NOVEL MECHANISMS IN ANGIOGENESIS: OXIDATIVE STRESS AS A PROMOTER OF MONOCYTE-TO-ENDOTHELIAL CELL DIFFERENTIATION AND ENDOTHELIAL CELL ACTIVATION

FILIPA LOPES COELHO

Tese para obtenção do grau de Doutor em Mecanismos de Doença e Medicina Regenerativa

Doutoramento em associação entre:

Universidade NOVA de Lisboa (Faculdade de Ciências Médicas | NOVA Medical School - FCM|NMS/UNL)

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The experimental data presented in this thesis are part of manuscripts previously published or in preparation for publishing. I hereby state that I have fully participated in the conception, execution and in the validation of the experimental work, interpretation of the collected data and in the manuscripts writing.

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Abstract

Cancer is one of the most burden diseases worldwide and every year millions of people around the world are diagnosed and eventually die from it. In the past 10 years, an increment in the efficacy of cancer treatment reduced the overall mortality from cancer. However, the metastatic disease and resistance to treatments are still largely responsible for cancer patients mortality and morbidity. Advances in cancer research have shed a light on the biology of cancer, pointing out that it cannot be studied only from a cancer cell point of view but the involvement of other non-cancerous cells during tumorigenesis should also be considered. For example, the contribution of endothelial cells (ECs) underlying tumor angiogenesis has revealed to be pivotal for cancer cell survival, tumor growth and metastatic spread.

The formation of tumor neovessels, supporting the delivery of oxygen and nutrients to the tumor is prompted by the generation of a pro-angiogenic and a pro-oxidant tumor microenvironment (TME). However, contrarily to normal vessels, the tumor vasculature is composed by a fragile and permeable vascular network, that enables cancer cell intravasation, tumor metastasis and compromises the delivery of chemotherapeutic agents and consequently their efficacy. For the past years, the development of strategies using anti-angiogenic drugs has tried to hinder tumor growth by disrupting the blood supply to tumors. However, recent evidence shows that instead of destroying tumor neovessels, the restoration of its normalization would increase the penetration of therapeutic agents to the tumor, improving their efficacy. Those evidence allied to the fact that the clinical results of anti-angiogenic agents have been disappointing, indicates that the mechanisms underlying tumor angiogenesis remain to be fully understood. For instance, the contribution of endothelial progenitor cells (EPCs) to tumor angiogenesis may be undervalued in part because, so far, the absence of specific markers to characterize EPCs may lead to the underestimated capacity of other cell subtypes in acting as EPCs.

As mentioned above, the pro-oxidant TME is known as an active player in the activation of the angiogenic switch. Furthermore, the role of reactive oxygen species (ROS)-dependent mechanisms, such as ferroptosis, recently described as a promoter of cellular phenomena beyond cell death; on the regulation of angiogenesis has not been explored so far.

Propranolol, a β-blocker, has shown efficacy in the treatment of vascular tumors, as hemangiomas and cavernomas although, the precise mechanism(s) by which it interferes with angiogenesis remains to be clarified. Recently, Propranolol has been

pointed out has having anti-ferroptotic properties and its use correlates with better prognosis of cancer patients and fewer metastasis.

The present thesis proposed to clarify that monocytes can act as EPCs and that a ferroptosis-like mechanism sustains the EC activation and further angiogenic switch, being possibly reverted by the anti-angiogenic effect of Propranolol.

We disclosed that monocytes are EPCs that upon a pro-angiogenic stimuli, differentiate into ECs and incorporate blood vessels. In a cancer scenario, monocytes contribute to cancer progression, in part by promoting the formation of the tumor neovasculature. As mentioned, and since the generation of a pro-oxidant TME favors angiogenesis, herein we observed that ROS push monocytes to differentiate into ECs, which in turn favor tumor neovasculature formation. The potential of translation of these data into the clinical context can be supported by the effect of Propranolol administration in a child with cerebral cavernous malformation (CCM). In fact, Propranolol administration decreased the levels of circulating cells expressing monocytic and ECs markers ($CD14^+/CD31^+$), and VEGF levels in the peripheral blood, indicating that Propranolol impairs the recruitment of $CD14^+/CD31^+$ monocytes to the vascular lesion.

In addition to the already described relevance of ROS in the promotion of angiogenesis, we describe that ROS-induced lipid peroxides generation induced by Erastin, at a non-lethal level, promotes ECs proliferation and migration and accounts for vessel-like structures formation. The stimulation of these ECs features where accompanied by ferroptosis hallmarks, which includes increased ROS generation, glutathione (GSH) depletion, abrogation of cyst(e)ine import and lipid peroxides accumulation. These alterations were accompanied by the generation of a leakier ECs monolayer, presenting increased intercellular junctional gaps in VE-Cadherin (VE-Cad) adherens junctions that facilitates the transendothelial cancer cells migration. The increased levels of selectins (ICAM and VCAM) were also observed, promoting cancer cells adhesion to ECs. The antioxidant and anti-ferroptotic Propranolol properties, mediated by the generation of hydrogen sulfide (H_2S), reverted the Erastin-induced ECs hyperactivation. Interestingly, since we observed that the antioxidant properties of Propranolol mediated by H_2S generation are EC-specific, we unraveled a putative anti-tumor and anti-angiogenic strategy using the pro-oxidant dendrimeric nanoparticles loaded with selenium chrysin (SeChry@$PURE_{G4}$) in combination with Propranolol. This strategy allowed the increase in oxidative stress induced-cancer cells death, potentiated by the action of Propranolol, while its protective effect in ECs avoided the generation of oxidative stress, which in turn impairs the generation of a leakier vascular structure and cancer cells extravasation.

Together, our results indicate that monocytes are EPCs that contribute to tumor angiogenesis and that a ferroptosis-like mechanism promotes the hyperactivation of ECs and the angiogenic switch with leakier vessel-like structures, which recapitulates the formation of the unstable tumor neovasculature that favors cancer cell's dissemination and metastasis. Moreover, a new therapeutic strategy was proposed, joining the anti-angiogenic and anti-tumor effects of Propranolol and the pro-oxidant effect of SeChry@PURE$_{G4}$, tackling cancer cells and stabilizing tumor neovessels, in order to impair metastasis and improve drug delivery.

Keywords: monocytes, endothelial progenitor cells (EPCs), endothelial cells (ECs), tumor angiogenesis, cerebral cavernous malformation (CCM), reactive oxygen species (ROS), lipid peroxidation, Propranolol, selenium-containing chrysin encapsulated in generation four polyurea dendrimer (SeChry@PURE$_{G4}$)

Resumo

A ocorrência de neoplasias malignas é considerada um dos principais problemas de saúde pública ao nível mundial, sendo a segunda causa de morte em países desenvolvidos. Nos últimos 10 anos, os avanços na terapêutica utilizada no tratamento do cancro veio aumentar a taxa de sobrevivência dos doentes oncológicos. Infelizmente, a doença metastática é ainda a principal responsável pela mortalidade e morbilidade associadas à doença.

Em 2000, Hanahan e Weinberg publicaram a sistematização das principais características da transformação maligna em seis categorias: autossuficiência para a produção de sinais ativadores da proliferação celular, insensibilidade para fatores inibidores da proliferação, capacidade replicativa ilimitada, capacidade de estimulação da angiogénese, capacidade de bloquear mecanismos desencadeadores de processos de morte celular e capacidade de invasão dos tecidos e de metastização noutros órgãos. Uma década mais tarde, surgiram duas novas categorias que vieram a revelar-se essenciais para a progressão tumoral, a reprogramação metabólica das células tumorais e a capacidade de evasão à ação do sistema imunitário. Durante a progressão tumoral, a formação de novos vasos sanguíneos é essencial para a oxigenação e suplementação de nutrientes às células tumorais, que consequentemente impulsiona a sobrevivência das células malignas e o crescimento tumoral, bem como o processo de metastização. Estudos na área da angiogénese tumoral demonstraram que contrariamente ao que acontece com os vasos sanguíneos normais, os vasos tumorais são frágeis e permeáveis, o que por sua vez favorece a extravasão de células tumorais para a corrente sanguínea e posteriormente a metastização, além de comprometer a entrega dos agentes quimioterápicos. Nos últimos anos, o desenvolvimento de estratégias terapêuticas anti-angiogénicas tem vindo a revelar uma baixa eficácia no tratamento de doentes oncológicos, sugerindo que os mecanismos subjacentes à angiogénese tumoral ainda não são totalmente conhecidos. Para além disso, os novos estudos indicam que estratégias que normalizam a vasculatura tumoral, em vez de a destruir, facilitam a chegada dos fármacos ao tumor, aumentando assim a sua eficácia. É de salientar ainda que a contribuição de células progenitoras endoteliais (EPCs) para o sucesso da angiogénese tumoral tem sido desvalorizada, em parte devido à inexistência de marcadores específicos que leva à má caracterização dessas células, o que impede e identificação de subtipos celulares que atuam como EPCs.

Durante a tumorigénese, a remodelação metabólica das células tumorais promove a geração de um microambiente pró-oxidante que atua como um estímulo pro-angiogénico responsável pela ativação das células endoteliais (ECs), que se traduz na

passagem das ECs de um estado de quiescência para uma fase proliferativa. O microambiente tumoral pró-oxidante também afeta mecanismos que atuam nas células malignas, tais como a ferroptose que tem como mecanismo subjacente a formação de peróxidos lipídicos devido à acumulação de espécies-reativas de oxigénio (ROS). Embora este processo tenha sido inicialmente descrito como um mecanismo de morte celular dependente de ferro, atualmente já é assumido também como responsável pela regulação de processos biológicos e fisiopatológicos. Ao nível molecular a ferroptose pode ser ativada pela angiopoietina 4 (ANGPTL4), uma molécula a jusante da via TAZ-ANGPTL4-NOX2. Por sua vez, a ativação desta via induz ferroptose através da geração de radicais superóxidos. Além da sua relevância na promoção da ferroptose, a ANGPTL4 também é um conhecido estimulador da angiogénese, o que sugere uma conexão entre o a ferroptose e a angiogénese.

Nos últimos anos, o β-bloqueador não seletivo Propranolol que é frequentemente prescrito para o tratamento de doenças cardiovasculares, hipertensão e enxaquecas, foi adaptado para o tratamento de tumores vasculares, como hemangiomas e cavernomas. Ao nível molecular sabe-se que o Propranolol tem a capacidade de interferir com a angiogénese através da diminuição dos níveis do fator de crescimento vascular (VEGF) e do fator de crescimento de fibroblasto (FGF). Recentemente, o Propranolol foi descrito como inibidor da ANGPTL4 em células de hemangioma e como tendo a capacidade inibitória da ferroptose. Porém, atualmente ainda é desconhecido o mecanismo exato pelo qual o Propranolol atua nas ECs e afeta a angiogénese. Alguns estudos retrospetivos em doentes com carcinoma da mama demonstram que a administração de Propranolol para outros fins terapêuticos correlacionam-se com um melhor prognóstico da doença oncológica. Os efeitos anti-angiogénicos do Propranolol em tumores vasculares, aliado aos estudos retrospetivos em doentes oncológicos, levam-nos a acreditar que o benefício da aplicação do Propranolol no tratamento do cancro poderá estar relacionado com a sua interferência com a angiogénese.

Os avanços ao nível dos tratamentos na área da oncologia têm vindo a aumentar a sobrevida dos doentes, porém, o uso destas terapêuticas está associado a toxicidade aguda e crónica. As novas evidências demonstram que a utilização de terapêuticas dirigidas às células tumorais poderão aumentar a especificidade dos tratamentos, diminuindo a toxicidade em células normais. Com os avanços científicos, o desenvolvimento de estratégias focadas em promover simultaneamente a morte celular de células tumorais e a normalização da vasculatura permitirá um aumento da eficácia do tratamento e uma diminuição dos efeitos adversos.

Na presente tese, o principal objetivo é investigar se os monócitos podem atuar como EPCs e se o stress oxidativo promove a ativação das ECs através de um mecanismo

semelhante à ferroptose, que poderá ser revertido pela ação anti-angiogénica do Propranolol. Assim, para avaliar a capacidade dos monócitos em diferenciar em ECs utilizámos monócitos isolados a partir de sangue periférico de dadores de sangue saudáveis e para e o estudo do mecanismo subjacente à ação do Propranolol e da ferroptose utilizámos ECs isoladas da veia do cordão umbilical humano (HUVECs) que foram expostas a Erastina, um ativador da ferroptose, na ausência e na presença de Propranolol.

No 1º capítulo de resultados experimentais desta tese foi demonstrado, em vários contextos biológicos, que os monócitos expostos a um estímulo pro-angiogénico diferenciam em ECs e incorporam os vasos sanguíneos. A angiogénese tumoral foi um dos fenómenos explorados que provou a capacidade de incorporação dos monócitos nos vasos e comprovou-se também que as ROS é um estímulo que promove a diferenciação de monócitos em ECs.

No 2º capítulo de resultados foi verificado que o Propranolol inibe as propriedades angiogénicas das ECs, como a proliferação, migração e capacidade *in vitro* e *ex vivo* de formar estruturas que mimetizam vasos sanguíneos *in vivo*. Numa criança com cavernomas cerebrais, também é responsável pela diminuição dos níveis de células do sangue que apresentam em simultâneo marcadores de monócitos e de ECs ($CD14^+/CD31^+$) e os níveis de VEGF no soro de sangue periférico. Estes resultados sugerem que o Propranolol inibe a ativação de ECs e a mobilização de células $CD14^+/CD31^+$ que promovem a expansão dos cavernomas cerebrais.

No 3º capítulo foi demonstrado que para além do efeito conhecido das ROS na promoção da angiogénese, a ativação não letal da ferroptose pela geração de peróxidos lipídicos, induzidos pela exposição à Erastina, também contribui para a ativação de ECs, com promoção da proliferação, da migração e da formação de estruturas semelhantes a vasos. Durante este processo existe um aumento das ROS, depleção do glutatião (GSH), inibição da importação de cist(e)ína e a acumulação de peróxidos lipídicos, que contribuem para a ferroptose. Além disso, a exposição de ECs a Erastina promove um aumento de lacunas nas junções aderentes da caderina endotelial vascular (VE-Cad) e a adesão de células cancerígenas ao endotélio pelo aumento da expressão de selectinas (ICAM e VCAM), favorecendo também a migração transendotelial de células cancerígenas. A ativação da ferroptose em ECs mimetiza as características dos vasos tumorais, indicando que este mecanismo também pode promover a angiogénese tumoral. Ainda neste contexto, observou-se que as propriedades antioxidantes e anti-angiogénicas do Propranolol mediadas pela produção de sulfureto de hidrogénio (H_2S) revertem o efeito do mecanismo semelhante à ferroptose na ativação de ECs, reforçando a sua utilidade como fármaco anti-angiogénico. Por um lado, foi provado que

o Propranolol tem a capacidade de inibir a ativação de ECs e a formação de estruturas que mimetizam vasos, características estas potenciadas pelo mecanismo semelhante à ferroptose. Por outro lado, o Propranolol induz a normalização das estruturas vasculares já formadas, eventualmente favorecendo uma maior eficiência na distribuição de agentes quimioterápicos.

No 4º capítulo de resultados, foi explorada uma estratégia terapêutica utilizando as propriedades antioxidantes do Propranolol seletivas para ECs juntamente com a utilização de nanopartículas dendriméricas que encapsuladas com seleno-crisina (SeChry@PURE$_{G4}$) com propriedades oxidantes. Esta estratégia promoveu a geração de stress oxidativo e consequente morte das células cancerígenas, enquanto que nas ECs o efeito protetor do Propranolol reverteu a geração de ROS e a permeabilidade das estruturas endoteliais pela diminuição de lacunas nas junções aderentes de VE-cad.

De um modo geral este projeto permitiu expandir o conhecimento dos mecanismos envolvidos no controlo da angiogénese tumoral e desvendou a contribuição dos monócitos como EPCs, bem como a sua contribuição para formação da vasculatura tumoral. Além disso, foi reforçada a utilização do Propranolol como um fármaco anti-angiogénico e a sua combinação com SeChry@PURE$_{G4}$ no contexto de uma nova estratégia terapêutica duplamente dirigida às células malignas e às ECs.

Palavras-chave: monócitos, célula progenitora endotelial (EPC), célula endotelial (EC), angiogénese tumoral, cavernomas cerebrais, espécies reativas de oxigénio (ROS), peróxidos lipídicos, Propranolol, nano partículas dendriméricas de quarta geração encapsuladas com seleno-crisina (SeChry@PURE$_{G4}$)

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List of publications

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Lopes-Coelho F (speaker), Silva F, Cardoso BA, Serpa J. HNF1β, an oncogene-like protein, is stabilized by acetylation, preventing its ubiquitination and further degradation. 8th International Conference on Tumor Microenvironment: progression, therapy and prevention (June, 2018), Lisbon, Portugal.

List of abbreviations

27HC - 27-Hydroxycholesterol
acLDL - Acetylated low-density lipoprotein
ALDH – Aldehyde dehydrogenase
ALK - Activin receptor-like kinase
ANG – Angiopoetin
ANGPTL4 - Angiopoietin-like 4
AR - β-adrenergic receptors
ATM - Ataxia-telangiectasia mutated kinase
AzMC - 7-Azido-4-Methylcoumarin probe
BCAA - Branched chain amino acids
BSA - Bovine serum albumin
CAA – Cancer associated adipocyte
CAF - Cancer associated fibroblast
CBS - Cystathionine beta synthase
CFU - colony-forming unit medium
Cys – Cysteine
cEPC – Circulating endothelial progenitor cell
CCM – Cerebral cavernoma malformation
CCR2 - C-C chemokine receptor type 2
CECR1 - Adenosine deaminase 2
CSF1 - Colony stimulating factor 1
$CHCl_3$ - Chloroform
CYP - Cytochrome P450
Cx - Connexins
CXCL - Chemokine (C-X-C motif) ligand
DFO – Deferoxamine
DIG - Digoxigenin
EBM-2 – Endothelial basal medium 2
EC – Endothelial cell
ECM – Extracellular matrix
BHA - Butylated hydroxyanisole
BM – Basement membrane
BMP - Bone morphogenic protein
CTCF - Corrected total cell fluorescence
ECM – Extracellular matrix

EFG – Epidermal growth factor

ELISA - Enzyme-linked immunosorbent assay

EMP - Erythro-myeloid progenitors

EMT - Epithelial to mesenchymal transition

eNOS – nitric oxide synthase

EPC - endothelial progenitor cell

Era – Erastin

ETS-1 - E26 transformation specific-1

EV – Extracelular vesicle

c-Src – Proto-oncogene tyrosine-protein kinase Src

Dll4 - Delta-like-4

FA - Folate

FBS – Fetal bovine serum

FDA – Food and Drug Administration

FELASA - Federation for Laboratory Animal Science Associations

FGF – Fibroblast growth factor

FGFR - Fibroblast growth factor receptor

FISH - Fluorescence *In Situ* Hybridization

FN – Fibronectin

GAG – glycosaminoglycans

G-CSF - Granulocyte-colony stimulating factor

GFP - Green fluorescent protein

GM- CSF - Granulocyte-macrophage colony-stimulating factor

GPX4 - Glutathione peroxidase 4

GSH – Glutathione

GSSG – GSH disulfide (oxidized GSH)

H_2O_2 - Hydrogen peroxide

H_2S – Hydrogen sulfide

HB-EGF - Heparin-binding epidermal growth factor

HDAC – Histone deacetylase

HGF - Hepatocyte growth factor

HMDB - Human metabolome database

HIF – Hypoxia inducible factor

HRE – Hypoxic-response elements

HUVECs - Human umbilical vein endothelial cells

ICAM - Intercellular adhesion molecules

IHC - Immunohistochemistry

IL – Interleukine

JNK - c-Jun N-terminal kinase

KRIT1 - Krev interaction trapped protein 1

LDL - Low-density lipoprotein

LPA – Lysophosphatidic acid

LPS - Lipopolysaccharide

MAPC - Rat multipotent adult progenitor cells

MAPK - Mitogen-activated protein kinase

MCP1 – Monocytes chemoattractant protein 1

miRNA – Micro RNA

MMP – Matrix metalloproteinases

MVD – Microvessel density

NAC - N-acetylcysteine

NaCl – Sodium chloride

NICD1 – Notch1 intracellular domain

NMR - Nuclear magnetic resonance

NO – nitric oxide

NOX - NADPH oxidase

Nrp – Neuropilin

NSCLC - Non-small cell lung carcinoma

$O_2^{\bullet -}$ – Superoxide

OS – Overall survival

PB – Peripheral blood

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate buffered saline

PDC10 - Programmed Cell Death 10

PDGFR – Platelet-derived growth factor receptor

PF4 – Platelet factor 4

PHD – HIF proxyl hydroxylases

PI – Propidium iodide

PI3K - Phosphoinositide 3-kinase

PIGF – Placental growth factor

PPP - Pentose phosphate pathway

Propranolol – Propranolol

PRPP - Phosphoribosyl diphosphate pathway

PTEN - Tensin homolog

PURE - Polyurea dendrimers

RB - retinoblastoma protein

ROS – Reactive oxygen species

RTK - Receptor tyrosine kinase inhibitor

RTKi – Receptor tyrosine kinase inhibitor

SDF1 - Stromal cell-derived factor 1

Se – Selenium

SeChry - Selenium-containing chrysin

SeChry@PURE$_{G4}$ - Dendrimeric nanoparticles loaded with Selenium-containing chrysin

SIGLEC1 - Sialic Acid Binding Ig Like Lectin 1

SMAD - Decapentaplegic homologue

SSC - Saline-sodium citrate

TAM – Tumor-associated macrophage

TASC – Tumor associated stromal cell

TBS - Tris buffered saline

TGFb – Transforming growth factor beta

TLC - Thin layer chromatography

TME – Tumor microenvironment

TNFα - Tumor necrosis factor alpha

TP53 - tumor protein p53

TSP - Transsulfuration pathway

TrxR - Thioredoxine reductase

UEA-1 - lectin

VCAM - Vascular cell adhesion molecule

VE-Cad – Vascular endothelial cadherin

VEGF – Vascular endothelial growth factor

VEGFR - Vascular endothelial growth factor receptor

vWF - Von Willebrand factor or VIII factor

vSMC – vascular smooth muscle cells

xCT - sodium-independent cystine/glutamate antiporter System xc-

ZO - Zonula occludens

Chapter I

General introduction

1. Cancer Biology

Cancer is considered as one of the major public health problems worldwide, being the second leading cause of death in developed countries. In US, the estimated cancer incidence in 2020 arounds 1,8006,590 individuals, with the most prevalent types of cancer associated with genital, digestive and respiratory system. Metastatic disease and resistance to treatments accounts for most of the cancer patient mortality and morbidity (Siegel *et al.*, 2019).

In 2000, Hanahan and Weinberg systematized the complexity of malignant transformation of cells into six hallmarks: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). A decade later, two novel emerging hallmarks emerged: reprogramming energy metabolism and evading immune response (Hanahan and Weinberg, 2011).

In normal cells, the balance between the production and the release of growth-promoting signals maintains tissue homeostasis, which is pivotal for the normal tissue architecture and function (Hanahan and Weinberg, 2011). However, cancer cells sustain the proliferative signaling through the overproduction of endogenous growth factors, the stimulation of normal cells within the tumor stroma to release growth factors and through the increased expression of cell surface receptors, which in turn promotes cancer cell hyperresponsiveness to otherwise-limiting amounts of growth factors (Hanahan and Weinberg, 2011; Feitelson *et al.*, 2015; Yadav and Mohite, 2020).

The increased expression or the occurrence of gain-of-function mutations in oncogenes (*e.g.* phosphoinositide 3-kinase (PI3K), KRAS and BRAF) or the downregulation or loss-of function mutations in canonical tumor suppressor genes, such as retinoblastoma protein (RB) and tumor protein p53 (TP53), increases the mitogenic capacity of cancer cells. Defects on the negative-feedback mechanisms, as the amplification of PI3K signaling as a resulting of phosphatase and tensin homolog (PTEN) loss, can also be responsible for enhancing the proliferative signaling in cancer cells and the escape from anti-growth signals (Jiang and Liu, 2008; Yuan and Cantley, 2008; Douglas Hanahan and Weinberg, 2011;). Those features allied with the escape from senesce and cell death, in part mediated by the telomere maintenance, or with the modulation of programmed cell death mechanisms, such as apoptosis, prompts the unlimited replicative potential of cancer cells (Fernald and Kurokawa, 2013). In which concerns to cell death, when Hanahan and Weinberg updated the cancer hallmarks in 2011, only apoptosis was the well-known programmed cell death, but today necroptosis and ferroptosis were already described as programmed cell death mechanisms, whose abrogation may contribute for tumor growth (Hanson, 2016; Mou *et al.*, 2019).

The metabolic remodeling of cancer cells, as a direct or an indirect consequence of oncogenic mutations, is crucial to fulfil a selective advantage to tumor initiation and progression. The cancer cell metabolic rewiring relies on the adjustment of metabolic and signaling pathways that allows tumor cells to consume and metabolize nutrients from a frequently nutrient-poor environment to maintain cell viability and build new biomass (Cairns and Mak, 2016; De Berardinis and Chandel, 2016; Serpa, 2020).

To ensure cancer development, cancer cells try to escape from the immune system through a process known as "cancer immunoediting", mediated by a decrease in immune recognition, an increased resistance to immune cells attack or by the development of an immunosuppressive tumor microenvironment (TME) (Beatty and Gladney, 2015; Muenst *et al.*, 2016). For instance, the elimination of anti-tumoral leucocytes and/or by recruiting immunosuppressive leukocytes promotes the generation of a TME that boosts a pro-tumor immune response (Beatty and Gladney, 2015; Muenst *et al.*, 2016).

The invasive and metastatic cancer cell potential depends on the gain of migratory and invasive abilities of the cancer cells from the primary tumor, relying on epithelial to mesenchymal transition (EMT) or in collective or cluster-based migration and invasion; on their intravasation into the bloodstream and further extravasation into the parenchyma of a distant organ or tissue; and on their survival and proliferation abilities in the metastatic niche (Chaffer and Weinberg, 2011; Lehúede *et al.*, 2016; Jolly *et al.*, 2017; Pastushenko and Blanpain, 2019). The efficient development of cancer metastases encompasses the compatibility between cancer cells and the metastatic microenvironment in which cancer cell seed (Yuneva *et al.*, 2012), however only a small fraction of the circulating cancer cells (0.01%) are capable of forming metastatic lesions (Schafer *et al.*, 2009; Stott *et al.*, 2010). Furthermore, the formation of tumor neovessels supports the delivery of oxygen and nutrients to the tumor, accounting for tumor cell survival, progression and metastatic spread (Chang *et al.*, 2002; Potente *et al.*, 2011; Hongwei Zhang *et al.*, 2020). For the last years, many strategies using anti-angiogenic drugs aim to abolish tumor blood supply to impair tumor growth, however the clinical results of those strategies have been disappointing, indicating that the mechanisms underlying tumor angiogenesis remain to be fully understood (Holohan *et al.*, 2013; Teleanu *et al.*, 2019).

The tumor angiogenesis, invasion and escape from the classical anti-angiogenic therapy has been the subject of a large research in the biomedical sciences field for many decades. A focus on concepts of tumor angiogenesis, how the pro-oxidative cancer metabolic remodeling influences the formation of tumor vessels, and how new therapeutic strategies tackling tumor and endothelial cell (EC) metabolism might improve the anti-cancer therapy, will be methodically presented in the next sections.

2. Angiogenesis: a brief overview

Blood vessels assemble into vascular networks that play an essential role in providing oxygen and nutrients for peripheral tissues, being crucial for the embryonic development, tissue renewal and organ physiology (Bergers and Benjamin, 2003; Adams and Alitalo, 2007). Tissue and organ homeostasis rely on the correct function of two hierarchically branched vessel networks, the vascular and the lymphatic systems (Fig I. 1) (Adams and Alitalo, 2007). Blood vasculature is essential for blood circulation, prompted by the heart, and for providing the transport of oxygen, nutrients, gases, metabolites, hormones and paracrine signaling molecules (Potente and Mäkinen, 2017). Lymphatic system is composed by lymphatic vessels, lymph nodes and lymphoid organs and is coupled to the vascular system, recovering extravasated fluid and macromolecules to blood circulation and contributing for immune cell trafficking, cholesterol clearance and fat absorption (Aspelund *et al.*, 2016).

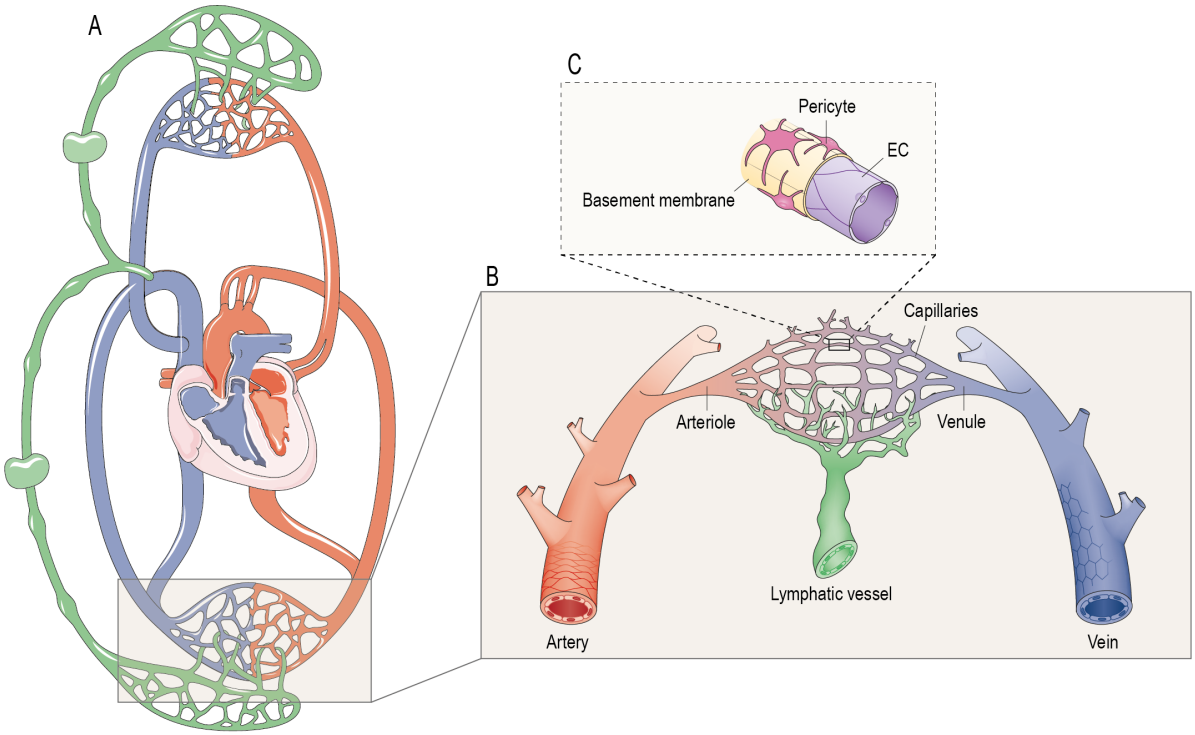


Figure I. 1 The vascular and the lymphatic systems.

A) Schematic representation of vascular network with heart, blood vessels (arterial (red) and venous (blue) circulation) and lymphatic vessels (green); B) The vascular network is hierarchically organized in arteries (red), veins (blue) and interconnected capillaries, as well as blind-ended lymphatic capillaries and collecting lymphatic vessels (green) that drain fluid into the venous circulation (Aspelund *et al.*, 2016; Carmeliet, 2005a; Herbert and Stainier, 2011). In the tree-like tubular network, the microvasculature is the site responsible for the most intercellular communications, being composed by arterioles, capillaries and venules. C) Endothelial cells (ECs) are the major cellular component of the vessels and mural cells (as pericytes) are essential for the stabilization of new connections, for vascular homeostasis and for barrier function (Potente *et al.*, 2011).

Basement membrane (BM) enveloping ECs and mural cells is fundamental for the histological structure of the vessel wall (Rhodes and Simons 2007; Xu and Shi 2014). Adapted from (Potente and Mäkinen, 2017).

During embryogenesis, blood vessels primarily arise from vasculogenesis, a process in which new vessels are formed by coalescence and assembly of angioblasts into functional vessels (Planas-Paz *et al.*, 2012; Domingues *et al.*, 2015b; Potente and Mäkinen, 2017). Angioblasts differentiate from hemangioblasts (multipotent mesodermal cells) and their function is controlled by tissue-derived factors, as fibroblast growth factor (FGF), bone morphogenic protein 4 (BMP4) and vascular endothelial growth factor (VEGF; also known as VEGFA). These central molecules are essential for the endothelial cell (EC) commitment, proliferation and survival (Marcelo *et al.*, 2013). Subsequently, this primitive vascular plexus progressively expands and remodels, leading to the formation of a mature and hierarchically organized vascular network, composed by arteries, veins and capillaries, a process defined as angiogenesis (Carmeliet, 2005a; Herbert and Stainier, 2011).

Angiogenesis is crucial during embryonic development, however it is also required in physiological and pathophysiological scenarios along life (Potente and Mäkinen, 2017; Eelen *et al.*, 2018). Physiological and active angiogenesis is associated with reproductive female cycle, tissue repair, wound healing and inflammation (Carmeliet, 2003; Ferrara and Kerbel, 2005; Chung *et al.*, 2010). In a pathophysiological context, angiogenesis is associated with cancer, diabetes, atherosclerosis and chronic inflammation (Carmeliet, 2003).

The formation of new blood vessels from a pre-existing one defines the concept of angiogenesis, however, this process can be mediated by two different and independent mechanisms: non-sprouting and sprouting angiogenesis (Bergers and Benjamin, 2003; Makanya *et al.*, 2009; Heinke *et al.*, 2012). Non-sprouting angiogenesis or intussusceptive angiogenesis involves the formation of transluminal pillars within a vessel, leading to the splitting of the vessel into two or more capillaries (Makanya *et al.*, 2009; Heinke *et al.*, 2012). In sprouting angiogenesis, ECs from a pre-existing vessel are activated in response to pro-angiogenic factors, generating sprouts responsible for the expand of the vascular network (Herbert and Stainier, 2011; Potente and Mäkinen, 2017). Additionally, new vessels can also grow through the recruitment of endothelial progenitor cells (EPCs) from the bone marrow, a process called neovasculogenesis (Hillen and Griffioen, 2007; Jung and Kleinheinz, 2013; Domingues *et al.*, 2015b). After, processes such as blood vessel maturation, extracellular matrix (ECM) deposition in the basement membrane (BM), vessel specialization and vascular quiescence will be

essential for the maintenance of a stable, functional, patterned and hierarchically organized vascular network (Conway *et al.*, 2001; Potente and Mäkinen, 2017).

2.1. Angiogenic signaling dynamics: the driving force of angiogenesis

The balance between pro- and anti-angiogenic stimuli determines the level of ongoing angiogenesis (Hillen and Griffioen, 2007). Among the positive regulators, VEGF is the most well-studied, being observed almost ubiquitously expressed in all active angiogenesis sites (Stacker and Achen, 2013). VEGF family consists of five secreted proteins, VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor (PlGF) (Holmes and Zachary, 2005), being the most well studied the VEGFA, from now on called VEGF. Moreover, VEGF, besides acting on tip cell activation, promotes vascular permeability that leads to the escape of plasma proteins (*e.g.* fibrinogen/fibrin), which are essential for the temporary supportive structure for migrating ECs and inflammatory cells (Conway *et al.*, 2001; J. Li *et al.*, 2003).

During embryonic development, the disruption of a single VEGF allele leads to embryonic lethality due to an impairment of vessels formation and function (Carmeliet *et al.*, 1996). VEGF is produced by a variety of cell types, such as ECs, haemopoietic and stromal cells and also cancer cells, being its expression directly correlated with regions of high vascular density (Bachelder *et al.*, 2001; Gerber and Ferrara, 2003; Stuttfeld and Ballmer-Hofer, 2009; Chen *et al.*, 2014b).

VEGF binds to its tyrosine kinase receptors (VEGFRs, type IV family of receptor tyrosine kinases; RTK), leading to the receptor dimerization and activation (Ferrara *et al.*, 2003; Shibuya, 2011; Blanco and Gerhardt, 2013;). VEGF activates VEGFR1 homodimers, VEGFR1/2 heterodimers and VEGFR2/3 heterodimers. VEGFB and PlGF can activate VEGFR1 homodimers, while VEGFC and VEGFD activate VEGFR2/3 heterodimers and VEGFR3 (fms related tyrosine kinase 4) homodimers, as well as VEGFR2 homodimers upon proteolysis (Ferrara *et al.*, 2003; Lohela *et al.*, 2009; Shibuya, 2011). VEGFRs are key regulators of angiogenesis and lymphangiogenesis (Holmes and Zachary, 2005; Shibuya and Claesson-Welsh, 2006Lohela *et al.*, 2009; Shibuya, 2011), being their activation a rate-limiting step in angiogenesis (Pugh and Ratcliffe, 2003; Carmeliet, 2005b). The VEGF binding to VEGFR2 activates downstream proteins, as Ras GTPases, proto-oncogene tyrosine-protein kinase Src (c-Src) and PI3K, fulcral for the activation of different signaling pathways important in the regulation of ECs proliferation, migration and differentiation and in the permeability of vessels (Kowanetz and Ferrara, 2006; Blanco and Gerhardt, 2013). Besides VEGFRs the type I transmembrane proteins neuropilin 1 and 2 (Nrp1 and 2) are responsible for transducing VEGFs signals together with the VEGFR family members. Nrp1 signaling is critical for

VEGF/VEGFR-2-mediated angiogenesis, being essential for angiogenesis and vascular patterning, and Nrp2 signaling is crucial for VEGF-C/VEGFR-2/R-3-mediated lymphangiogenesis (Guo and Vander Kooi, 2015; Tata *et al.*, 2015). Moreover, VEGF also induces the secretion of ECM degrading enzymes and protects neovessels from apoptosis (Carmeliet, 2005b).

Angiopoetin-1 (ANG1) is another molecule involved in the maturation and stabilization of blood vessels through Tie2 receptor activation (Type XII RTKs), by increasing perivascular-ECs interaction and ECs survival (Thomas and Augustin, 2009; Eklund *et al.*, 2017; Kiss and Saharinen, 2018). On the other hand, Angiopoetin-2 (ANG2) that is mostly synthesized and secreted by ECs, acts as an endogenous antagonist of ANG1, leading to remodeling processes or vascular sprouting in response to VEGF (Cascone and Heymach, 2012).

FGFs by binding and activating the fibroblast growth factor receptor (FGFR) family of RTK, increase the proliferation and the migration of ECs (Presta *et al.*, 2005; X. Yang *et al.*, 2015). FGF signaling pathway, a well-known angiogenic inducer, has been implicated as an activator of VEGF expression in ECs and as a strengthen of vascular endothelial cadherin (VE-Cad)/p120 catenin interactions, reducing VE-Cad internalization and increasing cell-cell adhesion (Cross and Claesson-Welsh, 2001; Murakami *et al.*, 2008; Murakami and Simons, 2008).

Hypoxia is one of the most well stablished angiogenic regulators, acting as a trigger for the release of pro-angiogenic factors, namely VEGF, which is a major driver of hypoxia responsiveness. VEGF leads to an “angiogenic switch” that stimulates the proliferation and migration of ECs to form new blood vessels (Domingues *et al.*, 2015b; Sliwinska *et al.*, 2018). Lower oxygen levels induce the expression of hypoxia-inducible factors (HIFs), essential for angiogenic and metabolic control and for the control of cell cycle genes (Pugh and Ratcliffe, 2003; Zimna and Kurpisz, 2015). During this process, HIF1α binds to hypoxic-response elements (HREs) in regulatory regions, mediating the transcriptional activation of target genes, as those encoding angiogenic molecules: VEGF, ANG1, ANG2, Tie-2, PDGF, FGF and monocyte chemoattractant protein 1 (MCP1) (Semenza, 2000). Under normoxic conditions, HIF prolyl hydroxylases 1-3 (PHD1-3) hydroxylate HIF1α for further proteasomal degradation, preventing the expression of its targets genes (Semenza, 2000; Rahimi, 2012). Therefore, HIF1α, by controlling pro-angiogenic molecules, links hypoxia to angiogenesis in a system regulated by the simple rule of demand and supply of nutrients and oxygen.

2.2. From a quiescent EC to a functional blood vessel: how angiogenesis works?

ECs, the cells that line the lumen of blood vessels, make part of a highly branched and tree-like tubular network, needed for oxygen and nutrient supply of peripheral tissues (Adams and Alitalo, 2007). Blood vessels, in addition to the continuous supply of nutrients and oxygen, control the systemic pH, temperature, homeostasis and mediate immune responses (Wilting and Chao, 2015).

Sprouting angiogenesis is a multi-step process that mediates the formation of new blood vessels. The release of a pro-angiogenic stimulus leads to the activation of endothelial tip cells, a specialized type of ECs that is characterized by its invasive and motile behavior due to the presence of dynamic filopodia protrusions that respond to a gradient of pro-angiogenic factors in the microenvironment (Domingues *et al.*, 2015b; Potente and Mäkinen, 2017). These cells are motile and guide the growing sprout, being followed by the stalk cells, that proliferate and elongate to form the new blood vessel and connect to the parental vessel (Domingues *et al.*, 2015b; Potente and Mäkinen, 2017). During this process, the justacrine delta-like-4 (Dll4)-Notch1 signaling pathway is pivotal for the establishment of tip-stalk specification in a VEGF-dependent manner (Fig I.2). VEGF, released by the microenvironment compartments, stimulates the expression of the Dll4 by tip cells that binds to the Notch1 receptor on neighboring cells, the stalk cells, activating Notch pathway with the cleavage of Notch1 intracellular domain (NICD1). NICD1 is a repressor of VEGFR2 expression, promoting the inhibition of stalk cells migration (Potente *et al.*, 2011; Blanco and Gerhardt, 2013; Stacker and Achen, 2013). Moreover, Dll4 defines tip cell identity by activating Notch signaling that regulate the expression of a number of cell surface receptors, including VEGFR2, VEGFR3, Nrp1, CD34 and platelet-derived growth factor receptor (PDGFR) (De Smet *et al.*, 2009; Siemerink *et al.*, 2012, 2013).

Dll4 is crucial in angiogenesis, since it is the only Notch ligand expressed predominantly in the vascular endothelium. Dll4 mutant mice have several vascular abnormalities, resulting in embryonic lethality in the most mouse strains (Duarte *et al.*, 2004; Gale *et al.*, 2004; Krebs *et al.*, 2004). Moreover, reduced levels of Dll4 or knockdown of active Notch signaling leads to the differentiation of excessive tip cells, resulting in dramatically increased sprouting, branching and fusion of blood vessels (Suchting *et al.*, 2007). Therefore, Notch1 mutants are able to form the primary vascular plexus, indicating that vasculogenesis is occurring, but they show deficits in angiogenesis, since embryos fail to induce the secondary vascular remodeling required to form a mature network of well-organized large and small blood vessels (Limbourg *et al.*, 2005). Additionally, in response to hindlimb ischemia, haplo-insufficient global or

endothelial-specific Notch1 mutant mice present compromised blood flow recovery and postnatal neovascularization (Takeshita *et al.*, 2007).

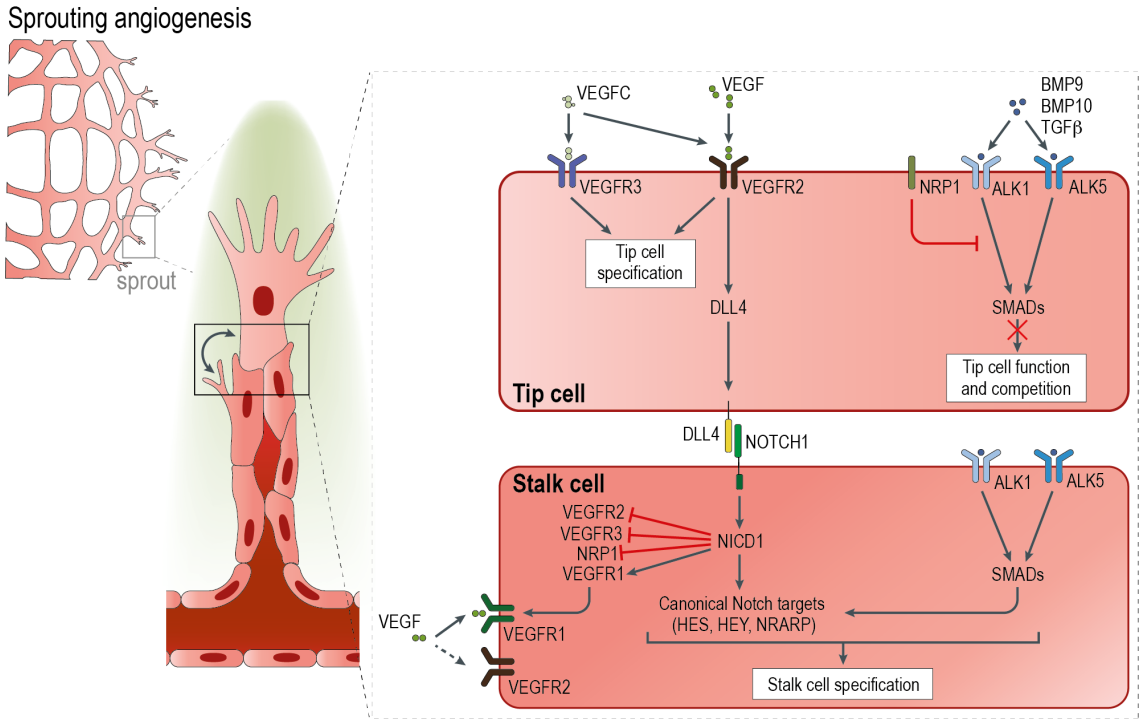


Figure I. 2 Sprouting angiogenesis is mediated by pro-angiogenic stimuli, leading to the formation of new blood vessels.

Vascular endothelial growth factor (VEGFA) and VEGFC bind to vascular endothelial growth factor receptors 2 and 3 (VEGFR2-3) respectively, activating endothelial tip cells, highly motile cells with filopodia that guide the capillary sprout towards the pro-angiogenic source (Domingues *et al.*, 2015b; Hillen and Griffioen, 2007; Potente *et al.*, 2011; Stacker and Achen, 2013). VEGFA and VEGFC stimulate the production of the ligand delta-like-4 (Dll4) by tip cells that binds to the Notch1 receptor on neighboring cells, the stalk cells, activating Notch1 intracellular domain (NICD1) that suppresses VEGFR2-3 and neuropilin 1 (NRP1) and promotes VEGFR1 expression, a decoy receptor that sequesters VEGF (Blanco and Gerhardt, 2013; Potente *et al.*, 2011; Stacker and Achen, 2013). Notch1 activation inhibits the migratory behavior of stalk cells. Also, NRP1, a co-receptor for VEGF, establishes differential responsiveness to transforming growth factor β (TGFβ)-bone morphogenetic protein (BMP) signaling through activin receptor-like kinase (ALK) signaling (Aspalter *et al.*, 2015). BMP9, BMP10 and TGFβ defines the stalk phenotype by the activation of inhibitors of decapentaplegic homologue (SMADs) and Notch target genes (Aspalter *et al.*, 2015). Adapted from (Potente and Mäkinen, 2017).

The acquisition of a tip or stalk cell phenotype involves the tight spatiotemporal coordination of gene expression. Furthermore, ECs can rapidly shift between tip and stalk cell positions during angiogenic sprouting. Tip and stalk fates are plastic and consequently, sprouting ECs are continuously shuffling and competing for the tip cell position by regulating their levels of VEGFR2 and VEGFR1 expression (Phng and Gerhardt, 2009; Jakobsson *et al.*, 2010; Potente *et al.*, 2011).

At the same time as tip and stalk cells are established, the BM and ECM that support the capillaries are degraded by proteases, mainly matrix metalloproteinases (MMPs),

expressed by ECs, allowing the formation of the new tube (Domingues *et al.*, 2015b). In addition to being an important structural support, ECM also acts as a reservoir of angiogenic growth factors, such as VEGF, FGF and transforming growth factor beta (TGFβ), which are released by proteolytic degradation of the ECM (Stetler-Stevenson, 1999; Liekens *et al.*, 2001; Kalluri, 2003; Stamenkovic, 2003;). *In vivo,* MMP7 expression promotes angiogenesis by enhancing ECs proliferation and upregulating the expression of MMP1 and MMP2 (Nishizuka *et al.*, 2001; Huo *et al.*, 2002). Moreover, MMPs are also responsible for the processing of growth factors and receptors (*e.g.* integrins) and for the generation of endogenous anti-angiogenic compounds (Stetler-Stevenson, 1999; Liekens *et al.*, 2001; Stamenkovic, 2003). For example, MMP3 and MMP7 cleave the membrane-bound precursor of heparin-binding epidermal growth factor (HB-EGF), releasing the active form, whereas tumor necrosis factor alpha (TNFα) is released from the cell surface by MMP1, 3, and 7 (Stamenkovic, 2003). MMP9 is also responsible for the cleavage of the pro-inflammatory and pro-angiogenic interleukine 8 (IL8), increasing tenfold its activity, and for the degradation and further inactivation of platelet factor 4 (PF4), an inhibitor of ECs proliferation and migration, *in vitro* and *in vivo* (Stricker *et al.*, 2001; Bikfalvi, 2004).

In an injured vessel, the adhesion and aggregation of platelets (coagulation process) promote the release of angiogenic growth factors (*e.g.* TGFβ, PDGF, and VEGF) and cytokines (*e.g.* chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL4, CXCL5, CXCL7, IL8), which are important players in the recruitment of macrophages and monocytes upon an inflammatory response (J. Li *et al.*, 2003; Yun *et al.*, 2016).

In the generation of new sprouts, the degradation of BM by MMPs occurs simultaneously with mural cell detachment. Mural cells, such as pericytes and vascular smooth muscle cells (vSMCs), act as supporting cells, and their detachment is controlled by ECs, through the release of ANG2. The disruption of ANG1 signaling by ANG2, via Tie2, destabilizes quiescent vessels and promotes sprouting angiogenesis (Maisonpierre *et al.*, 1997; Conway *et al.*, 2001; Augustin *et al.*, 2009;). The invasion of vascular sprouts is concomitant with vascular lumen morphogenesis, however newly formed vessels are immature and poorly functional. During this process, the sprouts contact with other sprouts or with other capillary connections, being their fusion (anastomosis) responsible for the generation of new vessel circuitries (Potente *et al.*, 2011). Moreover, in the newly formed vessels, after ECs migration and proliferation, inter-ECs junctions are formed via VE-cad and connexins (*e.g.* Cx37, Cx40, and Cx43) (Brisset *et al.*, 2009; Okamoto *et al.*, 2019).

During vessel formation, a stable vascular patterning (arteries, veins and capillaries specification) will be required for network functionality, and blood flow acts as an

instructor for blood vessels maturation (Hahn and Schwartz, 2009). Blood flow provokes mechanical/shear stress (frictional force per unit area) in ECs, inducing the secretion of PDGF, which in turn recruits pericytes and vSMCs (Beck and D'Amore, 1997; Risau, 1997; Conway *et al.*, 2001; Hahn and Schwartz, 2009). On the other hand, mural cells produce ANG1 that inhibits ECs proliferation and migration through Tie2 (Adams and Alitalo, 2007; Potente *et al.*, 2011). In addition, the interaction between mural cells and ECs induces the activation of TGFβ, promoting ECM deposition and contributing to vessel maturation and stabilization (Roberts and Sporn, 1989; Beck and D'Amore, 1997; Conway *et al.*, 2001). ECM of matured vessels are composed by proteins, as collagen IV, laminin and elastin; and by glycosaminoglycans (GAGs), that act as a scaffold for the maintenance of the histological structure of the vessel wall (Rhodes and Simons, 2007; Xu and Shi, 2014). For example, mutant embryos for collagen IV are inviable, dying in uterus with arteries and heart bleeding due to vessels rupture (Pöschl *et al.*, 2004).

Mural cells are recruited to stabilize the new connections, promoting the vascular homeostasis and barrier function of the newly formed vessels (Potente *et al.*, 2011). After stabilization, arteriovenous specialization will confer distinct hemodynamic properties. Arteries enlargement and remodeling is triggered mainly by high blood flow rates and signaling pathways dependent on Notch and EphrinB2 (Eichmann *et al.*, 2005; Kim *et al.*, 2008). Upon the establishment of a continuous and hierarchical vascular network, ECs undergo quiescence. The inhibition of the proliferative and migratory phenotype is regulated by PI3K-AKT and MAPK pathways, and when this process is blocked, vascular dysfunction occurs (Carmeliet and Jain, 2011a; Gimbrone and García-Cardeña, 2016; Marcelo *et al.*, 2013). The coordination of all these steps during angiogenesis is imperative for the maintenance of a fully functional vascular network.

2.2.1. Reactive oxygen species (ROS) as a mediator of angiogenesis

ROS are highly reactive molecules that derive from incomplete reduction of molecular oxygen, including free radicals as superoxide ($O_2^{\bullet-}$) and non-radicals, as hydrogen peroxide (H_2O_2) (Santoro, 2018). In ECs, superoxide-generating enzymes (NOX, also known as NADPH oxidases) are a major source of ROS (Manuneedhi Cholan *et al.*, 2017). In vascular cells there are four NOX isoforms (NOX1, NOX2, NOX4 and NOX5) but only NOX4 preferentially produces H_2O_2 (Panieri and Santoro, 2015; Manuneedhi Cholan *et al.*, 2017;).

High levels of ROS can promote oxidative stress and cause ECs dysfunction, however recent evidence support that physiological levels of ROS can be pro-angiogenic (Kim and Byzova, 2014; Manuneedhi Cholan *et al.*, 2017; Shafique *et al.*, 2017), stimulating essential stages during vascular formation, as proliferation, migration,

sprouting and tubule formation (Manuneedhi Cholan *et al.*, 2017). NOX4-derived H_2O_2 stimulates NOX2.22 and stabilizes HIF-1α, leading to the induction of VEGF expression that promotes tumor angiogenesis (Helfinger *et al.*, 2016).

VEGF signaling pathway is a target of ROS in ECs, promoting VEGFR2 dimerization and autophosphorylation, which in turn prompts angiogenesis (Colavitti *et al.*, 2002; Kim and Byzova, 2014). Moreover, VEGF-independent mechanisms of ROS-induced angiogenesis, as the activation of CEP/TLR2/MyD88 axis that generates lipid oxidation products or the downregulation of p38α phosphorylation mediated by activation of ataxia-telangiectasia mutated kinase (ATM), which has been showed to promote ECs proliferation and survival (West *et al.*, 2010; Okuno *et al.*, 2012; Kim *et al.*, 2013; Kim and Byzova, 2014; Huang and Nan, 2019).

2.2.2. Endothelial progenitor cells (EPCs): sustainers of angiogenesis

The identification of EPCs by Asahara and co-authors (1997) shifted the paradigm of angiogenic processes, since neovascularization in adult organisms was only attributed to sprouting angiogenesis at that time (Asahara *et al.*, 1997). EPCs comprises about 0.007% of total mononuclear cells and were originally isolated from adult peripheral blood (PB), being classified as cells with stem/progenitor characteristics that upon a pro-angiogenic stimuli were able to differentiate into EC lineages, promoting the reendothelization of injured arteries (Kim *et al.*, 2010; Balistreri *et al.*, 2015; Chong *et al.*, 2016; Yuan *et al.*, 2017; Ge *et al.*, 2018). Despite the absence of a precise panel of cell surface markers to characterize EPCs, most studies classified EPCs according to three prevalent panels of markers: CD133-CD34-VEGFR2; CD34-VEGFR2, or $CD114CD43^{low}$ (Richardson and Yoder, 2011; Yoder, 2012; Padfield *et al.*, 2013; Aragona *et al.*, 2016; Kutikhin *et al.*, 2018; Yuan *et al.*, 2017).

EPCs are recognized as a heterogenous population, with different stages of maturation (Fig I.3). Early outgrowth EPCs, also described as early EPCs, circulating angiogenic cells or myeloid angiogenic cells, have low proliferative capacities but they exhibit a paracrine role in inducing vascularization through the secretion of growth factors and cytokines, such as VEGF, hepatocyte growth factor (HGF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL8, ANG1, as well as releasing microvesicles containing microRNAs (miRNAs) (Rehman *et al.*, 2003; Cantaluppi *et al.*, 2012; Recchioni *et al.*, 2016; Alexandru *et al.*, 2020; Beltran-Camacho *et al.*, 2020). Hence, the early EPCs do not participate directly in the formation of blood vessels but induce the mobilization of autologous cells, responsible for improving revascularization and the maturation of a newly formed vasculature (Murayama *et al.*, 2002; Iwakura *et al.*, 2006; Miyamoto *et al.*, 2007;

Keighron *et al.*, 2018). Late outgrowth EPCs (or endothelial colony-forming cells) appear in peripheral blood (PB) cells culture with a cobblestone morphology after 14-21 days and present increased proliferative properties, being capable of incorporating neovessels and differentiating into mature ECs, replacing damaged ECs (Avogaro *et al.*, 2011; Madonna and De Caterina, 2015; Keighron *et al.*, 2018;).

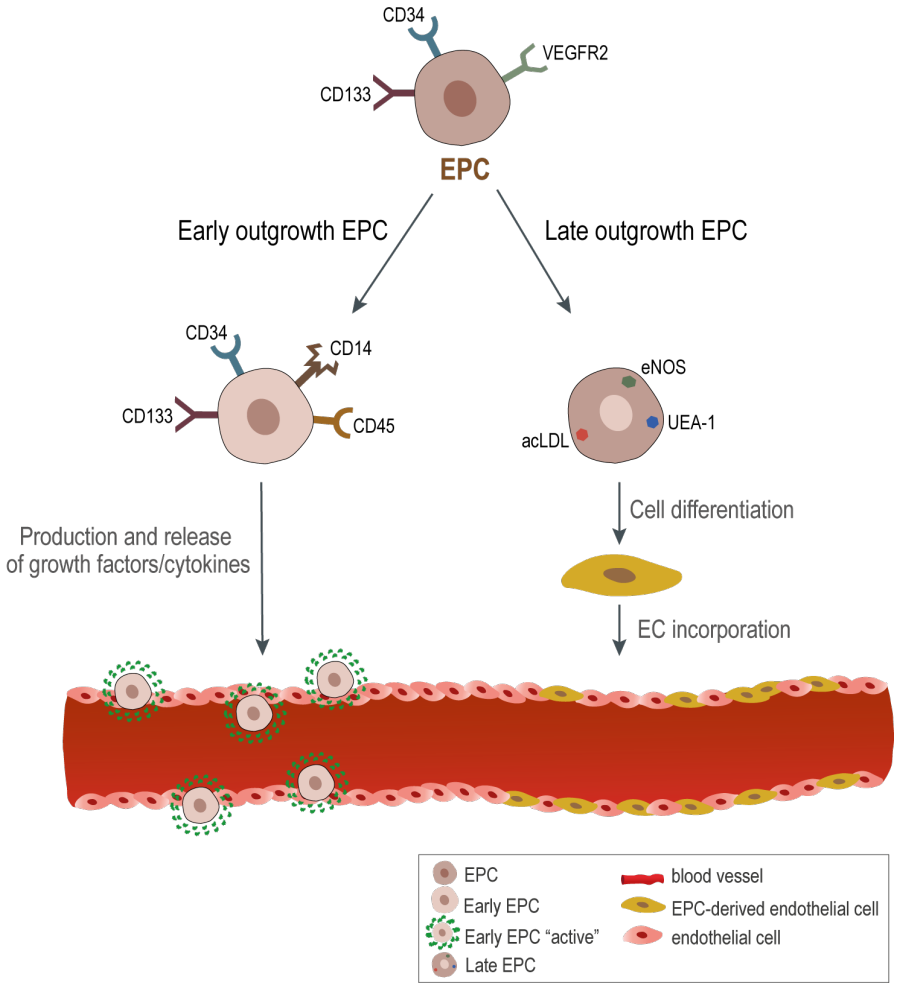


Figure I. 3 Endothelial progenitor cells (EPCs) participation in angiogenesis.

EPCs are characterized using CD133, CD34 and VEGFR2 (Padfield *et al.*, 2013; Peichev *et al.*, 2000; Richardson and Yoder, 2011; Urbich and Dimmeler, 2004; Yoder, 2012; J. jing Yuan *et al.*, 2017). Early outgrowth EPCs express CD133, CD34, CD45 and CD14 and promote EC proliferation and support the maturation of immature neovessels in a paracrine-manner, by secreting growth factors and microvesicles containing miRNAs (Iwakura *et al.*, 2006; Miyamoto *et al.*, 2007; Murayama *et al.*, 2002; Jalees Rehman *et al.*, 2003). Late outgrowth EPCs express acetylated low-density lipoprotein (acLDL) and lectin (UEA-1) as well as express nitric oxide synthase (eNOS) and produce nitric oxide (NO), supporting their differentiation into mature ECs that are further incorporated into neovessels (Asahara *et al.*, 1997; Avogaro *et al.*, 2011; Madonna and De Caterina, 2015).

Depending on their origin, EPCs are classified as tissue resident or circulating EPCs. Tissue resident EPCs show an adhesive nature and can be isolated directly from organ

tissues (Zhuo *et al.*, 2001; Bajenaru *et al.*, 2002; Malinverno *et al.*, 2017; Osman *et al.*, 2020; Royer *et al.*, 2020). Circulating EPCs are essentially isolated from PB or umbilical cord blood, being recruited and mobilized to the sites of vascular stress and transformed into tissue EPCs (Beltrami *et al.*, 2003; Ii *et al.*, 2009). The EPCs mobilization and recruitment is predominantly regulated by pro-angiogenic chemokines (CXCL1, CXCL7, CXCL12, CCL2) and their receptors (CXCR2, CXCR4, CCR2) (Bidzhekov, *et al.*, 2007; Liehn, *et al.*, 2007).

Independently of their origin, EPCs are essential for neovascularization processes and a better knowledge in EPCs characterization and in the mechanisms underlying their mobilization and homing will improve the therapeutic potential and effectiveness of EPCs-derived therapies in the treatment of angiogenic related diseases, as cancer.

2.3. Angiogenesis in cancer: tumor microenvironment (TME) as a promoter of endothelial cell activation

In 1971, Judah Folkman described for the first time that vessel formation (neoangiogenesis) is necessary for tumor growth (J Folkman, 1971), being later on associated with tumor malignancy, aggressiveness and metastasis (Gimbrone *et al.*, 1972; Zetter, 2008a). In cancer patients, the quantification of tumor angiogenesis through the determination of microvessel density (MVD) showed to be a useful prognosis factor, since the levels of expression of angiogenic factors can be correlated with higher tumor MVD, with advanced tumor stages or tumor invasiveness (Pang and Poon, 2006; Bačić *et al.*, 2018).

A pro-angiogenic microenvironment generated by cancer cells and tumor-associated stromal cells (TASCs), promotes a hyperactivation of the angiogenic switch in ECs. During this process, the pro-angiogenic phenotype of TME favors the hyperproliferation of ECs, mediated by PI3K/AKT activation, leading to the formation of a chaotic vessel network with perturbed blood flow that act as a metastatic facilitator (Chang *et al.*, 2002; R. K. Jain, 2014). Genetic alterations in cancer cells, as the constitutive activation of the oncoproteins RAS, RAF and DEK or the presence of p53 mutated variants, contributes to the angiogenic switch, through the increased expression and release of pro-angiogenic and pro-inflammatory factors, as VEGF and CXCL8 (Rak *et al.*, 2000; Sparmann and Bar-Sagi, 2004;Sharma *et al.*, 2005; Bottos *et al.*, 2012; Stantic *et al.*, 2015; Yanan Zhang *et al.*, 2016). Moreover, the detachment of pericytes from ECs, the absence of a continuous basement membrane and the disruption of ECs junctions contributes to tumor vessel fragility and hyper-permeability, pivotal for cancer cells intravasation and metastasis (Hashizume *et al.*, 2000; Shim *et al.*, 2007; Rohlenova *et al.*, 2018).

The uncontrolled proliferation of tumor cells concomitant with highly immature and unstable tumor vasculature promotes the generation of a highly hypoxic microenvironment, responsible for the expression of HIF1α and for the further release of pro-angiogenic factors, as VEGF and FGF (Domingues *et al.*, 2015b; Nowak-Sliwinska *et al.*, 2018). The inhibition of HIF1α abrogates the VEGF autocrine loop mediated by PI3K-AKT signaling pathway, impairing the angiogenic switch that drives ECs proliferation, vessels sprouting, vascular remodeling and tumor progression (Tang *et al.*, 2004; J. Wang *et al.*, 2015; Azoitei *et al.*, 2016; Cai *et al.*, 2018; Wang *et al.*, 2018). Interestingly, mutations in oxygen-sensing signaling pathways correlate with tumor progression and poor patient outcome, since they promote the constitutively HIF activation and increase the pool of pro-angiogenic factors in the TME (Miller *et al.*, 2005a; Giatromanolaki *et al.*, 2008).

The absence of a synchronized tumor growth and the chaotic vessel network prompt cycles of hypoxia–reperfusion, generating unbalanced reactive oxygen species (ROS) generation (Brown, 1979; Gorrini *et al.*, 2013). ROS can dynamically influence the TME and vice-versa, being already established that this crosstalk controls cancer angiogenesis, metastasis, and survival. Although ROS production in ECs may sustain tumor angiogenesis (Garrido-Urbani *et al.*, 2011), excessive ROS levels can abolish ECs responsiveness to extracellular VEGF by increasing VEGFR2 recycling and by inducing ECs-chromosomal abnormalities upon DNA damage (Warren *et al.*, 2014). Taking this into account, ROS-scavenging strategies could be applied to the treatment of diseases with pathological angiogenesis, as vascular diseases and cancer (Prieto-Bermejo and Hernández-Hernández, 2017). For instance, the use of dietary antioxidants such as vitamin C and E (Nespereira, 2003) could be an option, however it is difficult to determine the exact dosage since they are nonspecific and can also affect physiological angiogenesis. Other option could be pharmacological inhibitors of NADPH oxidase. However, *in vivo* studies using such inhibitors are scarce (Coso *et al.*, 2012). So far, it is known that ROS play an essential role in the induction of tumor angiogenesis, however it remains unclear how ROS generated by cancer cells and TASCs directly affect ECs and influence their angiogenic properties. The modulation of the behavior of TASCs, by tumor cells, is pivotal for the gain of a pro-angiogenic phenotype, crucial for supporting neoangiogenesis (Ribatti *et al.*, 2007; Shojaei *et al.*, 2008; Payne and Jones, 2011; Gacche, 2015). Tumor-associated macrophages (TAMs), neutrophils, mast cells, activated platelets, cancer-associated fibroblasts (CAFs) and cancer-associated adipocytes (CAAs) act as active sources of pro-angiogenic factors (*e.g.* VEGF, FGF, PlGF and CSF1) and membrane-bound or soluble proteases (*e.g.* MMP2, MMP9, MMP12 and cathepsins) that, in turn, promote ECM degradation, leading to the release

of pro-angiogenic factors sequestered in the perivascular ECM (Bergers and Benjamin, 2003; Arroyo and Iruela-Arispe, 2010; Bonnans *et al.*, 2014; Lugano *et al.*, 2018). The abrogation of the pro-angiogenic phenotype of TAMs and infiltrated mast cells delays the angiogenic switch and impairs the relapse of transplanted tumors after chemotherapy (Harney *et al.*, 2015; Hughes *et al.*, 2015) (Coussens *et al.*, 1999; Soucek *et al.*, 2007; Kabiraj *et al.*, 2018; W. Xu *et al.*, 2019). Moreover, the release of tissue inhibitor of metalloproteinase (TIMP)-free proMMP-9 by tumor-associated neutrophils and an increase in TAMs density correlates with increased vascular density, tumor cells' aggressiveness and cancer patients' poor survival (Leek *et al.*, 1996; Clear *et al.*, 2010; Bekes *et al.*, 2011; Koh *et al.*, 2014; Kovaleva *et al.*, 2016).

CAFs are the major components of the tumor stroma and the major source of non-derived cancer cell VEGF (Fukumura *et al.*, 1998; H. P. Gerber *et al.*, 2000). Moreover, CAFs can also drives tumor angiogenesis by attracting ECs and by recruiting monocytes from the bone marrow, through the CXCL12 (SDF1)–CXCR4 axis (Orimo *et al.*, 2005; Kalluri, 2016). Depending on the tumor site, CAAs can also be one the major stromal cells constituents, contributing to a rich milieu of cytokines, chemokines and hormones, termed adipokines, which have well-established pro-angiogenic functions (Cai *et al.*, 2016; Bougaret *et al.*, 2017). In accordance, tumors implanted in adipose tissue showed a denser vascular network and increased levels of VEGF, which promotes EPCs migration, differentiation and tube forming capacity (Lim *et al.*, 2016a)

The constitutive sharing of pro-angiogenic factors in the TME shapes ECs features to benefit the cancer cell needs. Interestingly, in addition to the release of soluble factor, cancer cells and TASCs also secrete extracellular vesicles (EVs) containing pro-angiogenic mediators (*e.g.* VEGFA, CXCL8, IL6 and FGF2) and ECM-remodeling enzymes (*e.g.* MMP2, MMP9 and uPA), pivotal for the promotion of ECs activation and tumor angiogenesis (Graves *et al.*, 2004; Taraboletti *et al.*, 2006).

2.3.1. The contribution of EPCs for tumor angiogenesis – partners in crime?

During carcinogenesis, the TME release pro-angiogenic factors (as VEGF, FGF, SDF1) and cytokines (as IL1β) that besides their role in promoting local angiogenesis by stimulating adjacent ECs, also support EPCs mobilization to the tumor (Gao *et al.*, 2008; Moccia *et al.*, 2014; Moschetta *et al.*, 2014; Chopra *et al.*, 2018). VEGF released by TME correlates with EPCs mobilization from bone marrow and with increased EPCs proliferation and further incorporation into tumoral endothelium (Mellick *et al.*, 2010; Chen *et al.*, 2011;), being the blockage of VEGF-induced migration of EPCs associated with angiogenesis abrogation (Ni *et al.*, 2019; Lee *et al.*, 2020). In non-small cell lung

carcinoma (NSCLC) patients with increased levels of circulating EPCs, bevacizumab (a humanized monoclonal antibody against VEGF) administration in combination with chemotherapy improves the clinical outcome, showing higher tumor reduction rate and longer progression-free survival, comparing with the group of NSCLC patient with low levels of circulating EPCs (Sudo *et al.*, 2017).

In addition to their role in incorporating tumor vasculature, EPCs are able to release stromal cell-derived factor 1 (SDF1) and VEGF, therefore promoting a gradient that may potentiate the extravasation and the development of a pre-metastatic niche (Kaplan *et al.*, 2005; Urbich *et al.*, 2005; Jin *et al.*, 2012). In line with this, the *in vivo* blockage of EPCs mobilization impairs macrometastases (Lyden *et al.*, 2001; Gao *et al.*, 2008; Moccia *et al.*, 2014).

Circulating EPCs (cEPCs), in PB of cancer patients (Zhang *et al.*, 2005; Yu *et al.*, 2007; Botelho and Alves, 2016; Chopra *et al.*, 2018), are positively correlated with disease progression and staging (Nowak *et al.*, 2010; Ziebart *et al.*, 2016;Tanaka *et al.*, 2017; Li *et al.*, 2018;). Interestingly, some clinical studies indicate that the levels of cEPCs may be indicative of the treatment response or the grade of malignancy, shedding a light on the therapeutic value of targeting EPCs in cancer patients (Zhang *et al.*, 2005; Dome *et al.*, 2006; Udi *et al.*, 2011; Wiessman *et al.*, 2019).

A better understanding of genetic and epigenetic changes in EPCs in response to TME will be essential for elucidating the exact cellular and molecular mechanisms of tumor angiogenesis. For instance, histone deacetylase 7 (HDAC7) acts as a regulator of the angiogenic properties of EPCs (Wei *et al.*, 2018). Moreover, the relevance of EPCs in tumor angiogenesis remains much unexplored, being the further identification of molecular players, chemokines/cytokines and tissue- specific ECM components, involved in EPCs mobilization essential to provide new targets for cancer treatment. The study of the clinical impact of EPCs mobilization in disease prognosis and EPCs therapeutic value will be also pivotal for improving patient's outcome. However, the main limitation is still the inconsistent and poorly reliable identification of EPCs.

2.3.2. Angiogenesis-like phenomena in cancer: another route to support tumor perfusion

During cancer progression, besides the development of vascular networks by sprouting angiogenesis (Fig I.4 A), other mechanisms can contribute to blood vessel formation (Rohlenova *et al.*, 2018).

The vascular co-option is a process in which cancer cells proliferate near the existing blood vessels, avoiding further angiogenic processes (Fig I.4 B) (Auguste *et al.*, 2005; Frentzas *et al.*, 2016). This phenomenon is essentially reported in highly vascularized

tumors, as brain, lung and liver (Auguste et al., 2005; Frentzas et al., 2016). The quiescent blood vessels co-opted by tumors suffer extreme changes over time. First, co-opted vessels regress by the action of Ang2, which promotes hypoxia and consequent tumor cell death (Holash *et al.*, 1999; Crinò and Metro, 2014; Seano and Jain, 2020). Second, HIF1α activation in the hypoxic TME leads to the release of pro-angiogenic factors that will promote the *de novo* angiogenesis, required for further tumor progression (Holash *et al.*, 1999; Ellis and Hicklin, 2008; Seano and Jain, 2020). Nevertheless, the exact mechanisms and the molecular players of vessel co-option need to be deeply explored.

Moreover, vascular mimicry relies on the blood supply of the tumor, independently of angiogenesis or ECs, and it correlates with cancer patients' poor survival. During this process, cancer cells acquire an EC-like phenotype and become organized into vascular-like structures that act as a perfusion system to canalize blood and obtain nutrients and oxygen (Fig I.4 C) (Maniotis et al., 1999; Angara et al., 2017; Fernández-Cortés et al., 2019). Hypoxia potentiates the *de novo* expression of VE-Cad in tumor cells, which in turn contributes to the acquisition of EC-like features and is accompanied by the formation and stabilization of a tubular structure (Giannotta *et al.*, 2013; Angara *et al.*, 2017; Delgado-Bellido *et al.*, 2017; Rezaei *et al.*, 2018).

Cytokines, adhesion molecules and growth factors shared in TME, besides their role in the stimulation of angiogenesis, has also a role in promoting lymphangiogenesis (Fig I.4 D). Lymphangiogenesis improves the metastatic spread through the lymphatic system, contributing to the morbidity and mortality of cancer patients. Moreover, a balance of pro- and anti-lymphangiogenesis factors, including VEGFC and their interactions with VEGFR3 and VEGFR2, regulates tumor-associated lymphangiogenesis (Karpanen et al., 2001; Mandriota et al., 2001; Stacker et al., 2001).

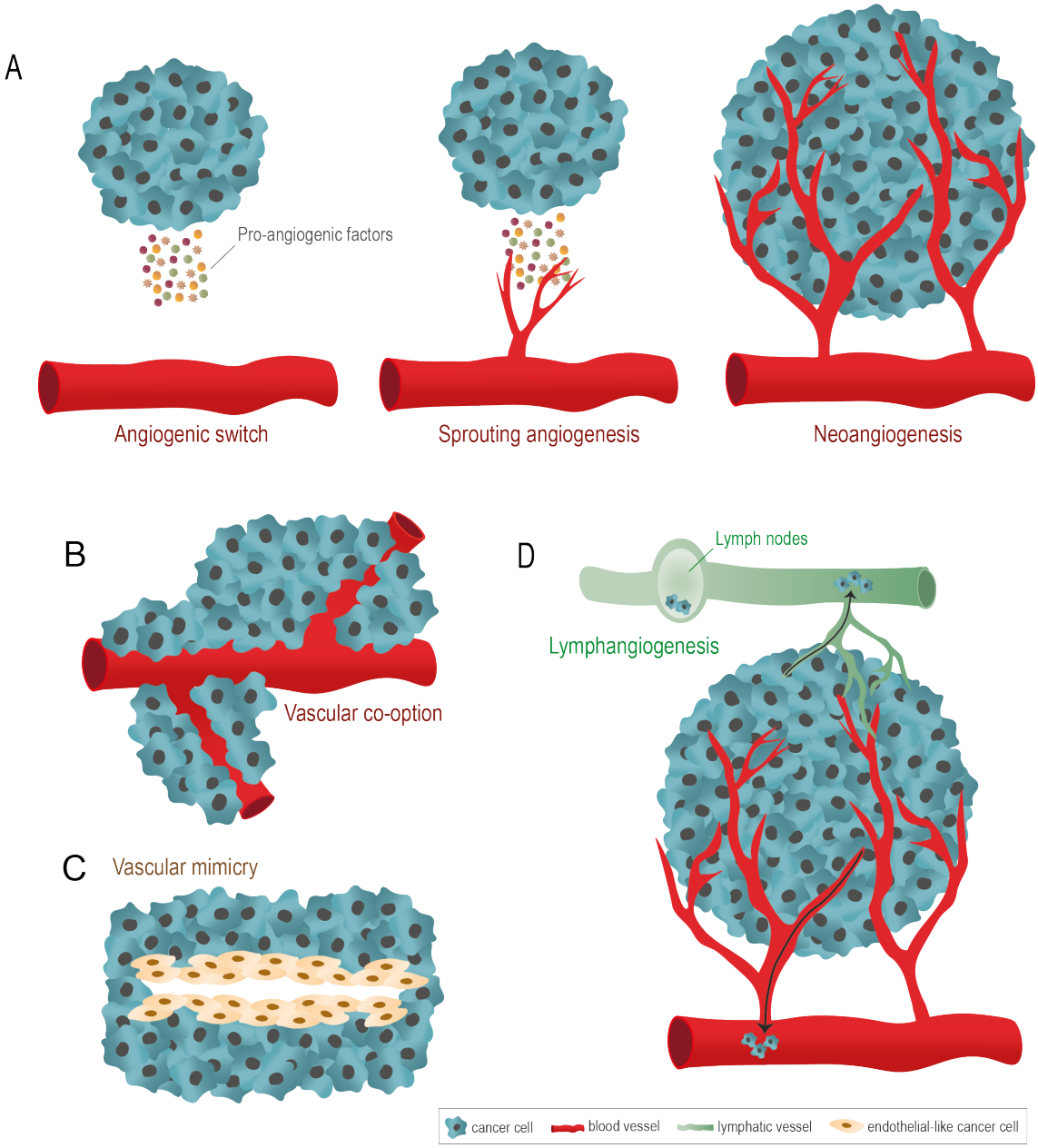


Figure I. 4 Vessels and vessels-like structures in cancer.

A) During tumor progression the release of pro-angiogenic factors by the tumor cells activates the angiogenic switch, leading to the migration and proliferation of endothelial cells (ECs) from the pre-existing blood vessels (Domingues *et al.*, 2015b; Nowak-Sliwinska *et al.*, 2018). Tumor cells rely on neoangiogenesis to obtain nutrients and oxygen to support tumor growth (Rohlenova *et al.*, 2017). B) During vascular co-option, tumor cells migrate along the preexistent blood vessels, taking over the existing vasculature to tumor blood supply (Auguste *et al.*, 2005; Frentzas *et al.*, 2016). C) In vascular mimicry cancer cells acquire EC-like properties and organize into vascular-like structures, acting as systems to obtain nutrients and oxygen, independently of ECs (Angara *et al.*, 2017; Fernández-Cortés *et al.*, 2019; Maniotis *et al.*, 1999). D) Lymphangiogenesis improves metastatic sprout to lymphatic system (Karpanen *et al.*, 2001; Mandriota *et al.*, 2001; Stacker *et al.*, 2001).

2.4. Monocytes as EPCs - an unexplored field

Cells from monocytic origin potentiate angiogenic processes. For instance, the *in vitro* culture of mononuclear cells (mainly monocytes and T-cells) have a paracrine role in the promotion of angiogenesis, as seen in models of carotid and femoral arterial injury, whose re-endothelization is improved by $CD14^+$ cells (Fujiyama *et al.*, 2003; Elsheikh *et al.*, 2005).

The relevance of TAMs during cancer progression is evident, since TAMs depletion by clodronate reduces *in vivo* tumor growth and neovascularization (Gazzaniga *et al.*, 2007; Kimura *et al.*, 2007; Halin *et al.*, 2009). TAMs are mainly linked to increased tumor invasion, migration, poor clinical outcome and tumor angiogenesis, being also positively associated with MVD, tumor stage and angiogenesis (Torisu *et al.*, 2000; Bingle *et al.*, 2006; Wu *et al.*, 2012; Jiang *et al.*, 2016). During TAMs differentiation processes, "immunoediting" promotes their modulation from an anti-tumor to a pro-malignant macrophage phenotype, characterized by the mixed M1 and M2 macrophage features.

In physiological and pathophysiological conditions, macrophages are essential and active players in angiogenesis (Rao *et al.*, 2007). In ischemic injury, ECs release CCL2, that drives monocyte recruitment to foster tissue reperfusion; however, macrophage deficient CSF1 mice fail to recover tissue reperfusion and decrease the formation of collateral vasculature (Bergmann *et al.*, 2006). After brain ischemic stroke, mice with C-C chemokine receptor type 2 (CCR2)-deficient monocytes present a worse behavioral performance accompanied by reduced angiogenesis (Pedragosa *et al.*, 2020), highlighting the essential role of monocytes during vascularization. Interestingly, in hepatocellular carcinoma, $CCR2^+$-TAMs accumulate at the vascularized border, but when they are pharmacological inhibited a reduction in neovascularization, hepatic blood volume and tumor burden is observed (Bartneck *et al.*, 2019).

In the tumor, TAMs density directly correlates with increased vessel density, hypoxic tumor regions and blood vessel regions (Ohno *et al.*, 2004; Prenen and Mazzone, 2019). Under hypoxia, the upregulation of HIF1α and HIF2α promotes the transcriptional TAMs phenotypic adaptation, leading to increased expression of pro-angiogenic genes (Murdoch *et al.*, 2008; Huang *et al.*, 2018). In metastatic liver cancer, $VEGFR1^+$-TAMs were found to be highly pro-angiogenic and the increased levels of circulating $VEGFR1^+$ monocytes are associated with worse disease outcome (Valls *et al.*, 2019). Interestingly, in a breast cancer model, TAMs revealed to be the main source of CCL8 and Sialic Acid Binding Ig Like Lectin 1 (SIGLEC1), through a TNFα regulatory loop; and breast cancer cells respond to CCL8 levels by producing CSF1, in order to promote monocyte infiltration into the tumor, accounting for an immunosuppressive microenvironment that will promote neoangiogenesis, tumor cell motility and malignancy (Cassetta *et al.*, 2019).

Moreover, IL8 released by TAMs increase *in vitro* ECs migration and tube formation and adenosine deaminase 2 (CECR1) stimulates pericytes recruitment and migration, leading to tumor angiogenesis (X. Wang *et al.*, 2013; C. Zhu *et al.*, 2017). In cancer patients, $CD14^+$ monocytes secrete VEGF in a NFκB signaling-dependent mechanism (Chittezhath *et al.*, 2014) and $Tie2^+$ monocytes stimulate the formation of tumor vasculature through the production of VEGF, TNFα and MMPs (Venneri *et al.*, 2007; Coffelt *et al.*, 2010; Harney *et al.*, 2015). Accordingly, the inhibition of angiopoietin/Tie2 signaling by rebastinib impaired tumor progression, due to the lack of angiopoietin/Tie2-dependent angiogenesis (Harney *et al.*, 2017).

The release of pro-angiogenic factors and immunosuppressive factors, besides their role in angiogenesis promotion, increase vascular permeability, which facilitates the extravasation of metastatic tumor cells (Wyckoff *et al.*, 2007). Interestingly, after platinum salts treatment, an increase in the density of pro-angiogenic TAMs is observed (Dijkgraaf *et al.*, 2013) and after radiotherapy TAMs were found in close proximity to the remaining tumor vasculature (De Palma and Lewis, 2013), suggesting a role of TAMs in vascular recovery. In addition to the production of pro-angiogenic and immunosuppressive factors, TAMs also activate angiogenesis by controlling the bioavailability of pro-angiogenic regulators capable of modulating ECM. The expression of membrane-bound or soluble proteases (*e.g.* MMP2, MMP9, MMP12 and cathepsins), by TAMs, promotes ECM degradation and consequently the release of pro-angiogenic growth factors sequestered in the perivascular ECM, benefiting the migration and proliferation of ECs (Moldovan, 2002; Bergers and Benjamin, 2003; Egeblad *et al.*, 2010).

The differentiation potential of monocytes has been firstly described as limited to phagocytic cells, as macrophages and dendritic cells (Geissmann *et al.*, 2010; Guilliams *et al.*, 2014; Kumar and Jack, 2006). However, accumulating studies showed that circulating monocytes can differentiate into a variety of cell types (*e.g.* EC, epithelial cell, adipocytes) (Zhao *et al.*, 2003; Seta and Kuwana, 2007, 2010). For instance, pluripotent stem cells isolated from a subset of colony stimulating factor 1 (CSF1)-stimulated monocytes have the potential to differentiate into different lineages, depending on the stimuli (Zhao *et al.*, 2003).

Over the past years, the disparity in the use of different markers to isolate and characterize EPCs instigated controversy about EPCs progeny and function. The role of monocytes as EPCs have been already proposed in cardiovascular (Jaipersad *et al.*, 2014) and inflammatory (Steinbichler *et al.*, 2014) diseases. In fact, monocyte levels correlate with increased capillary density in the calf microvasculature (Arras *et al.*, 1998) and in a mouse model of carotid injury, $CD14^+$ monocytes are capable of improving re-endothelialization (Fujiyama *et al.*, 2003). The lack of specific EPCs markers could

underestimated the contribution of monocytes as EPCs. Multiple studies highlighted the role of monocytes recruitment during neovascularization processes (Rehman *et al.*, 2003; Yamaguchi *et al.*, 2003; Hur *et al.*, 2004; Yoon *et al.*, 2005). Cell surface marker analysis of EPCs reveal that >95% of the cells expressed the monocytic marker CD14 and that the commonly used early and late outgrowth EPCs markers (described in detail in section 2.2.2.), as low-density lipoprotein (LDL) uptake, lectin binding and the expression of some endothelial-related markers (*e.g.* CD105, CD144) are intrinsic features of monocytes. Perhaps, some populations classified as EPCs are in fact monocytes, misjudging their contribution to angiogenesis. The major bone marrow cells recruited to VEGF-induced sites of neovascularization are $CD14^+$ (Grunewald *et al.*, 2006; Zentilin *et al.*, 2006) and monocytes recruited to hind limb ischemia express pro-angiogenic factors (*e.g.* FGF, VEGF, IL1; TNFα) (Yanagisawa-Miwa *et al.*, 1992), all common features of EPCs (Rehman *et al.*, 2003; Yamaguchi *et al.*, 2003; Hur *et al.*, 2004; Yoon *et al.*, 2005).

Some authors pointed that EPCs bone marrow-derived cells, comprising monocytes, contribute for neovascularization processes by differentiating into ECs, while other reports suggested their paracrine involvement during vascular remodeling (Asahara *et al.*, 1997; Murayama *et al.*, 2002; J Rehman *et al.*, 2003; Iwakura *et al.*, 2006; Avogaro *et al.*, 2011; Madonna and De Caterina, 2015). In a model of hind limb ischemia, $CD14^+$ monocytes after macrophage and dendritic cell differentiation were less efficient in improving neovascularization than monocytes maintained under an angiogenic culture (Urbich *et al.*, 2003), suggesting than monocytes upon a pro-angiogenic stimuli are active promoters in angiogenic processes, however their paracrine role or their contribution by directly differentiating into ECs has not been fully understood. Other studies also suggest a direct correlation between monocytes and the neoangiogenesis process in cancer. Monocytes exposed to PB serum from patients with multiple myeloma can acquire EC morphology and its specific gene expression (Haiming Chen *et al.*, 2011) and acute monoblastic leukaemia patients often show gingival hyperplasia, characterized by an excessive growth of vessels (Gallipoli and Leach, 2007). Furthermore, our group showed that in tumors and normal tissues some ECs simultaneously expressed CD14 and CD31 (EC marker), indicating mixed features between monocytes and ECs and the putative differentiation route of monocytes into ECs (Domingues *et al.*, 2015a).

3. The use of anti-angiogenic agents in cancer: a disappointing therapeutic strategy

The standard care of cancer patients with solid tumors, is based on surgical resection followed by chemotherapy and/or radiotherapy, in order to prevent cancer recurrence and the progression of occult microscopic tumors (Bellon *et al.*, 2005; Biondi *et al.*, 2010; Kajiyama *et al.*, 2018). However, one of the major obstacles for the optimal success of cancer therapy is therapy resistance (Holohan *et al.*, 2013).

Given the central role of VEGF in the promotion of tumor angiogenesis, targeting VEGF signaling pathway has emerged as the most promising therapeutic strategy for angiogenesis inhibition and cancer treatment. In 1971, Folkman hypothesized that anti-angiogenic therapy would promote tumor regression, by reducing tumor vasculature and consequently leading to tumor cells starvation and death. However, decades after Folkman statement, anti-angiogenic strategies were developed and, antibodies such as bevacizumab, the first VEGF-targeted agent approved by Food and Drug Administration (FDA) for cancer treatment, are available for cancer therapy (Zetter, 2008b). Nevertheless, so far these strategies have failed, in part because the precise molecular mechanisms of cancer neo-angiogenesis remain unclear. Also, a new paradigm emerge since some studies suggested that the abrogation of blood supply will restrict drug delivery (*e.g.* cytostatic agents) to the tumor, decreasing their clinical efficacy (Rohlenova *et al.*, 2017). In line, strategies focused on restoring tumor vessels normalization will increase the penetration of therapeutic agents into the tumor, improving the efficacy of drugs (Carmeliet and Jain, 2011b).

VEGF:VEGR axis is considered as the key mediator of pathophysiological angiogenesis. Overactivation of VEGF:VEGFR axis is a trait of many cancer types and correlates with increased MVD and metastatic spread (Kerbel, 2000; Yancopoulos *et al.*, 2000; Carmeliet, 2005b; Jain, 2005; Wu *et al.*, 2006; Y. Wu, Zhong, *et al.*, 2006; Fukumura and Jain, 2007;). The blockage of VEGF:VEGFR axis seems to be ineffective as monotherapy, and primary or *de novo* resistance is a common feature in cancer patients (Giantonio *et al.*, 2007; Holohan *et al.*, 2013). At molecular level, the disappointing clinical results obtained by the use of VEGFR inhibitors could be explained by VEGF-independent compensatory mechanisms, through the activation of other angiogenic signaling pathways (*e.g.* PDGF/PDGFR, FGF/FGFR, Ang/Tie2) and/or the upregulation of the expression of other pro-angiogenic factors (*e.g.* bFGF, PDGF) (Taylor *et al.*, 2003; Casanovas *et al.*, 2005; ; Shojaei *et al.*, 2007; Crawford *et al.*, 2009; Michaelsen *et al.*, 2018). This was clinically evident since cancer patients treated with bevacizumab increase de plasma levels of PIDF, FGF and PDGF concomitantly with

disease progression (Kopetz *et al.*, 2010; Lieu *et al.*, 2013). Accordingly, the *in vivo* blockage of PIGF or FGF pathway normalizes the tumor vessels and improves the efficacy of VEGF-targeted therapy (Casanovas *et al.*, 2005; Van de Veire *et al.*, 2010; Allen *et al.*, 2011; André *et al.*, 2013; Burbridge *et al.*, 2013; Lee *et al.*, 2015; Shen *et al.*, 2019). Strategies focused on the dual inhibition of VEGF and other pro-angiogenic signaling pathways might be pivotal for the improvement of anti-angiogenic cancer therapy. For instance, in bevacizumab-resistant tumors, brivanib (dual FGF/VEGF inhibitor) increases the overall survival (OS) in a mouse model of pancreatic neuroendocrine tumor, dovitinib (VEGFR, FGFR and PDGR inhibitor) delays tumor growth, and S49076 (MET, AXL, and FGFR kinase inhibitor) induces tumor growth arrest (Allen *et al.*, 2011; Burbridge *et al.*, 2013; C. K. Lee *et al.*, 2015). Also, in mice models of cancer, the blockage of VEGF/ANG2 suppresses revascularization and tumor progression and increases OS (Rigamonti *et al.*, 2014; Kloepper *et al.*, 2016; Peterson *et al.*, 2016). Although pre-clinical models showed increased efficacy in tackle tumor angiogenesis, unfortunately the results in clinical settings are not so favorable (Fig I.5). The limited success of the anti-antigenic approaches may be also related to the complexity of tumors vascularization processes, as the angiogenic-like phenomena (see section 2.3.2.) and the recruitment of EPCs (see section 2.3.1.) (Auguste *et al.*, 2005; Yoder, 2012; Frentzas *et al.*, 2016; Haider *et al.*, 2017). A better understanding on how tumors become vascularized and how they escape from anti-angiogenic therapy will for sure open new perspectives for the development of more effective anti-angiogenic approaches.

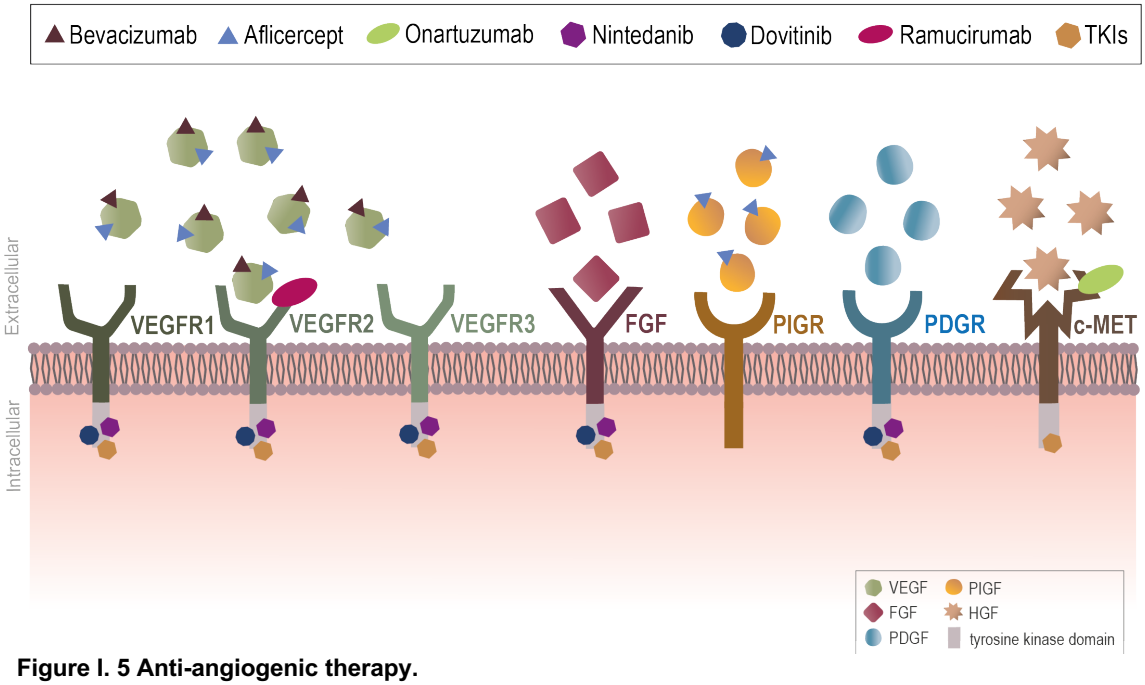


Figure I. 5 Anti-angiogenic therapy.

Bevacizumab was the first anti-angiogenic drug approved for human use, acting as a VEGF decoy agent (Kopetz *et al.*, 2010; Lieu *et al.*, 2013), however its use as monotherapy is clinically ineffective and when combined with conventional therapy only provides a modest survival benefit (Argiris *et al.*, 2013; Ferrara and Adamis, 2016; Mortimer *et al.*, 2012; Sennino and McDonald, 2012). Nowadays, novel drugs that block other effectors in angiogenic signaling pathways have become available as an attempt to improve the clinical efficacy of anti-angiogenic therapies. Aflibercept (decoy receptor for VEGF and PlGF) (Gomez-Manzano *et al.*, 2008), bovitinib (block VEGF, FGF and PDGFR), nintedanib (block VEGFR, FGFR and PDGFR) and ramucirumab (human Ig-G1 monoclonal antibody targeting the extracellular domain of VEGFR2) although are associated to less severe adverse drug reactions than bevacizumab their clinical results are not very promising (Norden *et al.*, 2015; Semrad *et al.*, 2017; Van Cutsem *et al.*, 2012; Wilke *et al.*, 2014). Tumors with resistance to bevacizumab increase the expression of c-MET, although the dual administration of onartuzumab (anti-c-MET) and bevacizumab has no additional clinical benefits (Cloughesy *et al.*, 2017; Jahangiri *et al.*, 2013). The use of TKIs downmodulates VEGF/VEGFR axis. However, due to adverse drug reactions unrelated to VEGF-blockage, the clinical resistance and toxicity of this drugs did not offered advantage over VEGF targeting (Bhargava and Robinson, 2011; Levitzki, 2013; K. D. Miller *et al.*, 2005b; H. Sun *et al.*, 2014a). FGF: fibroblast growth factor; FGFR: fibroblast growth factor receptor; HMG: hepatocyte growth factor; PDGF: platelet-derived growth factor; PDGFR: platelet-derived growth factor receptor; PlGF: placental growth factor; PlGFR: platelet-derived growth factor receptor; TKIs: tyrosine kinase inhibitors; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor.

3.1. The future in the use of classical anti-angiogenic agents: is it clear which is the best way to go?

In the scientific community there is still a debate in the paradox of the usefulness of anti-angiogenic therapy, since some authors defend that the abolishment of tumor blood supply would deaccelerate tumor growth, while others argue that those strategies would restrict drug delivery to the tumor, disabling clinical management. So far, it is difficult to elect the risks and the benefits of an improved anti-angiogenic therapy for cancer patients. A better understanding of the molecular mechanisms underlying neoangiogenesis and the role of TME components in its control will be pivotal to unravel new druggable biological targets and to design more efficient anti-angiogenic agents. Another fragility in this context is the lack of predictive biomarkers for anti-angiogenic treatment response (sensitivity *versus* resistance). For instance, circulating biomarkers (*e.g.* concentration of soluble angiogenic factors), genetic biomarkers (*e.g.* VEGF gene polymorphisms), tissue biomarkers (*e.g.* immunohistochemical staining of angiogenic receptors) and systemic biomarkers (*e.g.* changes in system blood flow), would be fulcral for the stratification of patients that would or not benefit from the anti-angiogenic therapy (Jain *et al.*, 2009; Wehland *et al.*, 2013).

4. The potential use of other therapeutic strategies targeting cancer cells and disturbing angiogenesis

The clinical results of the use of anti-angiogenic compounds, alone and in combination with conventional cancer therapy, are far from the remarkable successes obtained in pre-clinical settings. Since the efficacy and mechanism of action of the anti-

angiogenic drugs (inhibition *vs* normalization of tumor vasculature) is still a matter of debate, new efforts have been made for the development of new compounds with both anti-tumor and anti-angiogenic properties. For instance, ivermectin, a FDA-approved anti-parasitic drug for the treatment of intestinal worm infections, inhibit ovarian, breast and glioma cancer cells growth and promote cancer cell death (Hashimoto *et al.*, 2010; Draganov *et al.*, 2015; Dou *et al.*, 2016; Yingying Liu *et al.*, 2016) while it also targets angiogenesis through the inhibition of capillary network formation, proliferation and survival of human brain microvascular ECs (Liu *et al.*, 2016). In addition, carnosol and carnosic acid, the major components of rosemary extracts, inhibits tumor cell growth and affects several steps of the angiogenesis process, as EC differentiation, proliferation, migration and proteolytic capability (López-Jiménez *et al.*, 2013). Other compounds, as fucoidan and sulfated galactofucan have been shown to reduce tumor growth and inhibit tumor angiogenesis, in part through the inhibition of STAT3-regulated genes, as VEGF, Bcl-xL and cyclin D1 (Rui *et al.*, 2017; Jin *et al.*, 2019).

4.1. ROS-related drugs: a double-edge sword

The metabolic remodeling of tumor and TASCs drive the generation of a pro-oxidant rich-microenvironment, which in turn, favors tumor angiogenesis (Szatrowski and Nathan, 1991; Josson *et al.*, 2010; Martinez-Outschoorn *et al.*, 2011). The generation of a pro-oxidant and a pro-angiogenic TME seems to work synergistically in the promotion of the angiogenic switch and further tumor angiogenesis (Dewhirst *et al.*, 2008). The generation of ROS levels in cancer cells prompted by 27-hydroxycholesterol (27HC) and deferoxamine (DFO) activates STAT-3/VEGF and ERK1/2 / HIF1α signaling pathways, promoting tumor angiogenesis and metastasis (Liu *et al.*, 2014; Zhu *et al.*, 2016). Moreover, in human colon carcinoma cells the mutant p53 triggers angiogenesis through the ROS-mediated activation of VEGF and HiF1α (Khromova *et al.*, 2009).

The modulation of the ROS levels in cancer treatment is a double-edged sword. In one hand, the generation of a pro-oxidant microenvironment in early stages of tumor development and at moderate and non-toxic levels, activates cancer cell survival signaling cascades (*e.g.* MAPK/ERK1/2, p38, c-Jun N-terminal kinase (JNK), PI3K/Akt) and prompt tumor angiogenesis through the release of pro-angiogenic factors (*e.g.* VEGF, FGF) and ECM degrading enzymes (*e.g.* MMPs). On the other hand, the generation of high and toxic ROS levels by chemotherapy and radiotherapy promotes oxidative stress and further cell death and senescence (Assi, 2017; Raza *et al.*, 2017; Kumari *et al.*, 2018; Thyagarajan and Sahu, 2018; Aggarwal *et al.*, 2019). The *in vitro* exposure of breast cancer cells to the antioxidant resveratrol reduce the ROS accumulation, decreasing paclitaxel-induced cell death (Alexandre *et al.*, 2006; Fukui *et*

al., 2010). The tamoxifen-induced cytotoxicity in MCF-7 breast cancer cells was regulated by the intracellular concentration of the antioxidant vitamin C, decreasing the levels of ROS and lipid peroxides (Subramani *et al.*, 2014).

As referred, ROS generation in the TME, depending on the levels, can exerts pro-tumorigenic and pro-angiogenic effects or drive oxidative stress-mediated cell death, pointing out that strategies focused on the modulation of ROS levels and oxidative stress to impair tumor progression, angiogenesis and metastasis need to be carefully monitored. Baicalein, a phenolic flavonoid compound with antioxidant properties, inhibits cancer proliferation and migration, induces cancer cell death and disrupts the development of tumor vasculature (Kang *et al.*, 2012; Park *et al.*, 2017; Park *et al.*, 2019;). *OptiBerry*, a anthocyanins-rich berry extract with antioxidant properties, showed to inhibit H_2O_2 and TNFα-induced VEGF expression by keratinocytes and to diminish the ability of ECs to form hemangioma, suggesting a putative anti-angiogenic, antioxidant and anti-cancer effect (Bagchi *et al.*, 2004). Other compounds have also shown strong ROS scavenging activities and anti-cancer activity, as the polyphenol from *T. pallida* and white mulberry (*Morus alba*) (Alam *et al.*, 2016). However, the effects of this compound on the tumor vasculature have not been evaluated so far. Contradictory information comes from studies suggesting that strategies focused on the generation of ROS and further oxidative stress induce ECs dysfunction, impairing tumor angiogenesis (Incalza *et al.*, 2018; Yang *et al.*, 2019). Although most of the studies supports the angiogenic potential of ROS as a therapeutic target for anti-cancer and anti-angiogenic therapy, the effects of those strategies on tumor angiogenesis and their mechanisms of action not only in ECs, but also in the crosstalk between EC:cancer cells need to be explored. Further research to a better understanding of disease-specific ROS involvement and their potential as anti-tumor strategy will be pivotal for the discovery of new therapeutic targets and further drug development for cancer treatment.

4.2. β-adrenergic drugs: repurposing existing drugs for anti-cancer and anti-angiogenic clinical purposes

The activation of the β-adrenergic system by catecholamines, epinephrine and norepinephrine, has been related to the tumorigenic processes, as cancer cell proliferation and apoptosis and also with vascular events, as angiogenesis (Hulsurkar *et al.*, 2017; Zhao and Li, 2019). In ovarian cancer patients under social isolation, the increased levels of noradrenaline are correlated with tumor grade and stage, suggesting the contribution of the β-adrenergic receptors (AR) system in cancer progression (Lutgendorf *et al.*, 2011). Chronic behavioral stress hormones, as adrenaline and/or

noradrenaline, promotes pancreas, breast, colorectum and prostate cancer progression (Sastry *et al.*, 2007; Hermes *et al.*, 2009; Wong *et al.*, 2011; Schuller *et al.*, 2012; Hulsurkar *et al.*, 2017;). More recently, the expression of β2-AR has been proposed as a useful and novel prognostic factor for patients with clear cell renal carcinoma (Ha *et al.*, 2019). Besides the biobehavioral factors that contribute to the activation of β-AR system, some cancer cells (*e.g.* pancreatic, lung, colon) are able to synthesize and release adrenaline (Wong *et al.*, 2007; Al-Wadei and Schuller, 2009).

β-AR signaling activation via adrenaline and/or noradrenaline is implicated in the promotion of angiogenesis, through the upregulation of pro-angiogenic factors, as VEGF, IL6, IL8 and MMP2 and 9 and by the upregulation of VEGFR2 (Chen *et al.*, 2014b; Garg *et al.*, 2017; Chakroborty *et al.*, 2020;). In prostate cancer xenograft models, β-AR signaling promote tumor angiogenesis (Hulsurkar *et al.*, 2017). The crosstalk between ECs and cancer cells potentiates tumor angiogenesis mediated by β-AR signaling pathway, inducing the EC activation of Jagged1/Notch intercellular signaling and the metabolic shift of ECs from oxidative phosphorylation to aerobic glycolysis, which represents a critical step during the angiogenic switch (Chen *et al.*, 2014a; Zahalka *et al.*, 2017). Interestingly, in a mice model of ovarian cancer, dopamine administration (antagonize the effect of stress hormones) inhibits tumor angiogenesis and stabilizes the already formed tumor vasculature, enhancing cisplatin delivery and efficacy (Moreno-Smith *et al.*, 2013). These results reinforce that the activation of the AR signaling pathway by catecholamines might be a key event in the tumor angiogenesis cascade.

Considering the modulatory role of β-AR signaling in tumorigenesis, β-blockers showed a promising anti-angiogenic and anti-cancer therapeutic value. Preclinical and retrospective studies highlighted the beneficial actions of β-blockers administration in cancer patients, improving the relapse free survival and decreasing the tumor recurrence, cancer-specific mortality and metastasis (Powe *et al.*, 2010; Barron *et al.*, 2011). Moreover, the efficacy of Propranolol, a non-selective β-AR antagonist (in some tissues inverse agonist), that acts by competing with catecholamines for the binding to β-adrenergic receptors, was shown to be effective in the treatment of hemangioma, the most common infantile benign tumor that involves the accumulation, proliferation and differentiation of aberrant vascular structures (Lowenthal *et al.*, 1984; Sans *et al.*, 2009; Chim *et al.*, 2012; Chen *et al.*, 2017). Propranolol demonstrated anti-cancer and anti-angiogenic pharmacological properties since its administration in breast cancer patients with arterial hypertension significantly reduced the primary tumor development, nodal/metastatic occurrence and breast cancer-specific mortality (Barron *et al.*, 2011); abrogated the VEGF production (Park *et al.*, 2011); inhibited the noradrenaline-induced HIF1α expression in cancer cells (Park *et al.*, 2011) and inhibited

the catecholamine-induced signaling between macrophages and ECs (Xia *et al.*, 2019). In neuroblastoma, β-blockers (carvedilol, nebivolol and propranolol) independently of their selectivity, promoted vincristine-induced tumor regression, in part mediated by the inhibition of tumor angiogenesis (Pasquier *et al.*, 2013). Controversially, an *ex vivo* study using aortic rings showed that β-blockers pro-angiogenic or anti-angiogenic activity is independent of their ability to antagonize catecholamine action. For instance, forskolin (β-AR agonist) a direct activator o adenylate cyclase that increased AMPc production through β-signaling activation decreases VEGF-mediated microvessel sprouting while increases were observed with Propranolol (1st generation, non-selective β-AR), metoprolol and bisoprolol (2nd generation, β1-AR–selective antagonists) while carvedilol (3th generation, a nonselective β-AR antagonist) was unable to affect aortic sprouting (Stati *et al.*, 2014).

Although it has been suggested that β-blockers could be a putative anti-angiogenic drug, some studies have failed to observe this association, remaining unclear how β-blockers mechanistically affects and impairs tumor angiogenesis. Moreover, as referred, previous studies demonstrated that inhibitory neurotransmitters induce tumor vessel normalization, which might suggest that the inhibition of the β-adrenergic system by β-blockers will, in addition to the impairment of tumor angiogenesis, promote tumor vessel normalization, which in turn will increase the efficacy of the delivery of anti-cancer drugs. The favorable safety profile, the low cost and immediate clinical availability, together with the putative cancer patient welfares point the benefits of repositioning these old drugs for new clinical purposes.

5. Rationale, hypothesis, aims and thesis outline

Until 1971, it was thought that tumor survival and growth rely on oxygen and nutrients provided by the preexisting blood vasculature. However, in that time, Judy Folkman switch the paradox by showing that neoangiogenesis is pivotal for tumor growth, impacting tumor malignancy, aggressiveness and metastasis (Folkman, 1971; Folkman and Hanahan, 1991; Yadav *et al.*, 2015; Marmé, 2018;). The contribution of EPCs for the angiogenic processes has been already recognized, however, their inaccurate identification due to the lack of specific markers (Del Papa *et al.*, 2006; Allanore *et al.*, 2007; Avouac *et al.*, 2008; Nevskaya *et al.*, 2008; Richardson and Yoder, 2011; Yoder, 2012; Yuan *et al.*, 2017) may misjudge that other cell subtypes can act as EPCs. Our group have demonstrated that some cells in the vessel wall display an intermediate phenotype that fits with the differentiation route of monocytes into endothelium

(Domingues *et al.*, 2015a), leading us to explore if monocytes are underestimate sources of EPCs that can contribute for tumor angiogenesis.

During tumorigenesis, the metabolic remodeling of cancer cells drives the generation of a pro-oxidant rich TME, that in turn, act as a pro-angiogenic stimulus that switch ECs from a resting state (quiescence) to a rapid proliferative phase (Szatrowski and Nathan, 1991; Josson *et al.*, 2010; Martinez-Outschoorn *et al.*, 2011). ROS-dependent generation of lipid peroxidation triggers ferroptosis, that although was firstly described as an iron-dependent cell death process, nowadays it is revealed to contribute to the regulation of biological and pathophysiological processes (Szatrowski and Nathan, 1991; Josson *et al.*, 2010; Martinez-Outschoorn *et al.*, 2011).

Additionality, and considering that the effects of ferroptosis on ECs (dys)function and further regulation of angiogenesis have not been explored yet, we investigated if ferroptosis can act as a pro-angiogenic stimulus, promoting ECs activation and further angiogenesis. Moreover, Propranolol, a non-selective β-blocker, was shown to be efficient in the treatment of some vascular tumors, such as hemangiomas and cavernomas (Storch and Hoeger, 2010; Zabramski *et al.*, 2016a; Rotter and de Oliveira, 2017; Apra *et al.*, 2019). Propranolol administration also correlates with a better prognosis in cancer patients, it has been described as having a modulatory role in monocytic differentiation and anti-ferroptotic properties (Cardwell *et al.*, 2016; Pantziarka *et al.*, 2018; Hiller *et al.*, 2020; Mishima *et al.*, 2020). Taking all of this in consideration and since the clinical use of anti-angiogenic therapies have shown a limited of efficacy, we investigated whether Propranolol could act as an anti-angiogenic alternative drug in solid tumors.

Hence, our hypotheses are that monocytes are EPCs and that the anti-angiogenic effect of Propranolol relies on the abrogation of oxidative stress-related ferroptosis that sustains EC activation. Thus, to investigate this hypothesis the following specific objectives were purposed and are directly linked to the experimental and results chapters:

1. To disclose if monocytes act as EPCs and if ROS can trigger this process, contributing to cancer angiogenesis (chapter 2);

2. To understand if Propranolol administration in a child with cerebral cavernous malformation (CCM) has a role in circulating monocytes functioning as EPCs and their impact in the clinical outcome (chapter 3);

3. To unravel if ferroptosis has a role in ECs activation and in the differentiation pattern of monocytes functioning as EPCs and if Propranolol can interfere with these processes (chapter 4);

4. To explore if a dual anti-tumoral and anti-angiogenic strategy using SeChry-PUREG4 and Propranolol could be an alternative for cancer treatment (chapter 5).

This thesis is organized into 6 chapters. Chapter 1 presents a general introduction in order to provide insights into the theorical concepts addressed in the next chapters. Chapters 2 to 5 incorporate the experimental results obtained during the PhD project, being each chapter composed by an abstract, a short introduction, materials and methods, results and discussion. Chapter 6 comprises the general discussion and the main conclusions of this PhD project, highlighting the major contributions in understanding the processes underlying tumor angiogenesis and putative alternative anti-angiogenic and anti-tumoral strategies to improve cancer treatment. Moreover, in this last chapter we also included the future perspectives, in order to answer some research questions raised during this research project.

Chapter II

Monocytes as endothelial precursor cells (EPCs), another brick in the wall to disentangle tumor angiogenesis

This chapter was based on:

Lopes-Coelho F, Silva F, Gouveia-Fernandes S, Martins C, Lopes N, Domingues G, Brito C, Almeida AM, Pereira SA, Serpa J. Monocytes as Endothelial Progenitor Cells (EPCs), Another Brick in the Wall to Disentangle Tumor Angiogenesis. Cells. 2020 Jan 1;9(1):107. doi: 10.3390/cells9010107

Abstract

Endothelial progenitor cells (EPCs), upon pro-angiogenic stimuli, migrate and differentiate into endothelial cells (ECs) and contribute to re-endothelialization and neo-vascularization. Unfortunately, there are no specific markers to characterize EPCs, leading to their inaccurate identification. In a previous work, we showed in a panel of tumors and normal tissues that some cells on the vessel wall co-expressed CD14 (monocytic marker) and CD31 (EC marker), indicating a putative differentiation route of monocytes into ECs. Herein, using *in vitro* and *in vivo* models we unraveled monocytes as potential EPCs, in non-pathological and in cancer context. Upon a pro-angiogenic stimuli, monocytes acquired the expression of ECs markers and were able to incorporate blood vessels, contributing to cancer progression, in part, by incorporating the tumor neo-vasculature. Reactive oxygen species (ROS) pushed monocytes to ECs differentiation route and this phenotype could be reverted by cysteine (scavenger and precursor of glutathione) exposure, indicating that angiogenesis is controlled by the interplay between the oxidative stress and the scavenging capacity of tumor microenvironment.

Keywords: monocytes, endothelial progenitor cells (EPCs), endothelial cells (ECs), reactive oxygen species (ROS), angiogenesis, cancer

Introduction

Angiogenesis is essential during carcinogenesis, by promoting tumor growth, cell invasion and ultimately metastasis (Yadav *et al.*, 2015; Marmé, 2018). Tumor neovessels formation is essential to provide oxygen and nutrients to cancer cells and to remove waste products from tumor microenvironment (TME) (Yadav *et al.*, 2015; Marmé, 2018). The efficacy of anti-angiogenic therapies in cancer treatment has fallen short of expectations (Vasudev and Reynolds, 2014), showing that the mechanisms underlying neovascularization in cancer are not fully understood. This failure can be explained, at least in part, by the fact that the specific origin of EPCs is not yet determined and also because these therapies are focused on the disruption of pre-existent blood vessels, underestimating the role of EPCs in vascularization.

Over the last decade, it was demonstrated that EPCs are essential for restoring injured vessels. EPCs arise from a subset of hematopoietic stem cells in bone marrow and, upon a pro-angiogenic stimuli, they proliferate, migrate and differentiate into ECs (Richardson and Yoder, 2011; Yoder, 2012; J.Yuan *et al.*, 2017). Some reports focusing on EPCs and disease, like systemic sclerosis, showed contradictory and discrepant results about EPCs mobilization and further differentiation, in part because there is a lack of a precise panel of cell surface markers used for the characterization of this cell subset (Del Papa *et al.*, 2006; Allanore *et al.*, 2007; Avouac *et al.*, 2008; Nevskaya *et al.*, 2008; Richardson and Yoder, 2011; Yoder, 2012; Yuan *et al.*, 2017). In mouse embryonic vascular endothelium, erythro-myeloid progenitors (EMP) can differentiate into ECs (Plein *et al.*, 2018) and in a mouse model of carotid injury, monocytes ($CD14^+$ cells) are capable of improving re-endothelialization (Urbich *et al.*, 2003). Moreover, using *in vivo* and *in vitro* models targeting Tie2 monocytes decrease angiogenesis by regulating ECs proliferation (Lewis *et al.*, 2007; Mazzieri *et al.*, 2011; Nucera *et al.*, 2011) and *in vivo* chemokine (C-C motif) receptor 2 (CCR2) knockout impairs monocytes recruitment and VEGF expression, accompanied by a reduction in the angiogenesis rate (Willenborg *et al.*, 2012). The release of cytokines and pro-angiogenic factors (*e.g.,* VEGF, tumor necrosis factor α (TNFα), interleukin 8 (IL8) and fibroblast growth factor (FGF) and extracellular matrix (ECM) modifying proteins (*e.g.* metalloproteinase-9 (MMP9)) by macrophages enhance tissue ability to support capillary sprouting and vascular density (Zumsteg and Christofori, 2009; Deryugina and Quigley, 2015). The precise mechanism by which monocytes influence angiogenesis in tissue development, homeostasis and diseases is not fully understood. However, different studies, have shown that under *in vitro* pro-angiogenic pressure, blood mononuclear cells can acquire endothelial markers and morphology (Schmeisser *et al.*, 2001; Yoder *et al.*, 2007; Fadini *et al.*, 2012). In a previous study we showed that some ECs simultaneously expressed CD14 (monocytic

marker) and CD31 (EC marker), indicating mixed features between monocytes and ECs, in tumors and normal tissues (Domingues *et al.*, 2015a). Together, these scientific evidence, allied to the fact that EPCs are not a well unraveled cell type, indicate monocytes as a promising candidate to these heterogeneous endothelial-supplier cells.

In ECs, reactive oxygen species (ROS) seem to be needed for the maintenance of endothelium homeostasis by acting as a physiological regulator of intracellular signaling, although a redox imbalance can lead to vascular diseases (Cai, 2005; Coyle and Kader, 2007; Park, 2013; Bretón-Romero and Lamas, 2014; Satoh *et al.*, 2014; Qishan Chen *et al.*, 2018). In immature cells, as rat multipotent adult progenitor cells (MAPCs), bone marrow stem cells and EPCs, H_2O_2 (ROS) exposure inhibits ECs differentiation (Wang *et al.*, 2013; Chaudhari *et al.*, 2014; Xiao *et al.*, 2014). However, Zhou et al., (2018) reported that non-small cell lung cancer cells upon activation with H_2O_2 suffer EC-like differentiation, mediated by ETS-1 (E26 transformation specific-1) (Wilson *et al.*, 2005; Zhou *et al.*, 2018). This contradiction amongst biological models takes us to believe that the effects of ROS will depend on the cell lineage and its differentiation level.

Here we explore the role of monocytes as EPCs and their capacity of forming blood vessels triggered by ROS.

Materials and methods

Monocytes isolation and culture

Monocytes were isolated from peripheral blood (PB) collected under consent donation of healthy donors from *Serviço de Imuno-Hemoterapia* at *Instituto Português de Oncologia de Lisboa Francisco Gentil* (IPOLFG) (IPOLFG-Ethical committee UIC-1137). PB mononuclear cells (PBMCs) from blood samples were separated using Histopaque-1077 (10771, Sigma-Aldrich), followed by magnetic monocytes isolation using Monocyte isolation kit II (130-091-153, MACS Technology - MiltenyiBiotec), according to the manufacturers' protocols. Monocytes were cultured in plates coated with 0.2% of gelatine (G-1890, Sigma-Aldrich) or with Matrigel (354230, Corning) and maintained in colony-forming unit (CFU) medium (130-091-277, MACS Technology – MiltenyiBiotec) or endothelial basal medium 2 (EBM-2; CC-3156, Lonza) plus EGMTM-2 SingleQuotsTM Supplements (CC-4176, Lonza) and with 2% fetal bovine serum (FBS; CC4101A, Lonza), 50 ng/mL vascular endothelial growth factor (VEGF; V7259, Sigma-Aldrich) and 10 U/mL heparin (H3149, Sigma- Aldrich). Cells were maintained at 37°C, in a humidified atmosphere and 0.5% CO_2. 15 μM hydrogen peroxide (H_2O_2; 1.07210.0250, Merck) was used as ROS generator, 0.4 mM cysteine (Cys; 7048-04-6,

Merck) was used as antioxidant and 2 μM disulfiram as aldehyde dehydrogenase (ALDH) inhibitor (86720, Fluka).

Cell culture

Human umbilical vein ECs (HUVEC; ATCC ® CRL-1730™) were seeded in plates coated with 0.2% gelatine and cultured in EBM-2 medium supplemented with 2% FBS. Breast cancer cells (MDA-MB-231; ATCC® HTB-26™, and HCC1954; ATCC® CRL 2338™) were cultured, respectively, in DMEM (11965-092, Gibco - Thermo Fisher Scientific) supplemented with 10% FBS and 1% Antibiotic-Antimycotic (15240062, Invitrogen™ - Thermo Fisher Scientific) and RPMI1640, no phenol red (#11835-063, Invitrogen) supplemented with 10% FBS, 1% Penicillin and streptavidin (15140-163, Gibco - Thermo Fisher Scientific), 0.5mL 2-β-Mercaptoetanol (21985-023, Gibco - Thermo Fisher Scientific) and 3mL HEPES (15630-056, Gibco - Thermo Fisher Scientific) (respectively). Cells were maintained at 37°C, in a humidified atmosphere and 5% CO_2 and were detached with 0.05% Trypsin-EDTA 1X (25300-054, Invitrogen, Thermo Fisher Scientific) at 37°C for approximately 5 minutes (min). For each assay a Bürker counting chamber was used to determine the cell number.

Cell characterization by flow cytometry

Adherent monocytes-derived cells were detached with 2 mM EDTA-Phosphate-buffered saline (PBS; v/v) and incubated with anti-CD14 (1:100; 555397, BD Bioscience), anti-CD31 (1:100; FAB3567A, R&D Systems), anti-VE-Cadherin (1:100; FAB1002A, R&D Systems), anti-KDR (1:100; FAB357A, R&D Systems), anti-CD68 (1:100; 333810, Biolegend), anti-CD80 (1:100; 305206, BioLegend) and anti-CD163 (1:100; 333626, Biolegend), in 0.5% PBS-Bovine serum albumin (BSA; A9647, Sigma-Aldrich) (v/w), for 20 min, in dark at 4 ºC. Von Willebrand factor (vWF; factor VIII) detection was performed with anti-vWF (1:500; A0082, Dako – Agilent), for 60 min at 4 ºC with gently shaking. After incubation, cells were rinsed and re-incubated with a secondary antibody (Alexa Fluor® 488 anti-rabbit – A-11078, Invitrogen - Thermo Fisher Scientific) in 0.5% PBS-BSA, for 30 min at 4 ºC and in the dark with gently shaking. The immunolabelling was evaluated by flow cytometry (FACScalibur – Becton Dickinson) and data were analysed using *FlowJo X* v10.0.7 software (*https://www.flowjo.com/*).

Reverse transcription and quantifying PCR (RT-qPCR)

Total RNA was extracted using RNeasy Mini Extraction kit (74,104; Qiagen) and cDNA was synthesized using SuperScript II Reverse Transcriptase (18080e44,

Invitrogen - Thermo Fisher Scientific), according to the manufacturer's protocol. Relative quantification using qPCR was performed using Power SYBR Green PCR Master Mix (4367659, Applied Biosystems - Thermo Fisher Scientific), according to manufacturer's instructions, and carried out in a LightCycler 480 instrument (Roche). The primers used are presented in Table 1.

Table 1 List of primers used for RQ-PCR

Primer	Foward	Reverse
CXCR4	CTCCAAGCTGTCACACTCCA	TCGATGCTGATCCCAATGTA
CSF-1	GTCTTCCACCTGCTGGTGC	CCCTCTGGTTGCTCCAAGG
FLT1	CACCAAGAGCGACGTGTG	TTTTGGGTCTCTGTGCCAG
Hes1	ACGACACCGGATAAACCAAA	CGGAGGTGCTTCACTGTCAT
Ang1	GGGGAGGTTGGACTGTAATAC	GCATGTACTGCCTCTGACTG
Dll1	ATGCCTTCGGCCACTTCAC	CACATCCAGGCAGGCAGAT
Notch1	TGGCGGGAAGTGTGAAGCGG	GTGCTGAGGCACGGGTTGGG
Notch2	CCACAGGTGTCAGAATGGAG	GGCATTCATCCACATCCTCTG
Hey1	GACGAGACCGGATCAATAACAG	GGTCATCTGCAGGATCTCG
Hey2	GAGCGAGAACAATTACTCGGG	GTTATTTATCCGATCCCGACGC
FGFR4	GATGCTCAAAGACAACGCCTC	GACACCAAGCAGGTTGATGATG
18S	GCCCTATCAACTTTCGATGGT	CCGGAATCGAACCCTGATT

Nuclear magnetic resonance (NMR)

Monocytes were harvested with 1X PBS (washed twice), scraped and centrifuged at 155 *g* for 10 min. In cell extracts the methanol and chloroform was used to separate organic and aqueous phases. After cold methanol mixture (4 mL methanol/ 1 g weight pellet), 1 volume of water was added, mixed, and incubated for 5min on ice. Chloroform (1 volume) was added to the sample and mixed. Then, 1 volume of water was added and samples were incubated for 10 min on ice, following centrifugation at 4000 rpm for 15 min at 4 °C. Aqueous (upper) and organic (lower) phases were collected. Lyophilization of the aqueous phase was performed using Speed Vac Plus Scllon and then dissolved in deuterated water (D2O) and 4 % (v/v) sodium azide (NaN3)/4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) solution (1:10). 1H spectra were obtained in a magnetic field of 500 MHz in UltrashiedTM 500 Plus (Bruker) and compound identification was performed by resorting to the Human Metabolome database (HMDB) (http://www.hmdb.ca/) and ChemomxNMRsuite 7.6.

3D co-culture and microencapsulation

For the co-cultures, HCC1954 cells were inoculated at a density of $0.2x10^6$ cell/mL in a 125mL impeller spinner vessel (Corning) and aggregated at 80rpm for 4 days. The

spheroids were then co-cultured with human monocytes and encapsulated in 1.1% (w/v) Ultra-Pure Ca2+ MVG alginate (UP MVG NovaMatrix, Pronova Biomedical), prepared in NaCl 0.9% (w/v). The alginate microencapsulation was performed on VARV1 encapsulator (Nisco, Zurich, Switzerland) to obtain capsules of approximately 700µm and the polymerization occurred in a 20mM BaCl2 solution. After polymerization, the capsules were washed in 0.9% sodium chloride (NaCl; w/v) and cultured in RPMI 1640, in a 125mL vented cap Erlenmeyers (Corning) at 100rpm, in orbital agitation. After culture, samples were collected and fixed in 4% (w/v) formaldehyde with 4% (w/v) sucrose in 1X PBS for 20 min. Then, samples were dehydrated in 30% (w/v) sucrose for approximately 5 hours (h), embedded in Tissue-Tek® O.C.T. (Sakura) and frozen at -80˚C for cryosectioning. The frozen samples were sliced with a thickness of 10µm in a cryostat (Cryostat CM 3050 S, Leica).

Murine (BALB-c\SCID) model of neo-angiogenesis

The animal handling and experimental procedures were performed under the rules of Federation for Laboratory Animal Science Associations (FELASA), accomplishing the 3Rs through evidence-based guidelines. Female BALB-c/SCID mice (8 weeks) were maintained in a pathogen-free barrier room in the Animal Care Facility at Chronic Diseases Research Center (CEDOC)|NOVA-Medical School (NMS) (ethical committee NOVA Medical School Ref. 75/2019/CEFCM; DGAV Ref#0421/000/00/2016 - PI Duarte Barral).

In the plug assay, monocytes isolated from the PB of healthy donors ($1x10^6$ cells/mice) were previously cultured for 24 h in EBM-2 supplemented with 50 ng/mL VEGF plus 10 U/mL heparin. After, cells were embedded in 400 µL of matrigel supplemented with 50 ng/mL VEGF and subcutaneously inoculated in the right flank of the mice for 21 days. Control groups were inoculated with matrigel supplemented with VEGF. Plugs were removed by surgery and fixed with 2% paraformaldehyde for 10 min, followed by successive 15 min incubations in 100%, 95% and 60% ethanol and then processed into paraffin-embedded blocks.

Breast tumors were induced by inoculation of MDA-MB-231 cells in the mammary fat pad. Female mice were injected with 50 µL of matrigel with breast cancer cells (MDA-MB-231 - $1x10^6$ cells/mice) alone and in combination with monocytes previously cultured for 24 h in EBM-2 supplemented with 50 ng/mL VEGF (MDA-MB-231 - $1x10^6$ cells/mice plus monocytes $1x10^4$ cells/mice). Tumors were removed by surgery 45 days after inoculation and fixed with formaldehyde with further inclusion in paraffin-embedded blocks. The tumor volume was calculated using the formula: tumor volume = (length x width^2)/2.

Immunofluorescence

In vitro cells were cultured on glass slides with 0.2% gelatin coating and fixed in 2% paraformaldehyde, for 15 min at 4°C. After blocking, cells were incubated with primary antibodies diluted in 0.1% BSA-PBS (v/w), at 4 ºC overnight (1:100 -CD14, ab181470, Abcam; 1:50 - CD31, SCR023, Merck Millipore; 1:500 – vWF, SCR023, Merck Millipore; 1:500 - CD146, SCR023, Merck Millipore), followed by an incubation with secondary antibody for 2 h (1:1000 0.1 % BSA-PBS (v/w), Alexa Fluor® 594 goat anti-rabbit, A-11037, Invitrogen - Thermo Fisher Scientific; Alexa Fluor® 488 goat anti-rabbit, A-11078, Invitrogen - Thermo Fisher Scientific; Alexa Fluor® 488 goat anti-mouse, 115-545-003, Invitrogen - Thermo Fisher Scientific), at room temperature.

Paraffin sections (2 µm) were deparaffinized in xylol for 30 min and re-hydrated in decreased concentrations of ethanol (95%, 70%, 30%), followed by an immersion in water and antigen recovery using citrate buffer. Slides were blocked with 5% BSA-PBS (w/v) for 30 min to avoid non-specific interactions, followed by an incubation with 0.3 M glycine-goat serum-0.1% Triton x100, for 30 min. After, slides were incubated with primary antibodies diluted in 1 % BSA-PBS (v/w) (vWF – 1:200, A0082; Dako; FN – 1:50, SAB4500974, Sigma-Aldrich), for 1 h at room temperature followed by an incubation with secondary antibody (1:500 in 1% BSA-PBS (v/w); Alexa Fluor® 488 goat anti-rabbit; Alexa Fluor® 594 goat anti-rabbit - A-11037, Invitrogen - Thermo Fisher Scientific), for 2 h, at room temperature. Slides were mounted in VECTASHIELD media with DAPI (4′-6-diamidino-2-phenylindole; Vector Labs) and examined by standard fluorescence microscopy using an Axio Imager.Z1 microscope (Zeiss). Images were acquired and processed with CytoVision® software.

Fluorescence *In Situ* Hybridization (FISH)

After immunofluorescence analysis, FISH using an alphoid probe for centromere X (pLAX), and a probe for Yq12 region (pHY2.1) was applied. The X-centromere probe was labeled with digoxigenin (DIG) and the Yq12 probe with biotin, as previously described (C. Martins *et al.*, 2005). Briefly, slides were incubated twice in 2x saline-sodium citrate (SSC) buffer for 5 min at room temperature to wash away the DAPI antifade solution, followed by re-hydration in ethanol series and incubated in citrate buffer, in a water bath for 10 min at 90°C. Enzymatic digestion was carried out with 4mg/mL pepsin in 0.2N HCl (pH 2.0) for 30 min at 37 °C. After that, 10 µL diluted probes were co-denatured with the tissues at 90°C for 5 min and hybridized at 37 °C, overnight. Post-hybridization wash was performed in 2×SSC/0.3%NP-40 for 3 min at 73°C followed by a wash in 2×SSC/0.1%NP-40 for 2min at room temperature. X-centromere DIG-

labeled probe was detected by anti-digoxigenin-FITC (Anti-digoxigenin- fluorescein, Fab fragments; 11270741910, Roche), biotinylated Yq12 probe was detected by Cy3-streptavidin (CyTM3-conjugated Streptavidin; 016-160-084, Jacksons Lab), and nuclei were counterstained with DAPI-vectashield mounting solution. FISH signals, in previously recorded immunofluorescence vWF and FN positive cells, were captured and analyzed using a Zeiss Epifluorescence microscope linked to a Cytovision FISH Probe software program (Cytovision version 7.4, Leica Biosystems, Richmond, VA, USA).

Immunohistochemistry (IHC)

Paraffin sections (2 µm) from paraffin-embedded tissue blocks were deparaffinized, re-hydrated, as mentioned above, and antigen recovery was performed using citrate buffer. Slides were rinsed with 1x Tris Buffered Saline (TBS) followed by the blockage of endogenous peroxidase (K4011, Dako) for 10 min. Anti-vWF (1:200 in IHC diluent) was incubated for 3 h and anti- fibronectin (FN; 1:50 in IHC diluent, RE7133-CE, Leica) for 1h, at room temperature. Staining was performed using ABC detection kit (ab93677, Merck Millipore), according to the manufacturer's protocol. For nuclear staining, slides were immersed in Mayer's Hematoxylin (RE7164, Leica) for 5 min. Slides were dehydrated and mounted with Entellan (1079610500, Merck) and digitalized using Aperio ImageScope - Pathology Slide Viewing software version 12.4.

Data statistical analysis

Data were analyzed using t-test and one-way ANOVA tests in GraphPad Prism 7 software. Statistically significant changes were determined at the p value ≤ 0.05.

Results

In vitro monocytes cultured upon a pro-angiogenic stimuli increased the expression of endothelial cell (EC) markers and acquire an EC-like phenotype, suggesting an endothelial differentiation route of monocytes.

Freshly isolated monocytes from healthy donors and HUVECs showed an identical profile of EC and macrophage markers, with the exception of CD14 (monocytic marker) and vWF that were more expressed in monocytes and HUVECs, respectively (Fig II.1 A and B, supplementary Fig II.1). These results pointed out that monocytes cultured in a pro-endothelial medium share molecular features with ECs. Notably, monocytes cultured in CFU media, a media for the maintenance of stem and progenitor cells, had lower

expression of EC and macrophage markers (Fig II.1 A), indicating the maintenance of a resting and more undifferentiated state.

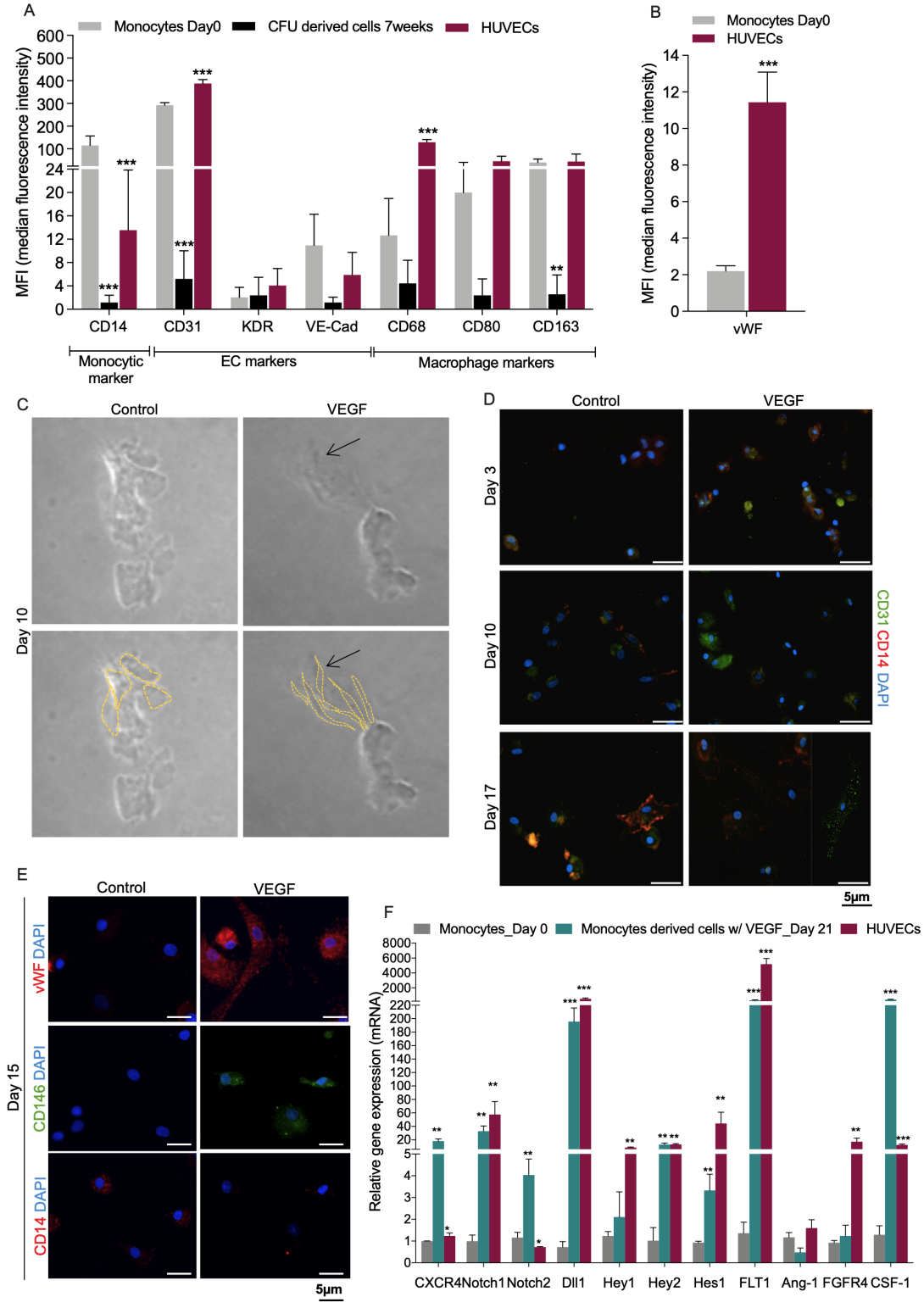


Figure II. 1 Cultured monocytes undergo an increase in the expression of endothelial cells (ECs) markers and acquire spindle cell like morphology, indicating EC differentiation of monocytes.

A) MIF (median intensity fluorescence) values from FACS analysis of CD14 - monocytic marker, CD31, KDR, VE-Cadherin (VE-Cad) – EC markers and CD68, CD80, CD163 – macrophage markers in monocytes

freshly isolated (Day0), monocytes maintained in CFU media and in Human umbilical vein ECs (HUVECs). B) Monocytes cultured for 10 days in Matrigel in EBM-2 medium with or without VEGF. Images taken under optical microscopy, magnification 200x; arrow shows spindle shape cells. C) Immunofluorescence for CD14 (red) and CD31 (green) in monocytes cultured in EBM-2 medium with or without VEGF for 3, 10 and 17 days. DAPI (blue) stains nuclei, magnification 400x. F) Relative quantification of typical endothelial genes in monocytes freshly isolated (Day0), in monocytes-derived cells cultivated for 21 days in the presence of VEGF and in HUVECs. **p<0.01, ***p<0.001

Monocytes cultured in matrigel plus VEGF presented a spindle-cell-like morphology (Fig II.1 B), typical of ECs. In the line with the morphological changes observed in cultured monocytes, a dynamic CD31 gain and CD14 loss was observed during 17 days in culture in the presence of VEGF (Fig II.1 C). Moreover, monocytes cultured with VEGF during 15 days increased the expression of the EC markers vWF and CD146, with a concomitant decreased expression of the monocytic marker CD14 (Fig II.1 E). In addition, VEGF stimulus drives an increase in the expression of EC genes (Fig II.1 F), which is in accordance with the morphological changes observed in monocytes-derived cells (Fig II.1 C).

Reactive oxygen species (ROS) stimulate monocytes differentiation into ECs and cysteine (Cys) plays a role in the control of EC-like differentiation

Short term exposure (30 min) of H_2O_2 (ROS) in monocytes-derived cells promotes a pattern of ECs differentiation, in which monocytes previously maintained in CFU for 4 days (Day 4) and in monocytes maintained in CFU for 4 days plus 1 day in EBM-2 media (Day 5) increased, respectively, 5.9 and 4.4-fold the vWF expression (Fig II.2 A). Moreover, day 5 monocyte derived-cells exposed to H_2O_2 also increased the expression of other ECs markers, as CD31 and CD146, while they lost the expression of monocytic marker (CD14) (Fig II.2 B).

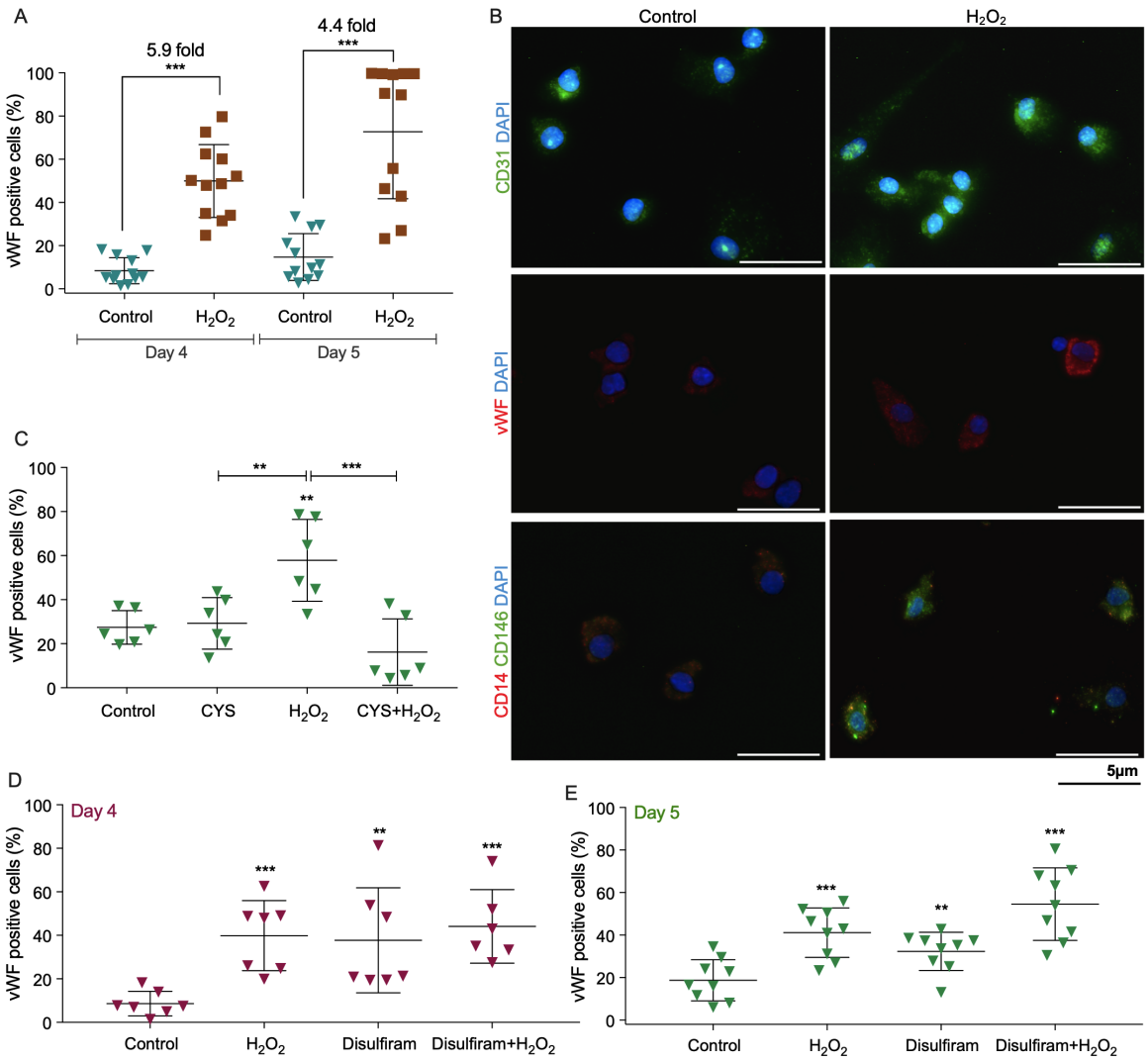


Figure II. 2 Monocyte-derived cells cultured in the presence of hydrogen peroxide (H_2O_2) increase the expression of endothelial cell (EC) markers that is abolished by the presence of cysteine (CYS).

A) vWF levels in monocytes-derived cells maintained in CFU media (Day 4) plus 1 day in EBM-2 plus VEGF (Day 5), in the presence or absence of H_2O_2. B) Immunofluorescence for CD31 (green), vWF (red), CD146 (green) and CD14 (red) in monocytes cultured during 5 days in EBM-2 medium with VEGF, in the presence or absence of H_2O_2. Nuclei is in blue (DAPI), magnification 400x. C) vWF levels in monocytes-derived cells maintained in EBM-2 plus VEGF, in the presence and/or absence of Cys and H_2O_2 for 1 day. D/E) vWF levels in monocytes-derived cells maintained in CFU media (Day 4) plus 1 day in EBM-2 plus VEGF (Day 5), in the presence or absence of H_2O_2 and/or disulfiram (inducer of rapid neovascularization). Each dot represents a healthy donor. **p<0.01, ***p<0.001

Cysteine, which is a thiol precursor of glutathione and itself an antioxidant, abrogates the H_2O_2-induced differentiation process, impairing the increase in H_2O_2-induced vWF expression (Fig II.2 C). The results indicate that redox potential of H_2O_2 pushes monocytes to EC-like differentiation while cysteine impair this phenotypic course and possibility plays a role in the control of EC-like differentiation.

Aldehyde dehydrogenase (ALDH) is used as a marker for populations enriched in stem and progenitor cells and a decrease in ALDH expression and activity is correlated with a differentiation status of EPCs to ECs (Blix *et al.*, 2015; Vassalli, 2019). Hence, the impact of ALDH inhibition by disulfiram (Koppaka *et al.*, 2012) was evaluated in the monocyte-EC differentiation. Monocytes from Day 4 (Fig II.2 D) and Day 5 (Fig II.2 E) exposed to disulfiram showed a gain of vWF expression similar to monocytes exposed to H_2O_2, reinforcing that monocytes act as EPCs and are differentiating into ECs.

Interestingly, using nuclear magnetic resonance (NMR) we observed that the differentiation pattern of monocytes upon H_2O_2 exposure drives a decrease in the concentration of branched chain amino acids (BCAA) (isoleucine, leucine and lysine), valine and glutamine (Fig II.3 A), indicating that H_2O_2 promotes the consumption and catabolism of amino acids to support monocytic to ECs differentiation. Also, cysteine exposure rescues a decrease in the concentration of those metabolites and using a multivariate analysis it is observed that cysteine exposure increases the levels of formate while it decreases the acetate levels (Fig II.3 B).

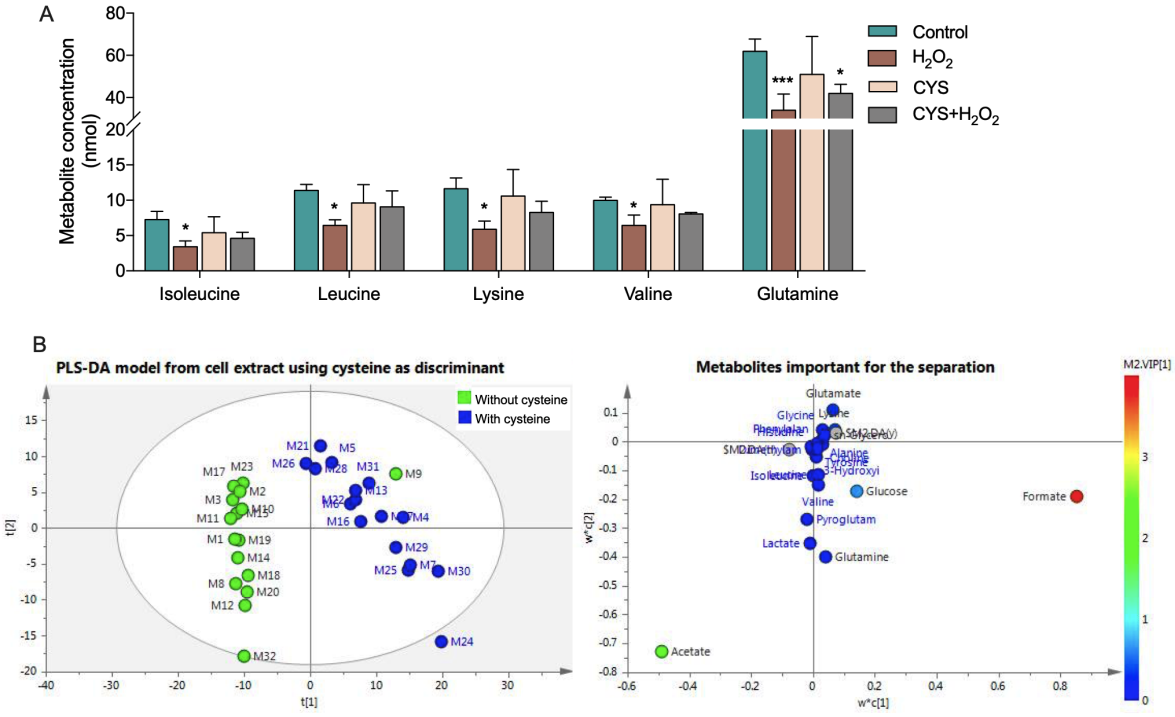


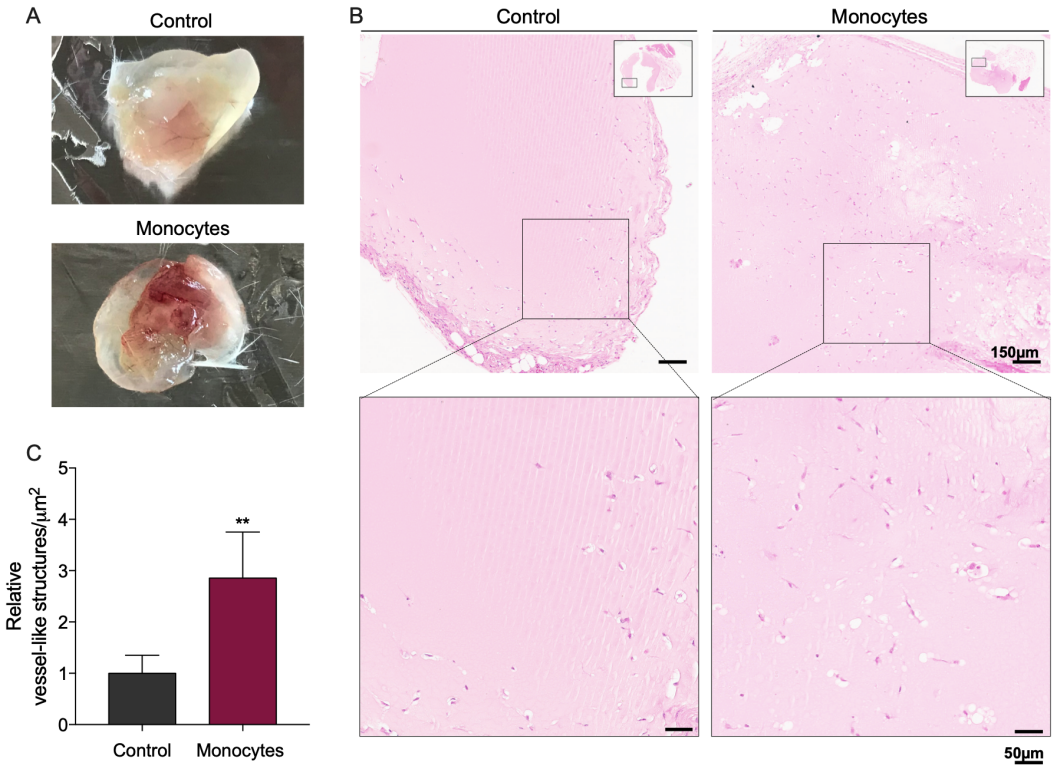
Figure II. 3 Monocytes exposed to H_2O_2 increases branched chain amino acids (BCAA), valine and glutamine catabolism while cysteine (Cys) promotes an increase in formate levels and a decrease in acetate.

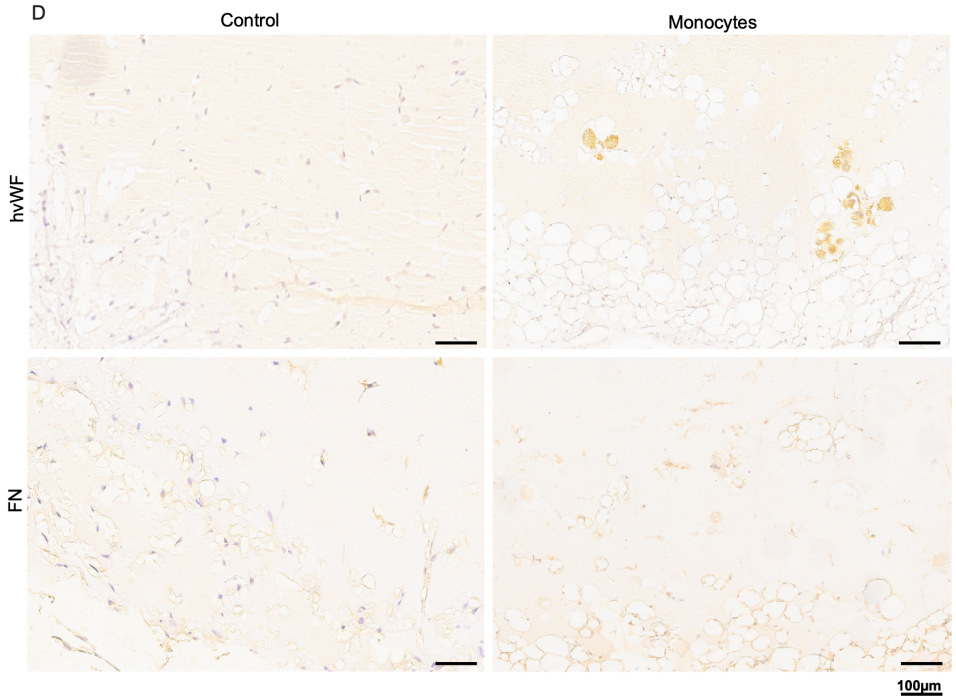
A) Metabolite concentration of BCAA (isoleucine, leucine and lysine), valine and glutamine of monocytes exposed to H_2O_2 and/or cysteine B) Multivariate analysis of monocytes cultured under H_2O_2 and/or cysteine. *p<0.05, ***p<0.001

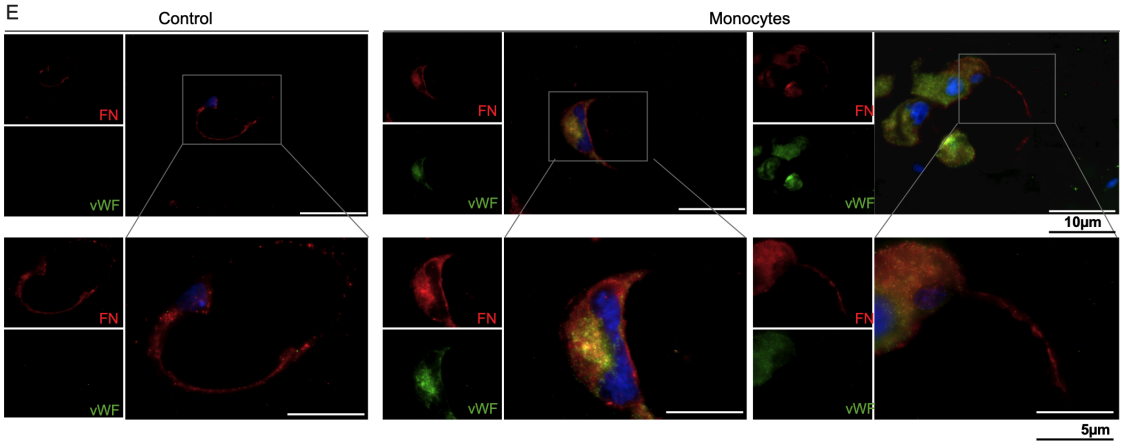
In vivo, monocytes differentiate into ECs and incorporate blood vessels, reinforcing the putative role of monocytes as EPCs

Matrigel plugs with embedded human monocytes from male healthy donors were inoculated subcutaneously in mice, whereas control plugs were induced with matrigel solely. Macroscopically, plugs inoculated with monocytes showed a more exuberant vasculature (Fig II.4 A), concomitantly with a microscopically increase in the density of vessel-like structures (Fig II.4 B and C). Some of the vessel-like structures in plugs containing monocytes were positive for the human vWF (hvWF) (Fig II.4 D and E, supplementary Fig 2), indicating that human monocytes differentiate into ECs and were incorporated into blood vessels. It is noteworthy to mention that the vessel-like structures in control plugs (Fig II.4 A, B and C), were negative for hvWF (Fig II.4 D and E), which confirmed their murine origin. Additionally, vessel-like structures of the control and monocytes group were positive for fibronectin (FN) (Fig II.4 D and E), a protein of the vessels' basement membrane that is expressed by active ECs (Hielscher *et al.*, 2016; Kolachala *et al.*, 2007), indicating that those structures are new-formed vessels.

Besides the hvWF expression in monocytes-derived ECs in vessel-like structures, the human and male origin was also confirmed by FISH (Fig II.4 F) performed with human specific probes to X and Y chromosomes. Thus, we confirmed that the hvWF positive cells, in plugs from monocytes group, were from human origin (X and Y positive; Fig II.4 F), proving that monocytes can directly differentiate into ECs.




D
Control
Monocytes
hvWF
FN
100μm


E
Control
Monocytes
FN
vWF
10μm
5μm

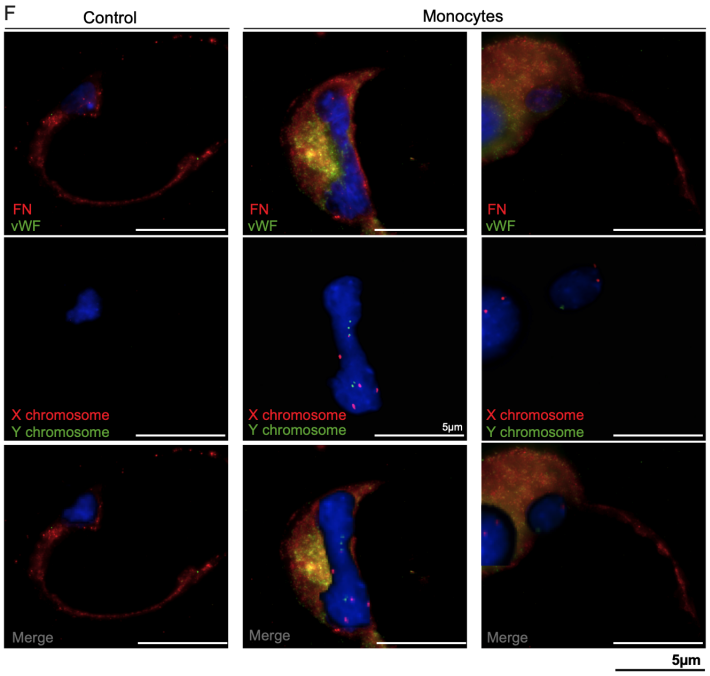

F
Control
Monocytes
FN
vWF
X chromosome
Y chromosome
Merge
5μm

Figure II. 4 Monocytes exposed to VEGF are able to form blood vessels *in vivo*.

A) Plugs images 21 days after monocytes inoculation in matrigel with VEGF. Control plugs were inoculated in the absence of monocytes. B) Hematoxylin and eosin staining from the paraffin embedded plugs. C) Relative density of vessel-like structures per area (μm) in plugs (n=4 per group). D) Human CD31 (hCD31) by immunohistochemistry. Optical microscopy, nuclei are blue (hematoxylin). E) Human vWF (hvWF; green) and fibronectin (FN, red) staining by immunofluorescence in plug blood vessels. F) FISH analysis for the human X (red) and Y (green) chromosome. Nuclei is blue (DAPI). **p<0.01

Monocytes improve the repair of injured aortas

In order to verify if monocytes were able to repair injured vessels, we used an *ex vivo* model of murine aorta injury, in which mice aorta were exposed to lysophosphatidic acid (LPA) and lipopolysaccharide (LPS) to induce endothelial dysfunction and injury (Cai *et al.*, 2017; Nogueras *et al.*, 2008; Teo *et al.*, 2009). Interestingly, we observed that monocytes acquired the expression of hvWF (Fig II.5), proving their differentiation route towards ECs and further incorporation into damaged aortas.

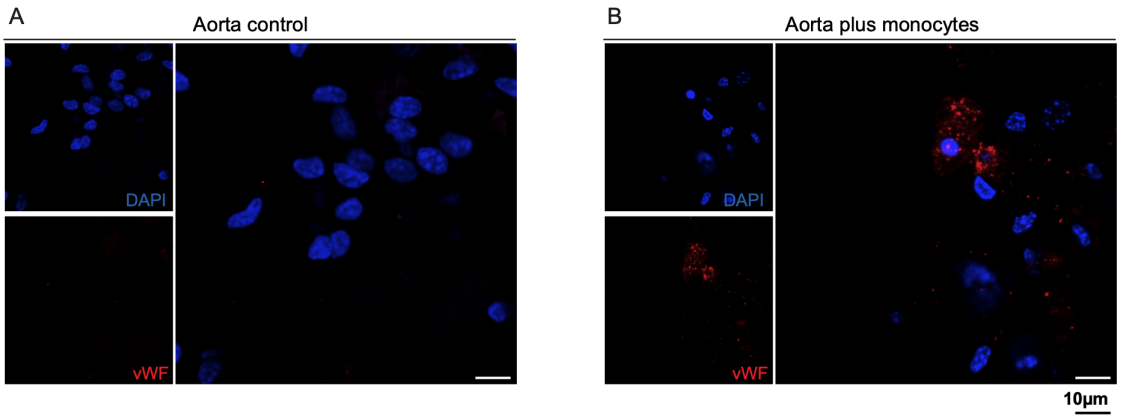


Figure II. 5 Monocytes are able to be incorporated in the aorta *ex vivo*, after endothelial Lipopolyssacharides/ Lysophosphatidic acid (LPS/LPA)-related injury.

The aortas were exposed *ex vivo* to LPS (0.5 μg/mL) plus LPA (0.5 μg/mL) to induce endothelial dysfunction. Afterwards LPS plus LPA were removed and monocytes were incubated with aortas. A) Murine aorta section incubated with LPS plus LPA, showing no positive cells for human vWF (bars 10 μm). B) Murine aorta section incubated with LPS plus LPA followed by incubation with monocytes, showing positive cells for human vWF (bars 10 μm). Nuclei are blue (DAPI).

Monocytes revealed to be active stakeholders in neo-vascular network formation during tumor development

To explore if monocytes can act as EPCs in cancer, we employed a 3D co-culture system (Rebelo *et al.*, 2018) of human breast cancer cells (HCC1954) and human monocytes. In co-culture, hvWF positive cells also expressed FN (Fig II.6 A), which suggested that monocytes are differentiating into ECs. In control cultures (monocultures of HCC1954), FN labeling was detected but not vWF.

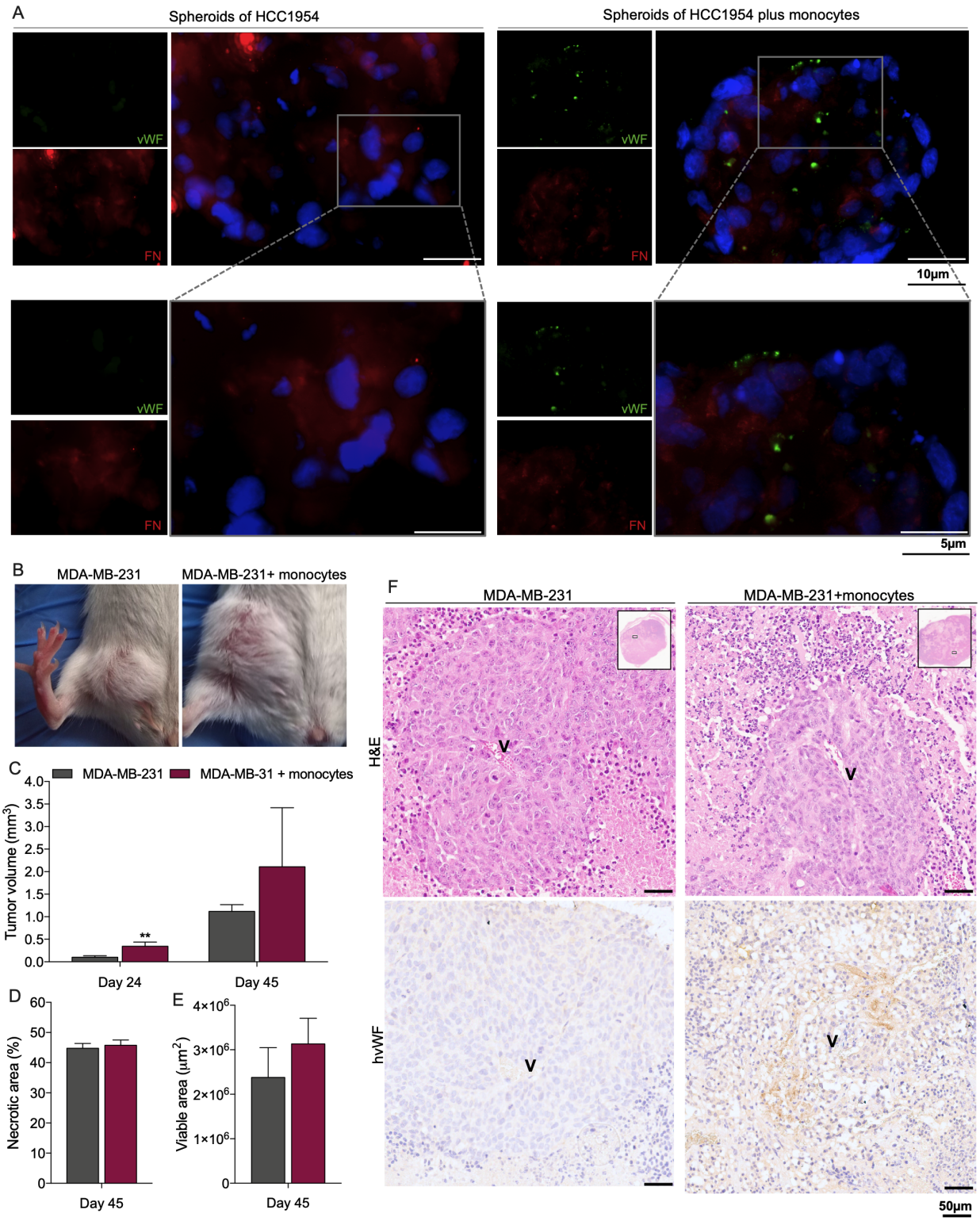


Figure II. 6 Tumors grown in the presence of monocytes have increased tumor volume and some vessels with human vWF (hvWF) staining

A) 3D spheroids of HCC1954 in co-culture with monocytes stained for hvWF (green) and FN (red). DAPI (blue) stains nuclei, magnification 400x. (B) Representative imagens of tumors from mice with breast cancer cells (MDA-MB-231), without and with monocytes previously cultured under VEGF. C) Tumor volume (mm^3) 24 and 45 after inoculated mice with breast cancer cells (MDA-MB-231), without and with monocytes previously cultured under VEGF. D) Quantification of tumor necrotic areas, 45 days after the inoculation of MDA-MB-231 with and without monocytes cultured in VEGF. E) Quantification of tumor viable areas, 45

days after the inoculation of MDA-MB-231 with and without monocytes cultured in VEGF. F) Immunohistochemistry (IHC) for hvWF (black arrow) in MDA-MB-231 tumors in the presence and absence of monocytes cultured in VEGF. Optical microscopy, nuclei are blue (hematoxylin). Blood vessels are signed with "V". **p<0.01

Furthermore, *in vivo*, we investigated if monocytes would be incorporated in the tumoral neo-vasculature, enhancing tumor development and growth. For that purpose, we established a cancer model in which human breast cancer cells (MDA-MB-231) were inoculated in the mammary fat pad of female mice, in the presence and in the absence of human male monocytes. Mice injected with MDA-MB-231 and human monocytes presented larger tumors (4 and 2-fold at days 24 and 45, respectively; Fig II. 6 B and C) in comparison to the mice inoculated only with MDA-MB-231. The percentage of necrotic area was similar in the two experimental groups (Fig II. 6 D), suggesting that monocytes act as helpers during tumor development since bigger tumors from the mice inoculated with MDA-MB-231 and monocytes showed equal necrotic areas in comparison to the smaller tumors of mice injected only with MDA-MB-231 cells. Remarkably, some ECs in tumor blood vessels of mice inoculated with MDA-MB-231 and monocytes were hvWF positive (Fig II.6 F; black arrow); indicating that during tumorigenesis monocytes can differentiate into ECs and be incorporated into the neovasculature.

Discussion

EPCs circulation, mobilization and differentiation play an essential role in the repair of injured vessels, besides their relevance in sprouting angiogenesis (Richardson and Yoder, 2011; Yoder, 2012; Yuan *et al.*, 2017). In the past years, the role of EPCs in the renewal of ECs integrity and function had gained attention from the scientific community, especially from the field of ischemia and cardiovascular disorders, opening new therapeutic perspectives for patients treatment (Haider *et al.*, 2017; Luo *et al.*, 2018; Montenegro *et al.*, 2018). However, so far, the lack of a precise panel of cell surface markers drives some heterogeneity in the methodologies used for classifying and isolating EPCs. Furthermore, the very low representativeness described in PB for EPCs represents a barrier for a better characterization of the molecular and biological processes underlying EPCs function, homing and differentiation (Basile and Yoder, 2014; Pelosi *et al.*, 2014). The exact classification of EPCs is still a matter of debate, so, we wonder if other cell subtypes that were not so far addressed in studies regarding angiogenesis, can also act as EPCs.

Reports in ischemia disclosed the involvement of BM-derived mononuclear cells as contributors for angiogenesis, by being incorporated into neocapillaries and by promoting

an increased vessel density (Kamihata *et al.*, 2001; Van Huyen *et al.*, 2008; Karcher and Greene, 2013;). A study from Nolan et al. (2007) correlated early stages of tumor development with the differentiation potential of BM-derived EPCs and their incorporation into a subset of tumor neovessels. As mentioned above, we have previously shown that some cells on the vessel wall co-expressed CD14 (monocytic marker) and CD31 (EC marker) in a panel of normal tissues and tumors from vascular, epithelial and embryonic origins (Domingues *et al.*, 2015a).

Herein we have observed *in vitro* that freshly isolated monocytes exhibited a mixed expression profile of EC and macrophages markers (Fig II.1 A, supplementary Fig 1), though expressing very low levels of vWF, an EC marker (Fig II.1 B). However, when monocytes were cultured in stem and progenitor cell medium (CFU), they reduced the expression of all those markers (Fig II.1 A), indicating that monocytes are in a more stem-like state. Monocytes are recognized as a cell type that expresses VEGF receptors (VEGFR) (Barleon *et al.*, 1996; Cuadrado *et al.*, 2006) and our results show that when cultured under the presence of VEGF, monocytes acquired a spindle-cell-like morphology (Fig 1 II. B), typical of ECs. This morphological remodeling was accompanied by the increased expression of CD31 (EC marker) and other EC specific genes (CD146, vWF), in concomitance with a decreased expression of CD14 (monocytic/macrophagic marker) (Fig II. 1 C and D). These *in vitro* results support that monocytes are close to a stem-cell like state, but, upon pro-angiogenic stimuli they can differentiate into ECs.

Pathologies like cardiac ischemia or cancer have defective tissue perfusion, and excessive ROS levels and deregulated redox signaling accounts for re-vascularization of tissues and diseases outcome (Gu *et al.*, 2018; Kumari *et al.*, 2018; Zhou *et al.*, 2018). In addition, ROS are essential for ECs homeostasis and can stimulate or inhibit cell differentiation, depending on the cell type (Ji *et al.*, 2010; Wang *e t al.*, 2013;Chaudhari *et al.*, 2014; Xiao *et al.*, 2014; Pashkovskaia *et al.*, 2018). Considering the relevance of EPCs during vascular remodeling in pathologies with alterations in redox balance, as cancer, we wondered if ROS would impact the monocytic - EC differentiation. Under exposure to H_2O_2 (ROS), monocytes increased the expression of specific EC markers in monocytes, such as vWF, CD31 and CD146 (Fig II.2 A and B) and a decrease in the expression of the monocytic marker CD14 (Fig II.2 B). Monocytes, both maintained in EBM2 and in CFU media, were able to react to H_2O_2 by increasing the expression of vWF (Fig II.2 A), highlighting the relevance of ROS in inducing the differentiation of monocyte-derived ECs. In addition and considering that the expression of some ECs markers (*e.g.* CD31) is often observed in macrophagic cells in cancer context and in vascular mimicry (McKenney *et al.*, 2001; Barnett *et al.*, 2016), we have also analyzed

the expression of vWF, a well-known ECs marker commonly used in other studies to ensure the endothelial differentiation (Eggermann *et al.*, 2003; Ge *et al.*, 2018; Chu *et al.*, 2019). vWF is expressed and accumulated in Weibel-Palade bodies, the endothelial storage granules (Castaman *et al.*, 2012). Accordingly, the immunodetection of vWF expression in ECs differentiating monocytes demonstrated a granular accumulation (Fig II.1 E and 2 B), typical of ECs. Moreover, we observed that the induction of monocytic differentiation into ECs promoted by H_2O_2 increase BCAA and glutamine catabolism (Fig II.3 A), indicating that their consumption and catabolism support ECs differentiation. The activation of metabolic pathways by the expression of genes involved in both metabolic and differentiation processes is pivotal for the onset of cell differentiation (Mathieu and Ruohola-Baker, 2017). In fact, increased BCAA and glutamine catabolism had been already linked with differentiation processes (*e.g.* myocytes, adipocytes) (Green *et al.*, 2016; Duan *et al.*, 2017; Lu *et al.*, 2019; Tao Zhou *et al.*, 2019).

Our team showed that cysteine has a protective role against ROS-related cytotoxic-induced cancer cell death (Nunes *et al.*, 2018a; Nunes *et al.*, 2018b). Interestingly, in our context, monocytes exposure to cysteine impaired their ROS-induced differentiation to ECs (Fig II.2 C). The abrogation of ECs differentiation upon cysteine exposure impaired BCAA, valine and glutamine catabolism induced by ROS exposure (Fig II.3 A), indicating that the antioxidant cysteine properties impair the metabolic readjustment that is occurring in the differentiation route of ROS-induced monocytes to ECs. Additionality, the increased formate levels and the decreased acetate levels (Fig II.3 A), observed in monocytes under cysteine supplementation, was previously linked with cell proliferation (Yoshimoto *et al.*, 2001; Meiser *et al.*, 2016; Yang and Vousden, 2016). In fact, we speculate that cysteine supplementation decreases one carbon metabolism, being glycine deviated to produce ribulose-1,5-biphosphate and afterwards formate instead of supplying folic acid cycle that together with methionine cycle contributes to the transsulfuration pathway (TSP) for cysteine synthesis (Fig II.7) (Yoshimoto *et al.*, 2001; Meiser *et al.*, 2016, 2018; Yang and Vousden, 2016). Also, acetate levels are decreased because in proliferating cells acetate supplies fatty acids synthesis (Fig II.7) (Yoshimoto *et al.*, 2001; Meiser *et al.*, 2016, 2018; Yang and Vousden, 2016). Together, the results suggest that a cysteine-rich environments favors cell proliferation through the glycine shuttle from folate/methionine cycle to pentose phosphate pathway (PPP)/ phosphoribosyl diphosphate pathway (PRPP) and fatty acid synthesis, indicating that perhaps cysteine promote monocytes proliferation, avoiding its differentiation to ECs under a metabolic rewiring.

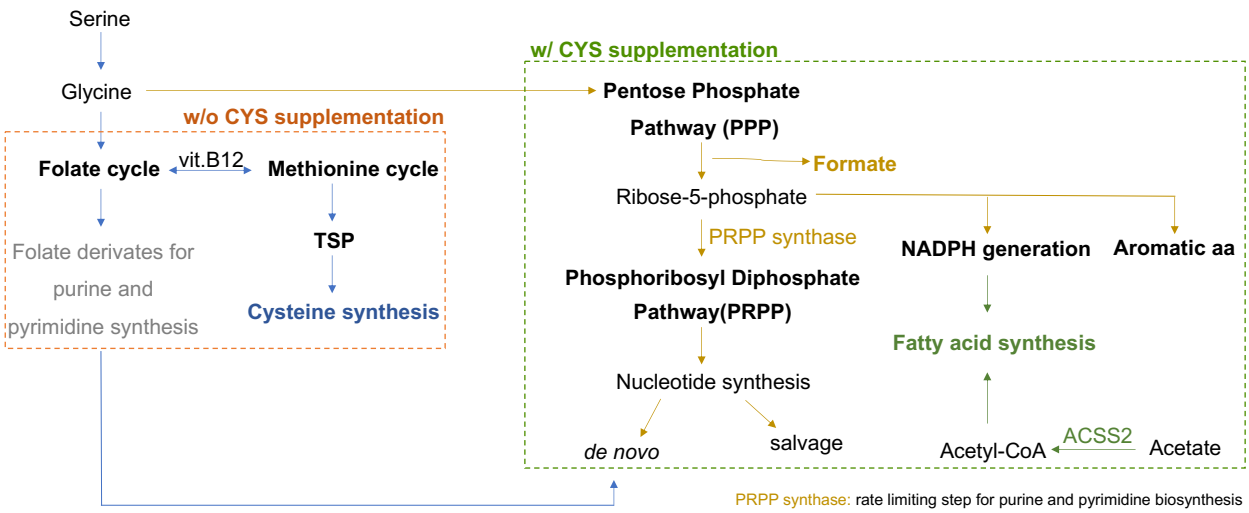


Figure II. 7 Cysteine supplementation may be involved in the promotion of cell proliferation.

In environments with increased cysteine bioavailability, the glycine flux will be deviated to the pentose phosphate pathway (PPP), increasing the levels of ribose-5-phosphate that contribute to nucleotide synthesis through phosphoribosyl diphosphate pathway (PRPP). Since the nucleotide synthesis (*de novo* or savage) is dependent on ribose-5-phosphate for PRPP, the shuttle from folate/methionine cycles to the PPP will sustain nucleotide demands essentially for cell proliferation. Although folate derivates are needed for *de novo* nucleotide synthesis, PRPP is the rate-limiting reaction. In theory, proliferating cells will prefer PPP instead of folate/methionine cycle, to sustain PRPP through R5P. Moreover, more active PPP leads to more formate production (metabolite increased in cysteine supplemented monocytes) and the NADPH generated from PPP will supply fatty acids synthesis, as well as acetyl-CoA derived from acetate (metabolite decreased in cysteine supplemented monocytes).

EPCs are characterized by having active ALDH and a decrease in its expression and activity is correlated with the differentiation from EPCs to ECs (Blix *et al.*, 2015; Vassalli, 2019). In a model of ischemia, decreased expression of ALDH is pointed out as a good strategy to induce rapid neovascularization and subsequent regeneration of ischemic tissues (Nagano *et al.*, 2007). Our results show that monocytes act as EPCs, since when they are exposed to disulfiram, an irreversible inhibitor of ALDH, present a similar pattern of vWF expression in comparison of monocytes exposed to H_2O_2 (Fig II.2 D and E). Thus, the decreased activity of ALDH prompts the monocytes-ECs differentiation process, in a similar manner than ROS (H_2O_2). The role of ROS (H_2O_2) as a stimulator of monocytes differentiation into ECs was reinforced by the observation that even when monocytes are shortly removed from CFU media, both disulfiram and H_2O_2 are able to induce the expression of vWF (Fig II.2 D). The ROS enriched microenvironment during disease progression is a feature already assumed in cancer (Weinberg *et al.*, 2019). Despite the already established ROS effect in tumor neo-angiogenesis (Kim and Byzova, 2014), here we observed that ROS stimuli allied with high plasticity of monocytes pushes them to differentiate into ECs.

In TME, stromal cells (*e.g.* fibroblasts, adipocytes, inflammatory and smooth muscle cells) and cancer cells secret monocyte chemoattractant protein 1 (MCP1) that leads to the recruitment of monocytes into the tumor (Deshmane *et al.*, 2009; S. Y. Lim *et al.*, 2016b; Yoshimura, 2018). Furthermore, the macrophage migration inhibitory factor (MIF), whose function as a regulator of inflammation remains controversial (Nishihira, 2000; Kasama *et al.*, 2010; Chen *et al.*, 2015), seems to promote cancer progression by stimulating the recruitment of myeloid cells into the tumor (Simpson *et al.*, 2012; He *et al.*, 2015; Wang *et al.*, 2017; Zhang *et al.*, 2017), and being associated with increased angiogenesis (Girard *et al.*, 2012; Mangano *et al.*, 2018). Since MIF have directly been implicated in the regulation of EC differentiation (Cui *et al.*, 2016), we believe that MIF as a tumor promoter proves again that monocytes- derived ECs can be a subpopulation of M2- tumor associated macrophages (TAMs), reaching the tumor to favor angiogenesis also by making part of blood vessels structure. Interestingly, an oxidized isoform of MIF has been identified as a prognostic biomarker and therapeutic target in inflammatory diseases and cancer (Mahalingam *et al.*, 2016; Schinagl *et al.*, 2016), showing again that the pro-oxidative TME is definitely playing a role in angiogenesis.

The participation of monocytes in angiogenesis, via the production and release of pro-angiogenic factors, has been reported in inflammatory diseases (Cursiefen *et al.*, 2004; Eubank *et al.*, 2003; Clark *et al.*, 2007). However, our results suggested that, in addition to their role in the secretion of cytokines, pro-angiogenic factors and ECM modifying proteins (Zumsteg and Christofori, 2009; Deryugina and Quigley, 2015), monocytes were also able to integrate new blood vessels by directly differentiating into ECs and incorporating the vessels structure (Fig II.4). This phenomenon was observed macroscopically by a higher density of blood vessels in plugs inoculated with monocytes (Fig II. 4A) with increased number of vessel-like structures (Fig II.4 B and C) expressing FN (Fig II. 4 D and E), indicating that those structures have a vessel basement membrane (Xu and Shi, 2014). Moreover, using two different *in vivo* models, we unraveled human monocytes capacity of differentiating into ECs and incorporating blood vessels (Fig II.4 B and C). The strategy we used by inoculating human male monocytes in plugs induced in female mice, undoubtedly proved the human monocytic origin of ECs (Fig II. 4 B, C and F). In addition, it was evident that not all vessel-like structures within the plug were from human origin (Fig II.4 D), since murine EPCs were also participating in the pro-angiogenic process (Malinda, 2009) and might react in a more efficient way as they were reacting to endogenous murine signaling. A recent study, confronting autologous and heterologous EPCs transplantation models stated that in the autologous context, cells more efficiently undergo homing and differentiation (Qin *et al.*, 2018).

The differentiation of monocytes into ECs, expressing vWF, was also induced in murine aortas *ex vivo* (Fig II.5), after EC dysfunction induced by LPS and LPA (Nogueras *et al.*, 2008; Teo *et al.*, 2009; Cai *et al.*, 2017). Again, the human origin of those cells incorporating injured aortas was assessed by using a specific anti-hvWF. Several studies have shown that injured arteries are repaired by both the recruitment of new cells and the activation of the proliferation of ECs (Zhao *et al.*, 2006; Nogueras *et al.*, 2008; Kong *et al.*, 2018; Zhao *et al.*, 2016). In this experiment, monocytes worked as recruited EPCs, being able to incorporate an already structured but injured vessel.

Thus, herein we provide new evidence to justify why the efficacy of the use of anti-angiogenic therapies in cancer treatment have been so far disappointing. We believe this failure relies on the missing pieces to construct the entire angiogenic route. Considering that monocytes recruitment is a well-established step during carcinogenesis (Karlmark *et al.*, 2012; Olingy *et al.*, 2019), we believe this recruitment favors tumor progression not only by differentiating into TAMs (Cortés *et al.*, 2017; Lopes-Coelho *et al.*, 2018; Niu *et al.*, 2017; C. Wei *et al.*, 2019), but also by acting as EPCs and differentiating into ECs. In a 3D co-culture system of breast cancer cells and monocytes, we detected hvWF positive cells that also expressed FN (Fig II.6 A); this suggested that also in *in vitro* cancer 3D-models, monocytes can differentiate into ECs. Once in the tumor and upon their activation, some monocytes can differentiate into macrophages that release cytokines and pro-angiogenic factors, while other monocytes can be targeted by this stimulation that will trigger their differentiation into ECs. This coordinated myeloid networking will favor tumor growth and disease progression. According to this, we observed, *in vivo,* that breast cancer cells inoculated in the presence of monocytes originated bigger tumors in comparison to control group (4 and 2-fold at day 24 and 45, respectively; Fig II. 6 B and C). The necrotic area was similar in the two experimental groups (Fig II. 6 D and E), showing that the viable area in tumors induced in the presence of monocytes is more extensive than the viable area in control tumors. This observation indicate that monocytes are acting as helpers for tumor growth. Interestingly, in the viable regions of tumors inoculated with monocytes, but not in the control group, some ECs and cells close to the vessels were hvWF positive (Fig II. 6 F). Therefore, in both the *in vivo* murine breast cancer model and the 3D *in vitro* model, we unraveled that monocytes have the potential to differentiate into ECs and be incorporated into the neo-vasculature, during tumor development.

Our study demonstrated that monocytes are in fact incorporated in blood vessels, strengthening their underestimation as a relevant stanchion of vascular growth. Due to their 2-10% prevalence in PB (Curry, 2015) and comparing to the estimated percentage of EPCs proposed by other studies (Richardson and Yoder, 2011; Yoder, 2012; Yuan *et*

al., 2017; Kaur *et al.*, 2018; Tal *et al.*, 2019), monocytes are putatively the most representative EPCs subgroup. Herein, we provided new evidence positioning a redox-dependence of monocytes under pro-angiogenic stimuli, which contributes for vascular growth (Fig II.8). A new point of view on neoangiogenesis and ECs sources must be explored further, since a better understanding of monocytes biology, as well as EPCs, different from the canonical angiogenic point of view, will contribute to a change in the paradigm of anti-angiogenic drugs in cancer therapy.

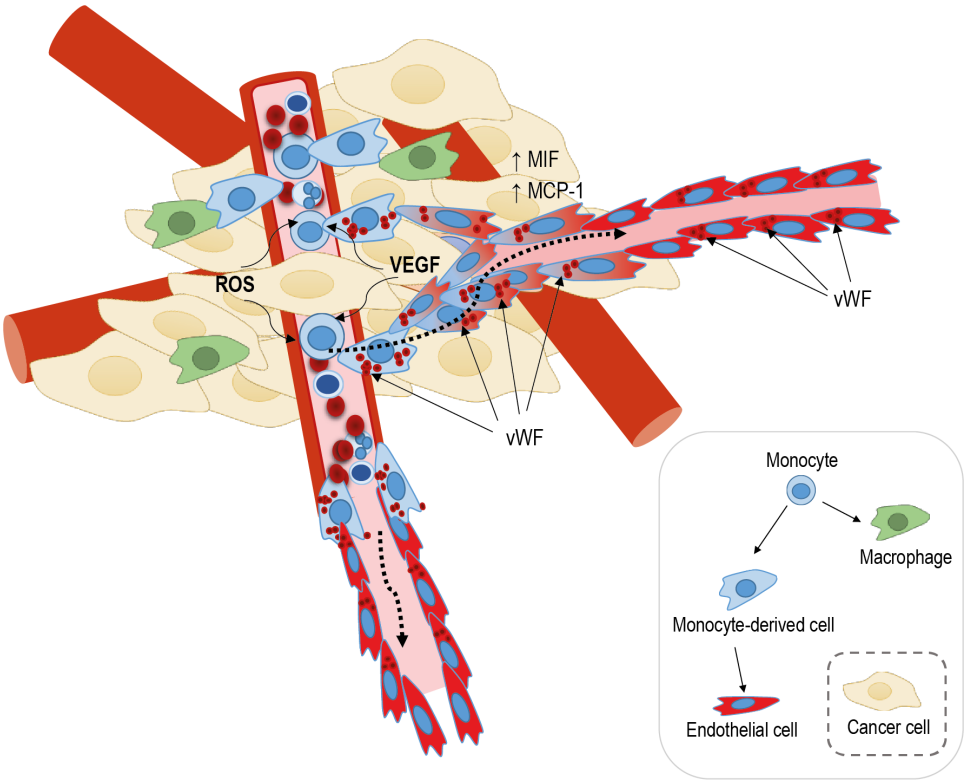
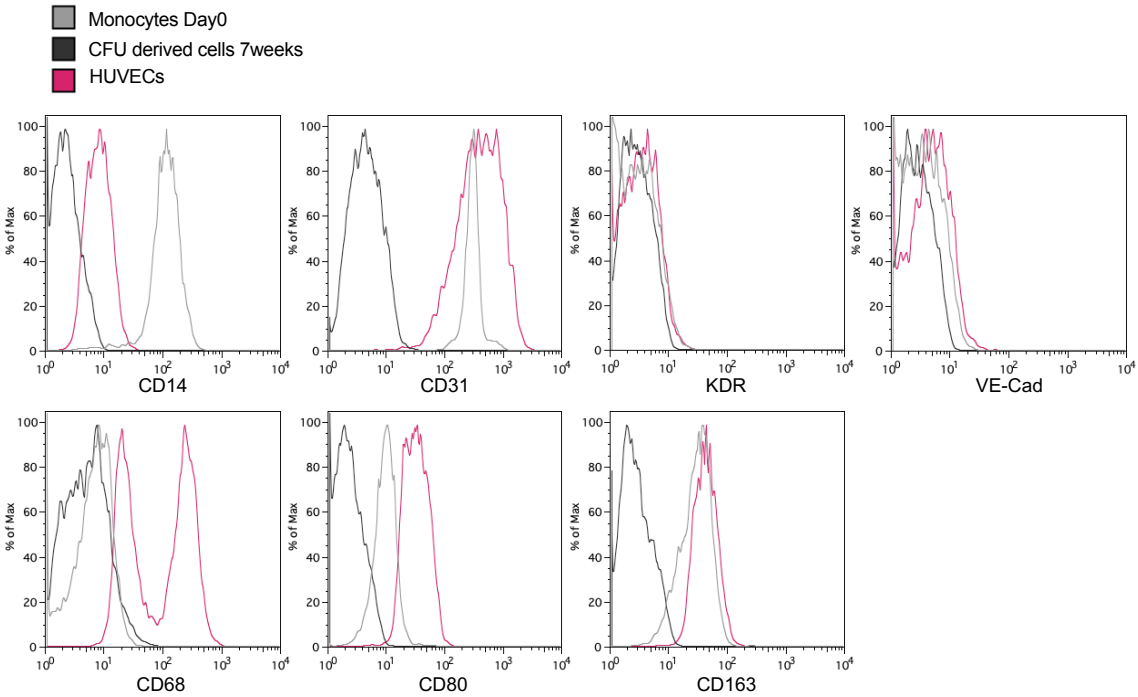


Figure II. 8 Monocytes recruitment into the tumor and endothelial differentiation.

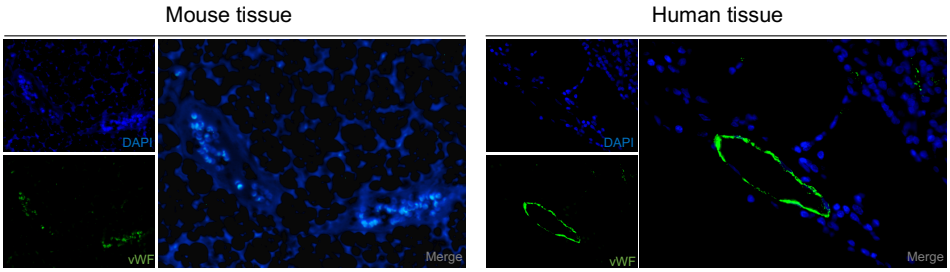
Our working model demonstrates that monocytes could act as EPC and be incorporated into neo-vasculature. Monocytes are a cell subtype characterized as having high plasticity, being able to differentiate into macrophages and we unraveled that endothelial differentiation can also be endpoint for monocytes. Monocytes-ECs differentiation depends on a pro-angiogenic stimulus (*e.g.* VEGF) and oxidative stress (ROS). The acquisition of the expression of vWF (EC marker) is an undoubtable evidence of this differentiation route accounting for tumor vascularization and growth.

Supplementary Figures



Supplementary Figure II. 1

Representative histograms of FACS analysis of CD14 - monocytic marker, CD31, KDR, VE-Cadherin (VE-Cad) – EC markers and CD68, CD80, CD163 – macrophage markers in monocytes freshly isolated (Day0), monocytes maintained in CFU media and in Human umbilical vein ECs (HUVECs).



Supplementary Figure II. 2

Confirmation of the specificity of anti- human vWF (anti-hvWF; A0082, Dako – Agilent) in lung section from mouse and human origin. Vessels (V) in mouse were negative for hvWF and human vessels were positive for hvWF (green) optimization in mouse and human tissues. Nuclei were counterstained with DAPI (blue).

Chapter III

Propranolol therapy is an efficient tool in the clinical management of cerebral cavernous malformations (CCM): a child case study

This chapter was based on:

Lopes-Coelho F, Nunes SG, Gouveia-Fernandes S, Domingues G, Melo BF, Sacramento JF, Conde SV, Pereira SA, Vinhais S, Salgado D*, Serpa J*. Propranolol therapy is an efficient tool in the clinical management of cerebral cavernous malformations (CCM): an infantile case study. Manuscript in preparation

* equal contribution

Abstract

Cerebral cavernous malformations (CCM) are vascular malformations characterized by the abnormal growth of vascular structures in the central nervous system. Unfortunately, the precise mechanism(s) responsible for the development of the CCM vascular abnormalities remains poorly explored. Propranolol administration in CCM, although it is not commonly prescribed, has revealed to be effective in children and seems to have a protective role in the prevention of CCM-derived hemorrhages in adults. Here, despite the effects of Propranolol on mature ECs by decreasing angiogenic related features, we observed that in the peripheral blood (PB) of a CCM child patient, Propranolol administration decreases the percentage of circulating cells sharing monocytic and ECs features ($CD14^+/CD31^+$) and the VEGF levels, concomitant with good prognosis and with the reversion of CCM lesions. A decrease in VEGF levels by Propranolol could be involved in the impairment of the recruitment of $CD14^+/CD31^+$ monocytes functioning as EPCs to sustain the vascular lesion. Our case study reinforced that Propranolol is useful in the clinical management of CCM and highlighted that the monitorization of the levels of $CD14^+/CD31^+$monocytes and VEGF could be used to predict the clinical efficacy of Propranolol in CCM patients.

Keywords: cerebral cavernous malformation, Propranolol, monocytes, endothelial cells, angiogenesis

Introduction

Cerebral cavernous malformations (CCM) present a relatively low prevalence (0,16-0.5%), accounting for approximately 5–15% of all central nervous system vascular malformations (Akers *et al.*, 2017; Flemming *et al.*, 2017; Goldstein and Solomon, 2017). The disease is characterized as low-flow vascular malformations composed by blood-filled sinusoidal locules known as “caverns”. At histological level, CCM is characterized by the lack of mural elements of mature vascular structures (Goldstein and Solomon, 2017).

The major clinical presentations are epilepsy, hemorrhage, headache or focal neurological deficits, however 30% of the patients are asymptomatic or present non-specific headache (Gross *et al.*, 2011). During disease progression, the growth of vascular malformations associates with recurrent hemorrhages (annual hemorrhage rate of 0.6-11% *per* patient-year) (Batra *et al.*, 2009; Goldstein and Solomon, 2017), which is believed to be a consequence of the immature leaking vascular network constituting the CCM lesions (Cox *et al.*, 2017). Depending on the anatomical localization of the CCM, patient management relies on surgical resection, observation and symptomatic treatment. In surgical inaccessible lesions, drugs such as statins, anti-angiogenic agents or vitamin D3 have been tested, although without clear benefits (Leblanc *et al.*, 2009; Wüstehube *et al.*, 2010; Gibson *et al.*, 2015; Shenkar *et al.*, 2017). The beneficial use of Propranolol in childhood hemangioma patients, a close pathologic counterpart of cavernous malformations, supports the putative use of Propranolol in the management of symptomatic CCM patients (Apra *et al.*, 2019). Propranolol administration in CCM patients, although it is not a commonly prescribed drug for this purpose, was effectively used in children and seems to have a protective role in the prevention of CCM-derived hemorrhages in adults (Moschovi *et al.*, 2010; Dotan and Lorber, 2013; Berti *et al.*, 2014; Miquel *et al.*, 2014; Cavalheiro *et al.*, 2016; Reinhard *et al.*, 2016).

A common feature of Propranolol-sensitive vascular tumors, as hemangioma and CCM, is the distinctive expression of CD15-positive “vasculogenic zones” (Ritter *et al.*, 2006; Navarrete *et al.*, 2014; Seidmann *et al.*, 2014). Interestingly, the *in vitro* gain of CD15 is followed by embryonic stem cell differentiation into endothelial cells (ECs) (Yue *et al.*, 2010), indicating the putative involvement of endothelial progenitor cells (EPCs) in the development of vascular malformations. The relationship between CD15-positive cells and neo-vessels formation is not new, it was described**,** at more than forty years ago**,** in immature vessels of the placenta (Cottrill *et al.*, 1977; Reed *et al.*, 1974), as well as the effect of Propranolol in placenta regression (Schoenfeld *et al.*, 1978).

The identification the EPCs subset in the peripheral blood (PB) is not clear. Our team showed that in tumors and normal tissues some ECs simultaneously expressed CD14

(monocytic marker) and CD31 (EC marker), indicating mixed features between monocytes and ECs (Domingues *et al.*, 2015a). In chapter II, we unraveled that monocytes can differentiate into ECs and be incorporated in blood vessels. Our studies strengthened the underestimation of monocytes as a relevant source for vascular growth. Moreover, some studies showed that CD15 is also expressed in monocytes (Nakayama *et al.*, 2001; Martin, 2011), being its levels increased in pathological conditions (Chung *et al.*, 2012). Interestingly, in tumors CD14 immune cells are also CD15 positive, clearly indicating a subset of monocytes/macrophages (Elliott *et al.*, 2017). These observations reinforce that CD15 cells in the "vasculogenic zone" are in fact monocytes functioning as EPCs, contributing for blood vessel formation in CCM.

Hemangiomas and CCM share phenotypical characteristics, being both composed of a mixture of abnormal dilated capillaries with disorganized ECs and pericytes (Frieden *et al.*, 2005; Kim, 2016; Ganmore and Achiron, 2017). Although some studies indicated that CCM lesions arises from EC loss of Krev interaction trapped protein 1/Cerebral Cavernous Malformations 1 (KRIT1/CCM1), Cerebral Cavernous Malformations 2 (CCM2) or Programmed Cell Death 10 (PDCD10), non-homologous proteins that form an adaptor complex (Plummer *et al.*, 2005), the exact mechanism that regulates the development of the vascular abnormalities still remains poorly explored.

From what it is known, during the growth phase of hemangiomas, the increased expression of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) correlates with ECs and interstitial cells proliferation (Frieden *et al.*, 2005). The effect of Propranolol in the reversion of vascular malformations is putatively associated with a decrease in the expression of FGF and VEGF, impairing ECs migration, proliferation and reorganization, which in turn leads to vasoconstriction (involution phase) (Léauté-Labrèze *et al.*, 2008; Annabi *et al.*, 2009; Lamy *et al.*, 2010; Apra *et al.*, 2019).

Since the natural evolution of CCM is chronic and unpredictable, the follow-up of CCM patients involves the long-term clinical and imagiological evaluation with Magnetic Resonance Imaging (MRI) (Apra *et al.*, 2019). In an attempt to find a suitable follow up method, we explored the monitorization of the levels of $CD14^+/CD31^+$ monocytes in the PB of CCM patients. Considering that in chapter II we demonstrated that monocytes are viable EPCs here, we hypothesize that circulating $CD14^+/CD31^+$ monocytes act as EPCs and contribute to the development of CCM lesions; and Propranolol administration, in addition to its effects on stablished CCM vessels, will interfere with the levels of circulating $CD14^+/CD31^+$ monocytes, functioning as EPCs.

Materials and methods

Peripheral blood (PB) processing and cell characterization

PB of a CMM child patient was collected under informed consent in Neuropediatrics department at *Instituto Português de Oncologia de Lisboa Francisco Gentil* (IPOLFG) (IPOLFG-Ethical committee UIC-1137). PB was centrifuged at 155 *x g* for 5 min and after, serum was stored at -20 ºC until further analysis and the cell pellet was resuspended in 45 mL of 1X RBC lysis buffer (786-1701, Biosciences) and incubated at 15 min, in dark. After, resuspended cells were centrifuged at 155 *x g* for 5 min, washed twice with 1X Phosphate-buffered saline (PBS) and incubated with anti-CD14-FITC (1:100; 555397, BD Bioscience) and with anti-CD3-APC (1:100; FAB3567A, R&D Systems) in 0.5% BSA-PBS (v/w), for 20 minutes (min), in dark at 4 ºC. The immunolabelling was evaluated by flow cytometry (FACScalibur – Becton Dickinson) and data were analyzed using *FlowJo X* v10.0.7 software (*https://www.flowjo.com/*). PB cells from healthy blood donors was used as normal controls.

Cell culture

Human umbilical vein endothelial cells (HUVECs: CRL-1730, ATCC) were cultured in Endothelial Cell Growth Basal Medium-2 (EBM-2: CC-3156, Lonza, Bioscience) supplemented with EGM-2 SingleQuots Supplements (CC-4176, Lonza, Bioscience) and maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were used until the passage 10 and were detached with 0.05% Trypsin-EDTA 1X (25300-054, Invitrogen, Thermo Fisher Scientific). For experimental conditions cells were cultured in the presence and in the absence of 100 μM Propranolol (P8688, Sigma Aldrich).

VEGF levels determination

The concentration of VEGF in PB serum and in the culture medium conditioned by monocytes, isolated as described in chapter II, was evaluated using Human VEGF Quantikine ELISA Kit (DVE00, R&D Systems), according to the manufacturer's instructions. PB serum from healthy blood donors was used as normal controls and for cell supernatants, cells in control conditions were maintained in H_2O_2 and Propranolol free media.

Proliferation assay

The determination of the cell proliferation was calculated by ratio of total and $ki67^+$ nuclei. Briefly, HUVECs ($5x10^4$ cells/well) were cultured on glass slides coated with 0.2% gelatin and fixed in 2% paraformaldehyde, for 15 min at 4°C, followed by blocking with

1% Bovine serum albumin (BSA)-1X PBS (w/v). Then cells were incubated with anti-Ki67 (1:100 in 1% BSA-0.1% triton X-100- 1X PBS (w/v/v); sc-15402, Santa Cruz), overnight at 4 ºC, followed by an incubation with secondary antibody (Alexa Fluor 488 goat anti-rabbit, 1:1000 in 1% BSA-0.1% triton x100-PBS; A-11078, Invitrogen - Thermo Fisher Scientific), for 2 h at room temperature. Slides were mounted in VECTASHIELD media with DAPI (4′-6-diamidino-2-phenylindole; H-1200, Vector Labs), examined by standard fluorescence microscopy, using an Axio Imager.Z1 microscope (Zeiss) with a CytoVision® software and analyzed using ImageJ software (*imagej.nih.gov/ij/*).

Wound healing assay

Cells were plated on 24-well plates ($1x10^5$ cells/well) until the formation of a confluent monolayer. Then, cells were incubated with mitomycin-C (M4287, Sigma), an antimitotic agent, for 3h. A linear scratch in each monolayer was made with a P20 pipette tip, creating a gap across the well diameter. The media was replaced to remove debris and cells in suspension and to expose cells to the experimental conditions. Bright-field images of each well at 10 h were acquired on the Olympus IX53 Inverted Microscope and images were analyzed and quantified by ImageJ software (*imagej.nih.gov/ij/*).

Tube forming assay

Matrigel was plated onto 48-well plates at 37 ºC for 30 minutes and HUVECs were incubated with calcein (2 µg/mL; C1430, Invitrogen), a fluorescent cell permeable dye, for 30 min at 37°C and 5% CO_2. After, trypsinized HUVECs ($3x10^4$ cells/well) were harvested, resuspended and seeded into matrigel and then incubated at 37 ºC and 5 % CO_2, for 6 h. Representative images of vessel-like structures formation were acquired in an Olympus IX53 Inverted Microscope.

Rat aortic rings sprouting assay

Aortas (thoracic and abdominal segments) were dissected from male Wistar rats (10 weeks old) and cleaned to remove external tissue (ethical committee NOVA Medical School Ref. 75/2019/CEFCM). After removing all extraneous fat, fibrotic tissue and *vasa vasorum* structures, the aorta was segmented into rings with approximately 1 mm length. The rings were transferred to a Petri dish and incubated overnight in FBS-free culture medium at 37°C, 5% CO_2. In the next day, rings were embedded in Matrigel in a 24-well plate with EBM-2, with and without 100 µM Propranolol. The medium was refreshed every 3-4 days, being the sprouts visible at 7-13 days. Representative images were acquired on the Olympus IX53 Inverted Microscope and the branch points (intersections between ECs) number per area were counted using ImageJ (*imagej.nih.gov/ij/*). The

density of vessel-like structures formation (branch points number/μm^2) was calculated as proxy of vascular density.

Results

CCM clinical management with Propranolol administration, in a child patient, decreases the percentage of $CD14^+/CD31^+$ cells and the VEGF levels

In the PB of the CCM child patient before Propranolol administration, the percentage of double positive $CD14^+/CD31^+$ cells was higher than in PB from healthy blood donors (Fig III.1 A and 1 B). Interestingly, along the follow up with Propranolol administration it was observed a decrease in $CD14^+/CD31^+$ levels in PB, becoming close to normal profiles (Fig III.1 A and 1 B). PB cells from healthy blood donors was used as normal controls.

The levels of VEGF, in PB, were higher before the treatment with Propranolol and decreased towards normal levels during follow up (Fig III.1 C). PB serum from healthy blood donors was used as normal controls.

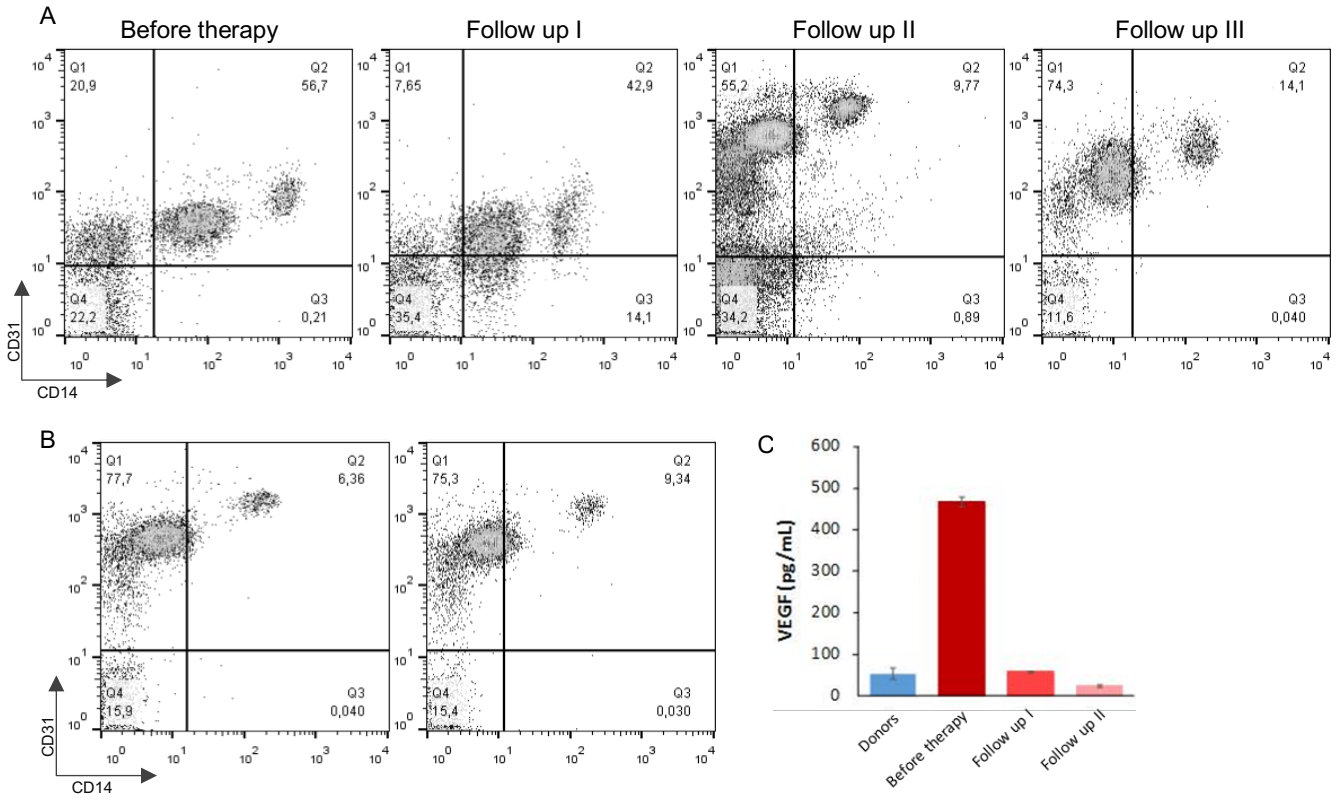


Figure III. 1 Dynamics of the percentage of $CD14^+$ and $CD31^+$ cells and of VEGF levels in peripheral blood (PB) of a child patient with a brain cavernoma before Propranolol (Prop) therapy and during the follow-up.

A) FACS analysis for CD14 and CD31 markers in total leukocytes; B) FACS analysis for CD14 and CD31 in healthy donors, and C) Enzyme-linked immunosorbent assay (ELISA) for VEGF measurement in PB serum.

Regarding monocytes exposed to H_2O_2 to undergo EC differentiation, it was seen that H_2O_2 decreases VEGF levels, but a long exposure to Propranolol reverts this tendency (Fig III.2), maybe because Propranolol decreases the use of VEGF by monocytes.

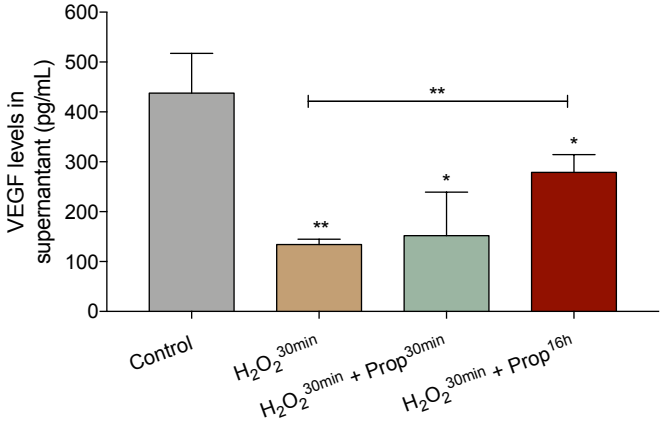


Figure III. 2 Monocytes exposed to ROS (H_2O_2) increase the use of VEGF, but Propranolol (Prop) exposure (16 h) partially reverted this phenotype.

Enzyme-linked immunosorbent assay (ELISA) for the measurement of VEGF levels in culture medium conditioned by monocytes isolated from healthy donors exposed to H_2O_2 in the presence and/or absence of Propranolol. *p<0.05; **p<0.01

Propranolol impairs ECs angiogenic features and inhibits vessel-like sprouting *ex vivo*

The activation of quiescent ECs is pivotal for angiogenic processes. Our *in vitro* results showed that Propranolol impairs ECs angiogenic properties through the decrease of ECs proliferation (Fig III.2 A) and migration (Fig III.2 B) and impairing their capacity to form vessel-like structures (Fig III.2 C). Moreover, using an aortic ring *ex vivo* model, mimicking the vessels microenvironment and the ECs response upon a stimuli, it was observed that Propranolol inhibits ECs activation and vessel-like sprouting (Fig III.2 D), abrogating the capacity of ECs to form vessel-like structures.

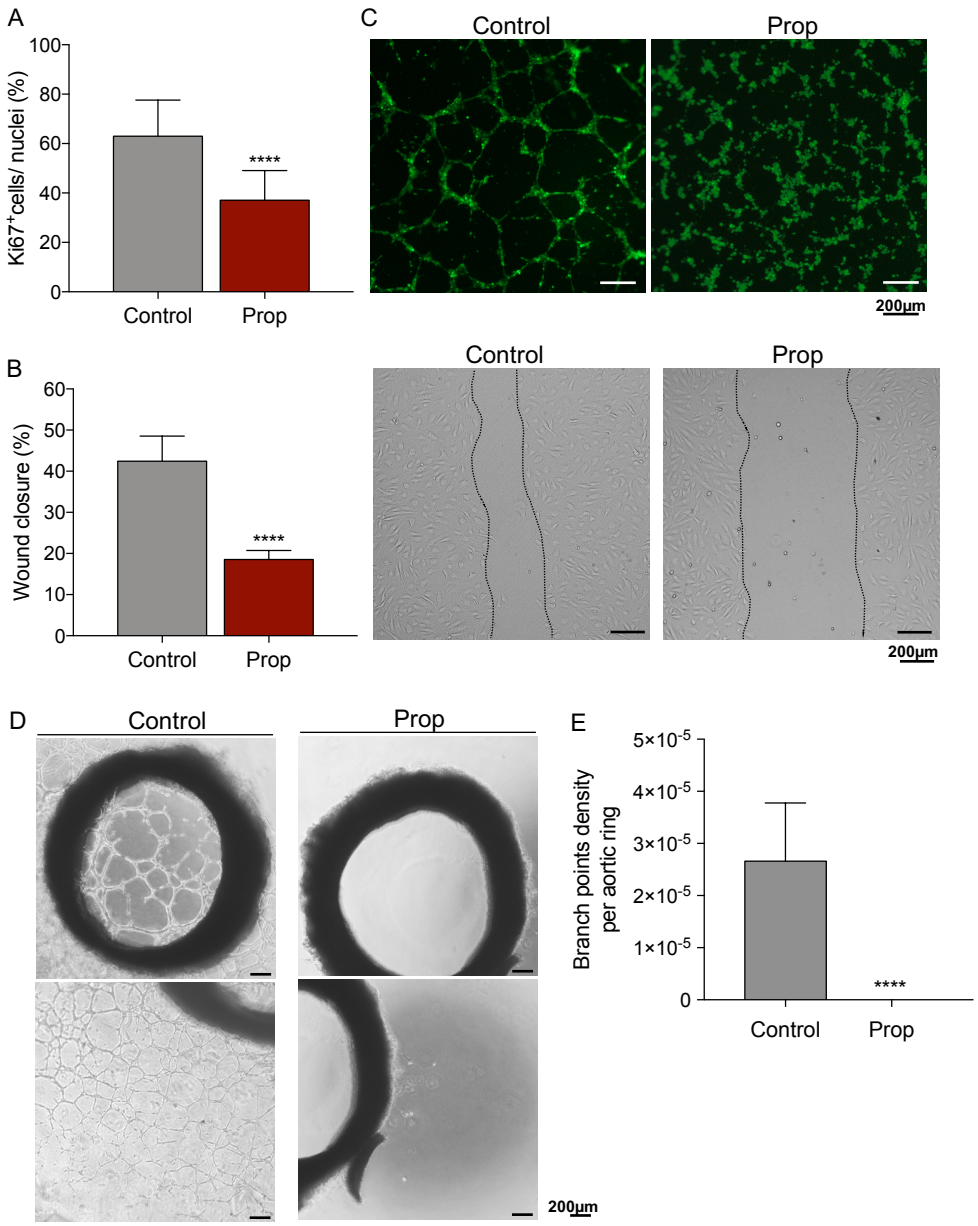


Figure III. 3 Propranolol (Prop) decreases ECs proliferation, migration and impaires the capacity to form vessel-like structures.

A) Proliferation analysis based on the percentage of Ki67^{+}nuclei/total nuclei of HUVECs cultured with and without Prop (100mg), for 16 h; B) Migration rate of HUVECs, previously exposed to mitomycin (3 h, 5mg/ml) to inhibit cell proliferation, in the absence and in presence of Propranolol, for 10 h; and C) Tube forming assay to evaluate the capacity of HUVECs cultured on the top of matrigel to form vessel-like structures, with and without Propranolol, for 6 h. D) Representative imagens at day 13 of aortic ring sprouting assay, in the presence and in the absence of Prop. E) Quantification of aortic ring sprouting assay (branch-point density), in the presence and in the absence of Propranolol. ****p<0.0001

Discussion

Propranolol is a liposoluble non-selective β-adrenergic blocker commonly used in the control of anxiety and cardiovascular conditions, such as hypertension, myocardial infarction and *angina pectoris*. In the last decade, Propranolol was re-discovered as an

effective drug in the treatment of some vascular tumors, inducing the rapid involution to quiescent residual lesions in 80% of cases (Storch and Hoeger, 2010; Zabramski *et al.*, 2016b; Al-Majed *et al.*, 2017; Rotter and de Oliveira, 2017; Apra *et al.*, 2019). The use of Propranolol in the treatment of infantile hemangiomas, the most common benign tumor of the skin, has been discovered accidentally in 2008 by Léauté-Labrèze et al, in which Propranolol administration demonstrated high efficacy and few adverse drug reactions (Léauté-Labrèze *et al.*, 2008). So far, the beneficial effect of Propranolol has been observed in the treatment of neonatal hemangiomatosis (Mazereeuw-Hautier *et al.*, 2010; H. Zhou *et al.*, 2014), placental chorioangioma (Padys *et al.*, 2015) and CCM (Moschovi *et al.*, 2010). Nevertheless, this selective therapeutic effect in a certain group of pediatric vascular tumors and the mechanisms that controls the development of the vascular abnormalities are not fully understood.

In this case study, the percentage of double positive $CD14^+/CD31^+$ cells was higher than in PB from healthy blood donors before Propranolol administration (Fig III.1 A and B), but during treatment's follow up a decrease in $CD14^+/CD31^+$ levels in PB of the patient was observed, presenting a final profile similar to healthy donors (Fig III.1 A and B). The observed normalization of the $CD14^+/CD31^+$ cell levels upon Propranolol administration suggested that the levels of circulating cells**,** sharing monocytic and ECs markers**,** are involved in CCM pathogenesis and are Propranolol-sensitive. Moreover, we speculate that circulating monocytes sharing ECs features ($CD14^+/CD31^+$) are acting as EPCs, contributing to CCM progression by being incorporated into CCM neovessels.

The exact mechanism by which Propranolol interferes with angiogenesis is not known, however some studies pointed that its anti-angiogenic effects are mediated by the downregulation of VEGF and FGF (Léauté-Labrèze *et al.*, 2008; Annabi *et al.*, 2009; Lamy *et al.*, 2010; Apra *et al.*, 2019). The dynamics of VEGF were also addressed in this case study, in an attempt to clarify if VEGF levels are linked to CCM regression. We observed, in this patient, a decrease in the levels of VEGF in the PB upon the Propranolol treatment (Fig III.1 C), coming close to the values observed in healthy donors (Fig III.1 C). Interestingly, *in vitro,* monocytes seems to use more VEGF upon H_2O_2 exposure, decreasing its free levels in conditioned culture medium, however longer exposure to Propranolol, rescues the observed decrease in VEGF levels due H_2O_2 exposure (Fig. III.2). This observation suggests that Propranolol, besides affecting the levels of circulating VEGF, it can also affect the way monocytes use VEGF *in vitro*, this way decreasing the overall pro-angiogenic capacity. Accordingly to the decreased stimulation of monocytes differentiation into ECs, we also observed that Propranolol affects the proliferation (Fig III.3 A), migration (Fig III.3 B) and vessel-like structure formation (Fig III.3 C) of mature ECs and impairs vessel-like sprouting in aortic rings (Fig III.3 D and E).

Since the VEGF levels could also be limiting in monocytes recruitment (Lee *et al.*, 2013; Jaipersad *et al.*, 2014; Wheeler *et al.*, 2018), suggests that the decreased VEGF levels, upon Propranolol treatment, is responsible, at least in part, by the decrease in the levels of circulating $CD14^+/CD31^+$ cells and CCM regression. However, further studies should be performed to understand how Propranolol is affecting the VEGF dynamics in monocytes and ECs.

Summing up, Propranolol, besides the promotion of CCM regression, impairs $CD14^+/CD31^+$ cells circulation (Fig III.1 A), in part by the decreased in VEGF levels (Fig III.1 C). Moreover, we observed *in vitro* that monocytes upon a pro-oxidant stimulus (H_2O_2), that pushes their differentiation towards ECs (Chapter II), decreases the levels of VEGF in cell supernatants. This indicates that VEGF is essential during the differentiation of monocytes into ECs. Considering that monocytes acting as EPCs may favor the development of CCM lesions and given that VEGF is pivotal for monocytic differentiation into ECs, the increased circulating VEGF levels observed in the CCM patient without treatment could be important for potentiating EC differentiation route and further, CCM pathogenesis.

Although Propranolol mechanism of action in CCM is not fully understood, the lack of a better therapeutic option for patients with surgically inaccessible CCM and the remarkable responses in a few patients makes it valuable to explore the exact efficacy of Propranolol in the treatment of CCM, as well as the associated adverse and side-effects (Apra *et al.*, 2019). In line, randomized prospective clinical trials with Propranolol *versus* placebo/nothing groups (NCT03523650, NCT03589014) are currently ongoing. Our case study reinforces the use of Propranolol in the clinical management of CCM and points out the monitorization of monocytes ($CD14^+/CD31^+$) and VEGF levels in PB as useful tools to predict the treatment effectiveness.

Chapter IV

Propranolol abrogates the ferroptosis-like mechanism pivotal in endothelial cells activation

This chapter was based on:

Lopes-Coelho F, Martins F, Hipólito A, Mendes C, Sequeira CO, Pires R, Almeida AM, Bonifácio VDB, Vicente JB, Pereira SA, Serpa J. The activation of endothelial cells relies on a ferroptosis-like mechanism: novel perspectives in management of angiogenesis and cancer therapy. Manuscript in preparation

Abstract

In cancer, the formation of blood vessels is crucial to sustain tumor growth and metastasis. It is known that the pro-oxidative tumor microenvironment (TME) favors angiogenesis and ferroptosis and it has been linked recently with the regulation of biological processes. However, so far, the effect of ferroptosis in endothelial cells (ECs) function and/or dysfunction has not been explored. Here, we show that Erastin-induced ferroptosis-like mechanism, at a non-lethal level, is involved in the activation of ECs features, such as proliferation, migration and vessel-like structures formation. The ECs activation is concomitant with GSH depletion and with the generation of oxidative stress and lipid peroxides. Moreover, the ferroptosis-like mechanism promotes VE-cadherin (VE-Cad) junctional gaps and potentiates cancer cells adhesion to ECs and transendothelial cancer cell migration. The Erastin effect is reversed by Propranolol, due to its anti-angiogenic and antioxidant properties mediated by the generation of hydrogen sulfide (H_2S). Our study shows that a ferroptosis-like mechanism underlies ECs activation and reinforces the use of Propranolol as an anti-angiogenic and anti-tumor drug.

Keywords: ferroptosis, oxidative stress, lipid peroxidation, endothelial cell hyperactivation, cancer invasion, Propranolol

Introduction

Tumor blood vessels are essential for providing nutrients and oxygen to cancer cells, and to eliminate waste products. In addition to the promotion of tumor growth, the neovasculature acts as gatekeeper for tumor cells invasion and metastasis (Potente *et al.*, 2011). In an ideal scenario, tackling tumor angiogenesis would be an efficient anti-cancer approach but, so far, the clinical application of the anti-angiogenic therapies have shown a lack of efficacy and drug resistance (Kim *et al.*, 1993; Vasudev and Reynolds, 2014).

During angiogenesis, the balance between pro-angiogenic factors (*e.g.* vascular endothelial growth factor - VEGF, fibroblast growth factors - FGFs, and angiopoietins - ANGs) and anti-angiogenic factors (*e.g.* endostatin, thrombospondin, and angiostatin) plays a vital role in the regulation of the angiogenic switch, a process characterized by the activation of quiescent endothelial cells (ECs) to form new blood vessels (Hanahan and Folkman, 1996). However, contrarily to physiological vessels networks, cancer neovessels are unorganized and leakier, suggesting that an imbalance in pro- and anti-angiogenic factors or the activation of unknown signaling pathways triggers an hyperactivation of the angiogenic switch and further formation of unstable cancer neovessels (Lugano *et al.*, 2020).

The oxidative stress acts as a pro-angiogenic stimulus, in both physiological and pathophysiological angiogenesis (Kim and Byzova, 2014; Prieto-Bermejo and Hernández-Hernández, 2017). Cancer neoangiogenesis seems to be more responsive to oxidative stress since the metabolic remodeling of malignant cells and tumor-associated stromal cells contributes for the generation of a pro-oxidative tumor microenvironment (TME) (Szatrowski and Nathan, 1991; Josson *et al.*, 2010; Ubaldo E. Martinez-Outschoorn *et al.*, 2011). At a molecular level, reactive oxygen species (ROS) inhibit PHDs (prolyl hydroxylases), leading to hypoxia-inducible factor 1α (HIF1α) stabilization and consequently to the transcription of VEGF and other pro-angiogenic factors (Kim and Byzova, 2014; Prieto-Bermejo and Hernández-Hernández, 2017).

The oxidative stress-dependent generation of lipid peroxides underlies ferroptosis, a recently discovered process of programmed cell death. In this process, a decrease in intracellular glutathione (GSH) levels and in the glutathione peroxidase 4 (GPX4) activity, alongside with increasing ROS levels, impairs lipid peroxide reduction by GPX4 and promotes lipid oxidation by Fe^{2+}, in a Fenton-like manner, which in turn, promotes ferroptosis (Friedmann Angeli *et al.*, 2014; Linkermann *et al.*, 2014; Yang *et al.*, 2014; Yu *et al.*, 2015; Lei *et al.*, 2019; J. Li *et al.*, 2020). The ROS-induced lipid peroxidation damages phospholipids directly and can also act as a cell death signal. Albeit, recent observations have shown that ferroptosis is not strictly a cell death type; it can also be

associated with the regulation of biological and pathophysiological processes, as carcinogenesis (Friedmann Angeli *et al.*, 2014; Linkermann *et al.*, 2014; Yang *et al.*, 2014; Yu *et al.*, 2015; Lei *et al.*, 2019; J. Li *et al.*, 2020). Ferroptosis can be induced by angiopoietin-like 4 (ANGPTL4), a potent angiogenesis mediator that activates the TAZ-ANGPTL4-NOX2 (transcriptional coactivator with PDZ-binding motif-ANGPTL4-NADPH oxidase 2) axis. This axis is responsible for the activation of NOX2 that induces the superoxide radical generation, which in turn, acts as an activator of ferroptosis (Dixon *et al.*, 2012; Xie *et al.*, 2016; Yang *et al.*, 2020), suggesting a correlation between ferroptosis and angiogenesis induction.

In the last years, Propranolol, a non-selective β-blocker (Al-Majed *et al.*, 2017) previously used in the context of cardiovascular diseases, was repurposed as a first-line therapy for vascular tumors, such as the child hemangiomas and cavernomas (Storch and Hoeger, 2010; Zabramski *et al.*, 2016a; Rotter and de Oliveira, 2017; Apra *et al.*, 2019). Retrospective studies demonstrated that breast cancer patients exposed to β-blockers prior or after diagnosis had a better disease prognosis and less metastases (Cardwell *et al.*, 2016; Pantziarka *et al.*, 2018; Hiller *et al.*, 2020). So far, it is recognized that angiogenesis impairment by Propranolol involves the downregulation of VEGF and FGF expression, and consequently the inhibition of MAPK signaling pathway (Pantziarka *et al.*, 2018; Cavalheiro *et al.*, 2016). Although it seems to be independent of its β-blocker action (Sasaki *et al.*, 2019), the specific mechanism(s) of action by which Propranolol affects endothelial cells (ECs) and angiogenesis remains to be clarified. A paper from Sasaki *et al*. demonstrated that Propranolol downregulates the expression of ANGPTL4 in hemangioma cells (Sasaki *et al.*, 2019). ANGPTL4, besides its involvement in the promotion of ferroptosis, regulates angiogenesis in a context-dependent manner, acting as a pro- or as an anti-angiogenic factor (Ito *et al.*, 2003; Le Jan *et al.*, 2003; Hato *et al.*, 2008; Chong *et al.*, 2014; Okochi-Takada *et al.*, 2014; Carbone *et al.*, 2018). Apart from the inhibition of ANGPTL4 expression and consequently abrogation of NOX2 activation, Propranolol could also interfere with ferroptosis through the inhibition of cytochrome P450 (CYP) enzymes, since some CYP-catalyzed reactions are involved in ROS and lipid peroxidation (Albertolle and Guengerich, 2018; Veith and Moorthy, 2018). Propranolol is a substrate of CYPs (Johnson *et al.*, 2000; Ayano, 2016; Mishima *et al.*, 2020), acting as an inhibitor of CYP2D6 (Rowland *et al.*, 1994), CYP2D19 (Lewis *et al.*, 2002) and CYP1A2 (Johnson *et al.*, 2000; Wang and Zhou, 2009). Recently, Mishima *et al*. identified the anti-ferroptotic properties of Propranolol, that promoted lipid peroxyl radicals scavenging, in a β1 activity-independent manner (Mishima *et al.*, 2020).

Here, we explore if the ferroptosis-like mechanism, at a non-lethal level, can promote ECs activation needed for angiogenesis, together with its reversion under the action of Propranolol.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs: CRL-1730, ATCC) were cultured in Endothelial Cell Growth Basal Medium-2 (EBM-2: CC-3156, Lonza, Bioscience) supplemented with EGM-2 SingleQuots Supplements (CC-4176, Lonza, Bioscience). All experiments were performed until the passage 10. Triple-negative breast cancer (MDA-MB-231: HTB-26™, ATCC) were used as tumor models, being cultured in Dulbecco's Modified Eagles Medium 1X (DMEM) (41965-039, Gibco, Life Technologies), supplemented with 10% fetal bovine serum (FBS; S 0615, Merck), 1% Antibiotic-Antimycotic (AA; P06-07300, PAN Biotech) and 50 µg/mL Gentamicin (15750-060, Gibco, Life Technologies). Cell cultures were maintained at 37°C in a humidified environment of 5% CO_2. Cells were detached with 0.05% Trypsin-EDTA 1X (25300-054, Invitrogen, Thermo Fisher Scientific) at 37°C for approximately 5 min. For each assay a Bürker counting chamber was used to determine the cell number necessary.

For experimental conditions, cells were cultured with 15 µM hydrogen peroxide (H_2O_2; 1.07210.0250, Merck), as a ROS generator, 1,5 µM Erastin (E7781, Sigma) as a ferroptosis inducer, 100 µM Propranolol (P8688, Sigma Aldrich), for 6 and 16 h.

Cell death analysis by flow cytometry

To analyze the effects of Propranolol and Era on EC death, HUVECs ($5x10^4$ cells/well) were seeded in 24-well plates. After exposure to the experimental conditions, supernatants and cells were collected and centrifuged at 155 *x g* for 5 min. Cell pellets were incubated with 0.5 µL FITC-labeled Annexin V (640906, BioLegend) in Annexin V binding buffer 1X (10 mM Hepes (pH 7.4) (391333, Millipore), 0.14 M sodium chloride (NaCl; 106404, Merck), 2.5 mM calcium chloride (CaCl2; 449709, Sigma Aldrich) and incubated at room temperature for 15 min, in dark. After incubation, cells were rinsed in 200 µL 1X Phosphate-buffered saline (PBS) / 0.1% (v/w) BSA and centrifuged at 155 *x g* for 2 min. The remaining pellet was resuspended in 200 µL of annexin V binding buffer 1X and 2.5 µL of 50 µg/mL propidium iodide (PI; P4170, Sigma Aldrich Aldrich) and analyzed by flow cytometry (FACScalibur – Becton Dickinson). *FlowJo X* v10.0.7 software (*https://www.flowjo.com/*) was used to analyze data.

Wound healing assay

Cells were plated on 24-well plates (1x10^5 cells/well) until the formation of a confluent monolayer. Once confluent, cells were incubated for 3 h with 5 µg/mL mitomycin-C (M4287, Sigma Aldrich), and a linear scratch in each monolayer was made with a P200 pipette tip, creating a wound across the well diameter. The media was replaced to remove debris and cells in suspension and the experimental conditions were added. Bright-field images of each well were acquired on the Olympus IX53 Inverted Microscope at the following timepoints: 0, 2, 4, 6, 8, 10 and 24 h. The wound closure was quantified using *ImageJ* software (*imagej.nih.gov/ij/*).

Tube-forming assay

A 48-well plate was coated with 100 µL matrigel (354230, Corning) and incubated at 37°C for 30 min until solidify. HUVECs were incubated with 2 µg/mL calcein (C1430, Invitrogen), a fluorescent cell permeable dye, for 30 min at 37°C and 5% CO2 and seeded (3x10^4 cells/well) on the top of matrigel. Cells were exposed to the experimental conditions for 6 h and representative images of the vessel-like structures formation were acquired in an Olympus IX53 Inverted Microscope and analyzed with *ImageJ* software (*imagej.nih.gov/ij/*). The density of vessel-like structures formation (branch points number/µm^2) was calculated as proxy of vascular density.

Reactive oxygen species (ROS) quantification by flow cytometry

HUVECs (5x10^4 cells/well) were plated in 24-well plates. The intracellular ROS were detected in cells incubated with 10 µM DCF-DA probe (D6883, Sigma Aldrich), and mitochondrial ROS were detected in cells incubated with 5 µM MitoSox Red probe (M36008, Invitrogen), both at 37°C for 30 min. The acquisition was performed with FACScalibur (Becton Dickinson) and data were analyzed with *FlowJo X* v10.0.7 software (*https://www.flowjo.com/*).

Lipid peroxides quantification by flow cytometry

HUVECs (5x10^4 cells/well) were plated in 24-well plates. After experimental conditions, cells were incubated with 2 µM C11-Bodipy 581/591 (D3861, Invitrogen), for 30 min at 37 ºC in dark. Excessive dye was removed by washing with 2% FBS-1X PBS and cell pellets were resuspended in 2% FBS-1X PBS for the acquisition by flow cytometry (FACScalibur – Becton Dickinson). *FlowJo X* v10.0.7 software (*https://www.flowjo.com/*) was used to analyze data.

High-performance liquid chromatography (HPLC)

HUVECs were plated 6-well plate ($2x10^5$ cells/well) and after collection, cell pellets were lysed with 0.01% triton-PBS and centrifuged at 20 000 *x g*, for 10 min at 4 ºC. The assessment of aminothiol profile (total, total free, free, protein bound and oxidized form) of lysed cells and supernatants was performed according to Grilo et al. adapted to cell culture (Grilo et al. 2017). The cysteine (Cys) and glutathione (GSH) metabolites were separated on a reversed-Phase C18 LiChroCART 250-4 column (LiChrospher 100 RP-18, 5 µm, VWR, USA), in an isocratic elution mode for 22 min, at a flow rate of 0.6 mL/min by HPLC system (Shimadzu Scientific Instruments Inc) with a RF 10AXL fluorescence detector, operating at excitation and emission wavelengths of 385 and 515 nm, respectively. The mobile phase consisted of 100 mM acetate buffer (pH 4.5) and methanol (98:2 (v/v)). The absolute thiols values were normalized to the protein assessed with Bradford method (500-0006, Bio Rad).

Quantification of H_2S in cell homogenates

HUVECs ($2x10^5$ cells/well) were seeded in 6-well plates and cultured in the experimental conditions, for 16 h. After, cells were scrapped in PBS 1X and centrifuged at 210 *x g* for 5 min. The cell pellet was homogenized in NP40 lysis buffer (1% NP40, 150 mM NaCl, 50 mM Tris-Cl, pH 8.0) on ice for 30 min and centrifuged for 5 min at 20,000 *x g* 4 ºC. Cell homogenates (20 µL) were incubated in black 96-well plates with 80 µL of 10 µM 7-Azido-4-Methylcoumarin probe (AzMC, L511455, Sigma Aldrich) and the protein concentration was determined using the Bradford method (500-0006, Bio Rad). The H_2S measurements were posteriorly normalized to total protein concentration and to a blank sample (cellular lysates without probe). H_2S production was monitored in a VIKTOR3 instrument from *PerkinElmer/ Wallac 1420 Software (version 3.0)* through the measurement of AzMC fluorescent signal (355 nm/460 nm) every 30 min for 2 h.

Immunofluorescence

For Ki67, ICAM and VCAM immunodetection, HUVECs ($5x10^4$ cells/well) were cultured on glass slides with 0.2% gelatin coating and fixed in 2% paraformaldehyde, for 15 min at 4°C. After blocking with 1% Bovine serum albumin (BSA)-1X PBS, cells were incubated with primary antibodies (anti-Ki67, 1:100 in 1% BSA-0.1% triton X-100- 1X PBS (w/v/v); sc-15402, Santa Cruz; anti-ICAM and anti-VCAM, 1:500 in 0.1% BSA-0.1% triton X-100-PBS (w/v/v); SRC023, Millipore), overnight at 4 ºC, followed by an incubation with secondary antibodies (Alexa Fluor 488 goat anti-rabbit, 1:1000 in 1% BSA-0.1% triton x100-PBS; A-11078, Invitrogen - Thermo Fisher Scientific; Alexa Fluor 488 goat anti-mouse ;115-545-003, Thermo Fisher Scientific and Alexa Fluor 594 donkey anti-

mouse; A21203, Thermo Fisher Scientific both at 1:1000 in 0.1% BSA-0.1% triton x100-PBS), for 2 h at room temperature.

For xCT immunodetection, after fixation cell were incubated with 50 mM ammonium chloride (NH_4Cl) for 10 min, followed by blocking and incubation with anti-xCT (1:100 in 0.5% BSA-0.1% saponin-PBS (w/v/v); ab1756, Millipore), for 30 min at room temperature.

For VE-Cadherin (VE-Cad) immunodetection, HUVECs ($1x10^5$ cells/well) were cultured in 24-well plate with glass slides coated with 0.2% gelatin, until the formation of a confluent monolayer. After fixation and blocking, cells were incubated anti-VE-Cad (1:50 in 3% BSA-0.1% triton X-100-PBS (w/v/v); AF938, R&D), for 2 h at room temperature, followed by an incubation with the secondary antibody (Alexa Fluor 488 donkey anti-goat, 1:500 in 3% BSA-0.1% triton X-100-PBS; A11055, Thermo Fisher Scientific), for 2 h at room temperature.

All slides were mounted in VECTASHIELD media with DAPI (4′-6-diamidino-2-phenylindole; H-1200, Vector Labs) and examined by standard fluorescence microscopy, using an Axio Imager.Z1 microscope (Zeiss) with a CytoVision® software.

The determination of cell proliferation rate was based on the ratio of total and $ki67^+$ nuclei and the quantification of ICAM and VCAM expression per cell was calculated according to the formula CTCF (corrected total cell fluorescence) = integrated density – (area of selected cell x mean fluorescence of background reading), both using ImageJ software. The quantification of VE-Cad junctional gaps was performed using *ImageJ* software (*imagej.nih.gov/ij/*).

Cancer cells endothelial adhesion

On a 24-well plate, calcein labelled-MDA-MB-231 ($5x10^4$ cells/well) were seeded on the top of a confluent HUVECs ($1x10^5$ cells/well) monolayer pretreated with 100ng/mL TNFα (H8916, Sigma), for 24 h. MDA-MB-231 were incubated with HUVECs (exposed previously to TNFα and experimental conditions) for 40min, at 37°C in a humidified environment of 5% CO_2. The non-adherent cells were removed by washing with 1X PBS and imagens were acquired in an Olympus IX53 Inverted Microscope and analyzed using *ImageJ* software (*imagej.nih.gov/ij/*). Three fields in each well were evaluated (10X magnification).

Transendothelial cancer cells migration

HUVECs ($5x10^4$ cells/well) were plated in 8 μm pore transwells (upper wells) (3422, Corning) until the formation of a confluent monolayer and exposed to 100ng/mL TNFα

for 24 h, and to experimental conditions, for 16h. MDA-MB-231 previously plated under starvation using serum-free DMEM, for 24 h, were incubated with calcein (2 µg/mL) and seeded ($1.5x10^4$ cells/well) in serum free DMEM on the top of the HUVECs monolayer, for 5 h. Complete media was added to the lower well and used as chemoattractant. Cells on the upper transwell surface were removed with a cotton swab and the invading MDA-MB-231-calcein labeled cells were photographed in an Olympus IX53 Inverted Microscope. Three fields in each well were counted (10X magnification) using *ImageJ* software (*imagej.nih.gov/ij/*).

Monocytes isolation, culture and characterization

Monocytes isolation and further cell characterization has been performed as described in chapter II. Briefly, cultured monocytes for 4 days in colony-forming unit (CFU) medium (130-091-277, MACS Technology) and for 1 day in complete EBM-2 were incubated with von Willebrand factor (vWF; 1:500 in 0.5% BSA-0.1% saponin-PBS; A0082, Dako), for 60 min at 4 ºC with gentle shaking followed by the incubation with Alexa Fluor 488 anti-rabbit), for 30 min at 4 ºC in dark, with gentle shaking. H_2O_2 (15 µM) was used as a promoter of monocytes differentiation into EC, as described in chapter II. vWF expression was detected by flow cytometry in a FACScalibur–Becton and data were analyzed using *FlowJo X* v10.0.7 software (*https://www.flowjo.com/*).

Statistical analysis

All data were analyzed using student's t-test, one-way ANOVA or two-way ANOVA in GraphPad Prism 7 software (*www.graphpad.com/).* The assays were performed with at least 3 biological replicates *per* condition and the differences were determined statistically significant at p value < 0.05.

Results

Erastin promotes the generation of ROS-induced lipid peroxides, while Propranolol (Prop) revert this effect through its antioxidant properties mediated by hydrogen sulfide (H_2S) levels

Erastin was used as a ferroptosis activator, since it inhibits the sodium-independent cystine/glutamate antiporter System xc- (xCT; encoded by *SLC7A11*), responsible for the import of cystine (Lo *et al.*, 2008; Lewerenz *et al.*, 2013), the main source of cysteine to sustain GSH synthesis (Sun *et al.*, 2018). Therefore, upon the inhibition of xCT the function of glutathione peroxidase 4 (GPX4) will be impaired, promoting an increase in lipid peroxidation, mediated by free active iron (Sato *et al.*, 2018), and further ferroptosis

(Koppula *et al.*, 2018). The expression of xCT was confirmed in HUVECs (Fig IV.1) and ferroptosis-related features were analyzed. HUVECs exposed to Erastin increased intracellular ROS (Fig IV.2 A) and lipid peroxides levels (Fig IV.2 C), without affecting mitochondrial ROS (Fig IV.2 B). Moreover, Erastin exposure decreases GSH levels (Fig IV.2 D) and their inhibitory effect in cyst(e)ine uptake was confirmed, since cells exposed to Erastin present high levels of total cysteine in culture medium (Fig IV.2 E). Interestingly, the increased generation of ROS (Fig 2 A), lipid peroxides (Fig IV.2 C) and the depletion of GSH (Fig IV.1 D) did not promote ferroptosis-induced ECs death (Fig IV.2 F). Considering that the ferroptosis-related features of HUVECs exposed to Erastin did not promote cell death we termed the effects of Erastin in HUVECs as ferroptosis-like mechanism.

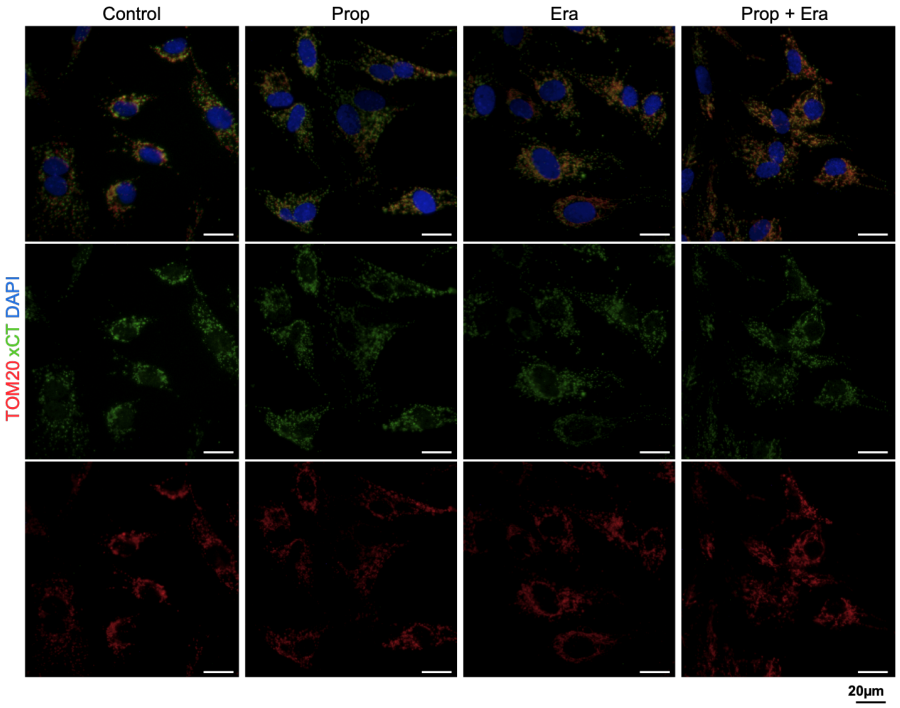


Figure IV. 1 xCT is expressed in endothelial cells (HUVECs).

HUVECs expresses xCT (green) mainly in mitochondria (TOM20, red), and its expression is not affected by Erastin (Era) and/or Propranolol (Prop), for 16 h (scale: 20µm).

Moreover, we observed that Propranolol alone has antioxidant properties that besides decreasing the intracellular ROS levels (Fig IV.2 A), also reverted the levels of ROS (Fig IV.2 A) and lipid peroxides (Fig IV.2 C) induced by Erastin. However, Propranolol also decreased GSH reduced and total levels, maintaining the oxidized GSH (GSSG) levels similar to the control condition, both alone or in combination with Erastin (Fig IV.2 D).

Since the Propranolol antioxidant effect is not due to increased GSH levels, we hypothesized that hydrogen sulfide (H_2S) generation may have a role in the antioxidant properties of Propranolol. H_2S, a product of cysteine degradation (Hipólito *et al.*, 2020; Nunes, Ramos, *et al.*, 2018), is a powerful antioxidant (Shefa *et al.*, 2018), with a reductive potential similar to the couple glutathione disulfide/glutathione (GSSG/GSH) (Giuffrè and Vicente, 2018). To disclose the involvement and the production of H_2S upon Propranolol exposure, H_2S production was quantified. Erastin did not affect H_2S levels while Propranolol exposure increased H_2S levels (Fig IV.2 G). Moreover, when Propranolol was combined with Erastin, the levels of H_2S come close to the control, suggesting that Propranolol's antioxidant effect is mediated by H_2S.

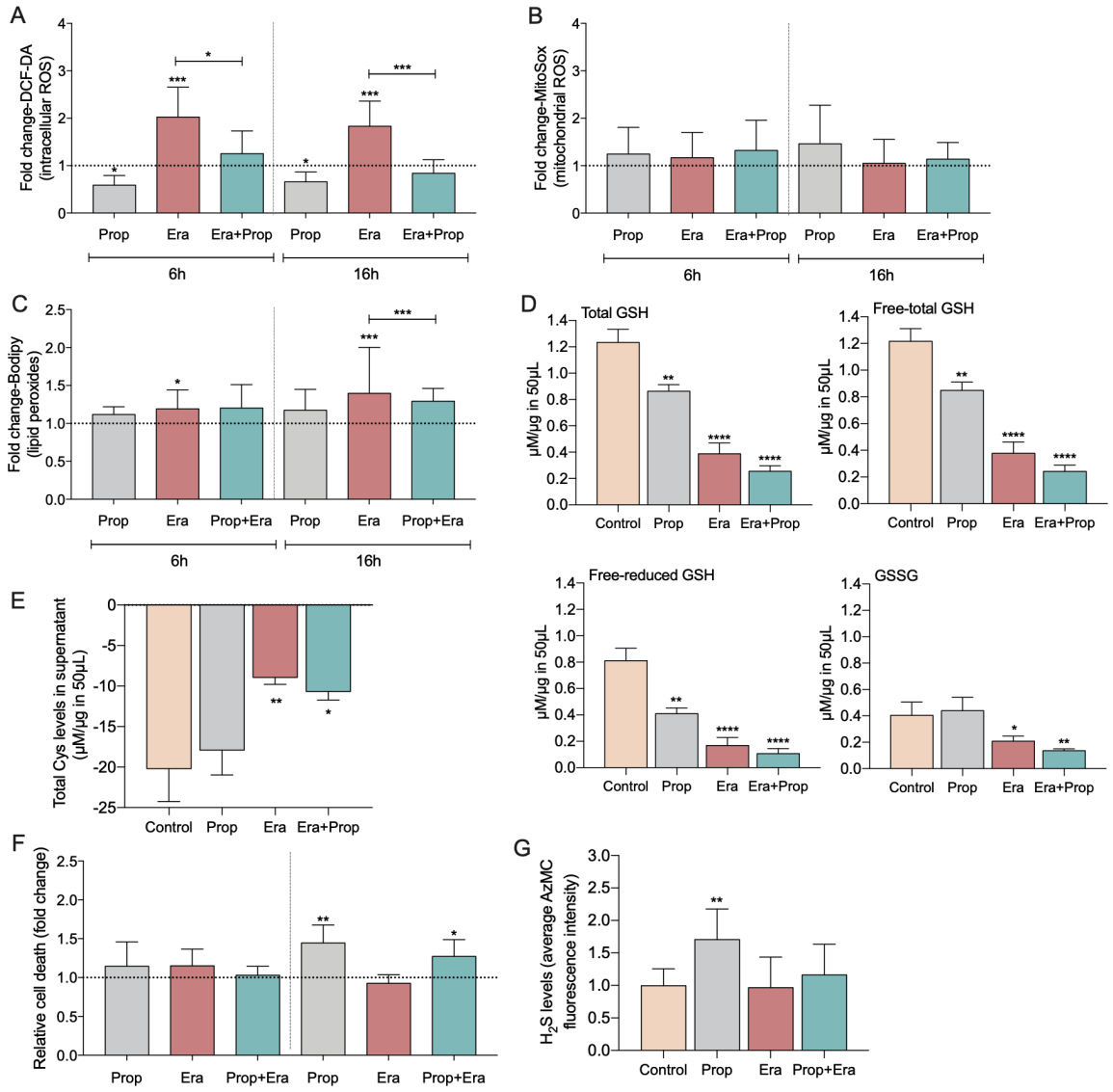


Figure IV. 2 Erastin (Era) promotes increased levels of ROS-induced lipid peroxides and Propranolol (Prop), through the generation of hydrogen sulfide (H_2S), is able to revert it.

A) The levels of intracellular ROS (DCF-DA) decreased upon Prop exposure, and Era, although increasing the ROS levels, when co-administrated with Prop the levels came close to the control, at 6 and 16 h. B) The

levels of mitochondrial ROS, assessed by MitoSox, were not affected by the presence of Era and/or Prop, at 6 and 16 h. C) Era induced lipid peroxides (C11-Bodipy) generation and although Prop alone did not affect the lipid peroxides content, its combination with Era reverted the levels generated by Era, being this effect more prominent at 16 h. D) The levels of GSH (total and free total) decreased upon exposure to Era and/or Prop for 16h. E) Era did not affect HUVECs death (annexin V plus PI positive cells), while 16 h of Prop exposure, with and without Era, increased the ratio of HUVECs death. F) Era did not affect H_2S levels of HUVECs while Prop increased, at 16 h. In graphs the dashed line represents the control condition. All data are normalized to the control condition and represented as mean ± SD. *p<0.5, **p<0.01, ***p<0.001, ****p<0.0001.

A ferroptosis-like mechanism promotes EC activation, which is impaired by the antioxidant properties of Propranolol

Hence, we investigate if the generation of ROS-induced lipid peroxides through this ferroptosis-like mechanism induces ECs activation. It was observed that Erastin exposure increased HUVECs proliferation (Fig IV.3 A) and migration (Fig IV.3 B), without affecting cell viability (Fig IV.2 E), suggesting that a ferroptosis-like mechanism has a role in the promotion of ECs activation,

Considering that Propranolol acts as an inhibitor of angiogenesis and ferroptosis (Sharifpanah *et al.*, 2014; Cavalheiro *et al.*, 2016), its interference with ferroptosis-mediated angiogenesis was evaluated. In fact, HUVECs exposed to Propranolol showed decreased proliferation (Fig IV.3 A) and migration (Fig IV.3 B) and increased cell death, at 16h (Fig IV.2 F). Moreover, Propranolol was able to revert the Erastin effect at 16h, leading to the inhibition of HUVECs proliferation (Fig IV.3 A) and migration (Fig IV.3 B), as well as disturbing cell viability (Fig IV.2 F).

Since the pro-oxidative microenvironment promotes angiogenesis (Kim and Byzova, 2014; Prieto-Bermejo and Hernández-Hernández, 2017), the HUVECs capacity to form vessels-like structures was tested under oxidative conditions simulated by hydrogen peroxide (H_2O_2) exposure, in an *in vitro* tube-forming assay. Erastin exposure increased the branch points density at the same range of H_2O_2 and no cumulative effect between Erastin and H_2O_2 was observed (Fig IV.3 C). Moreover, Propranolol decreased the vessel-like structure formation (Fig 3 C) and reverted the stimulation by H_2O_2 and Erastin (Fig IV.2 C), even when Propranolol was added to the vessel-like structures already formed in the presence of Erastin (Erastin+Propranolol (2h); Fig IV.3 D). These results indicate that the Propranolol anti-angiogenic effect is related, at least in part, to the abrogation of the ferroptosis-like mechanism induced by Erastin.

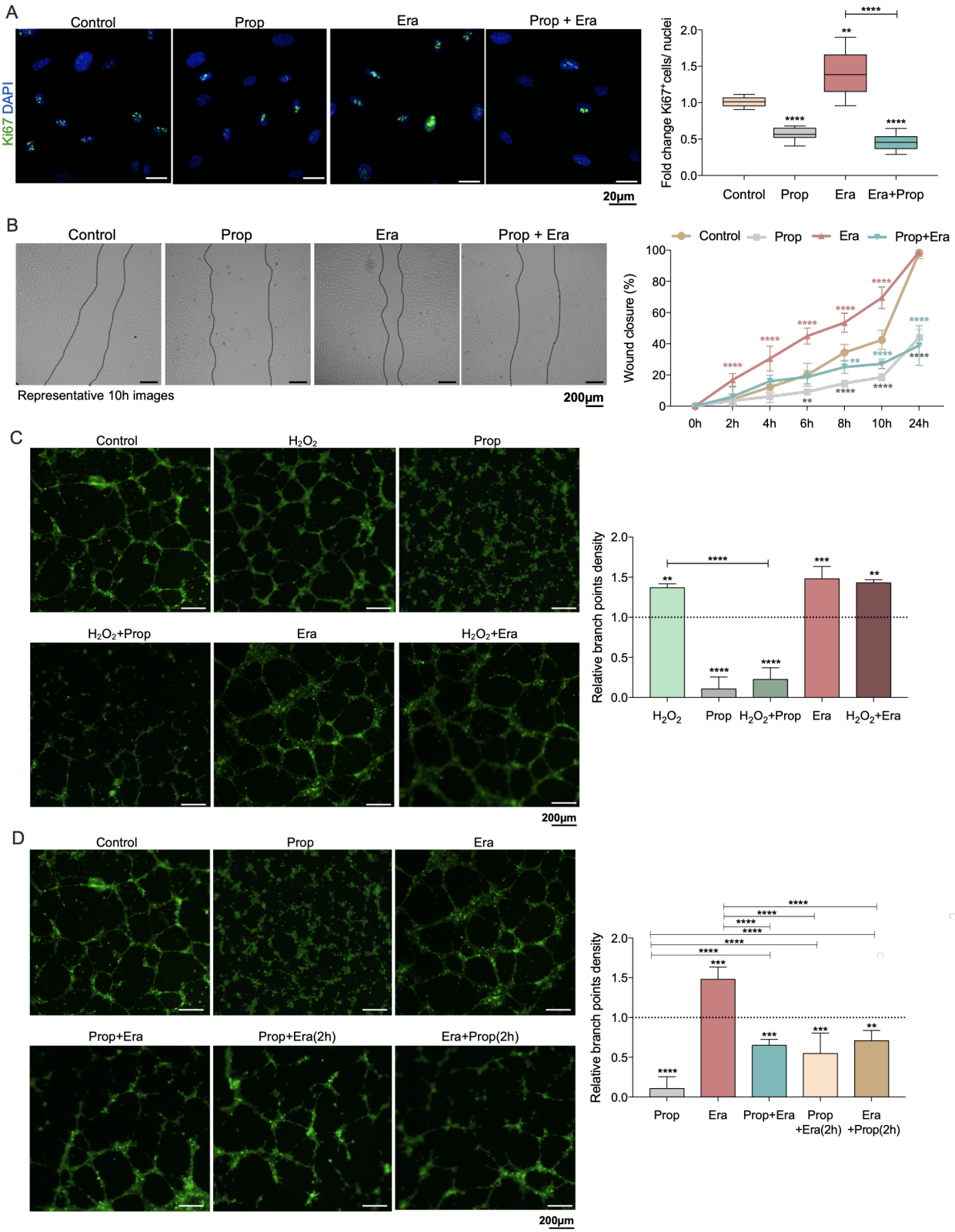


Figure IV. 3 The ferroptosis-like mechanism driven by Erastin (Era) promotes endothelial cells (ECs) activation and Propranolol (Prop) impairs the phenotype induced by Era exposure.

A) The ferroptosis-like mechanism, generated by Era exposure, promoted HUVECs proliferation (increased ratio of Ki67+ (green) nuclei/total nuclei), while Propranolol decreased the rate of HUVECs proliferation and impaired the phenotype induced by Era. The panel shows representative microscope images of the Ki67

staining (scale: 20 µm). B) The panel shows representative microscope images of wound closure at 10 h (scale: 200 µm). Era fostered HUVECs migration (increased % wound closure) and Prop inhibited and reverted the phenotype induced by Era. The panel shows representative microscope images of wound closure at 10h (scale: 200 µm). C/D) The panels show representative microscope images of tube-forming assay using HUVECs-calcein labelled cells (scale: 200 µm). Era increased the branch point density of vessel-like structures (proxy for vascular density) at the same range of H_2O_2 (ROS; positive control), without an additive effect. Prop, besides the impairment of vessel-like structures formation (decreased branch point density), inhibited the phenotype induced by Era, even when Prop was added to the vessel-like structures already formed in the presence of Era (Era+Prop (2 h)) and contrariwise (Prop +Era (2 h)). In graphs the dashed line represents the control condition. All data are normalized to the control condition and represented as mean ± SD. *p<0.5, **p<0.01, ***p<0.001, ****p<0.0001.

A ferroptosis-like mechanism induced by Erastin promotes the generation of leakier ECs structures, which are normalized by Propranolol, blocking cancer cell adhesion and transendothelial migration

The generation of a pro-oxidative microenvironment is correlated with the formation of disorganized and leakier vessel networks (Lugano *et al.*, 2020). Thus, the adhesion structures in HUVECs were evaluated, through vascular endothelial-cadherin (VE-Cad) immunodetection. VE-Cad is a component of ECs adherens junctions, crucial for the stability and function of the mature vessels (Harris and Nelson, 2010). Erastin affected the HUVECs monolayer stability by increasing VE-Cad intercellular junctional gaps and Propranolol reverted this effect (Fig IV.4 A and B). The ferroptosis like-mechanism mediates the proliferation of ECs (Fig IV.3 A), although with increased VE-Cad intercellular junctional gaps (Fig IV.4 A and B) between ECs, thereby contributing to a more instable vascular structure. Interestingly, Propranolol reverted this phenotype (Fig IV.4 A and B), inducing the stabilization of the ECs monolayer.

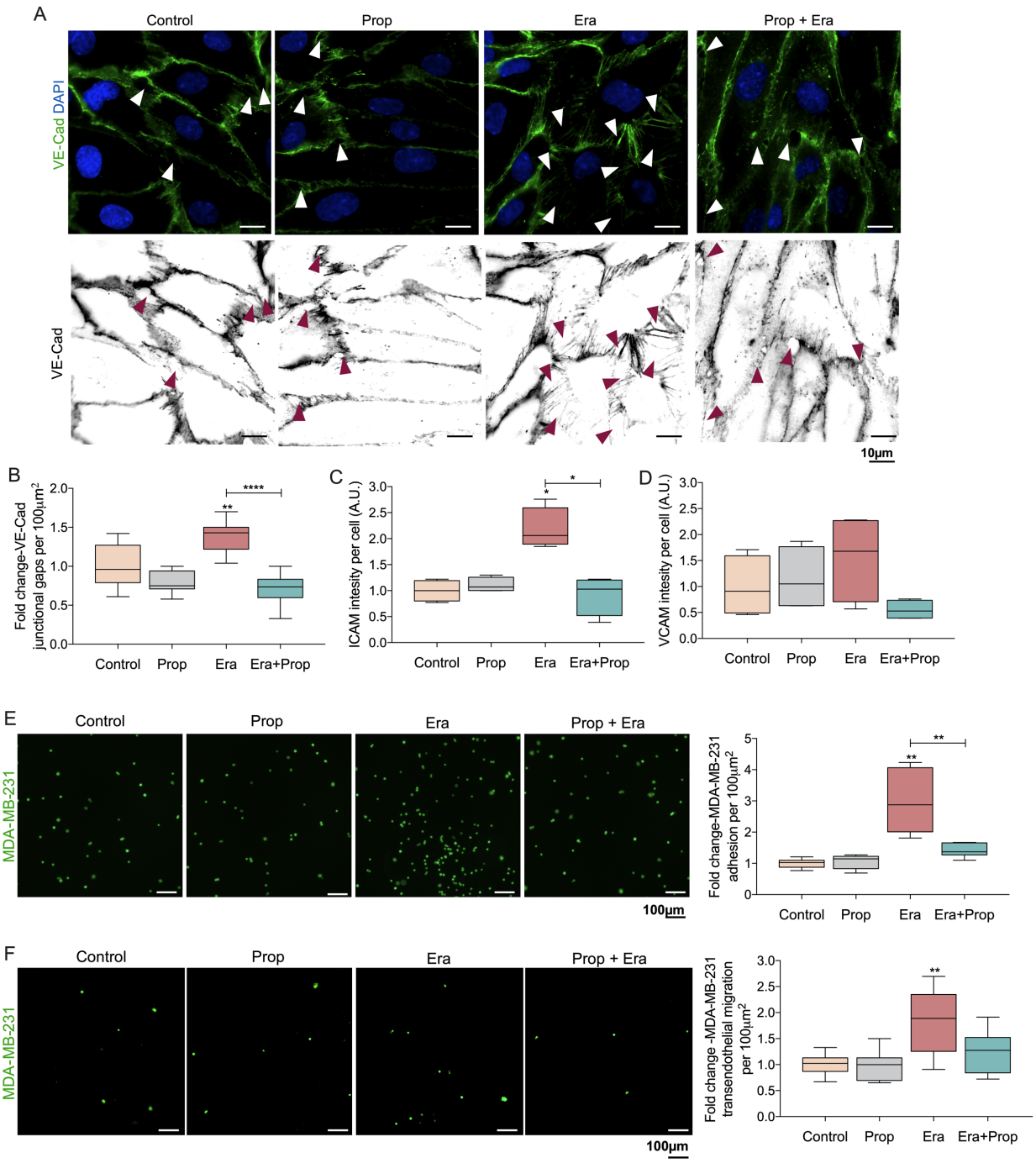


Figure IV. 4 Erastin (Era) promotes the generation of a leakier ECs monolayer while increases cancer cell-EC interaction and transendothelial migration.

A) The ferroptosis-like mechanism driven by Era promoted an increased generation of intercellular VE-Cadherin (VE-Cad) gaps *per* 100µm^2 , while Propranolol (Prop) was able to revert this phenotype. The panel shows representative images (scale: 10 µm) of VE-Cadherin (green) intercellular junctional gaps (arrows) in HUVECs exposed to Era and/or Prop for 16h. B) Intracellular adhesion molecule (ICAM) intensity *per* cell (HUVECs; A.U.: arbitrary units) increased upon Era exposure and although Prop alone did not affect ICAM expression, it was able to abrogate the expression induced by Era, at 16 h. Vascular cell adhesion protein (VCAM) intensity *per* cell (A.U.: arbitrary units) shows a tendency to increase under Era exposure. The ferroptosis-like mechanism, induced by Era, promoted cancer cell (MDA-MB-231-calcein labelled cells) adhesion to HUVECs (C) and transendothelial migration (D). Prop alone had no effect but impaired cancer cell adhesion and transendothelial migration induced by Era. The panels show representative microscope images (scale: 100 µm; MDA-MB-231-calcein labelled cells (green)). All data are normalized to the control condition and represented as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

Intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) expression in ECs is important for cancer cell-EC interaction during the metastatic cascade (Chen and Massagué, 2012; Zhang *et al.*, 2014; Shenoy and Lu, 2016). HUVECs exposed to Erastin significantly increased ICAM expression, as well as VCAM expression, though not in a statistically significant range (Fig IV.4 C). On the contrary, Propranolol alone did not alter ICAM and VCAM expression, but it was able to revert the increased expression induced by Erastin (Fig IV.4 C and D). Considering the increased expression of ICAM (Fig IV.4 C), we also explored the effect of Erastin and Propranolol on cancer cells adhesion to ECs and transendothelial migration. The triple negative breast cancer cell line MDA-MB-231 was co-cultured on the top of a previously stablished HUVECs monolayer exposed to Erastin and/or Propranolol. Erastin increased the number of cancer cells adherent to the ECs monolayer (Fig IV.4 E) and stimulated the transendothelial migration (Fig IV.4 F), while Propranolol reverted both Erastin effects (Fig IV.4 E and F). Our results indicate that a ferroptosis-like mechanism has also a role in the promotion of vessel stabilization and in cancer cells adhesion and extravasation. Summing up, Propranolol was able to revert the phenotype induced by Erastin, decreasing the intercellular junctional gaps (Fig IV.4 A and B), cancer cells adhesion (Fig IV.4 E) and transendothelial migration (Fig IV.4 F), therefore suggesting that Propranolol could eventually impair, or at least retard, the metastatic process (Hiller *et al.*, 2020).

Neither Erastin nor Propranolol affected monocytes differentiation to ECs

In chapter II, we showed that monocytes act as endothelial progenitor cells (EPCs), being their differentiation into ECs promoted by a ROS-enriched microenvironment simulated by H_2O_2. Considering the effect of a ferroptosis-like mechanism in ECs activation, we explored its impact in the differentiation route of monocytes into ECs, assessed by the gain of vWF (Von Willebrand factor or VIII factor). Erastin and/or Propranolol exposure had no effect in vWF expression and their concomitant exposure with a H_2O_2 did not change the vWF expression stimulated by H_2O_2 (Fig IV.5 A). Moreover, ROS and lipid peroxides (Fig IV.5 B and C) levels did not alter with Erastin exposure after H_2O_2 stimulation. In contrast to ECs, Propranolol did not affect ROS and lipid peroxides (Fig IV.5 B and C) levels in monocytes, suggesting that the antioxidant role of Propranolol verified during ECs activation did not interfere with the differentiation process of monocytes towards ECs.

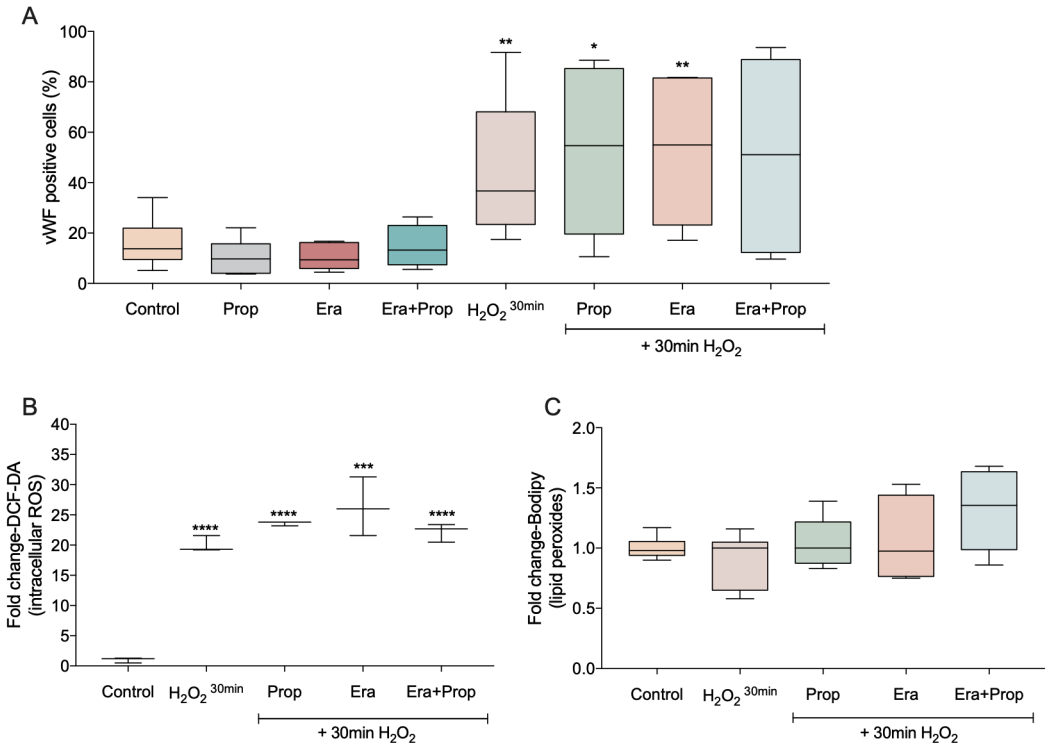


Figure IV. 5 Erastin (Era) and Propranolol (Prop) do not affect the differentiation route of monocytes into ECs.

A) Neither Era nor Prop affected the expression of vWF, even when co-exposed with a short H_2O_2 stimulation (positive control of the differentiation pattern of monocytes), indicating that Era and/or Prop had no impact in the differentiation process of monocytes-derived cells into ECs. Era and/or Prop exposure before the short H_2O_2 stimulation did not influence the intracellular ROS (DCF-DA; B) and lipid peroxides (11C-Bodipy; C) levels. In B and C data are normalized to the control condition and represented as mean ± SD. *p<0.5, **p<0.01, ***p<0.001, ****p<0.0001.

Discussion

During tumorigenesis, the increasing metabolic rate of cancer cells drive the generation of a pro-oxidative TME, which is responsible for the production and release of pro-angiogenic factors by cancer and tumor-associated stromal cells (Schito and Semenza, 2016; De Sá Junior *et al.*, 2017). The ROS production within the TME can be stimulated by VEGF and hypoxia, the main players of ECs activation (Brand *et al.*, 2004; Dewhirst *et al.*, 2008; Coso *et al.*, 2012; Lassègue *et al.*, 2012). The major production of ROS depends on superoxide-generating enzymes (NOX), which generate $O_2^{\cdot-}$ through the transfer of electrons from NADPH to oxygen (Brand *et al.*, 2004; Dewhirst *et al.*, 2008; Coso *et al.*, 2012; Lassègue *et al.*, 2012). The generation of NOX-dependent ROS increases VEGF secretion and angiogenesis, in a HIF1α-dependent manner (Xia *et al.*, 2007). Therefore, the generation of a pro-oxidative and pro-angiogenic TME seems to work synergistically in the promotion of tumor angiogenesis.

Ferroptosis has been firstly described as an iron-dependent programed cell death characterized by the accumulation of lipid peroxides, although new evidence have shown that ferroptosis is not a strict cell death mechanism (Hirschhorn and Stockwell, 2019). In fact, ferroptosis revealed to contribute to the regulation of biological and pathophysiological processes (Friedmann *et al.*, 2014; Linkermann *et al.*, 2014; Yang *et al.*, 2014; Yu *et al.*, 2015; Lei *et al.*, 2019; Li *et al.*, 2020;). Ferroptosis-inducing compounds, as Erastin, affects the antioxidant capacity of cells through the inhibition of xCT, that was proved to be expressed in ECs (HUVECs) (Fig IV.1). The impairment of xCT activity disturbs cystine import, the main source of cysteine for GSH synthesis. GSH acts as an electron donor and reduce lipid hydroperoxides upon GPX4 action (Yang *et al.*, 2014; Proneth and Conrad, 2019; Li *et al.*, 2020). So far, there are no specific markers for ferroptosis and since we are interested in disclosing the non-lethal effect of ferroptosis on angiogenesis, here we evaluated the levels of ROS-induced lipids peroxides underlying ferroptosis and GSH dynamics. In our experimental conditions, we observed that Erastin promotes the increase of intracellular ROS (Fig IV.2 A) and lipid peroxides levels (Fig IV.2 C), without affecting the mitochondrial ROS content (Fig IV.2 B). In agreement, a significantly decreased cyst(e)ine uptake (Fig IV.2 E) concomitant with GSH depletion was observed upon Erastin exposure (Fig IV.2 D). Since the aforementioned ferroptosis-like features did not account for ECs death (Fig IV.2 F), from now on we termed the effects of Erastin in HUVECs as ferroptosis-like mechanism.

The antiferroptotic properties of Propranolol has been recently described, in which its peroxyl radicals scavenging effect is independent of β1-blockade activity (Mishima *et al.*, 2020). Here, we observed that although Propranolol decreased reduced and total GSH levels and maintain GSSG (oxidized GSH) levels, in comparison to the control, it exerts antioxidant effects through the decrease of intracellular ROS levels (Fig IV.2 A) and the reversion of the accumulation of lipid peroxides induced by Erastin (Fig IV.2 A and C). In fact, the increased H_2S levels upon Propranolol exposure (Fig IV.2 G) indicates that the antioxidant Propranolol property might be mediated by H_2S, since it is a gasotransmitter capable of regulating oxidative stress by directly scavenging ROS (Olas, 2017; Shefa *et al.*, 2018).

Although the generation of a pro-oxidative TME is already implicated in the promotion of the angiogenic switch and further angiogenesis (Kim and Byzova, 2014; Prieto-Bermejo and Hernández-Hernández, 2017), here we unraveled for the first time the role of a ferroptosis-like mechanism in ECs activation. We showed that this mechanism induces ECs hyperactivation, by increasing cell proliferation and migration (Fig IV.3 A and B), and by promoting the formation of vessel-like structures (increased branch point

density, a proxy for vascular density; Fig IV.3 C)), mimicking the *in vivo* capacity of ECs to form vascular structures. The results strengthen the contribution of the ferroptosis-like mechanism in the promotion of tumor angiogenesis because tumor ECs are described as having a proliferation rate 50–200 times faster than normal ECs and have an increased capacity to migrate and organize into vascular tubules, thereby fueling the tumor mass (Azzi *et al.*, 2013; Folkman, 2006). Interestingly, Erastin stimulates the formation of vessel-like structures at the same range of H_2O_2 (ROS), without a synergistic effect (Fig IV.3 C), supporting again the involvement of the ferroptosis-like mechanism on angiogenic processes.

Propranolol acts as an inhibitor of angiogenesis and it has been recently described that it suppresses proliferation, migration and tube formation of hemangioma cells through the HIF-1α-VEGF-A axis (Chim *et al.*, 2012; Chen *et al.*, 2017) and decreases the expression of angiogenic growth factors, as VEGF and FGF (Sharifpanah *et al.*, 2014). However, the precise cellular mechanism underlying blood vessels disruption and angiogenesis impairment is still unknown. In this work, we observed that ECs proliferation, migration and vessel-like structure formation (Fig IV.3 A-D) are affected by the presence of Propranolol. Moreover, Propranolol, besides its anti-angiogenic effect under basal culture conditions, is able to abrogate the stimulation of ECs activation induced by Erastin and to disrupt already established vessel-like structures (Fig IV.3 A-D), suggesting that the Propranolol anti-angiogenic effect is related, at least in part, to the abrogation of the ferroptosis-like mechanism.

The cancer-associated vasculature is characterized by an increased permeability and interstitial fluid pressure due to the disruption of ECs junctions, which reveals to be pivotal for cancer cells extravasation and further metastasis (Hashizume *et al.*, 2000; Rohlenova *et al.*, 2018). As mentioned above, TME is characterized by increased ROS levels and some studies linked its levels with increased vessels permeability (Lum and Roebuck, 2001; Aghajanian *et al.*, 2008). In fact, ROS affects vascular permeability by regulating junctional protein phosphorylation and actin cytoskeleton organization (Moldovan *et al.*, 2000; van Wetering *et al.*, 2002; Sallee *et al.*, 2006). Increased ROS levels contributes for phosphatases inactivation at ECs junctions, leading to increased junction phosphorylation and further loss of junctional integrity (Moldovan *et al.*, 2000; Sallee *et al.*, 2006). EC:EC junctions are formed by different adhesive molecules; however, VE-Cad is considered the major EC-specific junctional protein that controls vascular integrity. A decrease in its expression in pathological conditions associates with a loss of vascular barrier integrity and permeability (Caveda *et al.*, 1996; Dejana, 2004; Gavard, 2014). The extracellular domain of VE-Cad mediates the homophilic adhesion and clustering between ECs while the intracellular domain binds to catenin's and to the

cytoskeleton, mediating VE-Cad complex stabilization and regulating intercellular permeability (Dejana, 2004; Gavard, 2014). Moreover, the increased VEGF levels in the TME, promotes VE-Cad phosphorylation and internalization in ECs, leadings to an impaired adhesiveness and non-functional vessels (Bentley *et al.*, 2009; Rohlenova *et al.*, 2018). Our results indicated that in addition to the role of the ferroptosis-like mechanism on ECs activation (Fig IV.3), ROS-induced lipid peroxides generation (Fig IV.2 A and C) also promotes the formation of VE-Cad intercellular junctional gaps (Fig IV.4 A and B), resulting in the generation of a leakier endothelium. However, its mechanism of action needs to be further explored. Furthermore, the exposure to antioxidants and free radical scavengers impairs ROS-induced EC permeability (Lum and Roebuck, 2001; Aghajanian *et al.*, 2008). Interestingly, we observed that the antioxidant properties of Propranolol induces the normalization of vascular structures (Fig IV.4 A and B) reinforcing its putative use in cancer therapy.

Despite VE-Cad function in the maintenance of vascular integrity, it is also known that VE-Cad plays a crucial role in cell-cell contact inhibition. For instance, VE–Cad-deficient ECs loose contact inhibition and have increased cell density (Carmeliet *et al.*, 1999; Lampugnani *et al.*, 2003; Wallez and Huber, 2008), suggesting that the disruption of VE-Cad intercellular junctions by the ferroptosis-like mechanism besides its effects in vascular integrity (Fig IV.4 A and B), could be implicated, at least in part, in the increased proliferation rate of ECs upon Erastin exposure (Fig IV.3 A).

EC:EC junctions, specially VE-Cad adherens junctions, controls the bidirectional passage of cells and molecules between blood vessel and irrigated tissues (Unger *et al.*, 2002). A hallmark of vascular pathologies, as tumor angiogenesis, is the disruption of VE-Cad junctions, leading to EC:EC junction weakening, transcellular holes formation and increased permeability (Hashizume *et al.*, 2000; Gavard, 2014). Tumor neovessels leakiness creates a hostile milieu to cancer cells invade and metastasize and limits the intravenous drug delivery of conventional chemotherapeutic agents (Carmeliet and Jain, 2011a, 2011b; Jain, 2014). Strategies focused in endothelium normalization associates with increased vessel barrier functions by the reorganization of the junctional molecules, as VE-cadherin, zonula occludens-1/2 (ZO-1, ZO-2) and claudin-5 (Mazzone *et al.*, 2009; Magrini *et al.*, 2014). The generation of a leakier endothelium with disturbed VE-Cad junctions by the ferroptosis-like mechanism (Fig IV.4 A and B) is accompanied by an increase in ICAM adhesion molecule expression (Fig IV.4 C), a crucial protein for cancer cell:EC interaction during the metastatic cascade (Chen and Massagué, 2012; Zhang *et al.*, 2014; Shenoy and Lu, 2016). Increased ICAM expression (Fig IV.4 C) fosters cancer cells:EC adhesion (Fig IV.4 E) and transendothelial migration (Fig IV.4 F), essential

steps during cancer cell systemic spread. Propranolol alone did not impact VE-Cad intercellular junctional gaps (Fig IV.4 A and B), ICAM expression (Fig IV.4 C) and cancer cell:EC adhesion (Fig IV.4 E) and transendothelial cancer cell migration (Fig IV.4 F), revealing its contribution for vessel stabilization and putatively a disturbance of metastasis. In fact, these results may explain why breast cancer patients exposed to β-blockers, as Propranolol, prior or after diagnosis had less metastases (Cardwell *et al.*, 2016; Pantziarka *et al.*, 2018; Hiller *et al.*, 2020). Accordingly, Propranolol reverted the pro-metastatic ECs phenotype induced by Erastin, by decreasing VE-Cad intercellular junctional gaps (Fig IV.4 A and B), ICAM expression (Fig IV.4 C) and by disturbing cancer cells adhesion to ECs and transendothelial cancer cells migration (Fig IV.4 E and F). Therefore, the effect of the ferroptosis-like mechanism, at a non-lethal level, mimics the pathophysiological angiogenic process in cancer, characterized by ECs hyperactivation that leads to the formation of a leakier vascular network (Hashizume *et al.*, 2000; Trédan *et al.*, 2007; Masiero *et al.*, 2013; Rohlenova *et al.*, 2018;). On the other hand, Propranolol administration will block the angiogenic switch, decelerating angiogenesis and further tumor growth, while preventing the generation of a leakier vasculature, decreasing metastasis and putatively increasing the delivery of cytotoxic drugs to the cancer cells.

In chapter II, we showed that monocytes act as EPCs and are capable of being incorporated into the tumor vasculature, contributing to cancer progression. Since pro-angiogenic and pro-oxidant stimulus (H_2O_2) pushes monocytes to ECs differentiation (chapter II) here, we explored if the differentiation route of monocytes towards ECs also benefits from the ferroptosis-like mechanism. We observed that Erastin does not interfere with monocytic differentiation into ECs (Fig IV.5) and does not impact monocytes differentiation under H_2O_2 exposure (Fig IV.5 A), as well as it does not change ROS and lipid peroxides levels (Fig IV.5 B and C). Moreover, Propranolol, that in ECs is able to impair the activation induced by the ferroptosis-like mechanism, has no impact on the monocytes differentiation process (Fig IV.5 A) and in intracellular ROS and lipid peroxides levels (Fig IV.5 B and C).

Together, the results suggested that the ferroptosis-like mechanism, mediated by Erastin, through GSH depletion and ROS-induced lipid peroxides generation is implicated in the regulation of some pathophysiological ECs features, promoting ECs hyperactivation, leakiness and cancer cell migration (Fig IV.6). Propranolol scavenging activity, mediated by H_2S, impairs the generation of oxidative stress, which in turn reverts the ECs phenotype observed under Erastin exposure and leads to the normalization of vascular structures (Fig IV.6). Additionally, despite the effects of the ferroptosis-like

mechanism in ECs activation, it did not affect the differentiation process of monocytes into ECs.

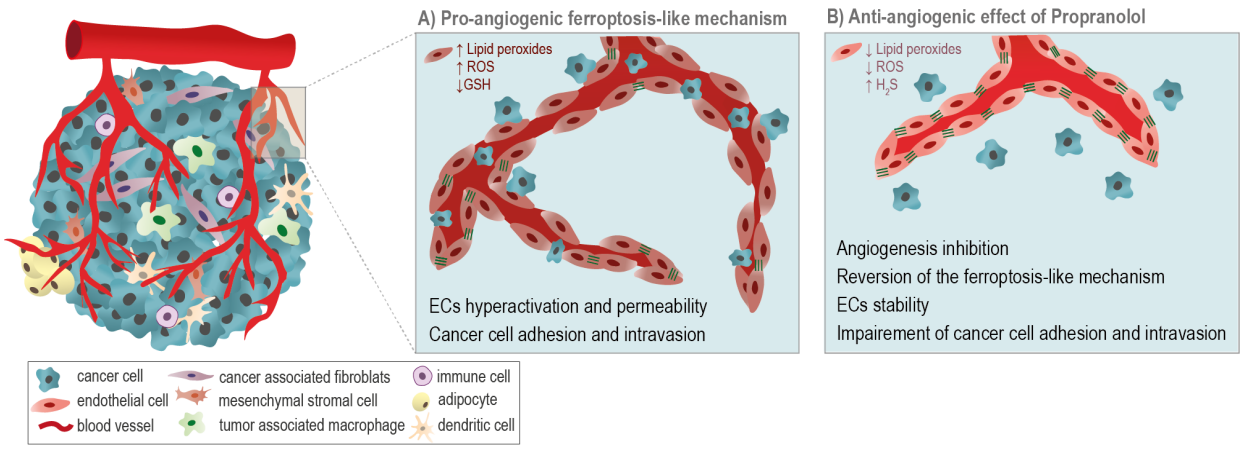


Figure IV. 6 The ferroptosis-like mechanism is involved in endothelial cells (ECs) activation and the antioxidant Propranolol (Prop) properties revert this process.

A) A pro-angiogenic ferroptosis-like mechanism, through the generation of ROS, the accumulation of lipid peroxides and glutathione (GSH) depletion is implicated in the promotion of ECs hyperactivation, vessels leakiness and cancer cells migration and intravasation. B) Prop scavenging activity is anti-angiogenic, since in one hand it impairs the ECs activation underlined by the ferroptosis-like mechanism and in other hand promote vessel normalization, pivotal to impair metastasis and improve the delivery of chemotherapeutic agents.

Chapter V

SeChry@PUREG4 and Propranolol as a promising strategy for cancer treatment: a way to simultaneously induce cancer cell death and stabilize tumor vasculature

This chapter was based on:

Lopes-Coelho F, Martins F, Hipólito A, Mendes C, Sequeira CO, Pires R, Almeida AM, Bonifácio VDB, Vicente JB, Pereira SA, Serpa J. The activation of endothelial cells relies on a ferroptosis-like mechanism: novel perspectives in management of angiogenesis and cancer therapy. Manuscript in preparation

Abstract

Selenium (Se) compounds and derivatives have known pro-oxidant properties, modulating the intracellular redox balance through the generation of reactive oxygen species (ROS) and, therefore, exerting a cytotoxic activity in cancer cells. We recently published that dendrimeric nanoparticles loaded with selenium chrysin (SeChry@PURE$_{G4}$) have anti-cancer effects. In chapter IV, we showed the potential use of Propranolol not only in the inhibition of the formation of new vessels, but also in the normalization of pre-existing endothelium, thereby decreasing cancer cell extravasation and improving the delivery of chemotherapeutic agents. Here, we tested the use of SeChry@PURE$_{G4}$ nanoparticles, with and without Propranolol, as a putative therapy to treat cancer by tackling cancer cells and endothelium. We observed that in cancer cells (MDA-MB-231), SeChry@PURE$_{G4}$ act as an oxidative stress mediated agent, inducing cancer cell death. Moreover, Propranolol potentiates the generation of ROS and lipid-peroxides induced by SeChry@PURE$_{G4}$, thereby promoting ferroptotic cell death. While in endothelial cells (ECs), the death levels upon concomitant exposure of SeChry@PURE$_{G4}$ and Propranolol are much lower and the ECs-specific Propranolol antioxidant properties revert ROS-generation induced by SeChry@PURE$_{G4}$, without affecting the stability of ECs networks. The combination of SeChry@PURE$_{G4}$ and Propranolol might be an efficient dual-effect therapy, by inducing cancer cells death and by stabilizing the tumor vasculature, impairing metastasis and improving the efficacy of chemotherapy.

Keywords: selenium-containing chrysin (SeChry), SeChry encapsulated in generation four polyurea dendrimer (SeChry@PURE$_{G4}$), Propranolol, oxidative stress-induced ferroptosis, cancer cell death, endothelium normalization

Introduction

Selenium (Se) is considered as an essential micronutrient in humans, being a co-factor in the production of selenoproteins, as glutathione peroxidase 4 (GPX4) and thioredoxine reductase (TrxR). Given its role in immune system, cancer prevention and its antioxidant properties, Se has been proposed as a potential anti-cancer agent (Rayman, 2000; Avery and Hoffmann, 2018; Gandin *et al.*, 2018) Nowadays, its already stated that Se properties and their mechanism of action is influenced by dose, species and presence of associated moieties (Wang *et al.*, 2007; Y. Yang *et al.*, 2009; Kong *et al.*, 2011; Luo *et al.*, 2012; Huang *et al.*, 2013).

The use of Se containing-small molecules and Se-derivates has revealed a wide range of biological functions, although their biological activity depends on their chemical formula (Jacob *et al.*, 2003; Battin and Brumaghim, 2009). Some chemical presentations of Se, as diselenides, selenomethionine, methylseleninic acid, dimethyldiselenide and organoselenium compounds have antioxidant properties, protecting cells against oxidative damage and modulating multiple biological functions implicated in cancer development and progression (Spallholz *et al.*, 2001; Rosa *et al.*, 2007; Sanmartin *et al.*, 2008; Brandão *et al.*, 2009; Plano *et al.*, 2010; Rahmanto and Davies, 2012). Contrarily, other compounds, as Se-containing chrysin (Sechry), reveal to be pro-oxidant and highly cytotoxic for cancer cells (IMartins *et al.*, 2015; Santos *et al.*, 2019). Therefore, some Se compounds are more effective on cancer prevention, mainly due to its antioxidant properties, while others, by its pro-oxidant properties, are more effective in killing cancer cells. The properties of Se compounds as modulators of the redox state and their increased selectivity and sensitivity for cancer cells (Weekley *et al.*, 2013; Fernandes and Gandin, 2015; Chaiswing *et al.*, 2018; Gandin *et al.*, 2018) make these compounds attractive for the development of anti-cancer formulations.

As mentioned, the cytotoxic activity of some Se compounds, in cancer cells, is mediated by their pro-oxidant properties that modulate the intracellular redox balance through the generation of reactive oxygen species (ROS), by the oxidation of intracellular thiols and/or by direct proteins oxidation, leading to protein unfolding and loss of biological functions (Cheong *et al.*, 2012; Selenius *et al.*, 2012; Longati *et al.*, 2013; Khalkar *et al.*, 2018;). For instance, NF-κB, p53, JNK and SP1 proteins are described as being directly modified by thiol oxidation (Hocevar *et al.*, 2001; Tseng *et al.*, 2001; Huang *et al.*, 2006; Eriksson *et al.*, 2015; Husbeck *et al.*, 2006). Moreover, Se compounds are selectively taken up, localized and accumulated in cancer cells, however the mechanisms of cancer selectivity and uptake are not fully understood (Esteban *et al.*, 1965; Jain, 2017; Ye *et al.*, 2017). Olm et al., demonstrated that the overexpression of sodium-independent cystine/glutamate antiporter System xc- (xCT; SLC7A11) in cancer

cells, promotes the extracellular reduction of selenite to selenide, generating a reductive microenvironment that in turn facilitates the Se uptake (Olma *et al.*, 2009). Other study purposed that the aberrant cancer cell metabolism that requires high amounts of Se, a rate-limiting substrate for selenoproteins synthesis such as GPX4, favors Se uptake (Carlisle *et al.*, 2020).

The cancer treatment has improved during the last years, however cancer patients still suffer from acute and chronic toxicity caused by the conventional cancer treatment (Cleeland *et al.*, 2012; Laviano *et al.*, 2012). New evidence point out that targeted anti-cancer therapies will be pivotal to increase cancer cell specificity and to avoid toxicity in healthy tissues (Sun *et al.*, 2014b; Zaimy *et al.*, 2017; Ahmad *et al.*, 2019). The development of anti-angiogenic drugs arises from Folkman theory, in 1971, which suggested an association between angiogenesis and tumor development (Folkman, 1971). Since then, numerous efforts have been made to develop anti-angiogenic drugs in order to improve disease outcome, however so far, the clinical results have been disappointing (Kim *et al.*, 1993; Vasudev and Reynolds, 2014; Teleanu *et al.*, 2019). In a theoretical scenario, the development of strategies focused in both cancer cell death and vasculature normalization would certainly improve the clinical efficacy of cancer treatment.

Here, we explore the use of the pro-oxidant SeChry compound encapsulated in a dendrimeric nanoparticle - SeChry@PURE$_{G4}$ -, in combination with Propranolol, as a strategy for cancer treatment. Given the effects of Propranolol in endothelial cells (ECs), as previously described in chapter III and IV, and the SeChry@PURE$_{G4}$ properties, we hypothesized that this approach would promote cancer cell death, mediated by oxidative stress, and ECs' stability ensured by Propranolol, a pivotal process for the abrogation of the metastatic cascade and the improvement of chemotherapy delivery.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs: CRL-1730, ATCC) were cultured in Endothelial Cell Growth Basal Medium-2 (EBM-2: CC-3156, Lonza, Bioscience) supplemented with EGM-2 SingleQuots Supplements (CC-4176, Lonza, Bioscience). All experiments were performed until the passage 10. Triple-negative breast cancer (MDA-MB-231: HTB-26™, ATCC) were used as tumor models, being cultured in Dulbecco's Modified Eagles Medium 1X (DMEM) (41965-039, Gibco, Life Technologies), supplemented with 10% fetal bovine serum (FBS) (S 0615, Merck), 1% Antibiotic-

Antimycotic (AA; P06-07300, PAN Biotech) and 50 µg/mL Gentamicin (15750-060, Gibco, Life Technologies). Cell cultures were maintained at 37°C in a humidified environment of 5% CO_2. Cells were detached with 0.05% Trypsin-EDTA 1X (25300-054, Invitrogen, Thermo Fisher Scientific) at 37°C and splitted to new plates according to the experimental procedure.

For experimental conditions cells were cultured with 100 µM Propranolol (P8688, Sigma Aldrich) and 160 and 200 µM SeChry, for 16 hours (h).

SeChry@PURE$_{G4}$ synthesis

Selenium-containing chrysin (SeChry) was synthesized following a reported protocol (I. L. Martins *et al.*, 2015). After purification, the formation of the product was confirmed by ^{1}H NMR. ^{1}H NMR (CDCl3, 400 MHz) δ (ppm): 7.96 (2H, d, *J*= 8.0 Hz), 7.76 (1H, s), 7.61 (1H, t, *J*= 8.0 Hz), 7.52 (2H, t, *J*= 8.0 Hz), 6.51 (1H, d, *J*= 4.0 Hz), 6.46 (1H, d, *J*= 4.0 Hz). Polyurea dendrimer generation four (PURE$_{G4}$) was obtained using our supercritical-assisted polymerization protocol (Restani *et al.*, 2012). SeChry was encapsulated in PURE$_{G4}$ nanoparticles following our protocol (Kunsang *et al.*, 2010). Briefly, SeChry (6.5 mg) was added to an aqueous solution (10 mL) of PURE$_{G4}$ (125 mg) and stirred overnight. Then, the aqueous solution was extracted with chloroform ($CHCl_3$) to remove non-encapsulated or surface bound SeChry. No SeChry was found in the $CHCl_3$ extracts (control by thin layer chromatography; TLC), thus confirming a full encapsulation. The release profile follows the usual profile reported for this nanodelivery system (Restani *et al.* 2015).

Cell death analysis by flow cytometry

To analyze the effects of Propranolol and SeChry@PURE$_{G4}$ on cell death, HUVECs ($5x10^4$ cells/well) and MDA-MB-231 ($5x10^4$ cells/well) were seeded in 24-well plates. After exposure to the experimental conditions, supernatants and cells were collected and centrifuged at 155 *x g* for 5 minutes (min). Cell pellets were incubated with 0.5 µL FITC-labeled Annexin V (640906, BioLegend) in Annexin V binding buffer 1X (10 mM Hepes (pH 7.4; 391333, Millipore), 0.14 M sodium chloride (NaCl; 106404, Merck), 2.5 mM calcium chloride ($CaCl_2$; 449709, Sigma Aldrich) and incubated at room temperature for 15 min, in dark. After incubation, cells were rinsed in 200 µL 1X Phosphate-buffered saline (PBS) / 0.1% (v/w) Bovine serum albumin (BSA) and centrifuged at 155 *x g* for 2 min. The remaining pellet was resuspended in 200 µL of annexin V binding buffer 1X and 2.5 µL of 50 µg/mL propidium iodide (PI; P4170, Sigma Aldrich Aldrich) and analyzed by flow cytometry (FACScalibur – Becton Dickinson). *FlowJo X* v10.0.7 software (*https://www.flowjo.com/*) was used to analyze data.

Reactive oxygen species (ROS) quantification by flow cytometry

HUVECs ($5x10^4$ cells/well) and MDA-MB-231 cells ($5x10^4$ cells/well) were plated in 24-well plates. The intracellular ROS were detected in cell pellets incubated with 10 µM DCF-DA probe (D6883, Sigma Aldrich) at 37°C for 30 min. The acquisition was performed with FACScalibur (Becton Dickinson) and data were analyzed with *FlowJo X* v10.0.7 software (*https://www.flowjo.com/*).

Lipid peroxides quantification by flow cytometry

HUVECs ($5x10^4$ cells/well) and MDA-MB-231 cells ($5x10^4$ cells/well) were plated in 24-well plates. After experimental conditions, cells were incubated with 2 µM C11-Bodipy 581/591 (D3861, Invitrogen), for 30 min at 37 ºC in dark. Excessive dye was removed by washing with 2% FBS-1X PBS and cell pellets were resuspended in 2% FBS-1X PBS for the acquisition by flow cytometry (FACScalibur – Becton Dickinson). *FlowJo X* v10.0.7 software (*https://www.flowjo.com/*) was used to analyze data.

Quantification of H_2S in cell homogenates

HUVECs ($5x10^5$ cells/well) and MDA-MB-231 HUVECs ($5x10^5$ cells/well) were seeded in 6-well plates and cultured in the experimental conditions, for 16 h. After, cells were scrapped in 1X PBS and centrifuged at 210 *x g* for 5 min. The cell pellet was homogenized in NP40 lysis buffer (1% NP40, 150 mM NaCl, 50 mM Tris-Cl, pH 8.0) on ice for 30 min and centrifuged for 5 min at 20,000 *x g* 4 ºC. Cell homogenates (20 µL) were incubated in black 96-well plates with 80 µL of 10 µM 7-Azido-4-Methylcoumarin probe (AzMC, L511455, Sigma Aldrich). The protein concentration was determined with Bradford method using protein assay dye reagent concentrate (500-0006, Bio Rad). The final H_2S measurements were normalized to total protein concentration and to a blank sample (cellular lysates without probe). H_2S production was monitored following fluorescent signal of AzMC (355 nm/460 nm) every 30 min for 2 h, in a VIKTOR3 instrument from *PerkinElmer/ Wallac 1420 Software (version 3.0)*.

Immunofluorescence for VE-Cadherin

For VE-Cadherin (VE-Cad) immunodetection, HUVECs ($1x10^5$ cells/well) were cultured in 24-well plate with glass slides coated with 0.2% gelatin, until the formation of a confluent monolayer that was posteriorly exposed to the experimental conditions, for 16 h. After fixation with 2% paraformaldehyde, for 15 min at 4°C, and blocking with 3% bovine serum albumin (BSA)- 1X PBS (w/v), cells were incubated with anti-VE-Cad (1:50 in 3% BSA-0.1% triton X-100- 1X PBS (w/v/v); AF938, R&D), for 2 h at room

temperature, followed by an incubation with the secondary antibody (Alexa Fluor 488 donkey anti-goat, 1:500 in 3% BSA-0.1% triton X-100-PBS; A11055, Thermo Fisher Scientific), for 2 h at room temperature. Slides were mounted in VECTASHIELD media with DAPI (4′-6-diamidino-2-phenylindole; H-1200, Vector Labs) and examined by standard fluorescence microscopy, using an Axio Imager.Z1 microscope (Zeiss) with a CytoVision® software. The quantification of VE-Cad junctional gaps was performed using *ImageJ* software (*imagej.nih.gov/ij/*).

Statistical analysis

All data were analyzed using student's t-test, one-way ANOVA or two-way ANOVA in GraphPad Prism 7 software (*www.graphpad.com/).* The assays were performed with at least 2 biological replicates *per* condition and the differences were determined statistically significant at p value < 0.05.

Results

SeChry@PURE$_{G4}$ alone and in combination with Propranolol has different effects in cancer cells and ECs

The efficacy of Sechry@PURE$_{G4}$ (Fig V.1) plus Propranolol dual-effect therapy was tested, in order to verify if this strategy would be highly selective and cytotoxic to cancer cells, as our team recently showed (Santos *et al.*, 2019); and if it would act on ECs and contribute for the stabilization of already existing ECs networks, as demonstrated in chapter IV.

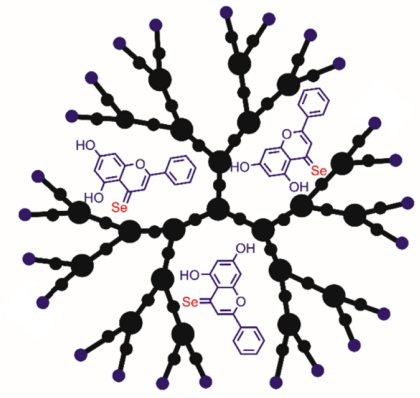


Figure V. 1 SeChry@PURE$_{G4}$- nanoformulation.

Representation of the nanoformulation, in which SeChry (chemical representation in purple color) is encapsulated in generation four polyurea dendrimer (cartoon representation in black color; PURE$_{G4}$). PURE dendrimers are a drug delivery system with increased biocompatibility and biodegradability and with low toxicity (Pires et al. 2016). SeChry formulation results from the replacement of the oxygen atom of the carbonyl moiety of 2 by a selenium (Se, in red color) atom in Chrysin (5,7-dihydroxylflavone; in purple color), a flavon derivate from the plant polyphenols with anti-inflammatory and anti-tumoral properties.

Hence, we tested the SeChry@PURE$_{G4}$ effect with and without Propranolol in triple negative breast cancer cells (MDA-MB-231) and in ECs (HUVECs). Propranolol induced a modest 1.4- fold increase in ECs death comparing to control, while in cancer cells its

effect increased 1.9-fold cell death (Fig V.2), meaning that Propranolol exposure almost duplicated cancer cell death in comparison with the control. The two tested SeChry@PURE$_{G4}$ concentrations (160 and 200 μM) significantly promoted cancer cell death, mainly when combined with Propranolol (Fig V.2 A). Interestingly, the levels of HUVECs death were not altered by 160 μM SeChry@PURE$_{G4}$ and only a slight increase was observed with 200 μM SeChry@PURE$_{G4}$ (Fig V.2 B), being these levels increased under Propranolol combined exposure (Fig V.2 B). The results confirmed the anti-tumoral SeChry@PURE$_{G4}$ effect and their differential consequences in malignant cells *vs* ECs.

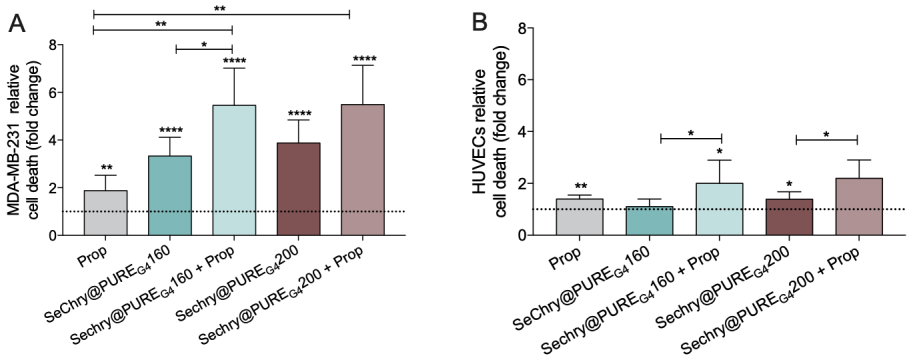


Figure V. 2 SeChry@PURE$_{G4}$ plus Propranolol (Prop) increase cancer cell death, indicating that this strategy has anti-tumoral effects.

A) SeChry@PURE$_{G4}$ (160 μM and 200 μM) exposure promoted cancer cell death (MDA-MB-231), being this effect boosted by Prop. B) ECs (HUVECs) were more resistant to SeChry@PUREG$_4$ -induced cell death, even under Prop exposure. In graphs the dashed line represents the control condition. All data are normalized to the control condition and represented as mean ± SD. *p<0.05, **p<0.01, ****p<0.0001.

Propranolol potentiates the effects of oxidative stress induced by SeChry@PURE$_{G4}$ in cancer cells, while in ECs Propranolol exerts its antioxidant properties

Regarding the effect of SeChry@PURE$_{G4}$ exposure in the intracellular ROS levels, in cancer cells the combination of SeChry@PURE$_{G4}$ and Propranolol induced a synergistic increase in ROS levels, while in HUVECs the addition of Propranolol had the opposite effect (Fig V.2 A and B), re-enforcing the previously observed antioxidant role of Propranolol in ECs (chapter IV). Accordingly, the ROS-induced lipid peroxides levels increased in MDA-MB-231 (Fig V.3 C), but not in HUVECs (Fig V.3 D), upon SeChry@PURE$_{G4}$, with or without Propranolol. Interestingly, in ECs, the antioxidant properties of Propranolol seem to be mediated by an increase in hydrogen sulfide (H_2S) levels (chapter IV), however, in MDA-MB-231 Propranolol did not increase the levels of H_2S (Fig V.3 E). The antioxidant H_2S role is supported by the fact that in cancer cells

Propranolol exposure, which has no impact in H_2S levels, it exerts a synergistic effect in the generation of ROS and lipid peroxides induced by SeChry@PURE$_{G4}$ (Fig V.3 A and C).

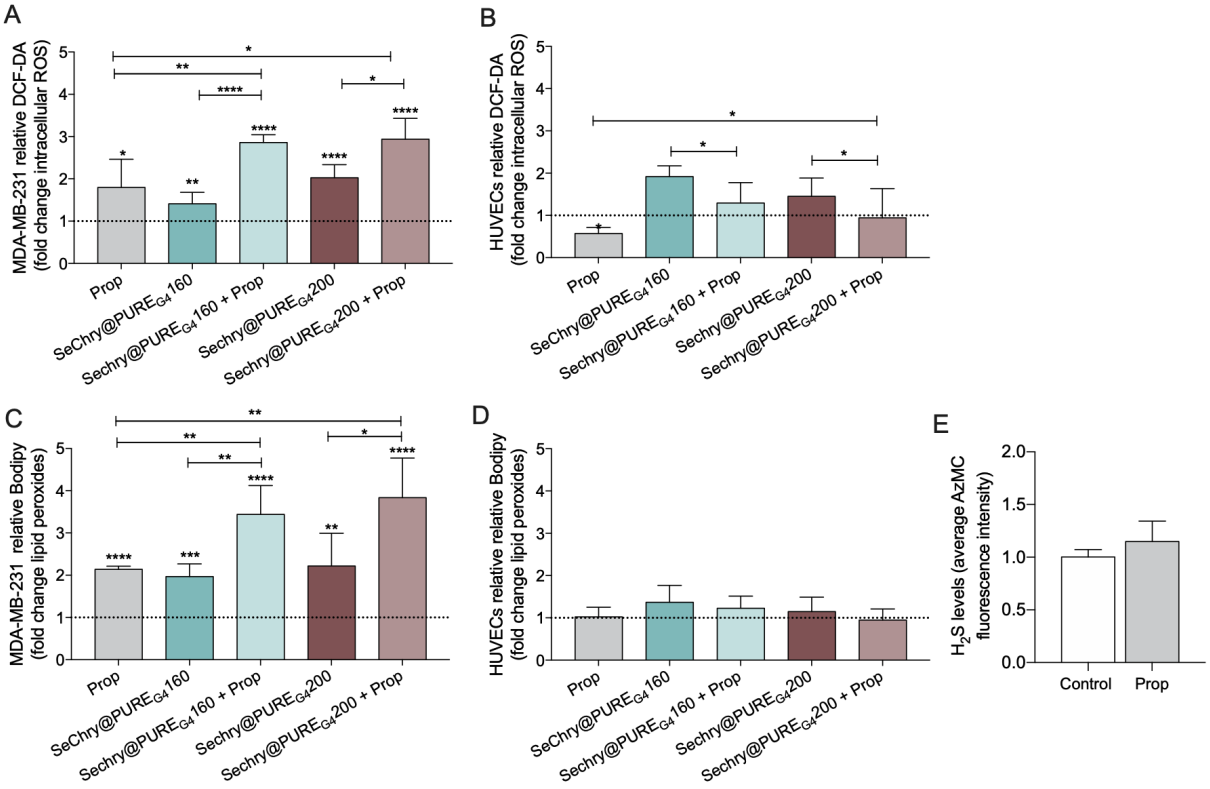


Figure V. 3 The combination of SeChry@PUREG$_4$ and Propranolol (Prop) mediates the generation of oxidative stress in cancer cells, while in ECs the antioxidant Prop properties revert the ROS levels induced by SeChry@PUREG$_4$.

A-D) Contrarily to HUVECs, in MDA-MB-231 Prop alone increased intracellular ROS (DCF-DA) and lipid peroxides (11C-Bodipy) levels and it did not revert the generation of ROS-induced lipid peroxidation induced by SeChry@PURE$_{G4}$. E) In MDA-MB-231 Prop did not affect the levels of the antioxidant H_2S (in contrast to the increase of H_2S levels upon Prop observed in HUVECs – shown in chapter IV), indicating that the selective antioxidant Prop effects in ECs were mediated by H_2S. In graphs the dashed line represents the control condition. All data are normalized to the control condition and represented as mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

SeChry@PURE$_{G4}$ and Propranolol exposure does not affect vascular integrity

HUVECs exposed to SeChry@PURE$_{G4}$, with or without Propranolol, presented a stable monolayer with reduced VE-Cad intercellular junctional gaps (Fig V.4), indicating that SeChry@PURE$_{G4}$ did not affect vascular permeability. Together, the results suggest that SeChry@PURE$_{G4}$ plus Propranolol could be an interesting strategy for cancer treatment, targeting both cancer cells and ECs. In cancer cells, SeChry@PURE$_{G4}$ and Propranolol would have anti-tumoral effects through the promotion of cell death mediated by a ferroptosis, whereas in ECs, Propranolol would impair oxidative stress-induced

mechanisms, impairing ECs hyperactivation and promoting stability (chapter IV), which in turn might decrease transendothelial cancer cell migration and impair metastasis.

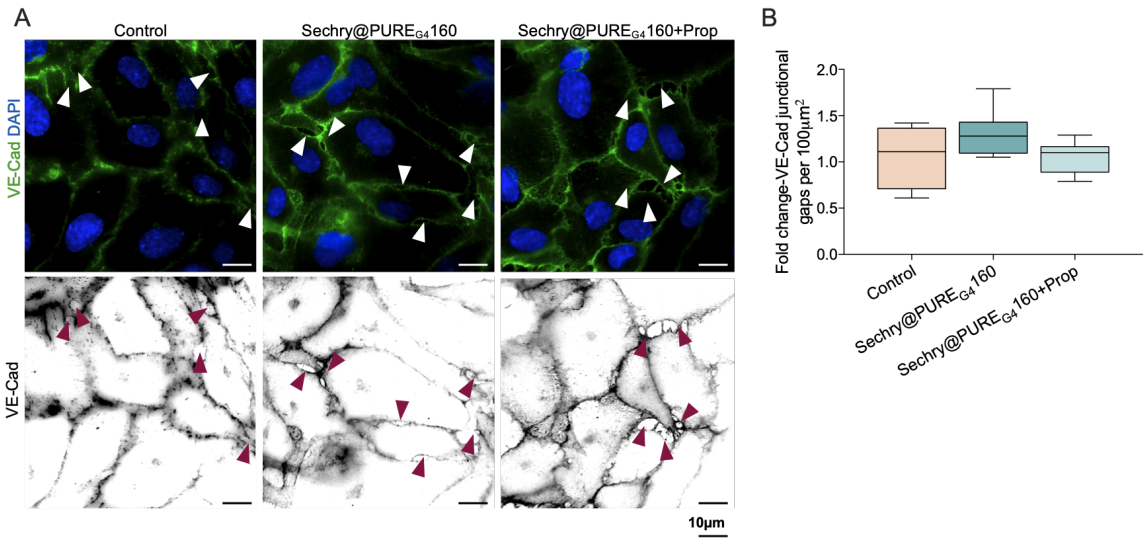


Figure V. 4 SeChry@PUREG$_4$ does not impact the generation of VE-Cadherin (VE-Cad) intercellular junctional gaps.

A) The panel shows representative images (scale: 10 µm) of VE-Cad (green) intercellular junctional gaps (arrows). B) SeChry@PURE$_{G4}$ had no effect in the number of VE-Cad gaps, reinforcing the potential use of this nanoformulation, in combination with Propranolol (Prop). All data are normalized to the control condition and represented as mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Discussion

The conventional drugs used in cancer treatment have low selectivity against cancer cells and cause toxicity in normal cells (Cleeland *et al.*, 2012; Laviano *et al.*, 2012). The development of new strategies to overcome the limitations of the conventional cancer chemotherapy urges. The abovementioned increased selectivity and sensitivity of Se compounds and derivates for cancer cells, allied with their anti-tumoral properties, makes them promising candidates for cancer therapeutics (Weekley *et al.*, 2013; Fernandes and Gandin, 2015;Chaiswing *et al.*, 2018; Gandin *et al.*, 2018).

Se compounds are active players in redox regulation through the modulation of ROS levels (Muecke *et al.*, 2010). In cells, the balance between the generation and the elimination of ROS plays a pivotal role in physiological processes, however excess free radical species and the disruption of normal cellular homeostasis by redox signaling promote pathological processes (Tafani *et al.*, 2016; Kumari *et al.*, 2018; Milkovic *et al.*, 2019). One of the proposed anti-tumoral mechanism of some Se compounds is the generation of oxidative stress, in which the generation of superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl ($OH\cdot$) radicals promotes cancer cell death (Jackson and Combs, 2008).

In cancer cells, contrarily to normal cells, the selenophosphate synthetase 2 (SEPHS2), an enzyme of the selenocysteine biosynthesis pathway, is essential for cell survival (Carlisle *et al.*, 2020). SEPHS2 is critical for selenide detoxification, a product of selenocysteine biosynthesis, and its expression is often elevated in breast cancer cells (Carlisle *et al.*, 2020). In line, some Se derivates interfere with the Se uptake, selenocysteine biosynthesis and with the production of selenoproteins, such as GPX4, therefore exerting cytotoxic effects and impairing cancer cell protection against ferroptosis (Singh *et al.*, 2014; Martins *et al.*, 2015; Rauf *et al.*, 2015; Carlisle *et al.*, 2020).

A previous study of our group showed that in ovarian cancer cells SeChry prompts glutathione (GSH) depletion and inhibits the action of the H_2S-generating enzyme cystathionine beta synthase (CBS), which in turn promotes cancer cell death (Santos *et al.*, 2019).

Given their drug delivery properties in several cancer cell models, the use of dendrimers drug-carriers such as polyurea dendrimers (PURE), emerge as a promising strategy for cancer treatment. The use of this strategy will overcome the chemotherapeutic non-specific distribution, the toxicity in non-malignant tissues and the low fraction of the drugs reaching the tumor (Castro *et al.*, 2018; Dias *et al.*, 2020). Dendrimers-based strategies showed *in vitro* anti-cancer activity against cancer cell lines from breast and lung adenocarcinomas and glioblastoma (Huang and Wu, 2018). Considering the aforementioned anti-tumoral and the pro-oxidant properties of SeChry (Martins et al. 2015), the anti-cancer effect of SeChry@$PURE_{G4}$ nanoparticles (Fig V.1) (Martins *et al.*, 2015; Santos *et al.*, 2019) and the anti-angiogenic and antioxidant properties of Propranolol in ECs (chapter III and IV), here we explored the effect of SeChry@$PURE_{G4}$ in combination with Propranolol in two core components of a malignant tumor: cancer cells and ECs. The breast cancer cells, MDA-MB-231, were more sensitive to SeChry@$PURE_{G4}$ than HUVECs, presenting higher cell death levels that increased upon the co-exposure with Propranolol (Fig V.2). Our results pointed out that SeChry@$PURE_{G4}$ induced cancer cells ferroptotic death, since it increased the generation of intracellular ROS and the concomitant accumulation of lipid peroxides (Fig V.3 A and C), features described as characteristics of ferroptosis mediated-cell death (Friedmann *et al.*, 2014; Linkermann *et al.*, 2014; Yang *et al.*, 2014; Yu *et al.*, 2015; Lei *et al.*, 2019; Li *et al.*, 2020). In HUVECs, SeChry@$PURE_{G4}$ has no effect on the generation of lipid peroxides (Fig V.3 C), reinforcing the previous evidence showing that SeChry has an increased selectivity index to cancer cells (Martins *et al.*, 2015).

Interestingly, in MDA-MB-231 cells, Propranolol alone is able to increase cancer cell death (Fig V.2 A), demonstrating that in addition to its role in preventing ECs

hyperactivation (chapter IV), Propranolol could also promote cancer cells death. Contrarily to the observed in HUVECs, in MDA-MB-231, Propranolol did not display antioxidant features, since the levels of ROS and ROS-induced lipid peroxides increased upon Propranolol exposure (Fig V.3 A-D), indicating that Propranolol can enhance ferroptosis cell death in cancer cells, but not in ECs. In chapter IV, we showed that antioxidant properties of Propranolol in ECs are mediated by an increase in the levels of hydrogen sulfite (H_2S), a gasomitter described as being as potent as GSH in detoxification processes, (Olas, 2017; Shefa *et al.*, 2018). However, in MDA-MB-231 the levels of H_2S did not increase under the presence of Propranolol (Fig V.3 E). Considering that in cancer cells SeChry exposure lead to GSH depletion (Santos et al., 2019) and that Propranolol exerted pro-oxidant features (Fig V.3 A and B), the dual administration of SeChry@PURE$_{G4}$ and Propranolol promoted oxidative stress-induced cancer cell death (Fig V.2 A), namely ferroptosis, while ECs benefit from the antioxidant Propranolol properties, preventing oxidative stress (Fig V.3 B and C).

The adherens junctions constitute strong adhesion structures between adjacent ECs, predominantly via the transmembrane protein VE-cadherin (VE-Cad). Besides its role in maintaining endothelium integrity, VE–cad is responsible for the control of vascular permeability (Aghajanian *et al.*, 2008; Wallez and Huber, 2008). In chapter IV, we demonstrate that Propranolol is able to normalize ECs leakiness mediated by ROS-induced lipid peroxides, indicating that Propranolol besides its role in preventing ECs hyperactivation, it also stabilizes established ECs structures through the inhibition of VE-Cad intercellular junctional gaps. In turn, less VE-Cad intercellular junctional gaps favors endothelium integrity that in cancer context impairs cancer extravasation and promotes the delivery of chemotherapeutic agents. Interestingly, in cancer, Se administration delays the development of various tumors via inhibition of VEGF expression and further angiogenesis, suggesting their putative anti-angiogenic applicability in tumor angiogenesis and in other pathological angiogenesis-mediated disorder (Mousa *et al.*, 2004; Stupin *et al.*, 2017). In HUVECs, SeChry@PURE$_{G4}$ did not affect the formation of VE-Cad intercellular junctional gaps (Fig V.4), suggesting that SeChry@PURE$_{G4}$ does not compromise vessels stability. However, the putative anti-angiogenic potential of this dual strategy through the prevention of ECs hyperactivation and further blood vessels formation should be explored in the future.

Together, the results demonstrated that SeChry@PURE$_{G4}$ plus Propranolol administration is a promising strategy for cancer treatment (Fig V.5), since this combination is able to induce cancer cell death through ferroptosis, while avoiding the formation of a leakier vasculature, which ultimately impairs cancer cell intravasation and

metastasis, as well as it improves drugs delivery and consequently enhances chemotherapy efficacy.

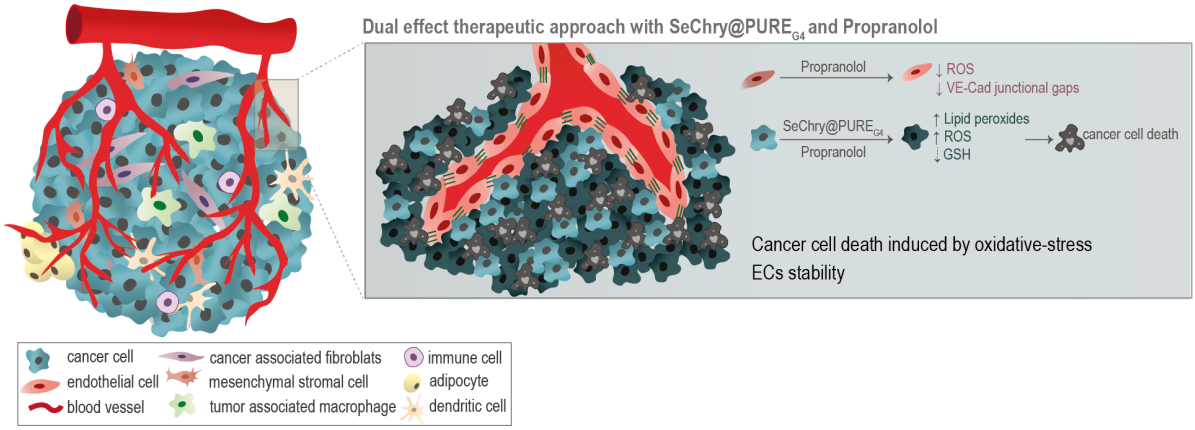


Figure V. 5 The combination of SeChry@PURE$_{G4}$ nanoparticles and Propranolol (Prop) is unraveled as a potential anti-cancer therapy.

Sehry@PURE$_{G4}$ induces cancer cell death mediated by pro-oxidative features (increased ROS and ROS-induced lipid peroxides levels and GSH depletion), being this effect potentiated by the action of Prop. In ECs, Prop through its antioxidant properties stabilizes ECs junctions which prevents the formation of a leakier vasculature. In turn, vessels stability is pivotal for impairing metastasis and improving the delivery of chemotherapeutic agents, tackling the remaining cancer cells that are not affected by oxidative stress-induced cancer cell death mediated by SeChry@PURE$_{G4}$ and Prop.

Chapter VI

General discussion

Final remarks

Future perspectives

General discussion

A better understanding of the mechanisms underlying tumor angiogenesis will open new perspectives on how it is regulated. Since the first evidence of the role of angiogenesis in cancer, by Folkman et al., 1971, several researchers developed strategies to abolish the nutrients and oxygen supply of the cancer cells by decreasing the vessels formation and avoiding the formation of new ones (Folkman, 1971; Vasudev and Reynolds, 2014). So far, most of the Food and Drug Administration (FDA) approved anti-angiogenic agents for cancer treatment rely on targeting vascular endothelial growth factor (VEGF) signaling pathway, however and since those strategies are inefficient in cancer patients, a new generation of drugs appeared. These drugs aim to tackle simultaneously the VEGF signaling pathway and other pro-angiogenic pathways and have been developed as an attempt to overcome drug resistance, due to adaptive and compensatory mechanisms (Taylor *et al.*, 2003; Casanovas *et al.*, 2005; Crawford *et al.*, 2009; Holohan *et al.*, 2013; D. Li *et al.*, 2014; Michaelsen *et al.*, 2018). In clinical settings, the use of anti-angiogenic therapies, alone and in combination with conventional cancer therapy, improves the disease outcome of cancer patients. In the other hand, some patients do not have additional benefits (Abdalla *et al.*, 2018), revealing that the precise mechanisms involved in the regulation of tumor angiogenesis remain unclear. Given those strategies in cancer patients are far from the successes obtained in pre-clinical settings, a new paradigm emerges as some studies suggested that the abrogation of blood supply will restrict drug delivery (*e.g. cytotoxic and* cytostatic agents) to the tumor, decreasing their clinical efficacy (Rohlenova *et al.*, 2018). The development of new strategies to restore tumor vessel normalization, instead of strategies to destroy it, will increase the penetration of therapeutic agents into the tumor, improving the efficacy of drugs (Carmeliet and Jain, 2011a). This thesis gives new insights on understanding alternative players in tumor angiogenesis and the mechanisms involved in tumor neovessels formation and purposes the development of new anti-angiogenic and anti-tumoral dual-therapeutic approaches.

Monocytes functioning as endothelial progenitor cells (EPCs) and endothelial cells (ECs) activation by a ferroptosis-like mechanism underlies tumor angiogenesis: novel perspectives in the mechanisms involved in angiogenesis

Tumor neovessels are characterized by being unorganized and leaky, due to the hyperactivation of the pro-angiogenic signaling cascades (Lugano *et al.*, 2020). In

addition to the release of pro-angiogenic factors by the tumor microenvironment (TME), the metabolic remodeling of cancer cells and cancer associated-stromal cells and hypoxia drives the generation of a pro-oxidant microenvironment, that works synergistically with the release of pro-angiogenic factors in the promotion of tumor angiogenesis, triggering ECs activation that is characterized *in vitro* by increased proliferation, migration and tube formation (Brand *et al.*, 2004; Dewhirst *et al.*, 2008; Coso *et al.*, 2012; Lassègue *et al.*, 2012; Schito and Semenza, 2016; De Sá Junior *et al.*, 2017). Reactive oxygen species (ROS) have a role in a number of cellular processes and although high levels of ROS lead to DNA and cellular damage, they also play a role in cell signaling contributing for the regulation of several cellular processes. The concept of 'homeostatic ROS levels' *versus* 'pathologic ROS levels' is raising and replacing the old dogma stating that ROS are prejudicial and toxic for the cells. In cancer context, the generation of ROS in the TME is involved in the angiogenic switch, allowing tumor angiogenesis (Kim and Byzova, 2014; Panieri and Santoro, 2015; Chen *et al.*, 2018; Huang and Nan, 2019). In fact, the angiogenic switch in tumors allows the passage of a low invasive and poorly vascularized tumor to a highly invasive and vascularized tumor (North *et al.*, 2005; Baeriswyl and Christofori, 2009). During this process, a switch in the equilibrium between positive and negative angiogenic regulators triggers a pro-angiogenic EC activation and further tumor angiogenesis (North *et al.*, 2005; Baeriswyl and Christofori, 2009). However, it is thought that this switch involves more than an imbalance between positive and negative regulators, being pointed that endothelial progenitor cells (EPCs) may also be a factor working on this switch. Conceptually EPCs are essential in the repair of damaged vessels and in the maintenance of endothelium integrity (Richardson and Yoder, 2011; Yoder, 2012; Yuan *et al.*, 2017), however the relevance of their contribution in tumor vessels is not completely clear. Although, in most studies have been presumed that only the activation of preexisting ECs proliferation accounts for tumor neovessels' formation, the EPCs-derived ECs may also be incorporated into growing tumor neovessels (North *et al.*, 2005; Baeriswyl and Christofori, 2009). In fact, EPCs have been underestimated as contributors for tumor angiogenesis, in part due to the lack of a molecular signature to identify EPCs, however some studies suggested that increased VEGF levels favor the differentiation of hematopoietic stem cells towards EPCs lineages and enhance their recruitment into the tumor vasculature (Asahara *et al.*, 1999; Gill *et al.*, 2001; Hattori *et al.*, 2001). Considering that monocytes arises from hematopoietic stem cells; their recruitment and paracrine involvement during neovascularization processes are already known in cancer and they are able to differentiate into non-macrophagic cell types, depending on the stimuli (Fujiyama *et al.*, 2003; Zhao *et al.*, 2003; Elsheikh *et al.*, 2005; Seta and Kuwana,

2007, 2010). Hence, we speculated that monocytes are misjudged EPCs and account for tumor angiogenesis. In the present thesis, we demonstrated in different contexts that monocytes act as EPCs. Our results showed that monocytes exposed to pro-angiogenic stimulation were able to differentiate into ECs and were incorporated into blood vessels *in vivo*, contributing for vessels repair and the angiogenic switch and further tumor angiogenesis.

In ECs the major sources of ROS are the NADPH oxidase that is activated by various pro-angiogenic factors (*e.g.* VEGF) and by hypoxia. The ROS derived from this oxidase are involved in VEGFR2 autophosphorylation and in the activation of several redox signaling pathways that triggers the induction of transcription factors and genes involved in angiogenesis (North *et al.*, 2005; Santoro, 2018). In fact, the activation of redox-dependent signaling pathways by endogenous or exogenous ROS leads to the transcription of genes involved in angiogenesis that therefore stimulate ECs proliferation, migration and tubular morphogenesis (Stone and Collins, 2002; Ushio-Fukai and Nakamura, 2008). In an *in vivo* tumor model, the administration of the thiol antioxidant N-acetylcysteine (NAC) attenuates EC invasion and inhibits angiogenesis (Cai *et al.*, 1999). Moreover, the ROS production by the TME is involved in the differentiation of monocytes into tumor-associated macrophages (TAMs), in which the continuous administration of the antioxidant butylated hydroxyanisole (BHA) suppresses tumorigenesis in mouse cancer models (Zhang *et al.*, 2013). Our study describes the differentiation route of monocytes into ECs induced by ROS, pointing out that ROS can promote angiogenesis by inducing the differentiation of monocytes into ECs. Our study is in agreement with other studies showing that the continuous exposure to ROS associates with decreased pluripotency of stem cells and additionally they prove a further promotion of their MAPK/ERK-dependent differentiation towards cardiomyogenic and vascular cell lineages (Ji *et al.*, 2010; Sauer and Wartenberg, 2005; Valko *et al.*, 2007). Also, the pro-vasculogenic effects of PDGF in embryoid bodies is mediated by ROS generation, resulting in the activation of ERK1,2 mediated signaling cascades (Lange *et al.*, 2009).

In addition to the novelty of monocytes functioning as EPCs and the relevance of ROS in the promotion of this differentiation pattern, we speculate that other ROS-dependent pathways could also be involved in the angiogenic switch and angiogenesis. Due to the regulation of ferroptosis by pro-angiogenic genes, as angiopoietin-like 4 (ANGPTL4) (Dixon *et al.*, 2012; Xie *et al.*, 2016; Yang *et al.*, 2020), we hypothesized that a non-lethal ferroptosis-like mechanism could be involved in ECs activation. The activation of ferroptosis is triggered by the accumulation of lipid peroxides in an iron-dependent ROS generation process due to the failure of the glutathione peroxidase 4 (GPX4) scavenging

system, and although most of the studies supports the role of ferroptosis as a type of cell death, its non-lethal effects can regulate physiological and pathophysiological processes, as cell proliferation, survival, invasion, and migration (Friedmann Angeli *et al.*, 2014; Linkermann *et al.*, 2014; Yang *et al.*, 2014; Yu *et al.*, 2015; Sakai *et al.*, 2017; Lei *et al.*, 2019; Li *et al.*, 2020; Y. Wang and Tang, 2019). In fact, we observed that the ferroptosis at a non-lethal levels recapitulates the pathophysiological features of tumor angiogenesis by promoting EC hyperactivation, characterized by increased proliferation, migration and vessel-like structure formation and triggering the formation of a leakier vasculature with increased VE-Cad junctional gaps, concomitantly with glutathione (GSH) depletion indicating the abrogation of GPX4 activity. These alterations favored cancer cell adhesion and transendothelial cancer cell migration. It is known that ROS levels driven by intermittent hypoxia activates HIF1α expression that in turn promotes vessels permeability by inducing VE-Cad cleavage (Harki *et al.*, 2019), suggesting that the activation of HIF1α modulates VE-Cad remodeling, controlling vascular permeability. Maybe, the ferroptosis-like mechanism promoting ECs activation could be regulated by HIF1α:VEGF signaling axis. The putative activation of HIF1α by ferroptosis in ECs could also lead to the production and further release of VEGF and other pro-angiogenic factors that stimulate the recruitment and the differentiation pattern of monocytes into ECs, contributing for the expansion of the vascular network. In fact, we observed in a child with cerebral cavernous malformation (CCM) that the levels of VEGF and circulating monocytes sharing monocytic and ECs markers ($CD14^+/CD31^+$) were increased in comparison to healthy donors, suggesting VEGF levels may be involved in monocytes mobilization for vascular CCM lesions that will contribute for the vascular malformations by functioning as EPCs.

Considering that the ferroptosis-like mechanisms and monocytes functioning as EPCs potentiate the angiogenic switch, strategies focused on the abrogation of oxidative-stress related ferroptosis and in monocytes recruitment and further differentiation in the tumor will bring new opportunities to the development of novel anti-angiogenic and anti-cancer strategies.

Repurposing Propranolol for anti-angiogenic and anti-tumoral strategies: novel perspectives for cancer treatment

The repurposing of old drugs for new therapeutic purposes offers an opportunity for cancer treatment, being cost-effective and time-efficient compared to new drugs in development and enabling the rapid clinical translation since the pharmacokinetic, pharmacodynamic and toxicity profiles of those drugs have been already established (Zhang *et al.*, 2020). However, several successful examples of drug repurposing do not

involve the same molecular mechanism underlying its effectiveness in different disease contexts (Moffat *et al.*, 2017). For instance, drugs that were not developed as anti-angiogenic agents can be repurposed and used in vascular diseases and cancer. An example is Propranolol, a non-selective β-blocker currently used in the treatment of some vascular tumors, due to its inhibitory effect of angiogenesis, which has been correlated with the inhibition of HIF1α expression (Chim *et al.*, 2012; Chen *et al.*, 2017). In our study, we also showed that Propranolol impairs ECs activation and rescues the ferroptosis-like phenotype needed for ECs activation. The reversion of the ferroptosis by Propranolol seems to be related to the increased production of the antioxidant hydrogen sulfide (H_2S). It is known that the activity of HIF1α, a master regulator of angiogenesis, is enhanced by ROS, thus the stimulation of H_2S production by Propranolol can interfere with HIF1α activity, as it was described for the antioxidant vitamin D (Gao *et al.*, 2007). Moreover, the exposure to antioxidants and free radical scavengers impairs ROS-induced ECs permeability and loss of junctional integrity mediated by VE-Cad cleavage driven by HIF1α (Lum and Roebuck, 2001; Aghajanian *et al.*, 2008; Harki *et al.*, 2019). Propranolol, maybe through H_2S, besides preventing ECs activation-induced by the ferroptosis-like mechanism it also, reverts the generation of VE-Cad intercellular junctional gaps, abrogating the pro-metastatic ECs phenotype that favors cancer cells intravasation to the vessels. The exogenous administration of antioxidants exert an inhibitory effect on neovascularization and may abrogate the ROS-induced impairment of endothelial barrier, suggesting that their application in therapy might impair excessive angiogenesis and normalize the existing vasculature (Radomska-Lesniewska *et al.*, 2017). In fact, our results pointed that the antioxidant Propranolol properties, maybe through H_2S production, the abrogates the oxidative stress-related ferroptosis and normalizes the vasculature. Another explanation for the anti-angiogenic Propranolol properties can be the fact that the β-adrenergic signaling pathway drives the metabolic shift of ECs to aerobic glycolysis, mediated by HIF1α. Thus upon Propranolol exposure, the interference with HIF1α activity in ECs by Propranolol could induce glycolysis inhibition, a vital metabolic pathway in ECs activation and rapid vascularization (De Bock *et al.*, 2013). Interestingly, the antioxidant properties mediated by H_2S levels are EC specific since we observed that in cancer cells Propranolol did not promote an increase in the H_2S levels and shows anti-tumoral effects, by inducing the generation of ROS and lipid peroxides and ultimately leading to cancer cell death. Moreover, the exposure to Propranolol, although has no effect in the *in vitro* differentiation pattern of monocytes into ECs, in the CCM patient it decreases the levels of circulating $CD14^+/CD31^+$ monocytes and VEGF in peripheral blood. These results indicate that, perhaps, the abrogation of

HIF1α activity in ECs and the induction of cancer cell death by Propranolol abrogates the release of VEGF that, in turn, might impair the levels of circulating monocytes, inhibiting its contribution for tumor angiogenesis. In fact, together these results showed that Propranolol has a promising applicability in cancer treatment, not only by its anti-angiogenic properties but also by its anti-tumoral effects.

Some studies pointed that the normalization of the tumor vasculature will improve the prognosis of cancer patients, because less permeable vessels will improve the delivery of chemotherapeutic agents and impair cancer cells spread. Interestingly, simvastatin, a statin with antioxidant properties, has been showed to reduces hypoxia-induced endothelium leakage and decreased ROS-induced HIF1α and VEGF expression, attenuating VEGF-derived tumor vessel hyperpermeability and improving cisplatin and cyclophosphamide efficacy (Chen *et al.*, 2013). In a theoretical scenario, cancer treatment might rely on multi-mechanisms targeting strategies focused on the induction of cancer cells death and in the promotion of tumor vascular regression events. At the same time, these strategies will transform the remaining vessels into a more functional vascular network with decreased permeability, promoting drug delivery and impairing metastasis. Cancer cells under a certain threshold have adaptive antioxidant mechanisms controlling oxidative stress, however above of this threshold, ROS accumulation disrupts redox homeostasis and causes severe damage in cancer cells, ultimately leading to cell death (Moloney and Cotter, 2018). Given these results, strategies to enhance lethal ROS production in cancer cells have a promising anti-cancer effect. As referred, the antioxidant Propranolol properties mediated by H_2S levels are ECs specific, so we take advantage of Propranolol properties to develop a strategy using the pro-oxidant selenium-chrysin loaded in dendrimers (SeChry@PURE$_{G4}$ nanoparticles) and induce cancer cells death through ferroptosis and Propranolol to impair ECs activation and induce vessel normalization. This strategy showed a promising application for cancer treatment, tackling cancer cell death through ferroptosis while avoiding the formation of a leakier vasculature, which ultimately impairs cancer cell extravasation and metastasis and improves drugs delivery. Furthermore, the normalized vasculature could be pivotal for the delivery of chemotherapeutic agents to induce cancer cells death in the remaining cancer cells that did not respond SeChry@PURE$_{G4}$ nanoparticles.

Final remarks

In this thesis we aimed to contribute to a better knowledge of the mechanisms involved in tumor angiogenesis. We investigated alternative players and mechanism(s) that contribute for tumor angiogenesis and we have, for the first time, unraveled the role of monocytes as EPCs and their contribution for tumorigenesis, by differentiating into ECs and being incorporated into the vessels structure (Figure VI.1 A). Moreover, the oxidative stress pushes the differentiation route of monocytes towards ECs.

In addition, we showed that a ROS-induced ferroptosis-like mechanism is pivotal for the hyperactivation of ECs and for the formation of leakier vessel-like structures, typical of tumor neovessels, driving the formation of an unstable and permeable neovasculature, favoring cancer cells dissemination and metastasis (Figure VI.1 A). Moreover, our findings support the repurpose of Propranolol as part of anti-cancer and anti-angiogenic clinical strategies. We showed that Propranolol impairs the ferroptosis-like-induced ECs hyperactivation and improves the anti-cancer effects of the pro-oxidant SeChry-PURE$_{G4}$ nanoformulation. In addition, it prevents the formation of leakier vascular structures, impairing metastatic disease and contributes for a better delivery of drugs (Figure VI.1 B). Our results pointed out that the clinical efficacy of Propranolol in the treatment of CCM patients could be monitored by the levels of circulating $CD14^+/CD31^+$monocytes and VEGF in peripheral blood (Figure VI.1 B), opening new perspectives for a novel therapeutic drug monitoring tool to support clinical decisions of the management of the chronic and unpredictable CCM disease.

This thesis provides insights on additional mechanisms regulating tumor angiogenesis, it unravels the role of monocytes as EPCs and crucial players during this process, and opens new perspectives for the development of alternative anti-angiogenic drugs and for the design of multitargeted strategies that simultaneously tackle cancer cells and tumor neovessels (Figure VI.1).

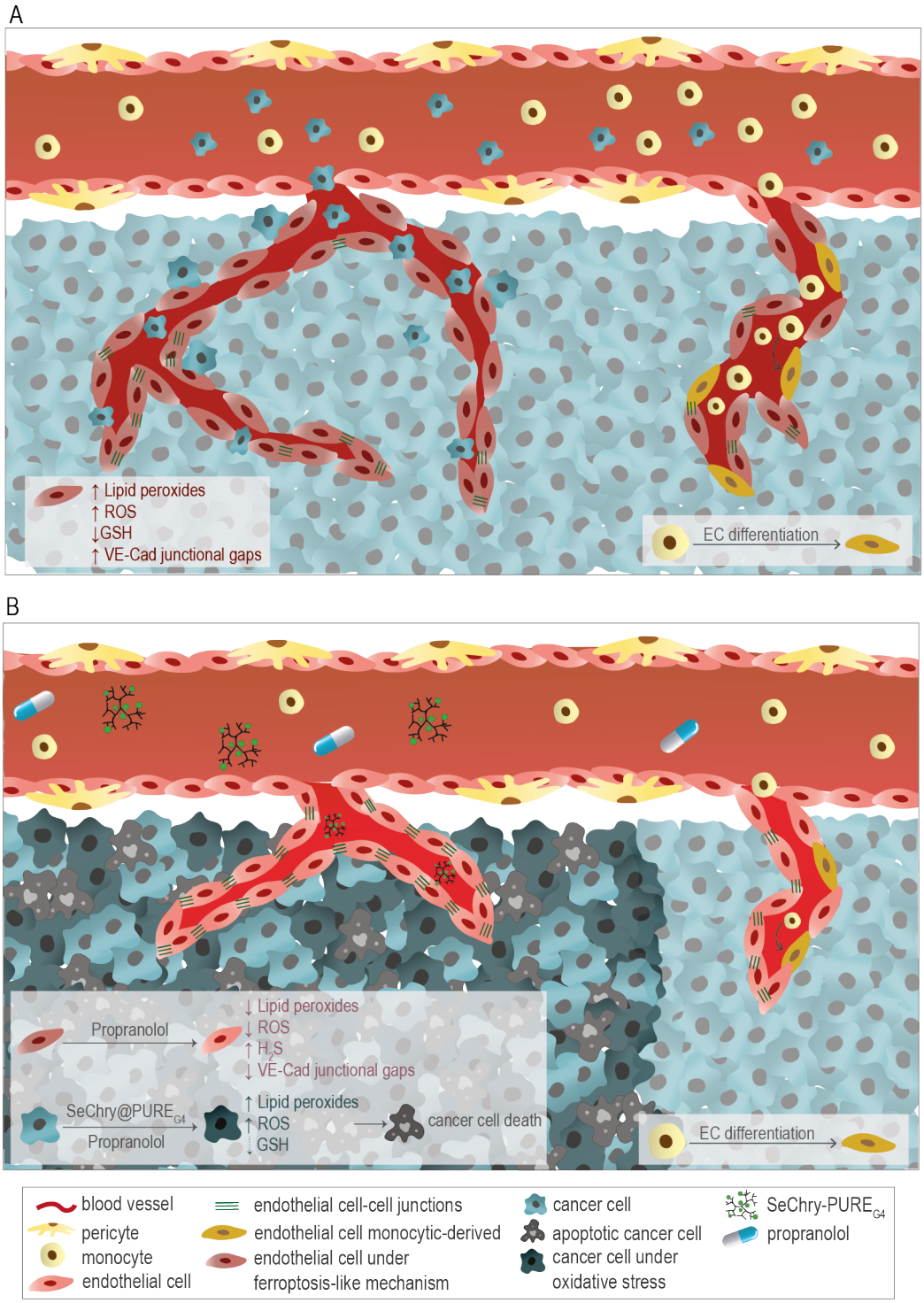


Figure VI. 1 Taking advantage of the differential oxidative stress response of cancer cells and endothelial cells (ECs).

A) The promotion of the pro-angiogenic ferroptosis-like mechanism, characterized by the accumulation of ROS-induced lipid peroxides and glutathione (GSH) depletion triggers endothelial cells (ECs) hyperactivation and vessel leakiness, which in turn is pivotal for increasing cancer cells migration and intravasation, fostering metastasis. Circulating monocytes, recruited to the tumor, are able to contribute to tumorigenesis by acting as endothelial progenitor cells (EPCs). Upon a pro-angiogenic and pro-oxidant stimulus, monocytes differentiate into EC and are incorporated into neovessels, accounting for tumor angiogenesis. B) The anti-angiogenic effect of Propranolol is mediated by increased levels of the antioxidant H_2S. Propranolol is able to impair the ECs hyperactivation induced by the ferroptosis-like mechanism, to induce vessel normalization through a decrease in intercellular gaps in the vascular endothelial cadherin

(VE-cadherin) junctions in ECs and to decrease the number of circulating $CD14^+/CD31^+$ monocytes. The combined strategy of SeChry@$PURE_{G4}$ with Propranolol has a potential anti-cancer therapy since SeChry@$PURE_{G4}$ induces cancer cell death mediated by pro-oxidative features that are potentiated by Propranolol, while the EC-specific antioxidant properties of Propranolol avoid the generation of oxidative stress, that in turn impairs the generation of a leakier vascular structure and cancer cell extravasation.

Future perspectives

The results obtain during this thesis contributed for a better understanding of the mechanisms involved in tumor angiogenesis, although several open questions deserve to be clarified.

The involvement of the ferroptosis-like mechanism in the promotion of tumor angiogenesis should be further explored *in vivo*. For that purpose, the analysis of ferroptotic-related genes/proteins, using RNA-seq or proteomic analysis, in ECs isolated from murine xenograft tumors should be performed. ECs isolated from tumors and normal tissues (control) and sorted by Fluorescence-Activated Cell Sorting (FACS) would be used for RNA-seq or proteomic analysis. If we detect an upregulation of genes or proteins involved in ferroptosis, such as glutathione synthase (GSS) or GPX4 (Stockwell *et al.*, 2017), in the tumor-associated ECs, this would be a good indication that a ferroptosis-like mechanism is occurring in the tumoral vasculature, *in vivo*. Additionally, these results should be merged with the immunohistological analysis of these tumors in order to confirm that the activation of the ferroptosis-like mechanism is not promoting ECs death, proving that this mechanism at a non-lethal level is involved in tumor angiogenesis.

The anti-cancer efficacy of Propranolol must be addressed *in vivo*, by evaluating tumor growth and metastasis formation. Moreover, in a subsequent experiment and considering the already described anti-ferroptotic properties of Propranolol, we can assess tumor ECs from mice treated with Propranolol and analyze the expression of genes involved in ferroptosis. We expect a downregulation, proving that Propranolol has anti-angiogenic properties in part mediated by the abrogation of ferroptosis in ECs. At the same time, we can use these mice, before sacrifice and analyze the ferroptosis effect in tumor vessel permeability using an *in vivo* vascular permeability assay, such as Evans blue technique or dextran-FITC injections into the mice tail vein.

For a better understanding of the putative applicability of Propranolol as an alternative anti-angiogenic drug, the effects on tumor vasculature regression and normalization should be explored *in vivo*. For that, we can take advantage of a transgenic mouse model (LifeAct $mCherry^+/CDH5.CRE^+$ mice) allied with intravital microscopy coupled to a window chamber technology, which permits a high-resolution imaging *in vivo* in a

temporal manner. Since in the LifeAct mCherry+/CDH5.CRE+ transgenic mice, actin from ECs is fluorescently labelled with mCherry, after tumor implantation (*e.g.* 4T1 mice cancer cells) it will be possible to monitor tumor neovessels formation. Moreover, using this same technology we can evaluate the effects of Propranolol administration during tumor vasculature formation and regression. Also, it should be examined the effects of Propranolol on cancer progression related-features, such as tumor growth, capacity to form metastasis and microvessel-density, which will help to disclose the role of Propranolol in improving cancer prognosis by interfering with tumor angiogenesis. For this we can take advantage of the transgenic mice and evaluate these parameters. Considering the results that we obtained *in vitro*, we expect that the anti-angiogenic and antioxidant Propranolol properties impair ECs activation and further tumor neovessels development, while in the tumor neovessels already formed it will decrease vessel permeability and their further normalization. The normalization will be essential for a better delivery of chemotherapeutic and cytotoxic agents and to decrease metastasis.

Moreover, considering that we observed that Propranolol administration in a child with CCM decreased the levels of circulating monocytes, a deeper *in vivo* characterization of the effects of Propranolol on monocytes recruitment to the tumor site should be performed. We can use an *in vivo* strategy with hCD68GFP monocytes from hCD68GFP reporter mouse, described to express a persistent green fluorescent protein (GFP) expression in both circulating monocytes and tissue macrophages (Iqbal *et al.*, 2014). They will be inoculated in a receptor mouse, previously depleted for monocytes using clodronate-loaded liposome (Côté *et al.*, 2013; Danenberg *et al.*, 2002; Z. Jiang *et al.*, 2017). After the tumor inoculation, the levels of circulating GFP+ monocytes during tumor progression in the presence and in the absence of Propranolol should be evaluated to disclose their influence in monocytes circulation. In fact, since we unraveled that monocytes function as EPCs contribute for tumor angiogenesis, Propranolol could also impair tumor angiogenesis not only by acting in ECs but also by inhibiting monocytes recruitment to the tumor. Looking at a more clinical point of view, it should be interesting to perform a retrospective study in cancer patients exposed to Propranolol in order to analyze if a better disease prognosis (Cardwell *et al.*, 2016; Pantziarka *et al.*, 2018; Hiller *et al.*, 2020) was correlated to a decrease in microvessel density, reinforcing a relationship between Propranolol and tumor angiogenesis. Together, those experiments will corroborate the applicability of Propranolol as an alternative anti-angiogenic agent for cancer treatment. Also, the follow-up analysis of blood samples from other patients with vascular tumors treated with Propranolol will be helpful for unravelling the clinical application of using the levels of circulating monocytes and VEGF as a predictive factor for the effectiveness of Propranolol treatment in vascular tumors.

The effect of SeChry@PURE$_{G4}$ in combination with Propranolol strategy should be also explored *in vivo*. Interestingly, our group previously showed that folate (FA)-functionalized SeChry-PURE$_{G4}$ nanoparticles increased its specificity for cancer cells (Santos *et al.*, 2019) since these cells express higher folate receptor levels in comparison to non-cancer cells (Fernández *et al.*, 2018). Considering that, we can optimize our dual-strategy to test *in vivo* by increasing the specificity of SeChry-PURE$_{G4}$ for cancer cells using folate, this would decrease its toxicity in adjacent non-cancer cells. For this we can use mice bearing xenograft tumors and treat them with SeChry-PURE$_{G4}$-FA in the presence or absence of Propranolol. Using these mice, we will monitor tumor growth and vascular permeability (using Evans blue or dextran-FITC) and analyze tumor necrosis and cancer cells proliferation and tumor vasculature (vessel structure and density). If significant differences were observed, we can assess survival of mice from the different groups.

Together, those experiments will be helpful to unravel *in vivo* the role of ferroptosis on tumor angiogenesis and the putative applicability of Propranolol as an alternative anti-angiogenic drug as well as their impact in monocytes circulation, that eventually will contribute to tumor angiogenesis by acting as EPCs.

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