients had a lower hematocrit and hemoglobin concentration ($p < 0.0001$), when compared to M0 patients or CFC (Figure 8A/B). Another parameter that was found altered was the Red Cell Distribution width (RDW) which indicates the variability in the size of the RBCs. RDW was higher in M1 patients (mean 14.96 ± 1.92) compared to the CFC group (mean 13.88 ± 0.77), ($p = 0.0174$), but not to M0 (mean 14.07 ± 0.93) ($p=0.0761$) (Figure 8C).

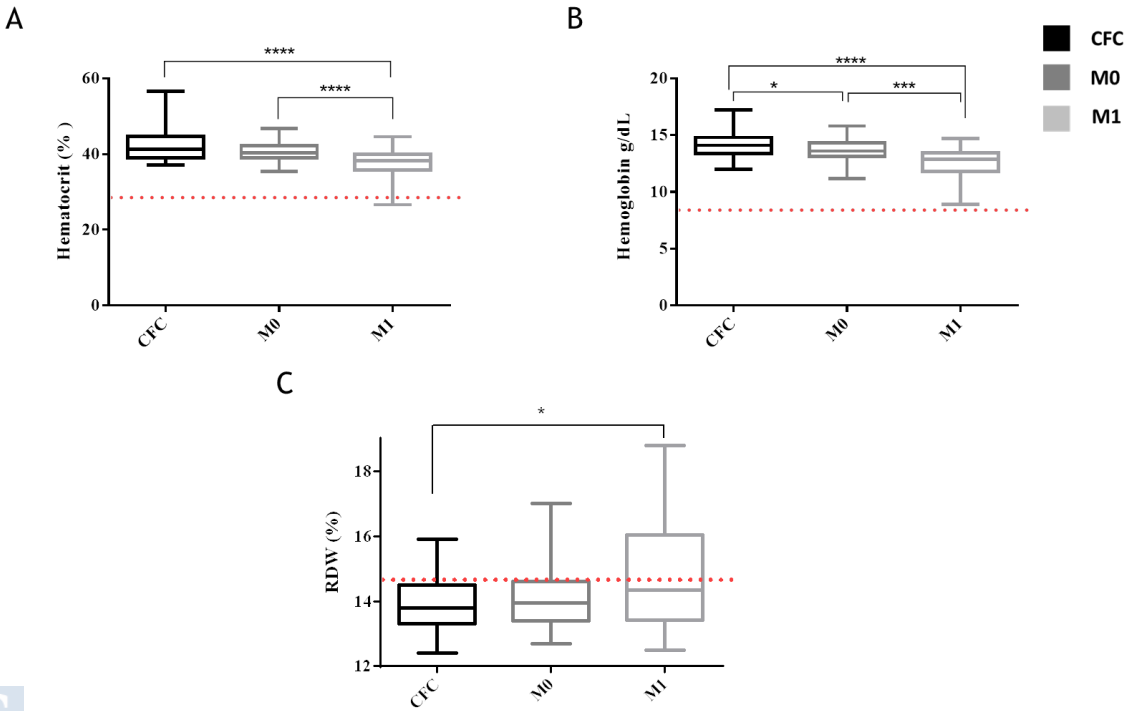


Figure 8. Blood parameters altered in metastatic (M1, n=44) (Grey) and non-metastatic (M0, n=34) (Dark Grey) breast cancer (BC) patients when compared to cancer-free controls (CFC, n=30) (Black). A. Hematocrit represents the percentage of RBCs in the whole blood. M1 BC patients have significantly lower hematocrit compared to CFC or M0 patients. The lower normal limit is 36.9%. B. Concentration of hemoglobin (Hb) in the blood. M1 BC patients had lower Hb when compared to CFC and M0. The lower normal limit is 12.2 g/dl. C. Red cell distribution width (RDW) is increased significantly in M1 patients when compared to CFC. The standard range is 11.5 to 14.5. The red dotted line represents the limit of the standard value for each parameter. Mann-Whitney test. P-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***), and p-value < 0.0001 (****).

1.2 LAMP2: Potential biomarker of the presence of metastasis

Previous results from our group described that RBCs from breast cancer patients have an altered protein profile compared to CFC[154]. Considering these data, we selected a set of proteins from M1 RBCs with increased levels (fold change ≥ 1.5) when compared to CFC to study if they could have value as biomarkers in BC. Among them, GNAQ, HBD, LAMP2, PNP and PECAM-1 were checked by ELISA in the cohort I. Protein expression from RBCs were detected for all the analyzed proteins, however significant differences between M1 patients and CFC were found only for LAMP2 (p=0.04) or PNP (p=0.02) (Figure 9A/C). Interestingly, a similar trend was found when comparing M1 and M0 for both proteins (p=0.02 for LAMP2 and p=0.02 for PNP), indicating that these changes were M1 specific. To guarantee that the expression of LAMP2 detected both at the proteomic and ELISA assays were RBC specific, LAMP2 levels were studied in paired plasma samples employed in the validation. No statistical differences were found between M0, M1 or CFC plasma samples (Figure 9B). The validation of these two proteins was especially interesting since high LAMP2 levels detected in the previous proteomic analysis were associated with worse Progression Free Survival (PFS) and Overall Survival (OS) in M1 patients, while PNP low levels in M1 patients correlated with bone metastasis[154].

To check if PNP expression was low in M1 patients with bone metastasis, M1 patients were divided into three groups depending on the location of the metastasis: Bone, visceral or mixed (both). As we can see in Figure 9D, although there is a tendency, there are no significant differences between PNP expression in RBCs from M1 BC patients and the metastatic site. Interestingly, compared to CFC, the M1 patients had significantly increased levels of PNP if they have visceral metastasis, (p=0.0185) or mixed (p=0.0365), and these differences cannot be seen for bone metastasis (p > 0.05) (Figure 9D). These results show that PNP levels are lower in patients with bone metastasis. Interestingly, survival analysis also showed that lower PNP was associated with worse OS (Figure S1).

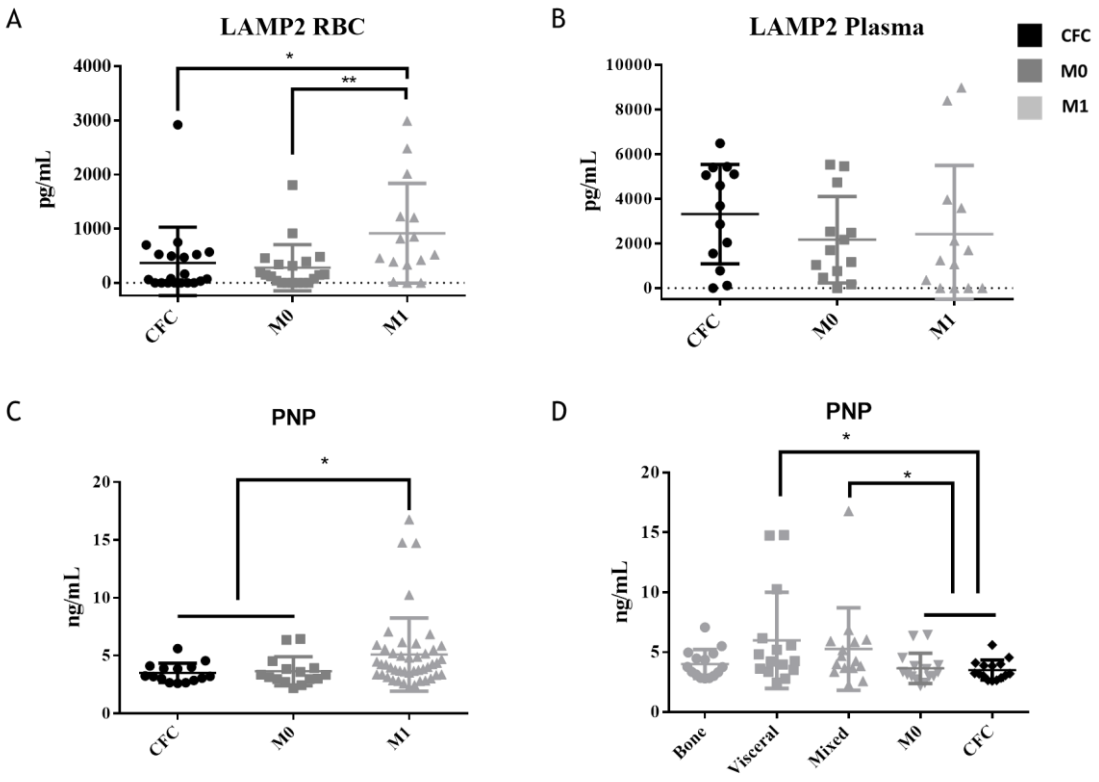


Figure 9. Protein concentration in RBCs using ELISA. Cancer-free controls (CFC) (Black), non-metastatic breast cancer (M0) (Dark grey) and metastatic Breast cancer (M1) (Grey). A. LAMP2 concentration in RBCs from CFC (n=20), M0 (n=20) and M1 (n=13). B. LAMP2 concentration in plasma from CFC (n=13), M0 (n=13) and M1 (n=13). C. Purine nucleoside phosphorylase (PNP) concentration in RBCs from CFC (n=15), M0 (n=15) and M1 (n=45). D. PNP concentration in CFC (n=15), M0 (n=15) and M1 with different metastasis locations (Visceral n=15, Mixed n=15 and bone n=15). Mann-Whitney. P-value < 0.05 (*); p-value < 0.01 (**).

Due to the variability in PNP expression depending on the location, we focused our studies on LAMP2. Our next step was to study if this protein could help to discriminate between patients with and without metastasis. To achieve this, we conducted a Receiver Operating Characteristic (ROC) analysis, yielding an area under the curve (AUC) value of 0.71, with 76 % sensitivity and 62% sensitivity (Figure 10A). Although promising, this value is not high enough to consider the use of LAMP2 as an independent biomarker of the presence of metastasis by itself. Since other blood parameters were also found altered in metastatic breast cancer patients, such as hematocrit and RDW, a combined signature of these clinical parameters and LAMP2 was analyzed, obtaining a more robust model with an AUC value of 0.89 that provides a sensitivity of 92.3 %, specificity of 80.5 % and 83,3 % of accuracy (Figure 10B).

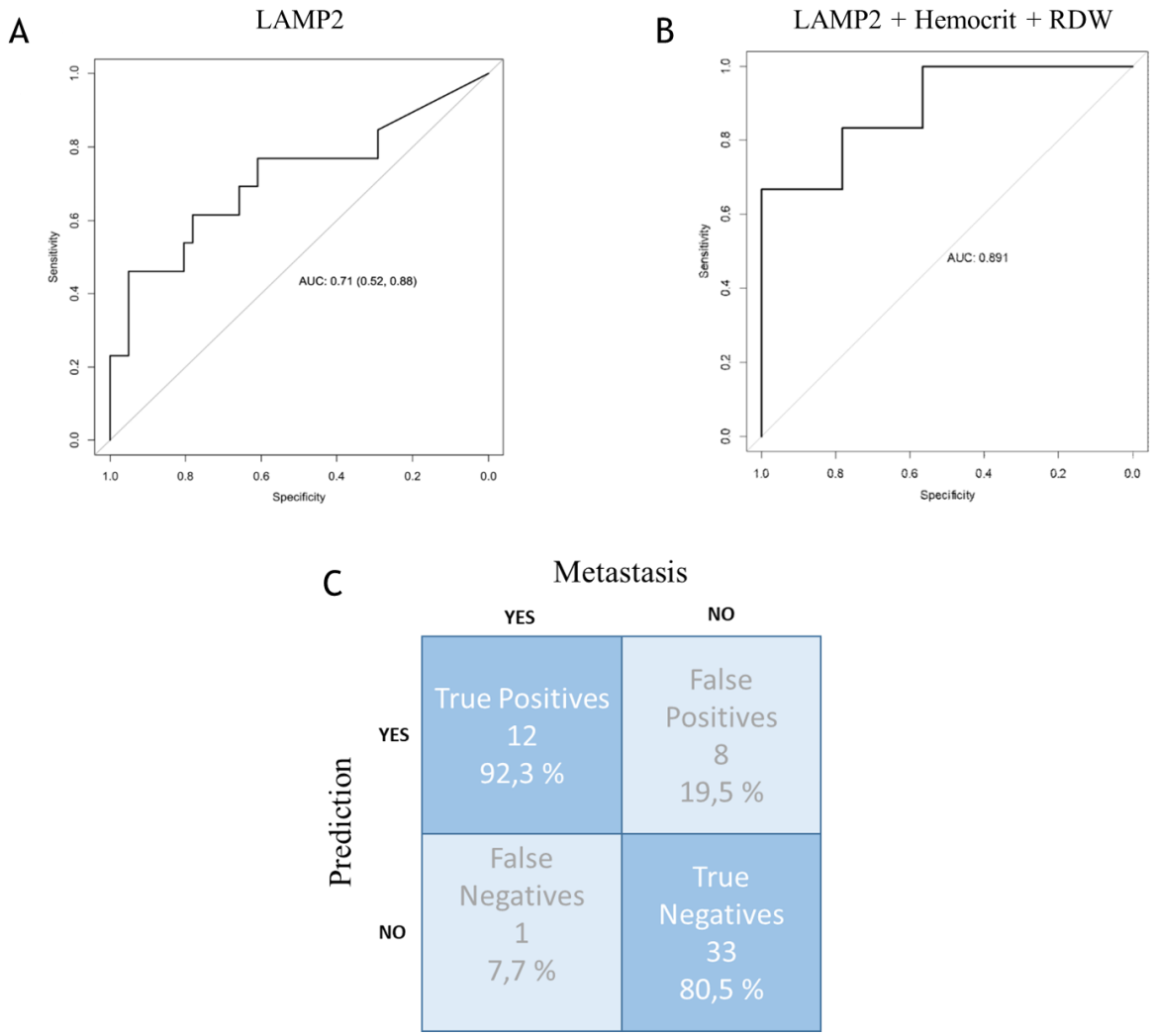


Figure 10. LAMP2 as a biomarker for predicting the presence of metastasis in BC patients. (M1 n=13, CFC n=20 and M0 n=20). A. ROC curve for LAMP2 concentration; AUC=0.71 B. ROC curve for the LAMP2 combined with hematocrit and RDW; AUC=0.89. C. Confusion matrix representing true positives and false negatives cases for the combined LAMP2 model.

2. Functional characterization of RBCs effects in BC cell lines.

2.1 RBC from M1 patients adhere to cancer cells

In a recent publication from our lab, RBCs were found attached to freshly isolated CTCs in M1 patients[156]. To check if this could be due to technical issues, spiking experiments were performed using healthy blood and BC cell lines. However, escort RBCs were not observed (data not shown), suggesting that RBCs from M1 have different behavior compared with control ones. As a proof of concept, RBCs from BC patients and CFC were isolated and co-cultured with two breast tumor cell lines of luminal and triple-negative subtypes (MCF7 and MDA-MB-231 respectively) in low attachment conditions. After 24h, wells were scanned with a contrast microscope to identify if RBC-tumor cell adhesion was taking place. MDA-MB-231 marked in GFP were used to make easier cell tracking and imaging. As can be observed in Figure 11A, RBCs from BC patients adhere with more frequency to cancer cells

than RBCs from CFC. Representative images of how RBC adhere to tumour cells are depicted in Figure 11C The number of samples showing RBCs adhesion to both MDA-MB-231 and MCF7 tumor cell lines was higher in the M1 group (p=0.0069 and 0.0059 respectively) (Table S2). Interestingly, it was also observed that despite performing the experiment in low adherence conditions, in some of the metastatic samples, tumor cells were able to adhere and even acquire their usual morphology, as we can see in Figure 11B (a phenomenon not observed with CFC). Considering these initial results, we planned to study the effect of the RBCs in the different steps of the metastatic cascade *in vitro*, from the primary tumor (proliferation, angiogenesis, migration) to the secondary site (adhesion to endothelia and extracellular matrix). Also, we aim to test if tumor-exposed RBCs condition this effect.

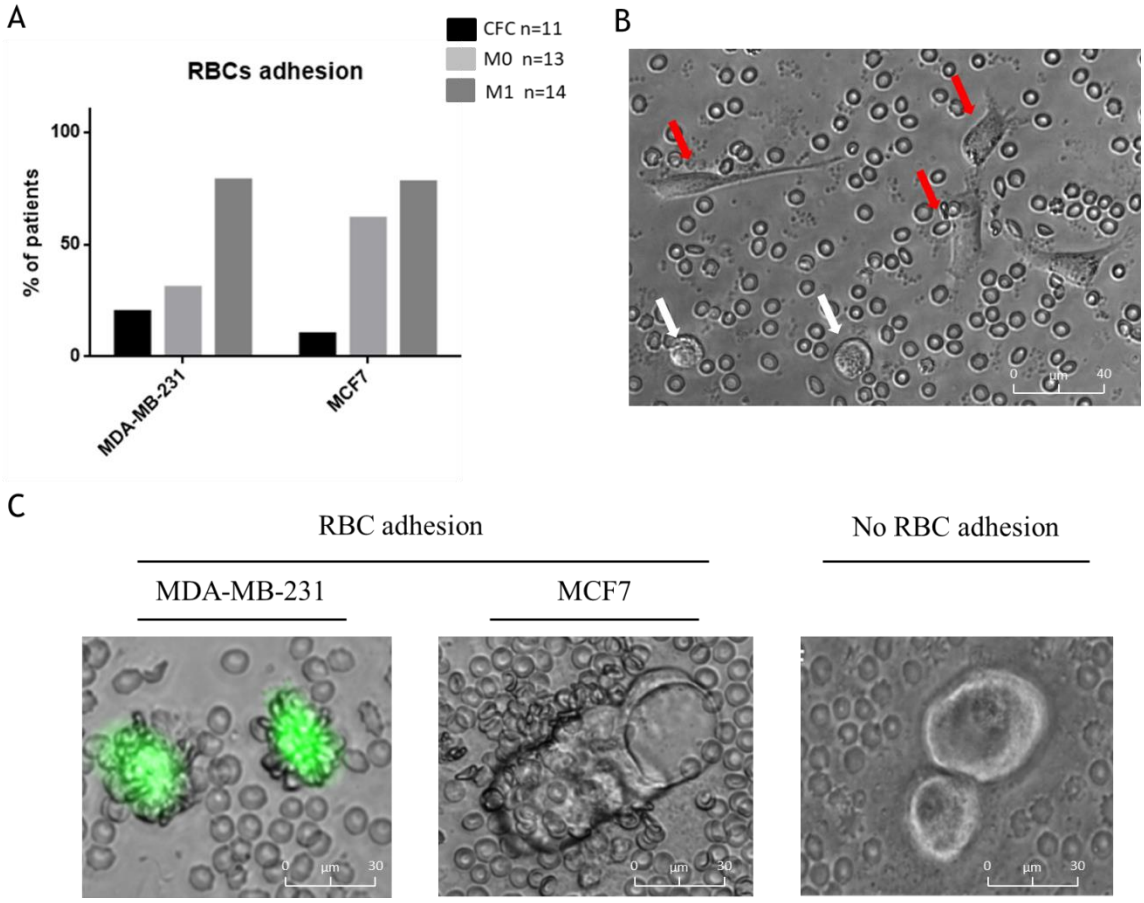


Figure 11. RBC adhesion to tumoural cells. A. Graph representing the percentages of patients showing RBC adhesion to cancer cells for non-metastatic breast cancer patients (M0, n=13 for MDA-MB-231 and MCF7)(Dark Grey), metastatic breast cancer patients (M1, n=14 for MDA-MB-231 and n=9 for MCF)(Grey), and cancer-free controls (CFC, n=10 for MDA-MB-231 and MCF)(Black). B. Representative image showing MDA-MB-231 adhesion to plate in low attachment conditions. Red arrows mark the attached tumoral cells whereas white arrows signal non-attached cells that remain in suspension. C. Representative images of BC RBC adhesion to tumoral cells and the absence of this adhesion with RBCs from CFC.

2.2 RBCs presence does not affect the proliferation of cancer cells

To know if RBC content influences tumor cell proliferation MDA-MB-231 and MCF7 were seeded in p96 wells and left to attach overnight. The next day RBCs from each sample (CFC or M1) were added to each well. This assay also included a negative control (CN) without RBCs. Next, the proliferation was checked at 24h, 48h and 72h through an MTT assay. The results obtained showed that RBCs did not influence cell proliferation, independently of the cell line or the origin of the RBCs (Figure 12).

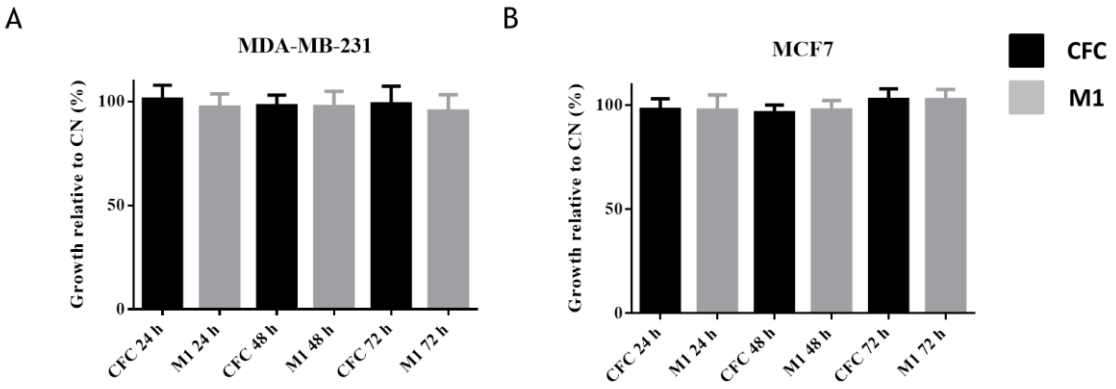


Figure 12. Proliferation assay. Proliferation is represented as relative growth to negative control (CN). A and B. Proliferation of MDA-MB-231 and MCF7 respectively after co-culture with cancer-free controls (CFC, n=5)(Black) and metastatic breast cancer patients (M1, n=5)(Grey) at different times.

2.3 Coculture with M1 RBCs alter tumour cell Cytokine profile

RBCs could also impact the cytokine production of tumour cells. To test this, MCF7 and MDA-MB-231 cells were co-cultured with RBCs from CFC or M1 for 24 h. Next, the resulting supernatants were mixed to prepare pools containing equal volumes of 3 different supernatants from each condition (M1, CN and CFC) and cell line (MCF7 or MDA-MB-231). Cytokine expression was detected with the commercial kit Proteome Profiler^{TM} (R&D SYSTEMS) using pools of samples.

The results, as we can see in the Figure 13, varied greatly depending on the cell line. For MDA-MB-231 cell line, many cytokines were detected in all of the supernatants, including CCL2, CXCL1, CXCL10, G-CSF, CCL5, ICAM-1, IL6, IL8, MIF and SERPIN R1 (Figure 13A). However, after performing dot density analysis, only CCL2 was increased in cells that were co-cultured with M1 RBCs compared with the other conditions. Nonetheless, this increase was quite significant, being up to 3-fold when compared to CN (Table S3). On the other hand, MCF7 supernatants did not contain a wide variety of cytokines (Figure 13B), finding only a very small amount of CCL7 in MCF7 exposed to M1 RBCs and IL-8, IL6 and CXCL1 in MCF7 co-cultured with CFC. No cytokines were detected for CN condition.

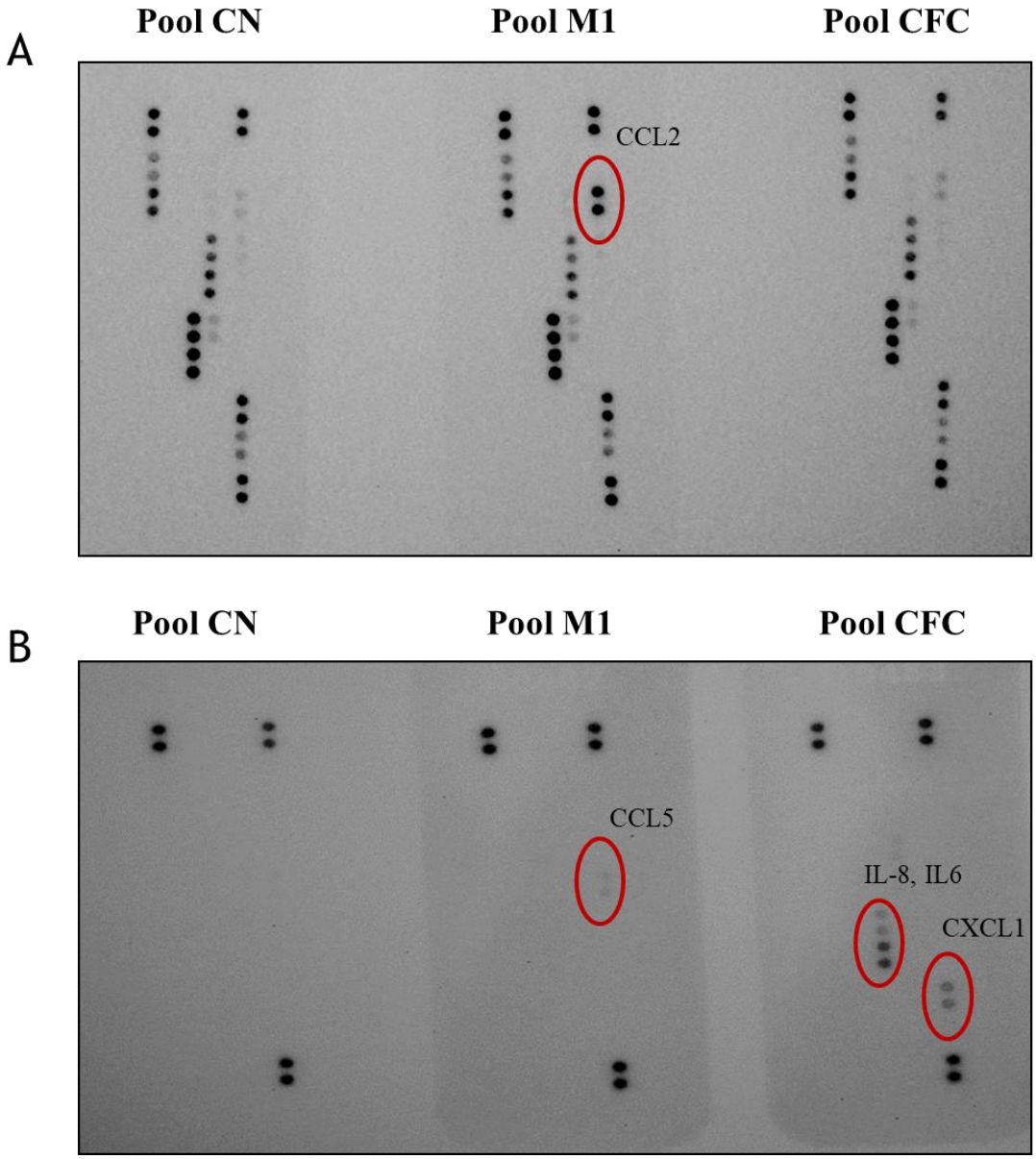


Figure 13. Cytokine array. Chemiluminiscence images obtained using ChemiDoc™ MP Imaging System for A. MDA-MB-231 cell line supernatant. CCL2 was increased after co-culture with metastatic breast cancer (M1) RBCs over 3-fold when compared to negative control (CN). B. for MCF7 cell line supernatant. CCL5 was only detected after co-culture with M1. IL-8, IL-6 and CXCL1 were detected after co-culture with cancer-free control (CFC) RBCs.

2.4 RBCs from M1 patients have a proangiogenic effect

To study if the RBCs from metastatic BC affect angiogenesis *in vitro*, HUVEC cells were seeded on an extracellular-matrix and cultured in basal Endothelial Growth Medium (EGM) containing lysates from M1 or CFC. Negative controls (CN) were included to assess basal levels of angiogenesis. As we can see in Figure 14A, RBCs from BC patients were able to induce the formation of larger and more complex structures than CFC RBCs and negative

control conditions. The quantification of the angiogenic process was based on the measurement of the tube length and the number of nodes per field. Tube length was significantly higher for M1 RBCs when compared to CFC or negative control (p=0.0019 and p=0.0002, respectively) (Figure 14B). On the other side, the number of nodes formed by the HUVECs was higher for M1 RBCs when compared only to negative controls (p=0.0173 (Figure 13C). No differences were found in terms of tube length or node formation when comparing CFC and CN. Overall, these results showed that M1 RBCs had a pro-angiogenic effect, not seen for CFC RBCs.

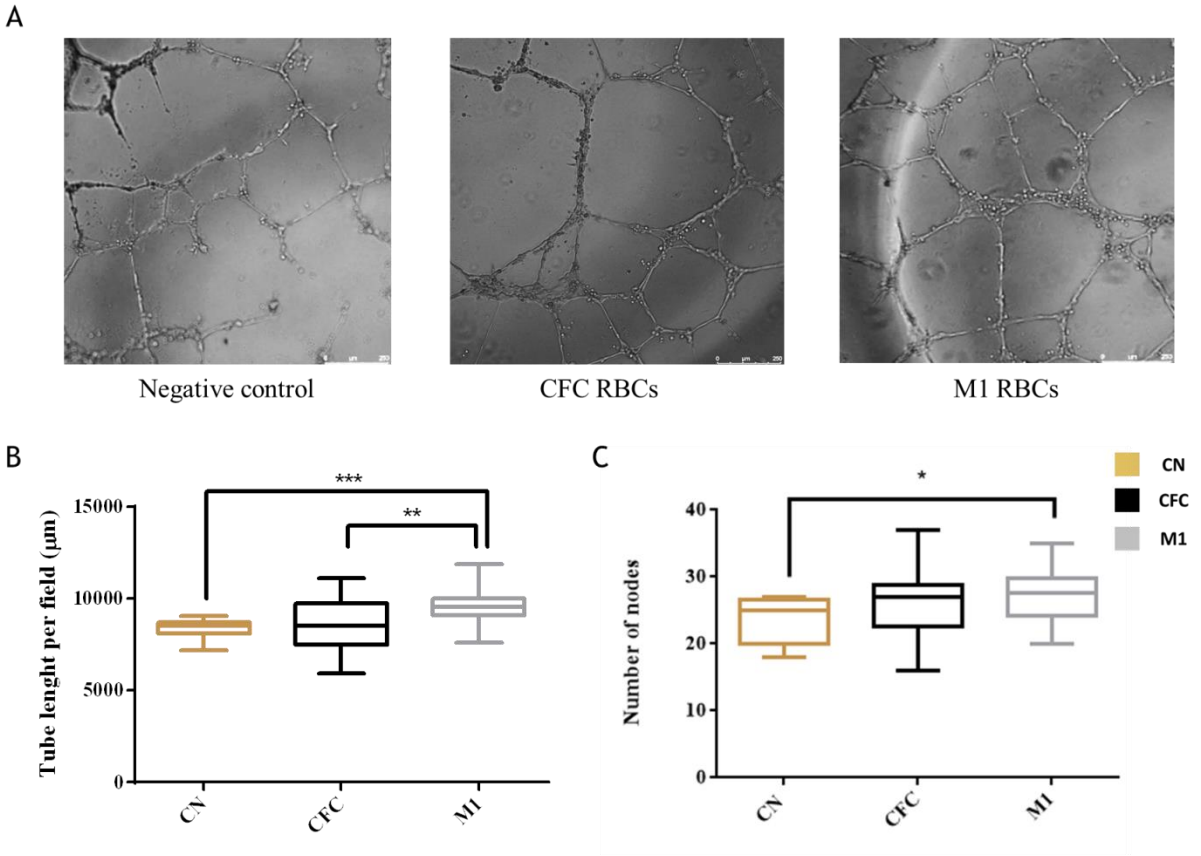


Figure 14. Characterization of the proangiogenic effects of RBCs. A. Exemplary images of the vascular network complexity of each condition, showing more developed structures when metastatic breast cancer patients (M1) RBCs were used. B. Graph representing the tube length per field that was measured for each condition. Conditions include RBCs from metastatic breast cancer patients (M1, n=6) (Grey), cancer-free control RBCs (CFC, n=6) (Black) and negative control without RBCs (CN, n=3) (Golden). Tube length was significantly higher for M1 RBCs. C. Graph representing the number of nodes formed during the angiogenic process. M1 RBCs formed a significantly higher number of nodes than negative controls. Mann-Whitney test. P-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***).

2.5. Effects of RBCs in migration of BC cell lines.

First, to study the possible changes in migration after co-culture with RBCs, wound healing assays were performed. Cells were grown until confluence was around 70-80%. At this point, RBCs from M1 and CFC patients were added to the media and cells were cultured

for 24 h. During this incubation, cells will end up forming a monolayer. After 24h, media was removed and a scratch was performed vertically with a pipette tip. Next, wells were washed using PBS and new fresh media with reduced FBS was added. Different times for imaging were used based on the cell line's ability to migrate. MDA-MB-231 required shorter times (8 h and 24 h), whereas MCF7 were slower and required longer times (24 h and 48 h). Wound healing % was calculated based on the ratio between the area of the scratch measured at 0 h and at 8, 24 and 48 h depending on the cell line.

As we can see in the Figure 15, the presence of RBCs has a different effect depending on the cell line. M1 and CFC RBCs reduced the migration of MDA-MB-231 both at 8 h (M1 35.4%±4.7, CFC 35.8%±3.8 compared to CN 39.4%± 4.9) and 24 h (65.4%±10.85, 66.6 % ± 10.6 compared to CN 70.2 % ± 5.9), meanwhile, no effect was seen in MCF7 independently from the precedence of the RBCs.

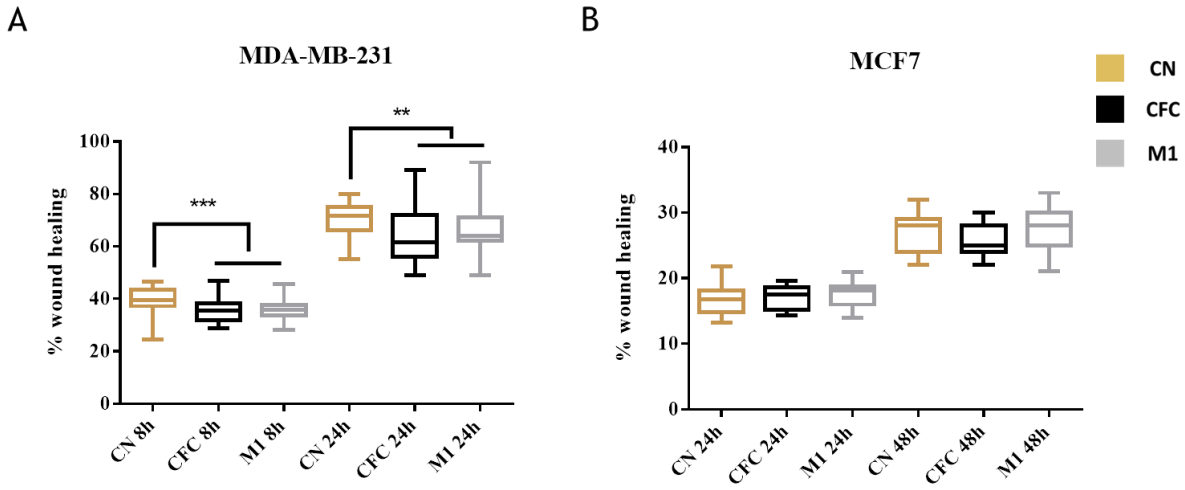


Figure 15. Wound healing assay to study of the 2D migration of tumour cells after coculture with RBCs. A and B. Graphs representing the % of wound healing at 8h and 24h for MDA-MB-231 (A) and 24h and 48h for MCF7 (B). Cancer-free control (CFC, n=5) (Black), metastatic Breast cancer (M1, n=5) (Grey) and negative control (CN, n=5) (Golden). Mann-Whitney test. P-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***).

Secondly, to extensively describe the effect of RBCs on the motility of cancer cells, migration was further studied using transwell assays. An effect of the RBCs on tumor cell migration was seen in a cell line-dependent manner. As can be seen in Figure 16A, there is no change in migration for MCF7 independently of RBCs origin. However, in MDA-MB-231 BC cells, the presence of RBCs increased the migration when compared to the CN. The increase in migration was more significant for M1 (p= < 0.0001) than M0 (p=0.0232). Moreover, M1 RBCs increased migration significantly more than CFC or M0 (p= < 0.0001 and p=0.0016 respectively) (Figure 16B). Although there are no significant differences between M0 and CFC, there is a trend that can be appreciated in the Figure 15B. These results showed that despite all BC RBCs can affect migration, M1 RBCs are the ones producing the most significant effect. To further study if this effect is sustained overtime, cells were cultured for a standard 24h or an extended 48h period before performing the assays as previously described. Results from this assay showed that even after 48h of incubation, the increase in migration was still significant, although it was less intense than after 24h of incubation

(Figure 16C). Next, to check if the RBCs could have a similar effect in a paracrine way, RBCs freshly isolated were seeded on the bottom of the well in the presence of an FBS gradient or plasma from the patients studied. CN conditions, without RBCs, were included for both approaches. As we can see in the Figure 16D, M1 RBCs increased migration significantly compared to the conditions without them independently of the use of FBS or patient plasma gradient ($p=0.0048$ and $p=0.0002$, respectively). Moreover, a migratory phenotype, characterized by the presence membrane protrusions that resemble lamellipodia, was observed more frequently in the FBS+ RBC M1 conditions compared to negative controls with just FBS (Figure 16G). No similar effect in migration or morphology was observed when using M0 RBCs on the bottom of the well (Figure S2).

Finally, since *in vitro* results shown that M1 RBCs increase migration of MDA-MB-231, we proceeded to study if those same effects could be seen also *in vivo*. For this purpose, MDA-MB-231 GFP cells co-cultured with M1 or CFC RBCs were injected in the Cuvier Duct of Zefrafish embryos. After 24h, fluorescence of migrated cells present in the tail of the fish was measured. Results showed that, similarly to *in vitro* assays, M1 RBCs increased significantly the migration of MDA-MB-231 compared to CFC ($p=0.0483$) (Figure 16H).

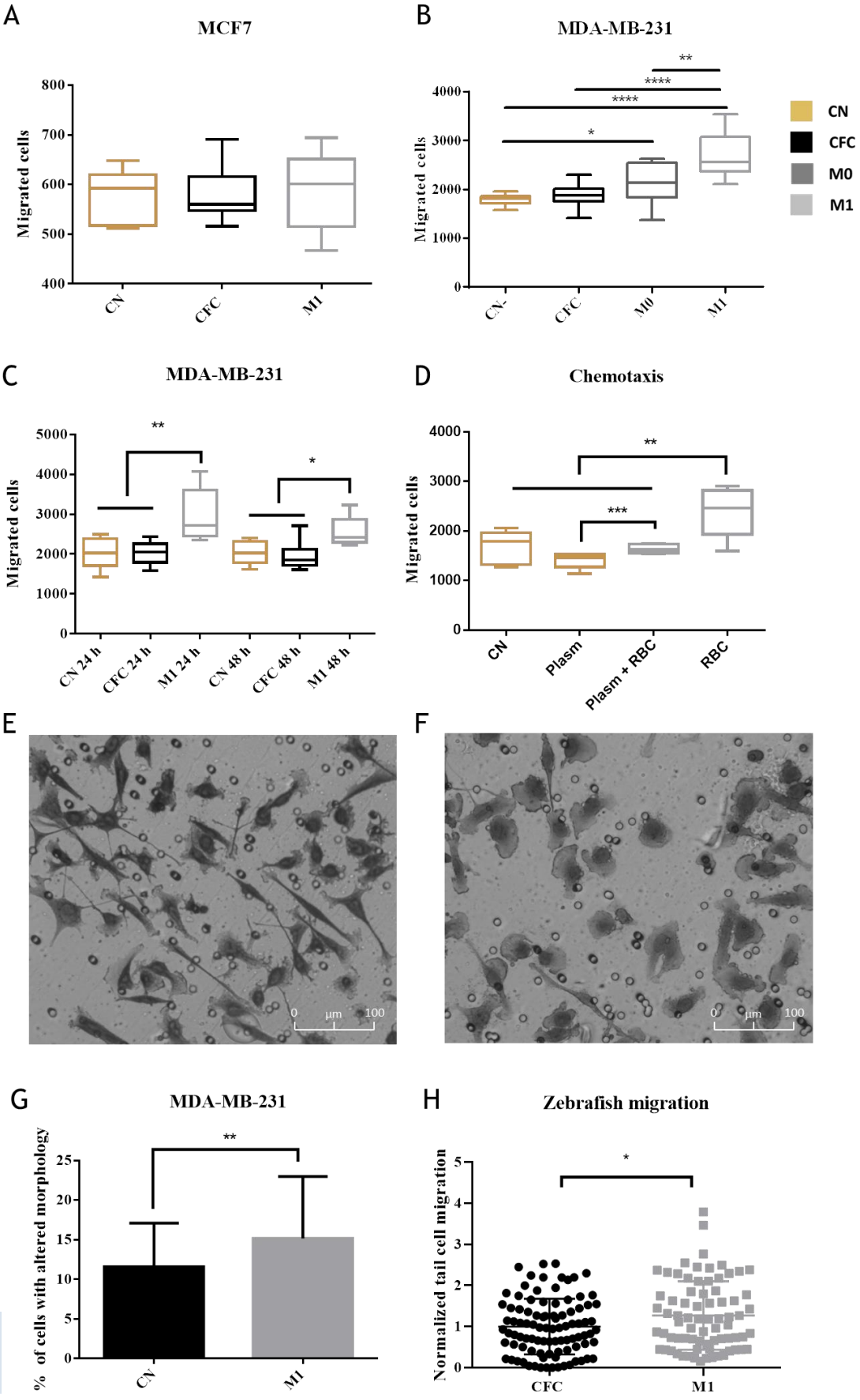


Figure 16. Migration of breast tumor cells after co-culture with RBCs *in vitro* and *in vivo*. A. Graph representing transwell migration of MCF7 co-cultured with Cancer-free control (CFC, n=5) (Black), metastatic breast cancer (M1, n=5) (Grey) and negative control (CN, n=5) (Golden) without RBCs in a transwell. B. Graph representing transwell migration of MDA-MB-231 co-cultured with CFC, M1, non-metastatic breast cancer (M0, Dark Grey) and CN (n=5 for all groups). C. Graph representing transwell migration of MDA-MB-231 co-cultured with M1 RBCs and CN (n=5 for each) after 24h co-culture (standard) and a 48h co-culture (extended). D. Graph representing transwell migration of MDA-MB-231 when M1 RBCs (n=5)(Gray both with plasma and FBS) were used as chemoattractant in the presence of FBS or plasma gradient. Paired CN conditions (n=5)(Golden both Plasma and FBS) were included for each condition. E-F. Representative images of the normal morphology (E) and the morphological change (F) of MDA-MB-231 observed in migrated cells in transwells from the chemotaxis assays. G. Graph representing the percentage of cells showing morphological change observed in chemotaxis assays in M1 (n=5) and CN (n=5) conditions. H. Graphic representing the normalized fluorescence intensity of MDA-MB-231 cocultured with RBCs from CFC (n=3 patients, n=95 fish) and M1 (n=3 patients, n=75 fish) that migrated to the tail of zebra fish embryos 24h after the injection. Mann-Whitney test. P-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***); p-value < 0.0001 (****).

2.6. Tumor-exposed RBCs enhanced tumor cell aggregation *in vitro*

To reach distal locations, cells must travel through the blood, and one of the most efficient ways to do it is forming clusters. We wanted to know if RBCs from M1 patients could increase the cell-to-cell adhesion that leads to the formation of clusters. For this purpose, a known number of MDA-MB-231 individualized cells were cultured in low attachment and agitation conditions for 1 h in the presence and absence of freshly isolated RBCs from different origins. The RBCs used came from CFC or M1 patients and also it was added another experimental group with pre-conditioned RBCs. This latter was obtained by co-culturing RBCs from CFC with conditioned media from a 48h MDA-MB-231 cell culture (cCFC). As we can see in Figure 17B, the results showed an increased number of clusterization for MDA-MB-231 in the presence of M1 and pre-conditioned RBCs than these cells without RBC ($p<0.0001$ for both) or in the presence of CFC RBCs ($p=0.0093$ and $p=0.0016$, respectively).

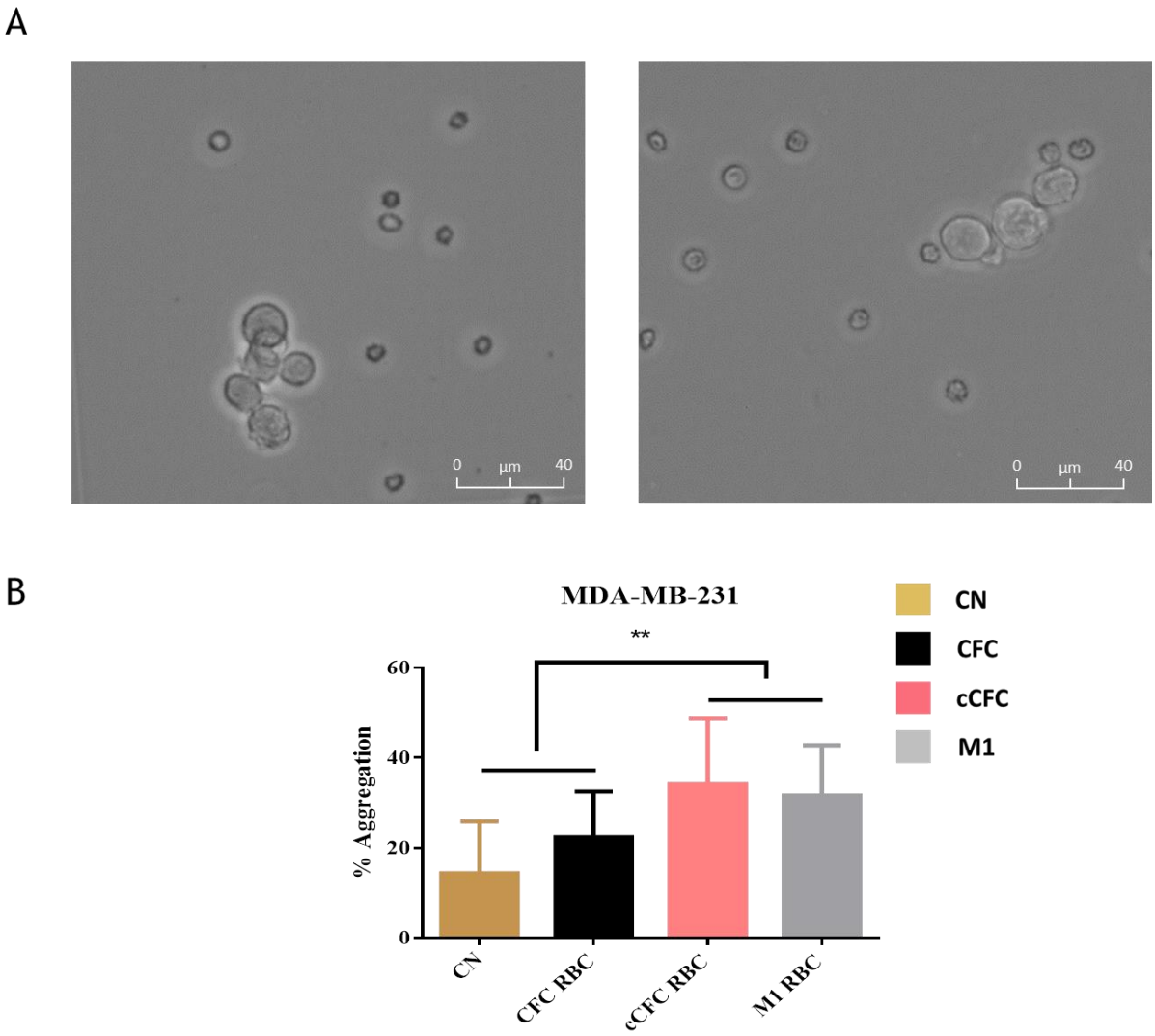


Figure 17. Aggregation of MDA-MB-231 in low attachment conditions in the presence of RBCs. A. Exemplary images showing the clusters formed after the assay in the presence of metastatic breast cancer (M1) and pre-conditioned cancer-free control (cCFC) RBCs. B. Graph representing the % of cell aggregation (measured indirectly by counting single cells) of negative control (CN) (Golden), CFC (Black), cCFC (Pink) and M1 (Grey) RBCs (n=5 for each group). Mann-Whitney test. P-value < 0.05 (*); p-value < 0.01 (**).

2.7 RBCs co-culture affects tumor cell adhesion

Continuing with the analysis of the different steps of the metastatic cascade, we proceeded to study how RBCs from BC could affect the ability of cancer cells to adhere to endothelial cells. The *in vitro* assay showed that MDA-MB-231 cells co-cultured with M1 RBCs had a significant increase in adhesion to HUVEC cells in comparison to CN (p=0.0263) or CFC (p=0.0013) (Figure 18A).

To further study the process of adhesion to the vasculature, we proceeded to focus on the adhesion of cancer cells to a major component of the extracellular matrix (ECM) present in vessels and tissues. For this purpose, similarly to what we did for endothelial cell adhesion, cancer cells (MDA-MB-231 and MCF7) were co-cultured with M1 and CFC RBCs prior to

the adhesion experiment. As we can see in the Figure 18B/C, only significant differences were found in MDA-MB-231, where M1 RBCs induced adhesion to collagen I more significantly than CFC (p=0.0145), M0 (p=0.002) or CN (p<0.0001). An increased adhesion was also found in M0 or CFC when compared to CN (p=0.0107 and p=0.0252, respectively). As a whole, these results show that the co-culture of the breast cancer cell line MDA-MB-231 with RBCs from M1 patients increased significantly the adhesion to both endothelial cells and collagen.

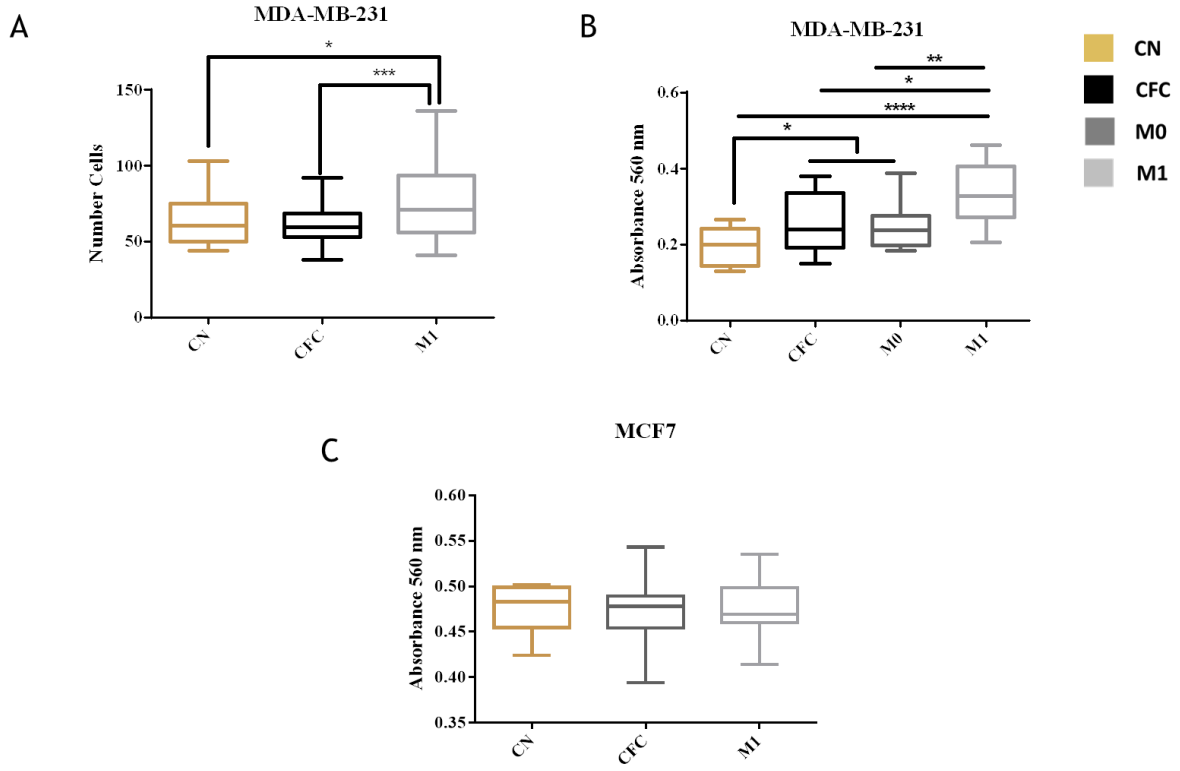


Figure 18. Adhesion of tumor cells to HUVEC and Collagen I after coculture with RBCs. A MDA-MB-231 number of cells adhered to HUVEC after being co-cultured with cancer-free control (CFC, n=5)(Black) RBCs, non-metastatic breast cancer (M0, n=5 only for MDA-MB-231)(Dark gray) and metastatic breast cancer (M1, n=5)(Gray) RBCs. Negative control (CN, n=5)(Golden) condition without RBCs was also added. B. Graph representing the absorbance values of MDA-MB-231 (CN, M1 and CFC, n=5 for all groups) adhered to collagen I after being cocultured with RBCs (M1, M0 and CFC, n=5) or without them (CN=5). C. MCF7 absorbance values of the cells adhered to collagen I after being co-cultured with RBCs (M1 and CFC, n=5) or without them (CN, n=5). Mann-Whitney test. P-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***); p-value < 0.0001 (****).

3. RBCs from M1 patients modify gene expression in MDA-MB-231

In vitro assays showed that RBCs were able to modulate the behaviour of cancer cells, affecting different stages of the metastatic cascade. To study the mechanisms through which RBCs can exert these effects on cancer cells, we focused on the molecular changes that may be happening in these cells after RBC priming. Since most of our results were mainly on the triple-negative breast cancer cell line MDA-MB-231, from this point onwards we will focus on these cells.

3.1 RNA-sequencing analysis of MDA-MB-231 cocultured with RBCs

To identify changes in gene expression after the co-culture of MDA-MB-231 with RBCs, an RNA-sequencing analysis was performed. Similarly to the previous *in vitro* assays, cells were co-cultured for 24 h in the presence of CFC or M1 RBCs or the absence of RBCs (CN). For this specific assay we included another extra condition where, instead of RBCs, we added liposomes, with similar lipid content to human RBCs, in the same concentration as the RBCs conditions. These liposomes acted as another control (LIPO), allowing us to rule out that any effect derived from the lipids present in the RBCs was mediating the changes in the cancer cells. Firstly, hierarchical clustering analysis of the data from the RNA-sequencing experiment showed that samples from MDA-MB-231 co-cultured with M1 RBCs segregated correctly from CFC and CN conditions (Figure 19). Interestingly, the use of liposomes had a reduced impact on the expression of MDA-MB-231, presenting a similar profile to CN conditions (Figure S3).

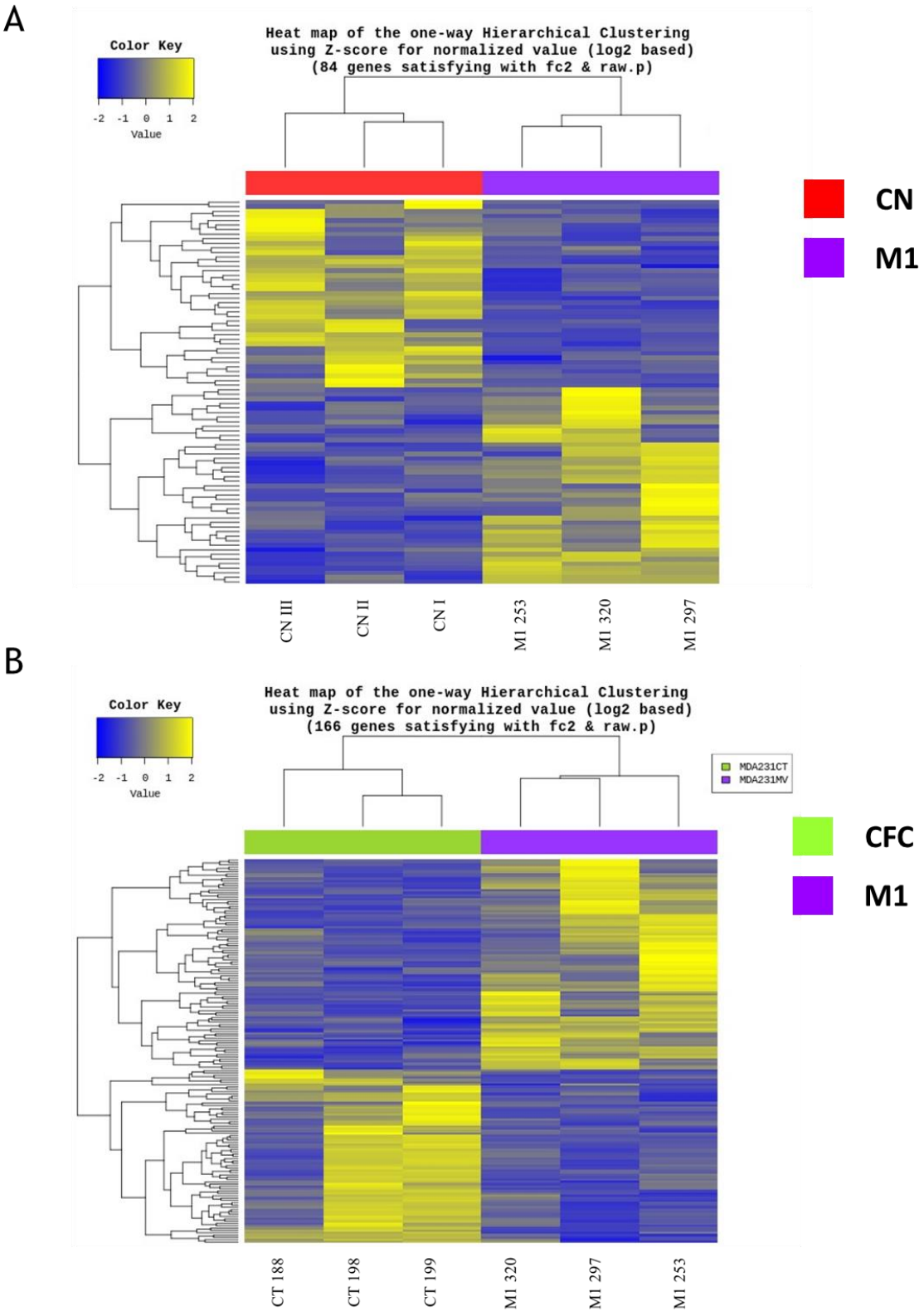


Figure 19. Heatmap showing results of the hierarchical clustering analysis of MDA-MB-231 (Euclidean Method, Complete Linkage) which clusters the similarity of genes and samples by expression level (normalized value) M1 corresponds to metastatic breast cancer RBCs (Purple), CFC to cancer-free control RBCs (Green) and CN (Red) to the negative control without RBCs.

As shown in the volcano plots from Figure 20 A/B, M1 RBCs are able to modify to of several genes in MDA-MB-231 cell line when compared to CN and CFC conditions. In order to explore the biological significance of the differentially expressed genes, a gene-enrichment and functional annotation analysis (Gene Ontology (GO) Analysis) was performed. Results from this analysis highlight that cell-cell adhesion, mitochondria metabolism; stress fiber assembly, cell adhesion and contractile actin filament assembly are the biological process mainly altered in MDA-MB-231 after co-culture with RBCs (Figure 20C). Amongst differentially expressed genes in MDA-MB-231 co-cultured with M1 RBCs compared to CN and CFC, a set of genes was selected, based on their p-value, fold change and their biological role in cancer disease. *Heme Oxygenase 1* (*HMOX1*), *Epoxide hydrolase 1* (*EPHX1*), *Delta-aminolevulinate synthase 1* (*ALAS1*) or *p21-activated kinase 4* (*PAK4*) were the genes selected.

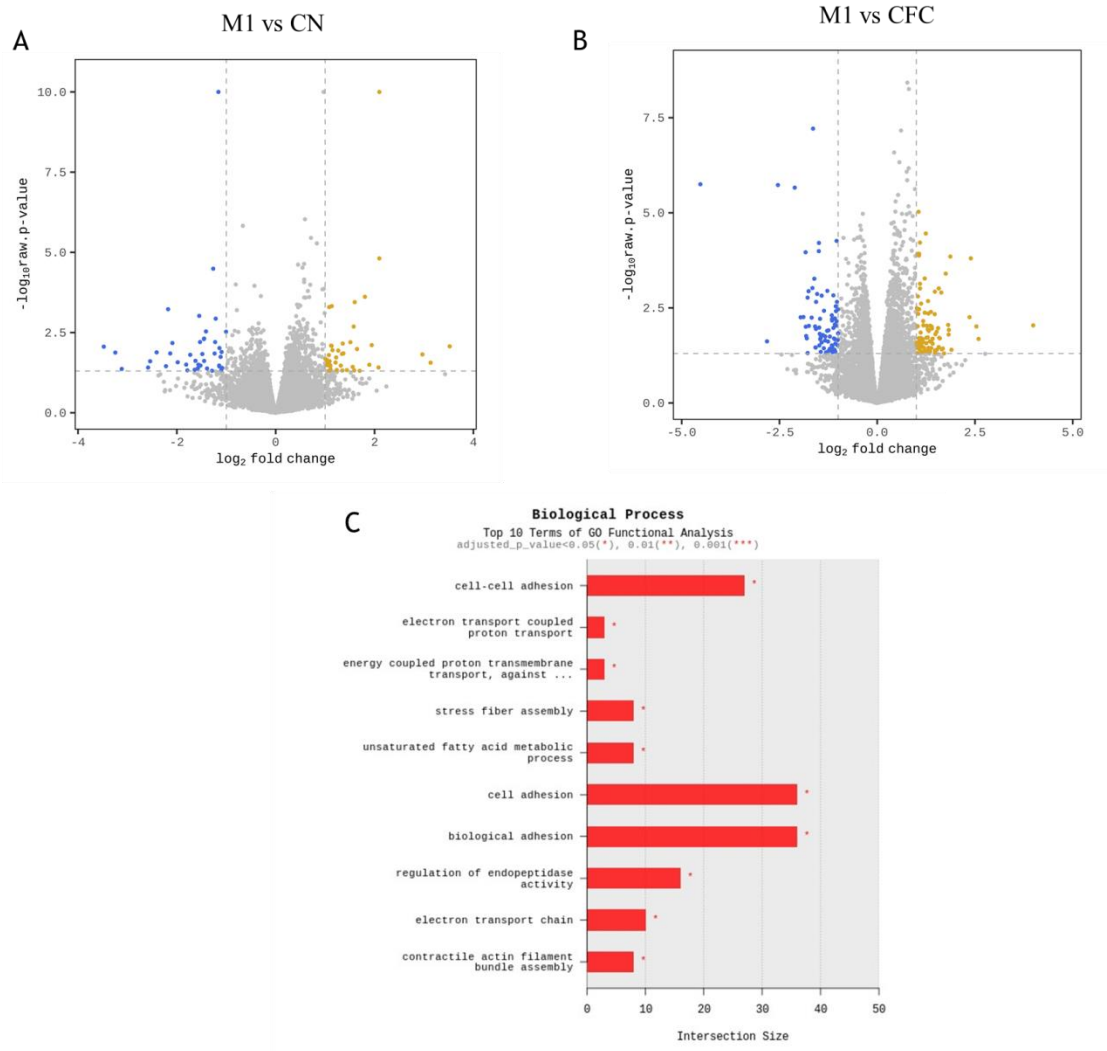


Figure 20. Differential gene expression analysis and altered biological processes. A-B Volcano plots representing the differentially expressed genes in MDA-MB-231 cocultured with metastatic breast cancer RBCs (M1) compared to cells cultured without RBCs CN (A) or with cancer free control RBCs CFC (B). Representation is based on the fold change and p-value of each gene. C. Gene ontology analysis of the differentially expressed genes has display the biological processes altered on MDA-MB-231 after co-cultivation with RBCs.

3.2 qRT-PCR analysis of genes upregulated by RBCs

As a next step, selected genes (*PAK4, HMOX1, EPHX1, ALAS1*) were analyzed by qRT-PCR in cells that were co-cultured with a larger cohort of BC patients (M1 and M0) or CFC (n=14 each). Paired MDA-MB-231 CN conditions without RBCs were added to act as a normalization control. In Figure 21 is depicted the analysis of the qRT-PCR results showing that *PAK4, HMOX1* and *EPHX1* were higher in MDA-MB-231 co-cultured with M1 RBCs compared to CFC (p=0.0004, p=0.0456 and p< 0.0001, respectively). When comparing cells co-cultured with M1 or M0 RBCs, *PAK4* and *EPHX1* were significantly higher in M1 (p=0.0030 and p=0.0005, respectively). Interestingly, *HMOX1* expression was also increased in M0 when compared to CFC (p=0.0137), showing the same trend as M1 RBCs.

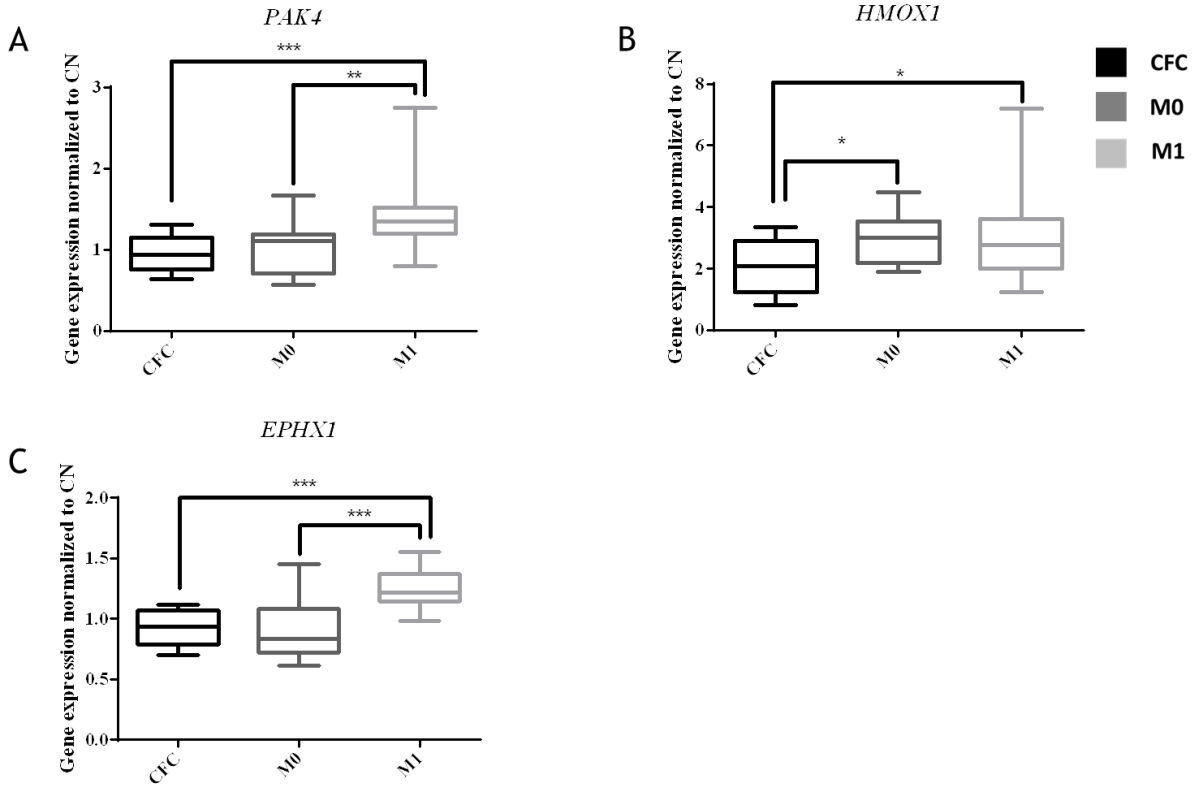


Figure 21. Gene expression was analyzed by qRT-PCR. Gene expression was relativized to B2M and normalized to Δct from negative control (CN) to reduce variability. A-C. Expression of *p21-activated kinase 4* (*PAK4*) (A), *Heme Oxygenase 1* (*HMOX1*) (B) and *Epoxide hydrolase 1* (*EPHX1*)(C) in MDA-MB-231 co-cultured with cancer-free control (CFC, n=14)(black), non-metastatic breast cancer (M0, n=14) (dark grey) and metastatic breast cancer (M1, n=14) (grey) RBCs. Mann-Whitney test. P-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***); p-value < 0.0001 (****).

PAK4 is an important regulator of the cell cytoskeleton, morphology and motility, and it has been associated in some publications with EMT. Despite no other markers of EMT being detected in the RNAseq analysis, a set of additional genes were included in the qRT-PCR analysis. *Signal transducer and activator of transcription 1* (*STAT1), Plastin 3* (*PLS3*) and *Vimentin* (*VIM*) were significantly higher for those MDA-MB-231 cells that were co-cultured with M1 RBCs when compared to CFC (p= 0.0002, p= 0.0021 and p < 0.0001, respectively).

STAT1 and *VIM* expression was also found higher in cells co-cultured with M1 RBCs compared to M0 RBCs (p=0.0091 and 0.0138, respectively) (Figure 22).

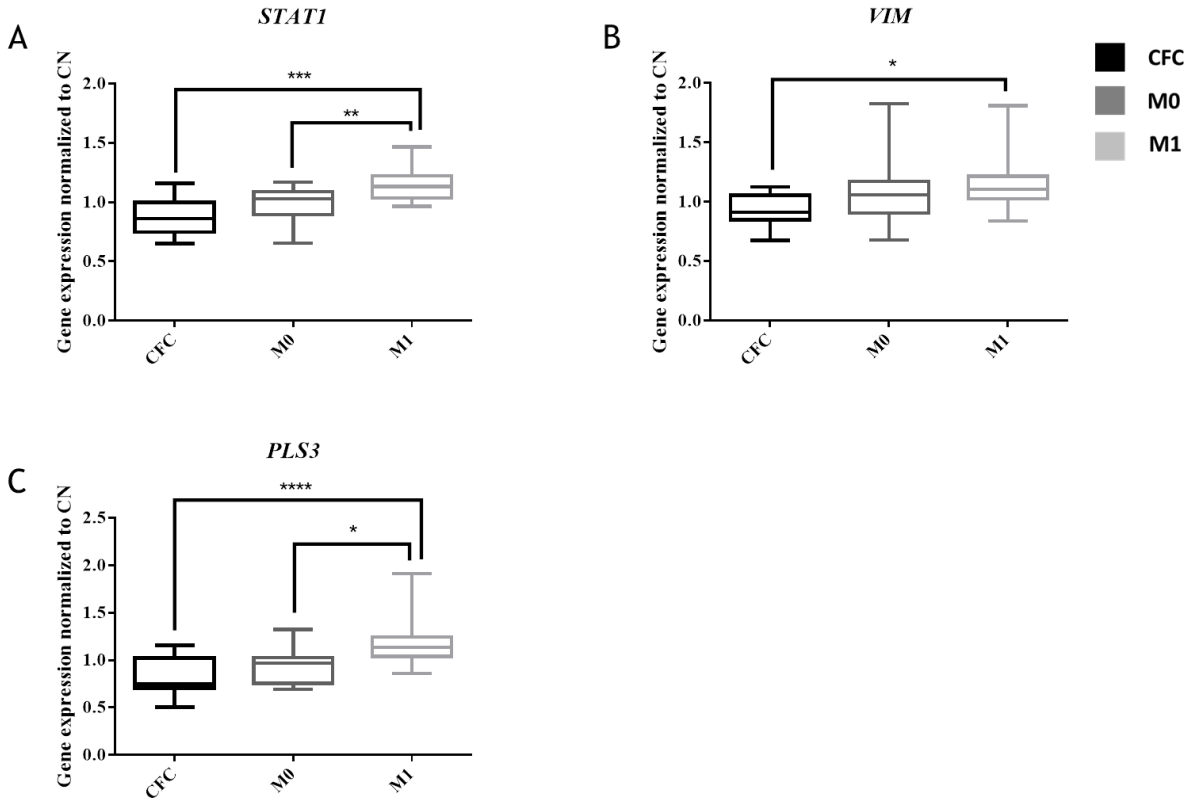


Figure 22. qRT-PCR analysis of genes related with EMT. Gene expression was relativized to B2M and normalized to Δct from negative control (CN) without cells to reduce variability A-C. Expression of *Signal transducer and activator of transcription 1* (*STAT1*), *Plastin-3* (*PLS3*) and *Vimentin* (*VIM*) in MDA-MB-231 co-cultured with cancer free control (CFC, n=14) (black), non-metastatic breast cancer (M0, n=14) (dark grey) and metastatic breast cancer (M1, n=14) (grey) RBCs. Mann-Whitney test. P-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***); p-value < 0.0001 (****).

4. Role of PAK4 in RBCs´ modulation of MDA-MB-231 functions

PAK4 is an important regulator of the cell morphology, migration and adhesion. The increased expression of *PAK4* in MDA-MB-231 after co-culture with RBCs from M1 patients suggested that the previously described alterations in the behavior of the cancer cells could be mediated by this protein. To check this, we employed a PAK4 inhibitor (LCH-7749944) on MDA-MB-231 cells for in vitro assays. Since *PAK4* is also involved partially in the regulation of the cell cycle, a proliferation assay based on an MTT test was performed. Different concentrations of this drug and times of exposure (24 h and 48 h) were included. Results from these assays shown that for concentrations above 20 μM the effect of the inhibitor in the proliferation of the tumor cells is very significant ($p < 0.05$) (Figure 23A/B). Concentrations of 10 and 20 μM do not decrease proliferation significantly.

Based on these results and the bibliography published on LCH-7749944, a concentration of 20 μM was chosen to perform further functional assays. Since transwell migration was significantly induced by M1 RBCs (see section 2.5 from Results), this setup was one of the

selected for testing LCH-7749944. As expected, migration of MDA-MB-231 cells co-cultured with M1 RBCs was higher than CN cells ($p > 0.0001$) (Figure 22C). PAK4 inhibition diminished the migration capacity of MDA-MB-231 cells in both CN and M1 settings when compared to untreated conditions ($p=0.0283$ CN, $p=0.0003$ M1) (Figure 23C). However, we can see that the inhibition of PAK4 had a more significant impact on those cells that were previously co-cultured with M1 RBCs. Moreover, no significant differences were found between untreated CN cells when compared with cells co-cultured with M1 and treated with LCH-7749944, indicating that the inhibitor is able to reverse the migratory capacity enhancement. Similar tendencies were observed when adhesion to collagen I was studied. Co-culture with M1 RBCs increased adhesion to collagen I ($p=0.0317$) when compared to CN, and cells of both conditions treated with LCH-7749944 adhered significantly less to the collagen I matrix ($p=0.0159$ for CN, $p=0.0079$ for M1). Importantly, inhibition of PAK4 in MDA-MB-231 co-cultured with M1 RBCs reduced adhesion to the same levels as MDA-MB-231 cells that were not exposed to RBCs (Figure 23D).

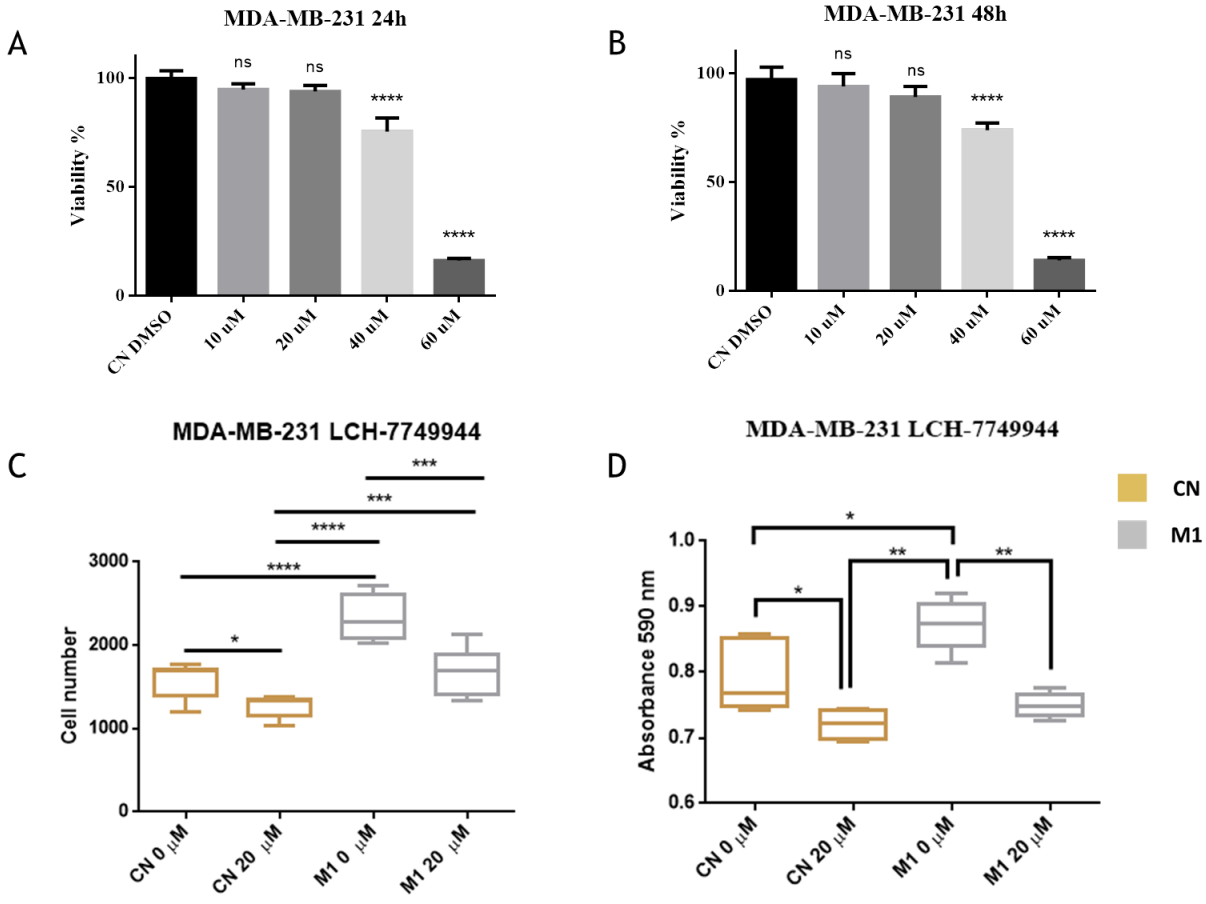


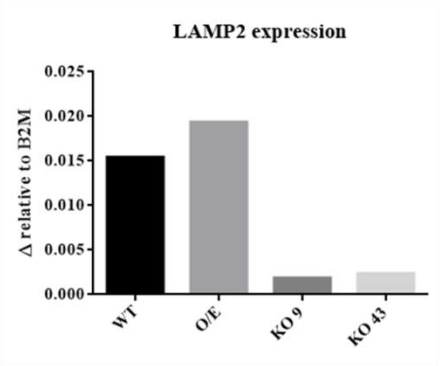
Figure 23. Effects of the inhibition of PAK4 in MDA-MB-231 proliferation, migration and adhesion. A-B. Graphs representing changes in proliferation of MDA-MB-231 at 24 and 48h after incubation with different concentrations of the PAK4 inhibitor (LCH-7749944). Absorbance values were normalized to negative control without DMSO and compared to CN with DMSO. C. Graph representing migration of MDA-MB-231 co-cultured with metastatic breast cancer RBCs (M1) (Grey) and without RBCs (CN) (Black) in presence or absence of PAK4 inhibitor. D. Graph representing the adhesion of MDA-MB-231 co-cultured with M1 RBCs and without them in presence or absence of PAK4 inhibitor. A-B Kruskal-Wallis and Dunn test (versus CN

DMSO). C-D. Mann-Whitney test. P-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***);p-value < 0.0001 (****).

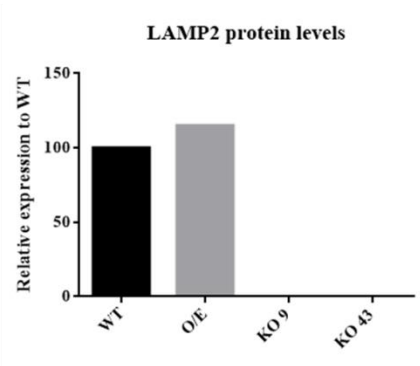
5. Study of LAMP2 involvement in vesicle biogenesis using erythoblast model

The results previously described showed that RBCs from M1 patients are able to modulate the BC cell line MDA-MB-231 behaviour. To further understand how RBCs exert this function, LAMP2, a protein overexpressed in M1 RBCs and involved in vesicle biogenesis and lysosomal activity, was studied. The objective was to determine whether LAMP2 participates in vesicle transport in RBCs. Alterations in LAMP2 expression could be linked to the release of extracellular vesicles (EVs), facilitating cell-to-cell communication. Thus, RBCs could load different factors that could potentially mediate the observed functional effects in *in vitro* assays. For this purpose, an immortalized erythroid cell line, the human umbilical cord blood-derived erythroid progenitor 2 (HUDEP-2) was chosen as a model. This cell line can be differentiated to immature RBCs and genetically edited. Thus, the expression of *LAMP2* in HUDEP-2 cell line was modulated to obtain knockout (KO) cells for *LAMP2*. The KO was obtained through CrisprCas9 editing. As shown in Figure 23, two complete KO were obtained after clonal selection. Expression of *LAMP2* mRNA is almost undetectable (Figure 24A) and no protein was detected (Figure 24 B/C).

A


LAMP2 expression
Δ relative to B2M
0.025
0.020
0.015
0.010
0.005
0.000
WT
O/E
KO 9
KO 43

B


LAMP2 protein levels
Relative expression to WT
150
100
50
0
WT
O/E
KO 9
KO 43

C

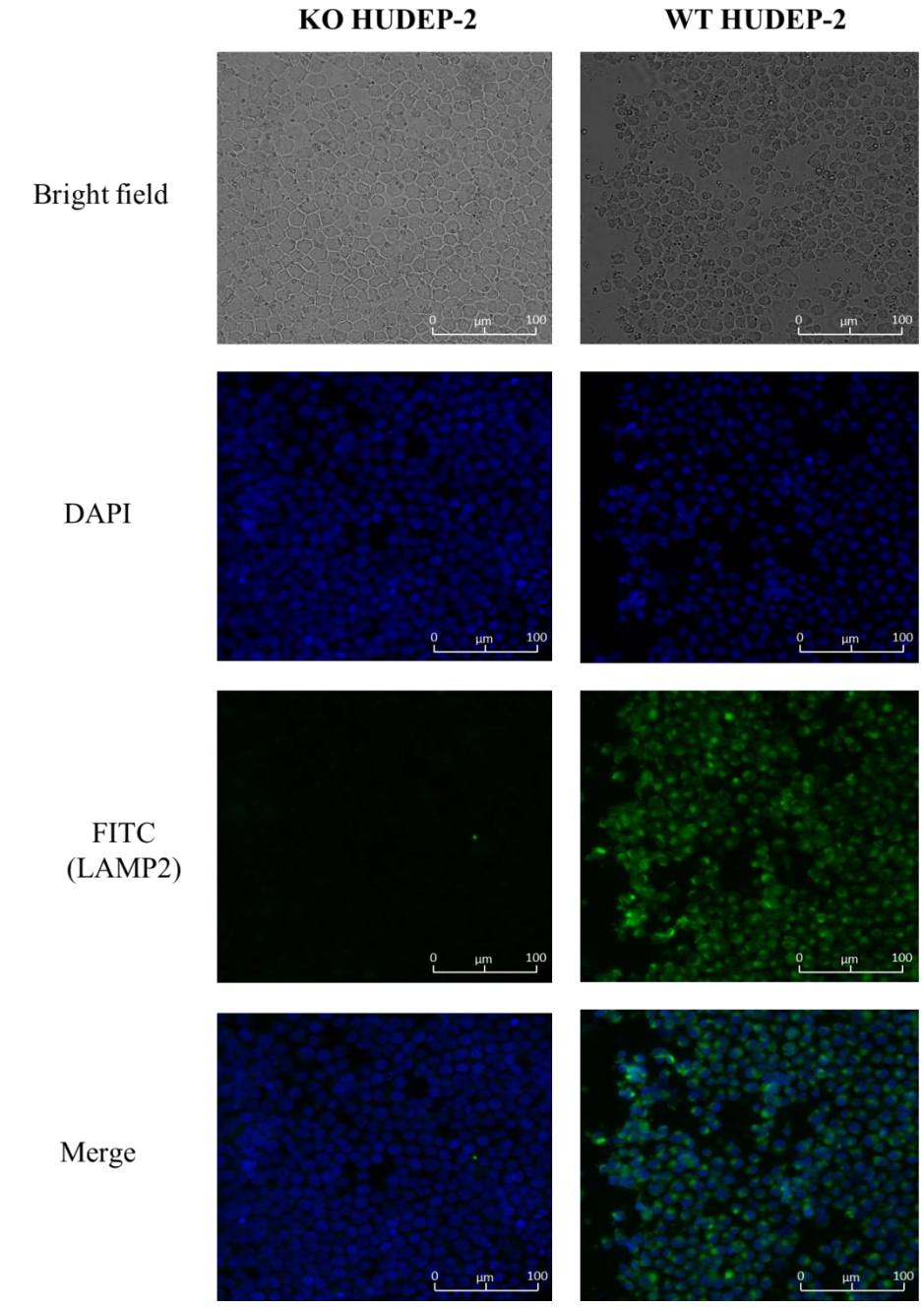
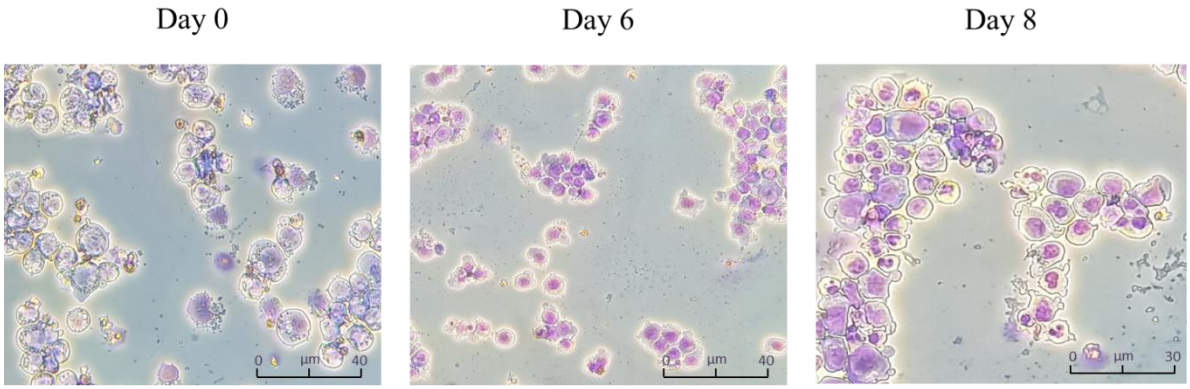

KO HUDEP-2
WT HUDEP-2
Bright field
DAPI
FITC
(LAMP2)
Merge
0 µm 100

Figure 24. HUDEP-2 Cell line genome editing. A. Graph representing the gene expression of LAMP2 in wild type (WT), LAMP2 knockout 9/43 (KO) HUDEP-2 cells. B. Graph representing protein levels of LAMP in WT and KO HUDEP-2 cells. C. Immunofluorescence images of WT and KO cells employed in HUDEPs assays, with the nucleus marked with DAPI (blue) and LAMP2 with FITC (Green).

5.1 Study of LAMP2 role in differentiated HUDEP cells

To obtain immature RBCs to study the role of LAMP2, HUDEP-2 cells have to undergo a differentiation process. This differentiation was achieved by culturing these cells in a specific HUDEP-2 differentiation base media. Along the differentiation process, cells were monitored every two days by studying the cell morphology and the expression of different membrane markers such as Band3 and α4-integrin. In the Figure 25A we can see Wright-Giemsa staining showing condensation of the nucleus (marked with black arrows) as well a small reduction in the size of the cells. Cytometry analysis indicated that with the differentiation, CD235+ cells (erythroid lineage marker) have increased expression of Band 3 (main structural protein of mature RBCs), and α4-integrin (an adhesion molecule present in erythroid precursors, not expressed in differentiated RBCs) expression has started to decline at day 8 (Figure 25B). Altogether these results guarantee that the differentiation is proceeding correctly and the cells employed in the following assay are in fact orthochromatic erythroblasts.

A



B

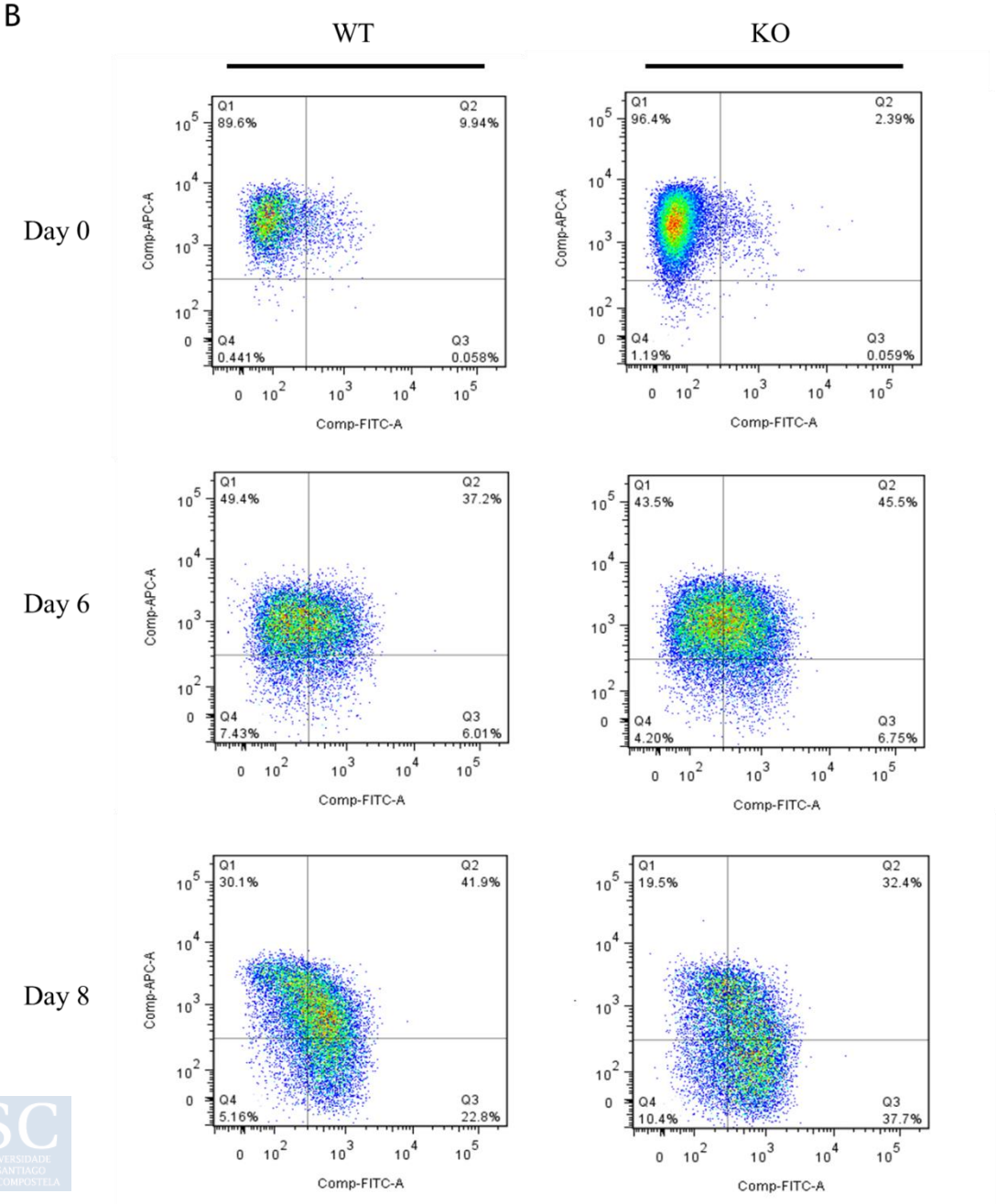


Figure 25. Differentiation of HUDEP-2 WT and KO cell line. A. Representative images from WT cytospined samples of day 0, 6 and 8 of the differentiation. Images display changes in cell morphology and nuclear structure along this process. B. Flow cytometry analysis of CD235+ HUDEP cells. Band 3 is marked with FITC and α4-integrin is marked with APC.

5.2 LAMP2 KO has an altered production of vesicles

In order to assess the involvement of LAMP2 in vesicle biogenesis, differentiated HUDEP-2 WT and KO were grown for two more days in differentiation media to assess the production of vesicles. After the culture, conditioned media containing the released vesicles was processed. Isolated vesicles were measured using a Nanoparticles Tracking Analysis (NTA) device. Results from this analysis showed that KO cells lacking LAMP2 produced fewer vesicles (concentration of 8.21 $x10^8$ ± 2.03 $x10^7$ particles/mL) compared to WT (concentration of $1.15x10^9$ ± $9.91x10^7$ particles/mL) (Figure 26A/B). Besides these vesicles were also smaller (Mo 27.5 ± 0.9 nm) than WT´s (Mo 100.6 ± 3.8 nm).

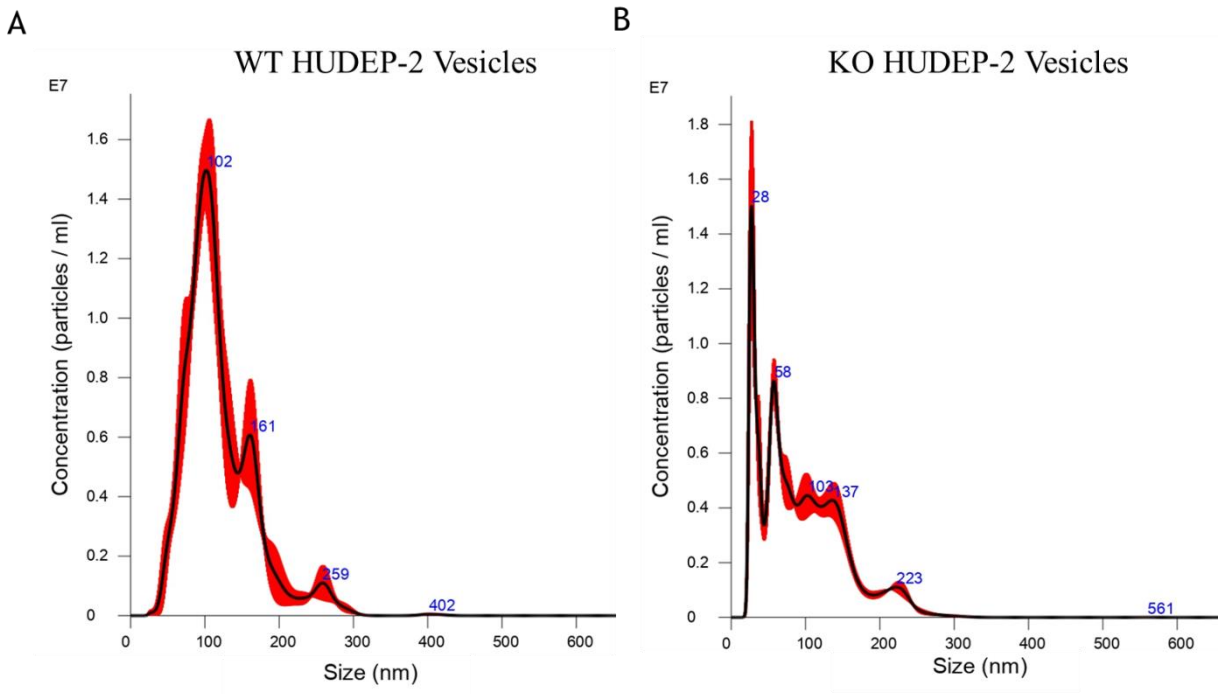


Figure 26. Impact of LAMP2 on the vesicle release. A-B. Graphical representation of the size of the vesicles produced by WT (A) and KO HUDEP-2 (B) cell lines at day 10 of the differentiation by NTA analysis.

5.3 LAMP2 play a role in paracrine communication between primed WT HUDEP-2 and MDA-MB-231

To study LAMP2's potential involvement in paracrine communication, we utilized a setup similar to the chemotaxis experiments conducted with M1 RBCs, adapted specifically for HUDEP-2 cell lines. Results from this experiment shown an increase in the migration capacity solely in HUDEP-2 LAMP2 WT cells when exposed to M1 plasma, demonstrating a significant increase in comparison to CN and HUDEP-2 CN (p=0.0007 and p=0.0016, respectively) or with WT HUDEPs exposed to CFC plasma (p=0.0002) (Figure 26).

Interestingly, the increase in migration is also noteworthy when compared with cells primed with LAMP2 KO cells exposed to either M1 or CFC plasma (p=0.0002 for both cases) (Figure 26).

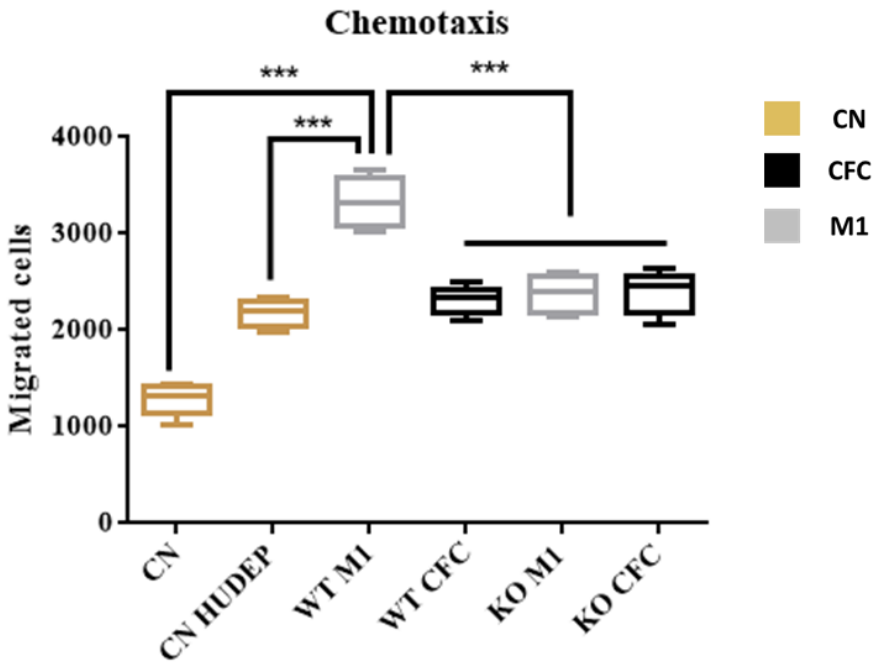


Figure 26. HUDEP-2 cells as chemoattractant. A. Graph representing the number of MDA-MB-231 cells that migrated when there was no HUDEPs (CN), HUDEPs without conditioning (CN HUDEP), Wild type (WT) and LAMP2 KO (KO) HUDEPs after conditioning with cancer free control (CFC) and metastatic breast cancer (M1) plasma. Mann-Whitney test. P-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***);

DISCUSSION

DISCUSSION

Breast cancer (BC) remains one of the leading health concerns for women in the world, affecting also a minor percentage of men. Despite the advances in therapies and monitoring, up to 20 % of the patients develop metastasis[184]. Moreover, between 5 to 10 % of the patients present metastasis at diagnosis[184]. At this point, BC turns into an incurable disease, with a 5-year overall survival rate of 27 % after the diagnosis of the metastasis when including all subtypes, having luminal A the better prognosis and TNBC the worse[185,186].Currently, the detection of metastasis is based exclusively on imaging methods such as ultrasound scan, mammography, x-rays, positron emission tomography (PET), conventional magnetic resonance (MRI) or computed tomography (CT)[187]. However, these techniques are only performed when the patient exhibits symptoms derived from the metastasis or in those with higher risk. These methods have proven to be inefficient, generally detecting metastasis in late stages of the formation of the secondary tumours, which are associated with a worse prognosis[187].In this regard, a better understanding of the metastatic cascade that leads to the formation of secondary tumours is essential for the development of new therapies and the finding of new markers to improve the survival and quality of life of these patients. This work is focused on defining the role of one of the most understudied potential interplayers of the metastatic cascade, the RBCs. Despite being the most abundant cell in the blood, RBCs were generally overlooked and considered mere oxygen transporters. However, it has been recently demonstrated that these cells are involved in the regulation of many processes including coagulation, immune response or angiogenesis[117,118,141]. Moreover, RBCs interact with cancer cells in the primary tumour as well as in the bloodstream[154,156,160,188]. Thus, in a recent publication, our laboratory demonstrated that the proteomic profile of RBCs from BC patients is modified by the presence of the disease[154]. Other similar works performed in hepatocellular carcinoma also detected alterations in the proteome of RBCs from cancer patients[188]. In another work from our laboratory it was also observed that CTCs from metastatic BC patients isolated with a negative enrichment technique, presented escorting RBCs attached to them, being a worse prognostic factor[156]. Adding to this, a work from Yin *et al* also showed that extravascular RBCs can promote tumour growth and therapeutic resistance[160]. These evidence demonstrate that, similarly to other "simpler" cells such as neutrophils or platelets[24,111], RBCs are altered in cancer patients. Considering this information, the study of the RBCs in BC could contribute to a better understanding of the metastatic cascade as well as provide a new surrogated biomarker for liquid biopsy.

To overcome some of the current limitations for detecting and diagnosing metastasis, it has been proposed the use of liquid biopsy (LB) as a complementary technique[189,190]. LB allows for a non-invasive and real-time approach by studying various materials derived from primary and/or secondary tumours present in blood and other fluids[83]. LB studies mostly focus on ctDNA and CTC, which provide information about the heterogeneity and characteristics of the tumour better than traditional tissue biopsies, on occasions unavailable[82]. Despite their advantages, the use of these materials has also certain limitations, since both ctDNA and CTCs presence is very scarce, making their isolation/purification challenging, especially in early disease and recurrence detection[81,82]. In contrast RBCs can be an interesting alternative due to their abundance in blood and easy isolation[154]. Although generally discarded and considered as mere bystanders, recent studies are showing that they can reflect the presence of the disease through changes in their RNA and protein content, similar to other cells from the tumour microenvironment (TME) such as neutrophils or

platelets[154,188]. Considering this, the use of RBCs as surrogated biomarkers could help in the diagnosis of metastatic BC and potentially, in early detection.

Previous data from a mass spectrometry analysis of RBCs showed that the proteomic profile of RBCs from BC patients is different than cancer-free controls (CFC) [154]. Interestingly, a set of proteins was up-regulated in RBCs from metastatic (M1) patients, suggesting that they could be used as biomarkers to detect the presence of advanced cancer stages. In line with this, the analysis of the blood parameters of BC patients showed that M1 patients had lower haematocrit and haemoglobin concentration compared to non-metastatic (M0) or CFC, indicating that RBCs are modified due the systemic disease. This was expected as many advanced stage BC patients experiment anaemia due to an increased loss of RBCs and a decreased erythropoiesis[125,152,191]. On one hand, the accumulation of pro-inflammatory cytokines in the blood leads to oxidative damage in RBCs and Phospatidil serine (PS) exposure, which increases RBC clearance through erythrophagocytosis of damaged cells[192]. On the other hand, this same accumulation of pro-inflammatory signals increase the expression of the iron master regulator Hepcidin, leading to a blockade of intestinal iron absorption as well as iron retention, resulting in iron-restricted erythropoiesis[152]. Overall, the accumulation of these cytokines contributes to disruptions in both RBCs function and iron homeostasis in the body. Another parameter found altered in the blood analysis was the RDW. This parameter, which reflects the variability in cell size and volume in the RBCs fraction, is higher in M1 patients when compared to M0 and CFC [154]. Similar results were found in other publications studying lung, breast or colorectal cancers[146,193,194]. Besides, high RDW was linked with worse outcome in cancer patients although there is some controversy in this regard[145]. Recent publications have shown that during RBCs´ lifespan, RBC's aging leads to a volume reduction around 30% and haemoglobin mass decreasing by 20 % [131]. Similarly, oxidative stress suffered by RBCs in cancer can lead to an accelerated aging of the RBCs, consequently increasing the amount of smaller aged RBCs[131,195]. These alterations in RBCs populations can be responsible for the increase in the RDW observed in our BC cohort as well as in other studies[131,147,192]. Importantly, some of these blood parameters could be altered in elder people. To prevent any bias, patients and controls recruited for our study had similar age. Interestingly, only RBCs-related blood parameters are altered in these patients.

Based on the identified differential proteins in M1 RBCs from BC patients, ELISA assays were conducted to assess their potential as predictors of the presence of metastasis through a technique more transferable to the clinic. ELISA results showed that both LAMP2 and PNP expression was increased in M1 RBCs, and was RBC specific. PNP is an important enzyme involved in the purine salvage pathway that is highly expressed in erythroid precursors and moderately expressed in RBCs[196,197]. The observed increase in PNP expression could be attributed to an increase in circulation of committed-erythroid precursors that have been associated with bone marrow dysfunction and extramedullary haematopoiesis[198]. Interestingly, previous mass data analysis of M1 RBCs showed that PNP levels were lower in M1 patients with bone metastasis. Analysis of the data obtained from ELISA showed a similar trend, however, PNP expression in only bone metastatic patients was not significantly higher than in CFC or M0 RBCs. Adding to this, low PNP expression is associated with shorter PFS in M1, further limiting its use as a prognostic biomarker. Regarding LAMP2, is a protein primarily located in late endosomes and lysosomes that have been associated with chaperone-mediated autophagy, selective degradation of cytosolic proteins and loading of proteins into exosomes[199,200]. It is expressed in many of the hematopoietic cells including RBCs and erythroid progenitors. Importantly, LAMP2 is highly expressed in the last ones and

plays an important role during enucleation and organelle clearance throughout RBCs differentiation[201]. After enucleation, RBCs retain large quantities of LAMP2 in their membrane that will decrease overtime in mature RBCs, where it is still expressed in low amounts[201]. Considering this information, the increase of LAMP2 in M1 RBCs could be attributed to an increase in recently matured RBCs in blood[201]. Interestingly, higher expression of PNP and LAMP2 could suggest that differences observed at proteomic level could be attributed to an increase in younger RBCs[198,201]. However, proteomic study of the smaller RBCs in M1 patients (older/damaged RBCs) presented the same altered pathways found when comparing M1 and CFC RBCs[154], so it remains unclear if these alterations are derived from the abnormal erythropoiesis or acquired during the RBCs lifetime in blood. Focusing on the predictive value of LAMP2, previous analysis of proteomic data from M1 RBCs showed that patients with high levels of LAMP2 in their RBCs associated with worse PFS and OS[154], however this association was not observed in the ELISA validation cohort, likely due to the low number of patients in the M1 group (n=13). Despite this ROC analysis of the predictive value of LAMP2 to detect metastasis revealed an acceptable level of predictiveness that was even higher when LAMP2 was combined with other altered blood parameters such as Haematocrit and RDW, reaching an accuracy of 83.3 %. Currently, the clinicians do not have LB tools to detect the metastasis and standard imaging techniques are often performed only when patients exhibit symptoms derived from the advanced disease[202]. Other researchers have proposed different signatures based on the study of circulating material, such as miRNA, EVs and proteins, with an accuracy for predicting metastasis ranging from 79-90%[203,204]. Implementation of these signatures is difficult due to the high cost and low accessibility of molecular techniques. On the contrary, combination of LAMP2, RDW and Haematocrit would require only the results of blood analysis, which are routinely performed in the clinic, along with the measurement of LAMP2 in RBCs isolated with a simple centrifugation and analysed by ELISA. This procedure would be similar to the detection of serum biomarker CA 15.3, commonly used in the clinics for metastatic BC patients monitoring. Although the use of CA 15.3 for predicting metastasis has been studied, its accuracy is below 62 %[205]. Altogether, these evidences suggest that the proposed signature is easily transferable to the clinic and provides better predictive value than other potential candidates. Despite promising results, it should be taken into account that all M1 patients included in this analysis were already diagnosed with metastatic disease using imaging techniques. It is necessary to study if this combination of markers can detect the presence of metastasis even before the traditional imaging techniques as could be a great tool for patient´s follow-up.

In our reported data we also described that the highlighted pathways deregulated in the metastatic BC RBCs were mainly regulation of amino acid metabolism or extracellular exosomes[154]. Taking this into account we were intrigued by the potential involvement of RBCs in the crosstalk between the tumour cells and the tumour microenvironment and to which extend RBCs may contribute to various metastatic steps. Also, after isolating CTCs for *ex vivo* culture, as mentioned above, RBCs were unspecific isolated. Interestingly, close observation of these cells under the microscope revealed that RBCs were in direct contact with the CTCs and they even form networks among them and containing CTCs[156]. *In vitro* study of this phenomena showed that RBCs from BC patients, especially M1, adhered more to cancer cells when compared to CFC RBCs. These observations point out that breast cancer not only modifies RBCs at proteomic level but also functionally. Despite scant information, it was reported that the adhesion between tumour cells and RBCs could be potentially mediated

by *GAL-4*, as it was described by Helwa *et al*. [157]. Another possible mechanism can be the interaction between RBCs PS and the receptor CD36, expressed in many cancer cells, including the cell line MDA-MB-231 used in this experiment or CTCs after culture as described by our group[156]. PS is externalized by a small subset of old or damaged RBCs, however in systemic diseases, such as metastatic cancer, accumulative damage mediated by the inflammation increases the amount of damaged RBCs exposing PS. Interestingly, this mechanism would explain the higher adhesion of M1 RBCs in comparison to M0 or CFC, since M1 patients have a higher number of damaged RBCs exposing PS in circulation[153,206,207]. Moreover, exposure of CFC RBCs to conditioned media from MDA-MB-231 (capable of inducing oxidative stress and PS exposure in the RBCs) increased adhesion of RBCs to cancer cells *in vitro* (data not shown).

To further characterise the role of BC cancer RBCs in the metastatic cascade, a set of experiments was performed to study the different stages of this process. From the primary tumour (proliferation, cytokine production, angiogenesis and migration) to the journey through the blood and establishing secondary metastasis (cell aggregation, cell adhesion to extracellular matrix and endothelial cells). We found that proliferation was not affected by RBC lysates no matter their provenance. This results contrast with the research performed by Yin *et al*. that demonstrated that naïve RBCs (comparable to CFC RBCs) were able to increase the proliferation of 4T1 murine BC cell line both *in vitro* and *in vivo*[160]. In this work it is hypothesized that the proliferative effect of the RBCs is mediated by the Hb contained in them[160]. Interestingly, the use of Hb instead of RBCs for *in vitro* proliferation promote the proliferation of 4T1[160]. Despite the differences with our results, it should be taken into account that the effect observed could be dependent on the cell line used and they are not considering the variability of using real human samples from cancer patients.

Results of cytokine production showed that M1 RBCs induced the release of CCL family cytokines (CCL2 and CCL5), which are inflammatory cytokines involved in the recruitment of a wide variety of immune cells such as monocytes, dendritic cells or granulocytes, and the polarization of macrophages to M2[26,30,208]. Interestingly, the previously mentioned work of Yin *et al.* also observed an increase in CCL2 in tumours that were injected with RBCs[160]. In our results, CFC RBCs did not produce any increase in CCL2 production; however, it should be considered that our *in vitro* approach does not take into account other cells present in the tumour microenvironment. Interestingly, CFC RBCs induced expression of CXCL1, IL-6 and IL-8 in the luminal BC cell line MCF7. These cytokines are related to EMT regulation, increased metastatic potential and immunosuppression[209–211]. Altogether, this assay demonstrated that RBCs priming is capable to influence the cytokine expression in different tumour cell lines. These results could be partially explained by the ability of RBCs to carry and retain cytokines using DARC receptors[138,159]. Patients with higher inflammation and circulating cytokines will present higher concentration of different cytokines bound to DARC[159]. It is also described in the bibliography that the union between DARC and cytokines is reversible, allowing this receptor to capture or release the cytokines as their concentration in blood varies [138,139]. Importantly, CFC RBCs were also able to induced expression of different cytokines. It should be taken into account that some of these controls suffer other aged related inflammatory disease such as arthritis, psoriasis, rheumatism or diabetes. Thus, in some cases, control RBCs could have an elevated number of cytokines bound to DARC due to the presence of non-cancer related diseases. Considering the results, it would be interesting to assess the cytokine expression directly on RBCs lysates to determine which

cytokines are more prominent in breast cancer patients. Also, it should be interesting to include for study inflammatory non-cancer cohorts.

RBC lysates from M1 patients produced a significant increase in angiogenesis when compared to CFC. The pro-angiogenic effect of the M1 RBCs, could be potentially explained by the aforementioned DARC receptors. Karsten *et al*. demonstrated that naïve RBCs that were exposed to the lung cancer cell line A549 sequestered high amounts of bFGF and VEGF, two well-known pro-angiogenic factors produced by this specific cell line[159]. Similarly, M1 RBCs from the BC patients could be uptaking pro-angiogenic factors produced by cancer cell as well as the tumour microenvironment to promote angiogenesis. Importantly, CFC RBCs do not increase angiogenesis, even when compared to the negative control which had the same conditions without RBCs. Regarding this observation, CFC RBCs could be uptaking cytokines present in the blood due to non-cancer-related diseases however proangiogenic cytokines seem to be specific to M1 patients. This could be of great value as a screening tool to identify suitable candidates for pro-angiogenic therapy in metastatic breast cancer patients from RBC analysis.

Continuing with the metastatic steps, transwell migration assays showed an increased migration of MDA-MB-231 after priming with M1 RBCs, even after extended co-culture time. However, wound healing assay results indicate a reduction in wound closure when MDA-MB-231 were primed with RBCs, independently of their provenance. Importantly, no differences in wound closure or transwell migration were observed for MCF7. It should be taken into account that MDA-MB-231 is a triple negative cell line with a more aggressive phenotype than the luminal BC cell line MCF7, which is not invasive[212]. These results could imply that RBCs effects are dependent on tumour cell subtype. Regarding MDA-MB-231, results from both assays seem to provide contradictory information about the effects of RBCs on migration. However, there are important differences between the technical aspects of each assay. Wound healing assays evaluates migration in a two-dimensional surface. Generation of the scratch is performed manually affecting to the reproducibility of this assay[213]. Indeed, a high dispersion was observed in the wound healing assays performed in this work. Moreover, some authors consider this technique may be unsuited for studying migration since the scratch causes alterations in the behaviour of the cells along the wound edge that would not be present in non-injured tissues[214]. Transwell migration assays evaluate the ability of tumour cells located in the upper chamber to actively migrate through a membrane with pores of small size (around 5 µm) using a gradient of FBS or a chemoattractant[213]. The presence of a stronger stimulus such as a gradient of FBS as well as the force of gravity (since migration is happening in vertical) are also technical differences that could be affecting. To elucidate the discrepancy between both techniques results, migration was assessed also *in vivo* using a Zefrafish model. Interestingly, data from these assays showed that MDA-MB-231 that were co-cultured with M1 RBCs migrated more to the caudal region of the Zebrafish embryo, supporting the results obtained using transwell assays. Altogether, these results suggest that M1 RBCs modify the breast cancer cell line MDA-MB-231 migratory and metastatic potential. To further characterise M1 RBCs effect, chemotaxis assays with fresh M1 RBCs were performed using similar conditions as the transwell migration assays. Interestingly, M1 RBCs located in the lower well were able to increase the migration of MDA-MB-231, both in presence of an FBS as well as a plasma gradient. Moreover, a morphological change was observed when M1 RBCs were used as chemoattractant. Under these conditions there was an increased number of cells with a rounded shape and spread cytoplasm that contrasted with the usual stretched and elongated shape of MDA-MB-231. Rounded cells presented migratory

phenotype with membrane protrusions that resemble lamellipodia, key membrane structures involved in chemo-sensing and migration[215–217]. Tumour cells migrate searching for new sites to establish, in their journey through the blood, CTCs can be presented as single cells or forming clusters, being these last ones associated with worse prognosis[64,218]. Clusterization assay showed that both RBCs M1 and conditioned CFC RBCs increased significantly cluster formation, meanwhile CFC did not. Interestingly, exposure of CFC RBCs to conditioned media modified these RBCs in a way that they behave similarly to those coming from M1 patients. Although previous results in this study showed increased adhesion of RBC to tumour cells, in this assay the presence of attached RBCs was not significant. However, it should be taken into consideration that the fixation used in this protocol is especially aggressive with RBCs, generally leading to lysis. Further optimization should be performed to elucidate the role of RBCs in clusterization. Also, it would be important to include platelets in this assay since they are known to attach both RBCs and tumour cells[118].

Finally, adhesion to endothelial cells and collagen I was analysed to study the role of RBCs in the extravasation, one of the last steps of the metastatic cascade. Adhesion to endothelial cells is essential for transendothelial migration, both in active migration, known as diapedesis, and passive migration, referred as angiopellosis, more common in CTCs cluster with an epithelial phenotype[26,68]. Similarly, adhesion to collagen I, the most common collagen type in the body and part of the perivascular ECM, is also relevant during the extravasation[219,220]. The results showed that M1 RBCs increased significantly the adhesion capability of tumour cells to HUVEC cells and collagen I.

Data from functional assays confirmed that RBCs had an impact on tumour cells, especially when they come from M1 patients, suggesting that they may play a role in the metastatic cascade. Besides, the RNA-sequencing analysis demonstrate that RBCs priming can modify tumour cell gene expression. Due to fatty acids can boost cancer cell metabolism[221], a liposome control was added. Importantly, hierarchical clustering analysis showed that RNA profile of MDA-MB-231 exposed to liposomes segregated from cells co-cultured with RBCs and it was similar to negative control conditions. Focusing on M1 RBCs effects on MDA-MB-231, gene ontology analysis of the differentially expressed genes identified cell-cell adhesion, mitochondria metabolism, cell adhesion, stress fiber and contractile actin filament assembly as the main biological process altered in MDA-MB-231. Interestingly, these results go in line with the observations from the functional assays, as genes involved in the regulation of migration and adhesion are affected. qRT-PCR analysis of the selected differentially expressed genes showed that *HMOX1*, *EPHX1* and *PAK4* were mainly overexpressed in MDA-MB-231 co-cultured with M1 RBCs when compared to CFC. Despite no differences were detected in functional assays due to tumour subtype, RBCs employed for the M1 group included an equal representation of those RBCs derived from luminal and TN BC patients. Importantly, no differences attributed to subtype were detected. *HMOX1* is an inducible intracellular enzyme involved in the degradation of the heme group that can be induced by oxidative stress, cytokines, prostaglandins or the presence of heme group. It is overexpressed in many cancers including BC both in malignant cells as well as in immune cells present in primary tumours[222]. Catabolites generated by *HMOX1* activity provide antioxidant, cytoprotective and anti-apoptotic effects to cancer cells[223]. Moreover, these molecules also modulate the microenvironment, promoting immunosuppression and anti-inflammatory response as well as tumour progression by facilitating angiogenesis and metastasis[222,224]. Interestingly, due to its involvement in the regulation of the immune system, *HMOX1* has been proposed as a potential target for immune-therapy in combination with

chemotherapy[225]. Despite the compelling evidence, the role of this protein remains controversial, as other works suggest an anti-metastatic effect of *HMOX1* by reducing tumour growth and inhibiting the expression of *MMP9*[226,227]. Regarding *EPHX1*, is an important xenobiotic catalytic enzyme that normally plays a role in the detoxification of potential carcinogens, converting them in less toxic metabolites. Certain isoforms of *EPHX1* have been associated with higher risk to develop cancer and to suffer toxicity from cisplatin derived therapies. Moreover, higher expression of *EPHX1* in human tissue associates with intrinsic drug resistance in BC[228,229]. The *PAK4* oncogene is overexpressed in a large number of cancers types, including BC, and its high expression has been associated with worse prognosis, being indicative of relapse and lower overall survival in BC patients[230–233]. Importantly, PAK4 is involved in many signalling pathways including proliferation, cytoskeletal organization, cell motility and EMT[234]. In agreement, EMT-related genes increased after co-culture with M1 RBCs. Among them, *Plastin 3* (*PLS3*) is an actin bundle protein involved in migration and invasion[235]. It has been described as a potential biomarker for the detection of breast and colorectal cancer CTCs, especially those with mesenchymal phenotype[236]. Overexpression of *PLS3* is associated with increased metastatic potential, EMT and stemness[237]. *VIM* is an important element of the intermediate filament protein family expressed in normal mesenchymal cells. Expression of vimentin in epithelial cancers plays a major role in the EMT and it has been linked to increased migratory and invasive potential. Interestingly, Winter *et al*. demonstrated that overexpression of *VIM* in triple-negative MDA-MB-231 cell line increased invasion and sphere formation. *STAT1* is a transcription factor that can be upregulated by *PAK4* overexpression[238]. Notably, Greenwood *et al.* described that in triple-negative breast tumours, high expression of *STAT1* correlated with increased lymph node metastasis[239]. Moreover, *in vitro* studies showed increased migration and invasion of MDA-MB-231 overexpressing *STAT1* after IFN-γ stimulation[239]. Altogether, the analysis of MDA-MB-231 expression after co-culture with M1 RBCs reveals significant alterations in genes linked to EMT, migration, and cytoskeletal regulation. These results are in line with the functional assays data, suggesting that M1 RBCs are increasing the aggressiveness of MDA-MB-231. Among the differentially expressed genes, *PAK4* stands out due to being an upstream regulator of EMT and migration. Moreover, expression of *PAK4* in MDA-MB-231 has been described as a mediator of the aggressiveness and metastatic potential of this cell line both *in vivo* and *in vitro*[240]. In this work, the use of PAK4 inhibitor reversed the effects mediated by M1 RBCs suggesting an involvement of this kinase in the changes observed both in phenotype and behaviour of MDA-MB-231. Nonetheless, more studies need to be conducted to assess PAK4 phosphorylation status after stimulation with RBCs.

Results from this research demonstrate that RBCs from M1 patients are modified by the presence of the disease and suggest that they are able to further promote the metastasis. RBCs effects on cancer cells could be potentially mediated by different factors released from the RBCs, such as cytokines or miRNA. In fact, as previously mentioned, RBCs express DARC receptors that allow them to capture a wide range of cytokines released from the tumour cell or the TME[138]. Also, recent research have been demonstrated that RBCs can express endosomal TLR9 and potentially, TLR8, allowing RBCs to capture circulating nucleic acids, including miRNA[117,137,241]. Moreover, RBCs themselves contain high amounts of miRNA and are major contributors to the miRNA circulating in blood[242,243]. Considering this information, defining the cytokine and miRNA profile of M1 RBCs and CFC would be of great importance to find the mediators of the effects observed and, potentially, find new targets for therapy. Besides this, it is also important to define the mechanism that leads these molecules in and out

of the RBCs. Due to their highly specialized phenotype and absence of organelles, RBCs were thought to be unable to perform vesicle transport[129]. However, over the last years, researchers have demonstrated that mature RBCs have endosomal compartment and retain the ability to perform endocytosis as well as to generate exosomes and micro vesicles, being major contributors to the EVs present in blood[129]. These RBC-derived EVs contain primarily lipids, proteins, such as haemoglobin, transporters or membrane associated proteins, and nucleic acids, including miRNA[129]. Interestingly, as previously mentioned, a protein involved in the regulation of exosome biogenesis, LAMP2, has been found elevated in RBCs from M1 patients[154,199]. Based on the results obtained in chemotaxis assays that demonstrated the ability of RBCs to act paracrine, further studies were performed to determine the role of LAMP2 in the vesicle production and intercellular communication in RBCs. Interestingly, knocking out LAMP2 in HUDEP-2 cells decreased the production of vesicles as well as reduced their size. Moreover, HUDEP-2 LAMP2 KO cells, which were preconditioned by exposing them to metastatic BC patient plasma, did not act as chemoattractant, in contrast with WT HUDEP-2 cells. Both of these results go in line with the bibliography and suggest that LAMP2 could be involved in the production and release of vesicles in the RBCs[199,200,244,245]. It is not clear whether it plays a role in the internalization of factors present in the media or not. However, the initial proof indicates that HUDEP-2 WT were able to be conditioned by patient´s plasma. Moreover, as reported by Thorn *et al*, RBCs are capable of capturing and releasing amino acids, which could be used to further study and define the role of LAMP2 in the internalization of extracellular molecules[246]. Despite the compelling evidence, certain limitations in these studies should be considered. The selected cell line for RBC modelling, HUDEP-2, has a very low percentage of enucleation. Inability to enucleate triggers apoptosis, limiting the stage of differentiation for functional assays to orthochromatic erythroblast (around day 8 of the differentiation)[173]. A potential alternative would be using primary CD34+ HSPC since they have high percentages of enucleation[173]. However, it should be taken into account that they have also a limited expansion potential *in vitro*, requiring the use of siRNA or CrisprCas9 editing for each isolation and experiment, increasing the cost and steps[247,248]. Another alternative would be the use of the immortalised cells line BEL-A that has increased levels of enucleation in comparison to HUDEP-2, however this levels are still lower that CD34+ HSPC[173].

In summary, this study highlights the importance of RBCs in BC, both as a potential cancer surrogated biomarker and as an interplayer in the metastatic cascade. Measurement of LAMP2 in RBCs could help in the diagnosis of metastasis in BC sooner, although further studies need to be developed to assess its ability to predict metastasis before the traditional imaging techniques. Complexity in the functions of RBCs is expanding and this work contributes to shed light on their role in the metastatic disease[118,119]. The results obtained provide evidence of M1 RBCs ability to induce angiogenesis, act as chemo-attractants and modify BC cell line MDA-MB-231 both at functional and expression levels. Importantly, alterations in RBCs are a reflection of a systemic disease, and their effects can be seen in other cancer types as well as in other inflammatory diseases [118,154,188,249,250]. Lastly, this study also showed the potential of CD34+ HSPC immortalised cell line HUDEP-2 for studying the role of proteins, such as LAMP2, in RBCs. Altogether, these results set the basis of a new line of research, providing further evidences that RBCs are not mere bystanders and they take part in systemic diseases such as cancer.

CONCLUSIONS

CONCLUSIONS

1. Blood test parameters such as RDW, Haematocrit or Haemoglobin, and protein expression of LAMP2 are altered in metastatic Breast Cancer. The signature including LAMP2_RDW_Haematocrit predicts metastasis with an accuracy of 83,3%, demonstrating the potential use of RBCs as biomarkers in cancer.

2. RBCs from metastatic Breast Cancer patients impact different stages of the metastatic cascade, including angiogenesis, migration, cluster formation and adhesion. The effects observed varied depending on the cell line used, having a great impact on the MDA-MB-231 cell line.

3. RBCs from cancer-free controls also exhibited certain effects on tumour cells even though these RBCs were not conditioned by the presence of the tumour.

4. MDA-MB-231 tumour cell line primed with RBCs from metastatic Breast Cancer patients have modified expression of genes mainly involved in cell adhesion and cytoskeletal dynamics. Thus, *PAK4, HMOX1* and *EPHX1*, as well as EMT-related genes such as *VIM, PLS3* and *STAT1* were overexpressed in MDA-MB-231 after co-culture with RBCs from metastatic BC patients, seen by qRT-PCR analysis.

5. RBCs from metastatic breast cancer patients were also chemoattractant for MDA-MB-231, producing a morphological change in migrated cells characterized by the formation of membrane protrusions similar to lamellipodia.

6. The use of PAK4 inhibitors can restore migration and adhesion of MDA-MB-231 cells primed with RBCs to basal levels. Due to its role as an upstream regulator of EMT, migration and adhesion, *PAK4* could be mediating the effects observed in MDA-MB-231 after co-culture with RBCs from metastatic BC patients.

7. HUDEP-2 cell line proved to be a good model for studying the role of proteins in RBCs, allowing to obtain differentiated RBCs with edited expression of the protein of interest that can be studied at genetic, proteomic and functional levels.

8. Knocking out LAMP2 on HUDEP-2 cells impairs vesicle production and migration of primed tumour cells, suggesting that RBC impact on tumour cells could be mediated by extracellular vesicles crosstalk.

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SUPPLEMENTARY MATERIAL

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Table S1. Inclusión and exclusion criteria employed in the collection of blood samples from breast cancer patients

Inclusion Criteria	Exclusion criteria
Breast cancer diagnosis of any subtype and stage with indication to start 1st line of treatment palliative systemic, endocrine, cytotoxic and/or with biologicals.	Known diagnosis of psychiatric illness that contraindicates treatment antineoplastic or prevent the patient from understanding and accepting the conditions of the study.
Disease-free interval ≥ 12 months	Life expectancy < 3 months.
Age ≥ 18 years	Radiotherapy on followable lesion (if less than 6 months)
Dated and signed informed consent	Systemic prior treatment for advanced breast cancer
Traceable disease (measurable or not)	Current malignant tumor or in the last 5 years, except cutaneous cell carcinoma basal or squamous cell or carcinoma *in situ* of the cervix appropriately treated
Ability and willingness to follow the study plan	

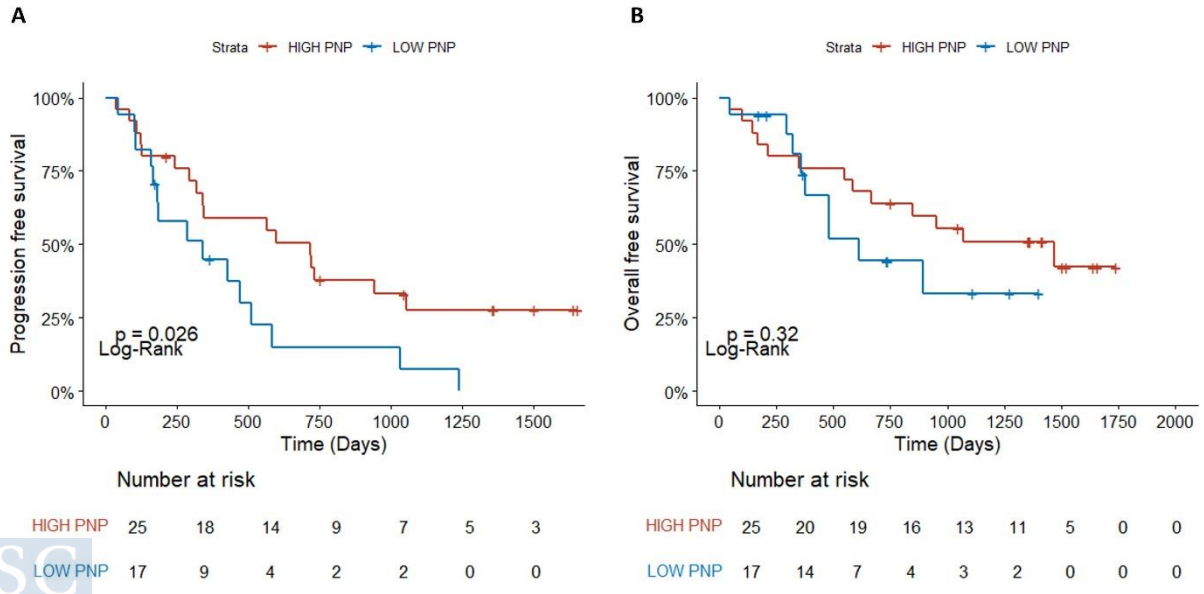

Figure S1. RBCs PNP levels from metastatic breast cancer patients associated with Progression-free survival. Kaplan-Meier plot for A) PFS (p =0.026) and B) OS (p> 0.05), in stage IV BC patients (n=42). Statistics were performed by log-rank test.

Table S2. Contingency table containing the data from RBC-tumor cell adhesion assay. YES/NO indicates the presence/absence of RBC-tumor cell adhesion. The number of BC patients andcontrols are depicted for each group. Fisher test was performed for comparisons between M1 and M0 or CFC for MDA-MB-231 and MCF7 cell lines.

MDA-MB-231						MCF7					
Group	NO	YES	% Patients with adhesion	*p value*	N		NO	YES	% adhesion	*p value*	N
CFC	8	2	11.8		10	CFC	9	1	6.2		10
M0	9	4	23.5	0.006909	13	M0	5	8	50	0.005923	13
M1	3	11	64.7		14	M1	2	7	43.8		9

Table S3. Fold change of MDA-MB-231 cytokine spots for supernatants from M1 and CFC. Fold change was calculated using a ratio of the densitometry measurements of negative control and M1/CFC foresights.

MDA-MB-231 Cytokine fold change							
M1				CFC			
CCL2/MCP-1	2,94 2,88	ICAM-1	1,30 1,17	CCL2/MCP-1	0,83 0,81	ICAM-1	1,23 1,09
CXCL1	1,06 1,12	IL6	1,04 1,11	CXCL1	1,30 1,19	IL6	1,01 1,10
CXCL10	1,06 1,15	IL8	1,09 1,12	CXCL10	1,18 1,12	IL8	1,09 1,07
G-CSF	0,96 0,92	MIF	0,94 1,03	G-CSF	1,14 1,10	MIF	0,93 1,05
CCL5	0,90 0,93	SERPIN R1/PAI-1	0,98 1,01	CCL5	1,09 1,05	SERPIN R1/PAI-1	0,90 1,06

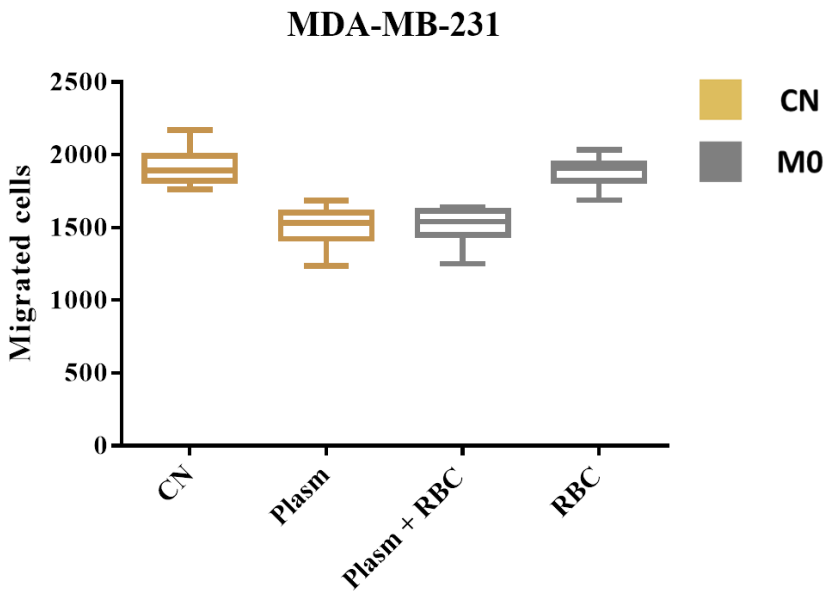

Figure S2Transweel m migration of MDA-MB-231 when using RBCs from non-metastatic breast cancer (M0, n=5) (Dark grey) patients as chemo-attractants. Negative controls (CN, n=5) (Golden) were included to compare. Mann-Whitney test. P-value < 0.05 (*).

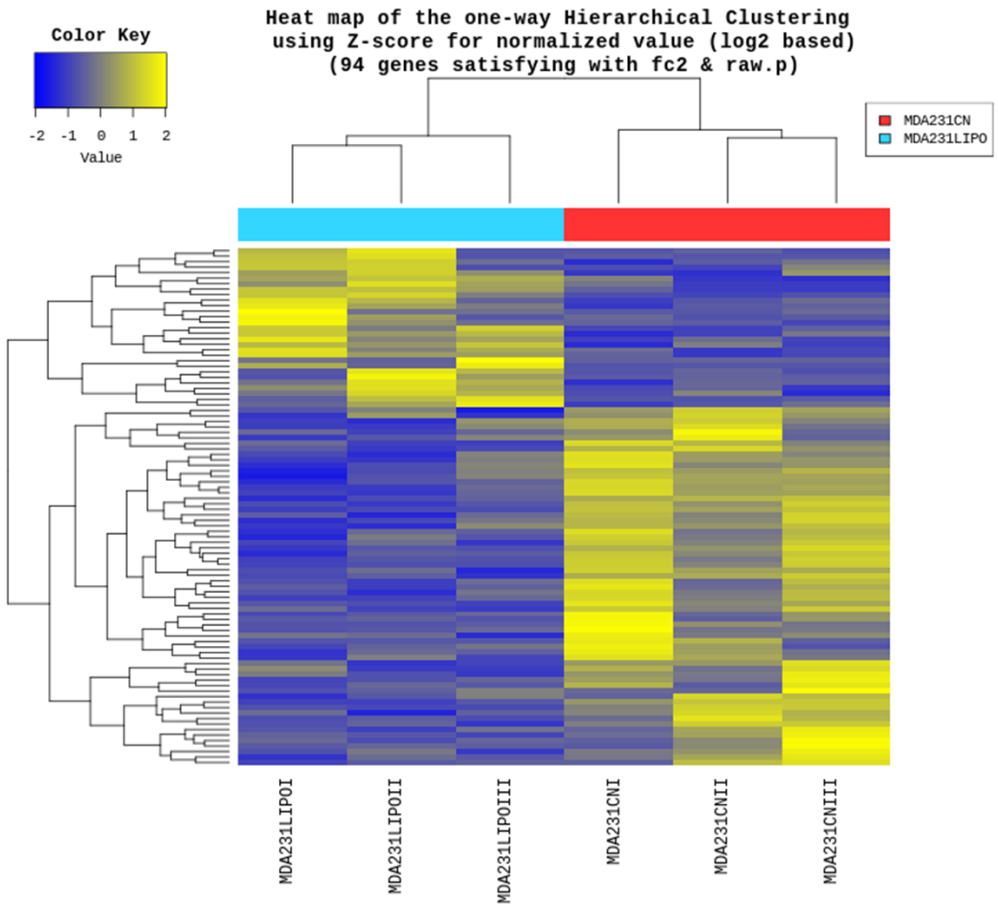

Figure S3. Heatmap showing results of the hierarchical clustering analysis (Euclidean Method, Complete Linkage) which clusters the similarity of genes and samples by expression level (normalized value). Negative control (CN) is represented in red, MDA-MB-231 co-cultured with Liposomes (LIPO) is represented in light blue.

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Y para terminar, igual que empecé esta tesis, gracias a ti Miriam. Por las horas en el menos 80 buscando eppendorf y dejándonos los dedos, por los días que estábamos mano a mano cuando venían todas las muestras a la vez, por los días malos en los que te preocupabas por mí, por las risas y las bromas, por los viajes y por haberte conocido.

Gracias a todos, pues este tesis no es mia, sino nuestra.

ANNEXES

Favorable reports from the clinical research ethics comitte of Galicia

XUNTA DE GALICIA
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DICTAMEN DEL COMITÉ AUTONÓMICO DE ÉTICA DE LA INVESTIGACIÓN DE GALICIA

Paula M. López Vázquez, Secretaria del Comité Autonómico de Ética de la Investigación de Galicia

CERTIFICA:

Que este Comité evaluó en su reunión del día 22/12/15 :

Título: Biopsia líquida para oncología de precisión
Promotor: Rafael López López
Tipo de estudio:EPA-SP
Version: versión 30 de noviembre de 2015 y HIP/CI (cultivo, control biopsia, y biopsia líquida) de la misma fecha
Código del Promotor:RLL-BL-2015_01
Código de Registro: 2015/772

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables, y en particular la Ley 14/2007, de investigación biomédica, el Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humano, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica, la ORDEN SAS/3470/2009, de 16 de diciembre, por la que se publican las Directrices sobre estudios Posautorización de Tipo Observacional para medicamentos de uso humano, y la Circular nº 07 / 2004, investigaciones clínicas con productos sanitarios.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos de la Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del CEIC de Galicia

Emite un **INFORME FAVORABLE** para la realización del estudio por el/la investigador/a del centro:

Centros	Investigadores Principales
C.H. Universitario de Santiago	Clotilde Costa Nogueira

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Y HACE CONSTAR QUE:

1 El CAEIG cumple los requisitos legales vigentes (R.D 223/2004 por el que se regulan los ensayos clínicos con medicamentos, y la Ley 14/2007 de Investigación Biomédica).
2 El CAEIG tanto en su composición como en sus PNTs cumple las Normas de Buena Práctica Clínica (CPMP/ICH/135/95).
3 La composición actual del CAEIG es:

Manuel Portela Romero. (Presidente). Médico Especialista en Medicina Familiar y Comunitaria.
Irene Zarra Ferro. (Vicepresidenta). Farmacéutica de Atención Especializada.
Paula Mª López Vázquez, (Secretaria). Médico Especialista en Farmacología Clínica.
Juan Vázquez Lago (Secretario Suplente). Médico Especialista en Medicina Preventiva y Salud Pública.
Jesús Alberdi Sudupe. Médico especialista en Psiquiatría.
Rosendo Bugarín González. Médico Especialista en Medicina Familiar y Comunitaria.
Juan Casariego Rosón. Médico Especialista en Cardiología.
Xoán X. Casas Rodríguez. Médico Especialista en Medicina Familiar y Comunitaria.
Juana Mª Cruz del Río. Trabajadora Social.
Juan Fernando Cueva Bañuelos. Médico Especialista en Oncología Médica.
José Álvaro Fernández Rial. Médico Especialista en Medicina Interna.
José Luis Fernández Trisac. Médico Especialista en Pediatría.
Mª José Ferreira Díaz. Diplomada Universitaria de Enfermería
Pablo Nimo Ríos. Licenciado en Derecho. Miembro externo
Pilar Gayoso Diz. Médico Especialista en Medicina Familiar y Comunitaria.
Agustín Pía Morandeira. Farmacéutico de Atención Primaria
Salvador Pita Fernández. Médico Especialista en Medicina Familiar y Comunitaria.
Carmen Rodríguez-Tenreiro Sánchez. Licenciada en Farmacia.
Susana María Romero Yuste. Médico Especialista en Reumatología.
Mª Asunción Verdejo González. Médico Especialista en Farmacología Clínica.

En Santiago de Compostela, a 05 de enero de 2015

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Nombre de reconocimiento (DN): c=ES, o=XUNTA DE GALICIA, ou=certificado electrónico de empleado público, serialNumber=46900339G, sn=LOPEZ VAZQUEZ, givenName=PAULA MARIA, cn=LOPEZ VAZQUEZ PAULA MARIA - DNI 46900339G
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Favorable report from the clinical research with farmaceutics ethics comitte of Galicia

SERVIZO GALEGO DE SAÚDE

DICTAMEN DEL Comité de ética de la investigación con medicamentos de Galicia (CEIm-G)

2015/772

Paula M. López Vázquez, Secretaria del Comité de ética de la investigación con medicamentos de Galicia (CEIm-G)

CERTIFICA:

Que este Comité evaluó en su reunión del día 27/06/23 la modificación del estudio:

Título: Biopsia líquida para oncología de precisión
Versión modificación: protocolo Versión 5: 15 de Junio de 2023
Tipo de estudio: EPA-SP
Promotor: Rafael López López
Código del Promotor: RLL-BL-2015_04
Código de Registro: 2015/772

Y que este Comité acepta de conformidad con sus procedimientos normalizados de trabajo y tomando en cuenta los requisitos éticos, metodológicos y legales exigibles, que dicha enmienda sea incorporada al estudio de investigación.

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SERVIZO GALEGO DE SAÚDE

Y HACE CONSTAR QUE:

1 El comité cumple los requisitos legales vigentes aplicables a los Comités de ética de investigación.
2 El comité tanto en su composición como en sus PNTs cumple las Normas de Buena Práctica Clínica (CPMP/ICH/135/95).
3 La composición actual del comité es:

Susana María Romero Yuste (Presidenta). Médico Especialista en Reumatología.
Diego Santos García (Vicepresidente). Médico Especialista en Neurología.
Paula Mª López Vázquez (Secretaria). Médico Especialista en Farmacología Clínica.
Martina Lema Oreiro (Vicesecretaria). Farmacéutica de Hospital.
Rosendo Bugarín González. Médico Especialista en Medicina Familiar y Comunitaria.
Nuria Carballeda Feijóo. Miembro lego. Representante de los intereses de los pacientes.
Juana Mª Cruz del Río. Trabajadora Social.
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Agustín Pía Morandeira. Farmacéutico de Atención Primaria
Jorge Prado Casal. Licenciado en CC. Físicas. Experto en Protección de Datos.
Carmen Rodríguez-Tenreiro Sánchez. Licenciada en Farmacia.
Rafael Carlos Vidal Pérez. Médico Especialista en Cardiología.
Mª Asunción Verdejo González. Médico Especialista en Farmacología Clínica.
Irene Zarra Ferro. Farmacéutica de Hospital

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Figures Autorship and list of publications used in this thesis

Autorship disclosure:

All figures have been created by the author using biorender.com

List of publicactions

Red Blood Cells Protein Profile Is Modified in Breast Cancer Patients

Thais Pereira-Veiga , Susana Bravo, Antonio Gómez-Tato, **Celso Yáñez-Gómez**, Carmen Abuín , Vanesa Varela , Juan Cueva, Patricia Palacios, Ana B Dávila-Ibáñez, Roberto Piñeiro, Ana Vilar, María Del Pilar Chantada-Vázquez, Rafael López-López, Clotilde Costa

Mol Cell Proteomics . 2022 Dec;21(12):100435.

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Journal Citation Reports (JCR) category rank: Q1. Biochemical Research methods
IF:6.1 (2024)

Contribution to this work: I, Celso Yáñez Gómez, was involved in the methodology, validation, investigation, visualization, writing, review and editing of the manuscript.

Breast cancer is the most common cancer diagnosed in women around the world. Around 30% of the patients will eventually develop metastasis, which is responsible for 90 % of cancer-related deaths. In order to find new biomarkers and therapy targets for these patients, it is important to study the metastatic process and the cells that take part in it. This thesis focused on the study of red blood cells, one of the most overlooked interplayer in cancer and metastasis. The results shown that RBCs from metastatic breast cancer patients were capable of interacting, and more importantly, modifying breast cancer cells, increasing their aggressiveness. Moreover, this thesis provided evidence of the RBCs potential to be used as predictive biomarkers of the presence of metastasis.