Universidade de Coimbra

Faculdade de Medicina

UNIVERSIDADE DE COIMBRA

Low Doses of Ionizing Radiation induce Angiogenesis after Radiotherapy

Joana Filipa Pereira Ferreira

Mestrado em Investigação Biomédica

2016

Dedico esta tese ao meu grande amigo

Gerson que deixará para sempre Saudade.

Acknowledgements

Este espaço que aqui dedico para agradecer a todos os que me apoiaram nesta jornada torna-se pequeno. Tenho plena consciência que, sem a ajuda de todos os que me acompanham, não teria sido possível chegar aqui.

Começo por agradecer à minha orientadora Professora Doutora Susana Constantino, por me ter recebido no laboratório e ter tornado tudo isto possível. Agradeço também todo o apoio e disponibilidade durante o tempo em que mais precisei de orientação para continuar.

Gostaria de agradecer ao serviço de Radioterapia, nomeadamente à Doutora Filomena Pina, Diretora do Serviço, à Doutora Esmeralda Poli, Diretora da Unidade de Física Médica, e à Técnica Ana Duarte. Gostaria ainda de agradecer ao serviço de Cirurgia do Centro Hospitalar de Lisboa Norte – Hospital de Santa Maria, em particular ao Professor Doutor José Mendes de Almeida, Diretor do Serviço, e ao Doutor João Malaquias pelo empenho demonstrado diariamente que, em estrita colaboração, tornaram este projeto possível.

Gostaria de agradecer também à unidade de Histologia e Anatomia Comparada do Instituto de Medicina Molecular pela disponibilidade e empenho demonstrado ao longo de todo o projeto.

Agradeço também às minhas colegas do grupo de Angiogénese por todos os bons momentos partilhados durante este ano, obrigada tornaram o trabalho muito mais fácil. Não posso deixar de fazer um agradecimento especial à minha colega Filipa Marques que, mesmo quando tudo parecia estar ao contrário, me ajudou a superar as adversidades e se empenhou tanto quanto eu para que nada falhasse.

À minha família deixo o maior agradecimento, agradeço o amor incondicional e os valores que sempre me transmitiram e me fizeram chegar até aqui. Agradeço aos meus pais que investiram em mim desde sempre, dando-me as ferramentas necessárias para o futuro e acreditando sempre em mim (mesmo quando eu não acreditava!) e às minhas irmãs que acrescentam um quê de loucura à minha vida!

Agradeço ao meu namorado pelo apoio, carinho e cumplicidade que partilha comigo diariamente! Sem ti, Jorge, tudo ficaria menos brilhante!

Finalmente agradeço à minha colega de mestrado Ana Leal por estar sempre à distância de um telefonema para ouvir os meus desabafos! Às minhas amigas de sempre, um obrigada não chega.

Abstract

Radiotherapy is widely used to treat human malignant tumors, characterized by uncontrolled growth, capacity to invade adjacent tissues and produce distant metastasis. The goal of radiotherapy is to reduce or eliminate the primary tumor and its metastasis.

However, ionizing radiation also alters the tumor microenvironment. While there is evidence that these changes might contribute to the anti-tumor effects of radiotherapy, it was demonstrated that the irradiated stroma might exert tumor-promoting effects. Therefore, a careful analysis of the putative pro-metastatic effect of radiotherapy is imperative as radiotherapy is an essential part of cancer treatment.

Furthermore, during radiotherapy not only the tumoral target volume is irradiated but also the peritumoral tissues are exposed to low doses of ionizing radiation (LDIR). The biological effects of these LDIR on the peritumoral tissues remain to be determined. In our lab it was found that LDIR enhance angiogenesis and consequently promote tumor growth and metastasis in a mechanism dependent of the activation of VEGF receptor. This work was focused in the peritumoral vasculature that is exposed to LDIR. These findings are relevant in a tumoral context, since the vascular activation in these areas may contribute to tumor re-growth and metastasis after or during radiotherapy.

In this work, we are interested in validating these findings in humans, our main goal. Tissues exposed or not to LDIR from patients with rectal cancer that received neoadjuvant radiotherapy were used. Two distinct biopsies of parietal peritoneum were surgically removed 8 weeks after the end of radiotherapy: i) a specimen exposed to doses from 5 to 30% of the therapeutic dose (100%), located in the vicinity of the tumor; and ii) an unirradiated specimen that will be used as an internal calibrator for each patient.

Endothelial cells (ECs) were isolated from both of these species by laser capture microdissection (LCM) microscope followed by mRNA extraction, cDNA synthesis and quantitative RT-PCR analysis. According to our results (n=16), the level of expression of several pro-angiogenic factors (*VEGFR1*, *VEGFR2*, *ANGPT2*, *TGFB2*, *VWF*, *FGF2*, *PDGFC*, and *HGF*) was significantly up-regulated in ECs exposed to LDIR when compared to non-irradiated ECs suggesting, that ECs are activated after LDIR exposure. Simultaneously, an

immunohistochemistry for vWF was performed (n=14) and we found a significant increase in microvessel density in parietal peritoneum exposed to LDIR when compared to the unirradiated one. According to our results, we discussed the vascular response (activation state of ECs and microvessel density) of the oldest patients included in the study after LDIR exposure. Moreover, we discussed the data obtained between EC activation state and microvessel density suggesting that the effect of LDIR could be dynamic and temporally different in different patients.

This truly translational study will surely provide novel insights into the cellular effects of low-dose IR and be of use in the improvement of the current radiation oncology protocols.

Keywords: angiogenesis; radiotherapy; ionizing radiation; microvasculature; endothelial activation

Resumo

A radioterapia é usada no tratamento de tumores malignos caracterizados por um crescimento descontrolado e capacidade de invadir tecidos adjacentes e metastizar. O objectivo desta terapêutica é reduzir ou eliminar o tumor primário evitando que haja formação de metástases. Tal é conseguido pela acção da radiação ionizante nas células tumorais e também no microambiente tumoral. Contudo, há observações experimentais e clínicas que indicam que a radiação ionizante poderá promover um comportamento metastático por parte das células tumorais e que o microambiente irradiado poderá promover a tumorigénese. Assim, é importante estudar os possíveis efeitos da radiação ionizante que promovem o crescimento tumoral e metastização, uma vez que a radioterapia é essencial no tratamento do cancro.

É, também, importante ter noção que durante a radioterapia não só o volume tumoral é exposto a radiação ionizante mas também os tecidos peri-tumorais são expostos a doses mais baixas de radiação ionizante. Os efeitos biológicos e moleculares destas baixas doses de radiação ionizante nos tecidos que rodeiam a área a tratar são desconhecidos. Recentemente o nosso laboratório demonstrou que baixas doses de radiação ionizante induzem angiogénese e consequentemente promovem progressão tumoral e metastização após ou durante a radioterapia.

Neste trabalho, o nosso principal objectivo é de validar estes resultados em humanos. Para tal, utilizaremos tecidos expostos ou não a baixas doses de radiação ionizante de doentes com cancro do recto submetidos a radioterapia neoadjuvante. Duas biópsias distintas de peritoneu parietal foram removidas no momento da cirurgia: i) uma em que os tecidos foram expostos a doses baixas de radiação ionizante (5 a 30% da dose terapêutica), localizada na região peri-tumoral e ii) outra que corresponde a tecido não irradiado e que será usada como calibrador interno para cada doente.

As células endoteliais serão isoladas destes dois tipos de biópsias por microscopia de microdissecção e captura a laser, o ARNm será extraído, o ADNc sintetizado e será efetuada uma análise por RT-PCR quantitativo. De acordo com os nossos resultados (n=16), o nível de expressão de vários factores pró-angiogénicos (*VEGFR1*, *VEGFR2*, *ANGPT2*, *TGFB2*, *VWF*, *FGF2*, *PDGFC*, and *HGF*) está significativamente aumentado nas células endoteliais expostas

a baixas doses de radiação ionizante quando comparado com aquele que é encontrado em células endoteliais provenientes de tecidos não irradiados. Simultaneamente, após imunohistoquimica para vWF (n=14), verifica-se um aumento significativo da densidade microvascular no peritoneu parietal exposto a baixas doses de radiação ionizante quando comparado com peritoneu parietal não irradiado. De acordo com os nossos resultados, também discutiremos neste trabalho a resposta vascular (nível de activação endotelial e densidade microvascular) após exposição a baixas doses de radiação ionizante nos doentes mais idosos que foram incluídos no estudo. Discutiremos ainda os resultados obtidos para o mesmo doente de activação endotelial e densidade microvascular sugerindo que o efeito de baixas doses de radiação ionizante poderá ser dinâmico e temporalmente diferente para diferentes doentes.

Os resultados obtidos são cruciais para revelar novos mecanismos que permitirão compreender o efeito pro-metastático de baixas doses de radiação ionizante, contribuindo para um avanço do conhecimento na área e para a optimização dos protocolos de radioterapia.

Palavras-chave: angiogénese; radioterapia; radiação ionizante; microvasculatura; activação endotelial

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Abbreviations and Symbols List

ANG/ANGPT 1/2 – Angiopoetin-1 and -2

CHLN – Centro Hospitalar de Lisboa Norte

cDNA – complementary deoxyribonucleic acid

EC(s) – Endothelial cell(s)

ECD – Extracellular Domain

ECM – Extracellular Matrix

EPC – Endothelial Progenitor Cell

bFGF/FGF-2 – Basic Fibroblast Growth Factor

FVIII – Factor VIII

HIF – Hypoxic Inducible Factor

HUVECs – Human Umbilical Vein Endothelial Cells

(LD)IR – (Low doses of) Ionizing Radiation

LCM – Laser Capture Microdissection

MMP – Metalloproteinase

mRNA – messenger ribonucleic acid

PlGF – Placenta Growth Factor

RNA - ribonucleic acid

RNS - Reactive Nitrogen Species

ROS - Reactive Oxygen Species

RT – Radiotherapy

RTK – Tyrosine Kinase Receptor

TAM - Tumor associated macrophages

TGFB – Transforming Growth Factor Beta

VEGF – Vascular Endothelial Growth Factor

VEGFR1/Flt-1 – Vascular Endothelial Growth Factor Receptor 1

VEGFR2/KDR/Flk-1 – Vascular Endothelial Growth Factor Receptor 2

VEGFR3/Flt-4 – Vascular Endothelial Growth Factor Receptor 3

VPF – Vascular Permeability Growth Factor

vWF – von Willebrand Factor

Introduction

Introduction

Physiological Angiogenesis

In order to survive, mammalian cells need nutrients and oxygen provided by blood vessels. Thus, for multicellular organisms, the recruitment of new blood vessels, by vasculogenesis and angiogenesis, is essential for their growth and survival[1]. In the embryo, vessels are formed primarily by mesoderm-derived endothelial precursors - angioblasts - which origin vascular cords. Then, through a process known as vasculogenesis, vascular cords acquire a lumen and shape the first vascular labyrinth (Figure 1). Afterwards, angiogenesis starts and by vessel sprouting a more complex network is created remodeling subsequently into arteries and veins. Finally occurs arteriogenesis which consists in the recruitment of pericytes and vascular smooth muscle cells that enwrap new endothelial cells (ECs) tubules providing stability and regulating perfusion[2].

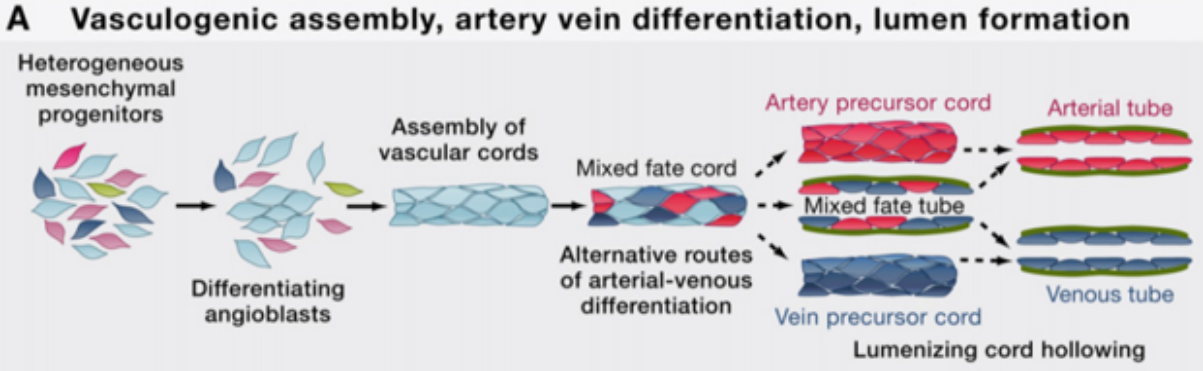

Figure 1. Vasculogenesis. Angioblasts differentiate into ECS, which form vascular cords, acquire a lumen and are pre specified to arterial or venous phenotypes. (Adapted from Potente, M. *et al.* Basic and therapeutic aspects of angiogenesis).

The angiogenic process starts with production of angiogenic growth factors that diffuse into nearby tissues and bind to specific receptors located in the ECs of preexisting blood vessels. Those ECs become activated and begin to produce new molecules and enzymes that dissolve small holes in the surrounding basement membrane. Afterwards, adhesion molecules like integrins lead the sprouting front forward and metalloproteinases (MMPs) dissolve the tissue in front the sprouting vessel tip in order to accommodate it[3].

Pro- and Anti-Angiogenic Factors

The angiogenic process is essential for homeostasis in the healthy organism since blood vessel malformation or dysfunction contributes to the pathogenesis of many diseases. Therefore, it is crucial to maintain a balance between pro- and anti-angiogenic factors thereby regulating angiogenesis[4]. Angiogenesis-dependent diseases such as cancer, atherosclerosis, age-related macular degeneration and rheumatoid arthritis are related with shifts in the finely balanced equilibrium between angiogenic stimulators and inhibitors[5].

Vascular Endothelial Growth Factor Family

In humans, the Vascular Endothelial Growth Factor (VEGF) family consists in VEGF-A, -B, -C, -D and Placenta Growth Factor (PlGF) which arise from alternative splicing and proteolytic processing of N- and C-Terminal, leading to distinct VEGF variants with specific functions[6].

Mammalian VEGFs bind to three different Tyrosine Kinase Receptors (RTKs): Endothelial Growth Factor Receptor-1 (VEGFR1/Flt-1), Endothelial Growth Factor Receptor-2 (VEGFR2/KDR/Flk-1), and Endothelial Growth Factor Receptor-3 (VEGFR3/Flt-4). They belong to class V RTKs, which carry seven Ig-like domains in the extracellular domain (ECD). VEGF-A binds to VEGFR-1 and VEGFR-2, VEGF-B and PlGF bind to VEGFR-1 and VEGF-C and –D bind to VEGFR-2 and VEGFR-3. VEGFRs become activated when a dimer (ligand) links covalently to the ECD, promoting receptor homo- and heterodimerization followed by phosphorylation of specific tyrosine residues located in the intracellular domain and in carboxy-terminal tail of the receptor. This interaction with the ligand leads to a downstream signaling mediated by intracellular signaling effectors[7]. Furthermore, VEGFs interact with several co-receptors such as heparin sulfate proteoglycans and neurophilin-1 and -2[8] (Figure 2).

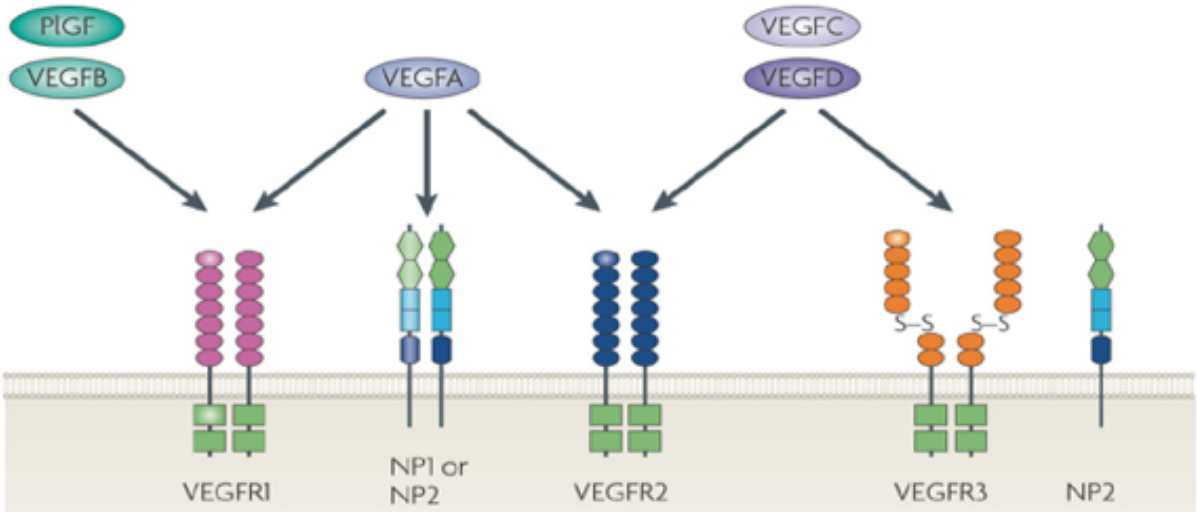

Figure 2. VEGF Family. The mammalian family of VEGF ligands consists of five family members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and PGF. The 5 ligands bind to 5 different receptors: VEGFR-1, VEGFR-2, VEGFR-3, NP1 and NP2 leading to downstream signaling. (Adapted from Lee M. Ellis & Daniel J. Hicklin, VEGF-targeted therapy: mechanisms of anti-tumor activity).

VEGF, also known as vascular permeability factor (VPF), is a potent mitogen for micro- and macrovascular ECs but it is devoided of consistent and appreciable mitogenic activity in other cell types. VEGF is produced by many cell types, including tumor cells, macrophages, platelets, keratinocytes and renal mesangial cells[9].

Studies demonstrated that VEGF promotes angiogenesis in tridimensional in vitro models[10], inducing confluent microvascular ECs to invade collagen gels and form capillary-like structures. These studies suggest a strong synergism between VEGF and Basic Fibroblast Growth Factor (bFGF) to induce this effect. Furthermore, *in vitro, it was shown that* VEGF prevents apoptosis of ECs by two different mechanisms: it promotes the activation of the PI3K/Akt signaling pathway that leads to survival signals and it increases the expression of some anti-apoptotic proteins, such as Bcl-2 and A-1. *In vivo*, VEGF effects depend on the developmental stage and maturation level of the vessels. In neonatal mice, vessels are very dependent on VEGF and the inhibition of this factor can lead to endothelial cell death and the destruction of some vessels. However, in adult mice the inhibition of VEGF appears to have no significant effects in endothelial cell survival and vessels maintenance. In addition, newly formed tumor vessels are much more VEGF dependent than already established vessels. In several experimental models, such as, the rabbit cornea, the primate iris and the rabbit bone it was demonstrated that VEGF induces a strong angiogenic response[11].

However, VEGF activity is not exclusive to the vascular system, it also plays a role in other normal physiological functions such as hematopoiesis, wound healing and bone formation[9].

VEGF is a major factor in the cardiovascular system. It is present in cardiac myofibroblasts, which are non- ECs and are morphologically similar to fibroblasts. It is responsible for the growth, development and repair of normal cardiac tissue and it is found at the site of myocardial infarction. Diverse techniques demonstrated that myofibroblasts at the site of infarction co-expressed VEGF and its receptors as well, suggesting a huge contribution to tissue remodeling in an autocrine manner[12].

The binding affinity of VEGFR-1 for VEGFA is at least 10 fold higher than that of VEGFR-2. Nevertheless, despite binding VEGF with high affinity, VEGFR-1 presents weak tyrosine kinase phosphorylation activity following VEGF stimulation[13]. Studies using mice lacking *Vegfr-1* revealed that this receptor acts as a negative regulator of angiogenesis during embryonic development, as animals exhibit a severe disorganization of the vasculature and an increased number of ECs[14]. The phenotype observed was due to an increased mesenchymal to hemangioblast commitment resulting in an excess of the EC population that leads to the development of a disorganized vascular plexus. Therefore, it was proposed that VEGFR1 could be a negative regulator of the VEGF activity, acting as a "decoy" receptor to sequester VEGF, thus rendering it less available for interacting with VEGFR2[15].

In adult, VEGFR-1 plays a role in activating VEGFR-2 and thereby in angiogenesis, binding of PlGF[16]. This mechanism gains importance in angiogenesis-associated pathologies, where PlGF has often been described upregulated[17]. Furthermore, VEGFR-1 is involved in the preparation of the metastatic niche, since VEGFR-1-positive haematopoietic progenitor cells were shown to colonize tumour specific pre-metastatic sites prior to the arrival of tumour cells[18].

VEGFR-2 is considered the major mediator of the VEGF signalling during vasculogenesis and angiogenesis[19]. Phosphorylated VEGFR-2-tyrosine residues serve as docking sites for molecules that initiate different signalling cascades leading to cellular responses such as proliferation, migration, survival and permeability[19]. In pathologic conditions, VEGFR-2 promotes tumour angiogenesis, being highly expressed by several human cancer cells[20]. It

has been shown that the blockage of VEGF activity leads to an inhibitory effect on the growth of many tumour cell lines in nude mice[20].

Due to these findings, VEGF has been at the center stage of antiangiogenic therapy and so far many patients with cancer have benefited from VEGF therapy. However, this anticancer strategy is challenged by insufficient efficacy and resistance. Even in responsive patients, antiangiogenic drugs generally prolong survival only in order of months[21]. Clinical efficacy is lower than the observed in the pre-clinical cancer models. Multiple mechanisms of resistance to anti-angiogenic therapy were already invoked in different tumor contexts[22,23]. Furthermore, certain preclinical studies show enhanced metastasis in tumor-bearing mice treated with VEGF-blocking drugs, such as sunitinib[24-26]. However, these findings remain debated because other preclinical studies did not detected increased metastasis[27,28] and large meta-analysis have not shown more metastatic dissemination in patients[29].

von Willebrand Factor

Von Willebrand Factor (vWF) is an adhesive plasma glycoprotein that binds to Factor VIII (FVIII), platelets surface glycoproteins and constituents of connective tissue to perform its haemostatic role. Factor VIII is degraded by activated protein C unless it is non-covalently linked to vWF, establishing a stable complex. The biosynthesis of vWF is restricted to ECs and megakaryocytes. ECs synthetize vWF as a pre-pro-vWF (signal peptide, pro peptide and mature vWF subunit) which is posteriorly modified in the endoplasmic reticulum and in the Golgi apparatus[30,31]. The mature vWF is directly released to the plasma through a constitutive secretory pathway or tubulised and stored into Weible-Palade bodies which are unique to ECs[31].

vWF deficiency or dysfunction leads to von Willebrand disease which is the most common congenital bleeding disorder in humans. Usually, vWF dysfunction causes angiodysplasia, a common vascular lesion of the gastrointestinal tract characterized by a fragile vascular network with a disrupted architecture, increased permeability and susceptibility to rupture[32,33].

Regarding angiogenesis, it was found that the absence of vWF increases endothelial cell proliferation *in vitro*. Accordingly, vWF-deficient mice display an increased vessel density of the vasculature in the ears when compared to vWF-expressing mice[34].

According to the local cellular microenvironment, it was described that vWF may exert a proliferative effect. Upon damage of the vascular endothelial layer, vWF is able to penetrate into the intima of large peripheral vessels and the deposition of vWF in the intima coincides with intimal thickening. This suggests that vWF plays a role in the pathogenesis of intimal hyperplasia by promoting smooth muscle cell proliferation. This is supported by the results obtained in *in vitro* experiments where it was found that vWF directly stimulates smooth muscle cell proliferation[34].

Moreover, it was shown that high plasma vWF concentrations are correlated with advanced tumor stage, the presence of multiple metastasis and significantly poor prognosis of patients with metastatic colorectal carcinoma. High vWF plasma concentrations have also been reported in patients with various types of cancer, such as squamous cell carcinoma of the larynx and the cervix. This effect is associated with tumor-related angiogenesis and the metastatic process[35].

Importantly, it was found that FGF2 and VEGF, alone or in combination, up-regulate vWF mRNA and protein in human ECs. Therefore, the expression of an endothelial cell marker is controlled by angiogenesis factors and this aspect makes vWF mRNA particularly useful to detect activation of the endothelium, an early sign of angiogenesis, in tumors[36].

Angiopoietins

Angiopoietin 1 (ANG1) and Angiopoietin (ANG2) are two proteins composed by an N-terminal super clustering domain, a linker peptide and a carboxy-terminal fibrinogen-homology domain. The fibrinogen-homology domain mediates receptor binding whereas the N-terminal is required for dimerization or oligomerization. ANG1 is mainly produced by perivascular cells whilst ANG2 is primarily produced by ECs. Both proteins bind to Tie receptors which are highly homologous cell surface molecules that are almost exclusively expressed in ECs and hematopoietic cells. ANGPT ligands act on Tie2 receptor in either paracrine (ANG1) or autocrine (ANG2) manner[37].

ANG1 is generally considered a Tie2 agonist which promotes vessel maturity and stability, and reduces leakiness[38]. *In vivo,* studies suggest that ANG1 is essential for maturation and stabilization of the developing vasculature and for normal remodelling, since mice lacking *Angpt1* start to develop a primary vasculature which fails to stabilize or remodel leading to embryonic lethality[37]. Moreover, overexpression of ANG1 produces enlarged and leakage-resistant vessels in adult mice. It was also found that ANG1 act synergistically with VEGF-A to promote angiogenesis[39].

On the other hand, ANG2 has been considered to have the opposite effect of ANG1 since it disrupts the connections between the endothelium and perivascular cells and promotes cell death and vascular regression by blocking ANG1-mediated Tie2 receptor activation[40].

However, a number of studies of ANG2 function have suggested a more complex situation. Corneal pocket assays have shown that both ANG1 and ANG2 had similar effects acting synergistically with VEGF-A to promote the growth of new blood vessels, suggesting a proangiogenic role for ANG2[40].

Moreover, it was found that *in vitro* and at high concentrations ANG2 can also be pro-angiogenic, suggesting the possibility that there was a dose-dependent endothelial response[41]. High levels of ANG2 can induce TIE2 phosphorylation in human umbilical vein ECs (HUVEC), stimulating cell proliferation, cell differentiation and protection against induced cell death[41]. It was also demonstrated that in the presence of VEGF, ANG2 is responsible for an increase in capillary diameter, migration and proliferation of ECs, and sprouting of new blood vessels[42]. ANG2-induced TIE2 phosphorylation has also been demonstrated in murine brain capillary ECs, promoting migration and tube-like structure formation[43].

Transforming Growth Factor Beta

Transforming Growth Factor Beta (TGFB) is a 55kDa cytokine produced and secreted by most cell types in a latent form, which needs to be cleaved to become active and exert its proper function. TGFB has 3 isoforms expressed in mammals: TGF-β1, TGF-β2 and TGF-β3. To be active this cytokine molecule needs to be in a dimer composed of a previously cleaved polypeptide chain from a precursor. The active TGFB binds to two pairs of receptors

serine/threonine kinases known as type I and type II receptors, respectively. Seven type I receptors and five type II receptors paired in different combinations provide the receptor system for the entire TGFB family[44].

In physiological conditions, TGF-β can influence the angiogenic process in different ways, depending on its concentration and the presence of other cytokines in the microenvironment. At low doses it contributes to the angiogenic switch, either indirectly, by inducing the upregulation of angiogenic factors (e.g. VEGF, FGF and PDGF, and proteinases)[45] or directly, through the binding to the two types of TGFβR1: ALK1 and ALK5 (activin receptor-like kinase -1 and -5, respectively) and consequent activation of proangiogenic or maturation-specific genes[46]. At high doses, it inhibits endothelial cell growth, promotes the reorganization of the basement membrane and stimulates smooth muscle cells differentiation and recruitment[45].

In a tumoral context, TGF-β signaling has been shown to act as a strong activator of tumor growth and metastasis by acting directly in tumor cells and local environment. TGF-β contributes for immunosupression, modification of the extracellular matrix and induction of angiogenesis. TGF-β1 and β2 induce cancer cells to produce VEGF and PAI-1, promoting endothelial cell proliferation and vascular remodeling[47,48]. Some studies demonstrated that hypoxia and TGF-β signalling pathways can synergize in the regulation of VEGF gene expression at the transcriptional level and cooperate in the induction of the promoter activity of VEGF[49]. Blocking of TGF-β action inhibits tumour viability, migration, metastasis in mammary cancer, melanoma and prostate cancer. Reduction of TGF-β production and activity may be a promising target of therapeutic strategies to control tumour growth[50].

Interestingly, it was also found that low doses of ionizing radiation (LDIR), 0.3 Gy, induce a significant increase in TGF-β1 circulating levels and this effect may contribute to the anti-inflammatory effect mediated by low-dose IR[51].

Fibroblast Growth Factor

Fibroblast Growth Factor (FGF) was the first pro-angiogenic molecule to be identified. The FGF family contains 20 factors. The classical FGFs (FGF-1 and FGF-2) lack cytoplasmic sequences for extracellular export and, for that reason, its acceptance as a pro-angiogenic

factor with crucial role in angiogenesis was contested[52]. However, several studies have been done in order to find alternative modes of transport out of the cell rather than via the classical secretory apparatus. FGFs bind with high affinity to heparin sulfate proteoglycans which are located on the surface of most cells and within the ECM, thus constituting a reservoir of the growth factor that can be released in a regulated manner[53].

FGF biological effects depend on four RTK (FGFR-1, -2, -3 and -4) which display broad expression patterns. FGFRs consist of three extracellular immunoglobulin-like (Ig) domains (D1-3), a single transmembrane helix domain and an intracellular Tyrosine Kinase domain[54]. Studies have showed that mouse embryos with dominant negative FGFR-1 don't develop or maintain vasculature[55]. By contrast, inactivating FGF-2 results in mice apparently normal but with decreased vascular tone and low blood pressure[56].

In physiological conditions, FGF-2 participates in the repair phase of wound healing through regulation of cell-surface adhesion molecules, most notably the αVβ3 integrin complex. Expression of the αVβ3 integrin complex on the surface of human microvascular ECs is increased by treatment with FGF-2. The αVβ3 integrin complex, also known as the vitronectin receptor, mediates endothelial cell binding to extracellular components such as vitronectin and fibrinogen[57]. Moreover, in experimental models, it was shown that wound healing is accelerated after topical application of FGF1 and FGF2[58].

FGF2 and *FGF1*/*FGF2* knockout mice exhibit delay in the remodeling of damaged blood vessels during wound healing and tumor angiogenesis[59].

Moreover, FGF is involved in tumor progression by favoring tumor angiogenesis. Moreover, it can act directly on tumor cells via paracrine or autocrine loops of stimulation. Thus, targeting the FGF/FGFR system through anti-FGF/FGFR agents may provide benefits not only in terms of neovascularization inhibition but also by an anti-tumoral effect on malignant cells[54].

Hepatocyte Growth Factor

Hepatocyte Growth Factor (HGF) is a disulfide-linked heterodimer of a heavy (α) subunit of 55-65 kDa and a light subunit of 32-36 kDa. HGF is secreted by different cell types in an

inactive form, becoming mature at extracellular environment. In a mature form HGF is able to activate its tyrosine kinase receptor – MET – and induce biological activity[60].

HGF was identified as a mitogen for hepatocytes in primary cultures and, currently, it is considered the major mediator of liver regeneration *in vivo*[61]. It is a mitogen for several cell types including hepatocytes, melanocytes and ECs [60].

The Met protein (HGF receptor) is largely expressed in ECs and it has been shown that its interaction with HGF can stimulate these cells to proliferate and migrate *in vitro*[62]. Moreover, it was found that, in vitro, endothelial wound healing is induced by HGF[62]. In vivo, highly purified HGF promotes neovascularization at sub-nanomolar concentrations[62]. It was also shown that HGF stimulates the expression of urokinase by ECs. Urokinase binds to their surface receptors and mediates endothelial cell invasion and migration during the early stages of angiogenesis[63]. Interestingly, other studies show that a much more robust endothelial proliferative and chemotactic response is obtained when combining HGF and VEGF than either growth factor alone[64]. These results were corroborated by a gene expression analysis demonstrating that the combination of the two growth factors synergistically induces a number of genes involved in the regulation of the cell cycle[65].

Moreover, several carcinomas of the colon and rectum, lung, stomach, kidney and pancreas were positive for Met receptor. The expression of Met receptor has been correlated with an aggressive phenotype[60] corroborating the findings that HGF promotes tumoral angiogenesis.

Platelet Derived Growth Factor

Platelet Derived Growth Factor (PDGF) was first identified as a growth factor for fibroblasts, smooth muscle cells and glia cells. It consists of a disulfide-linked dimer with two different polypeptide chains, A and B. PDGF family used to comprise PDGF-AA, PDGF-BB and PDGF-AB encoded by to genes *PDGFA* and *PDGFB*. More recently, two additional PDGF proteins and genes have been discovered – PDGF-C and PDGF-D[66]. PDGF receptors are RTK, receptor β and receptor α. Both PDGF receptors are expressed in fibroblasts and Smooth Muscle Cells, whereas other cell types express only β receptors, such as ECs [66].

In general, PDGF does not appear to be of importance for the initial formation of blood vessels, since no apparent vascular abnormality was observed during early embryogenesis in mice with genes for PDGF or PDGF receptors inactivated. However, in specific organs, the effect of PDGF on angiogenesis may be significant[67] For instance, stimulation of PDGF-AB production in cardiac microvascular cells leads to induction of both von Willebrand factor and VEGF and VEGF receptor-2, suggesting an important role of PDGF in cardiac angiogenesis[67]. Moreover, in different experimental models such as the chick embryo chorioallantoic membrane and in the mouse cornea pocket assay, the pro-angiogenic effects of different PDGF isoforms have been demonstrated[68,69].

Both in normal and pathological conditions, it was shown that PDGF-B and PDGFRβ are mainly expressed in the developing vasculature including tumor angiogenesis. It was shown that PDGF-B produced by quiescent ECs could activate perivascular cells that express PDGFRβ[70]. However, if this paracrine signaling is disrupted, the recruitment of perivascular cells does not occur and consequently ECs proliferate irregularly, leading to improper vessel formation and hemorrhage[70]. Therefore, PDGF plays an important role in inducing the formation and stabilization of new vessels by recruitment of perivascular cells during angiogenesis and vasculogenesis.

The expression of PDGF is associated with several diseases and pathological conditions such as cancer[67], lung fibrosis[71], retinochoroidal vascular diseases[72], and atherosclerosis[73]. Due to its pathological implications, potent and specific PDGF antagonists, such as PDGF receptors inhibitors, are therefore clinically useful[66]. Ongoing clinical trials are using PDGF antagonists to block the effects of PDGF in retinochoroidal vascular disease[72]. PDGF has been associated as an autocrine growth factor in the development of spontaneous tumors and its hyperactivity in multiple types of human solid tumors. Moreover, chromosomal translocation involving PDGF genes has been observed to cause dysregulation of PDGF expression in certain solid tumors[66].

Tumor Angiogenesis

Tumor formation is based on a combination of genetic and epigenetic alterations. Although genetic instability is recognized as crucial to achieve a malignant phenotype, cancer cells also acquire other capacities overall designated as hallmarks of cancer, which confer them

growth advantage, increased survival and proliferation as well as dissemination ability resulting in early growth[74]. Once the tumor mass has reached a critical size tumor cells start to lack an appropriate supply of oxygen and nutrients, inducing cellular necrosis and apoptosis. To overcome this growth problem the tumor can induce new blood vessels formation from pre-existing ones, a process known as tumoral angiogenesis[75] (Figure 3).

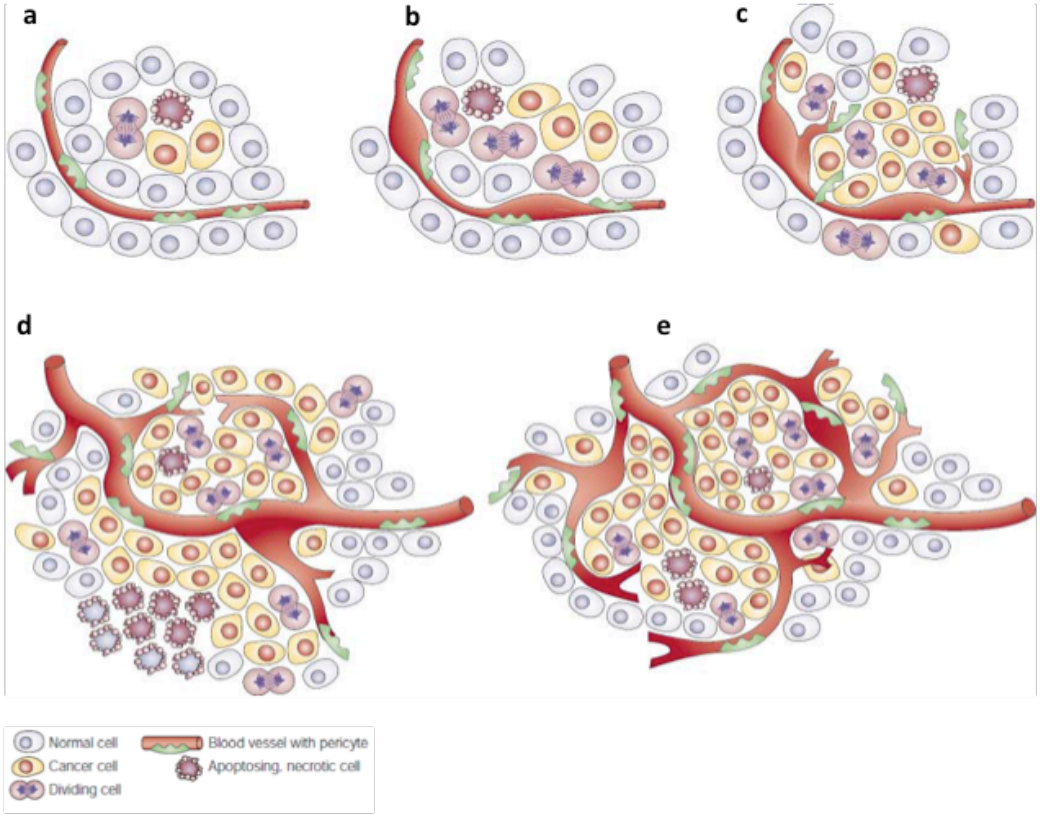

Figure 3. Tumor Angiogenesis. Most tumors start growing as avascular nodules (dormant) (a) until they reach a steady-state level of proliferating and apoptosing cells. The initiation of angiogenesis has to occur to ensure exponential tumor growth. The switch begins with perivascular detachment and vessel dilation (b), followed by angiogenic sprouting (c), new vessel formation and maturation, and the recruitment of perivascular cells (d). Blood-vessel formation will continue as long as the tumor grows, and the blood vessels specifically feed hypoxic and necrotic areas of the tumor to provide it with essential nutrients and oxygen (e). (Adapted from Bergers & Benjamin 2003, Tumorigenesis and the angiogenic switch).

The process by which tumoral angiogenesis is induced is called “angiogenic switch” that allows a permanent induction of angiogenesis, required to support increased nutrients and oxygen needs, as well as higher evacuation of metabolic wastes and carbon dioxide[76]. In tumoral angiogenesis the balance between pro- and anti-angiogenic factors is unsettled tending to up-regulation of pro-angiogenic factors, such as VEGF, FGF, vWF, ANGPT and HGF, and inhibition of anti-angiogenic factors (Figure 4). This switch is responsible for

activate quiescent vasculature which begins capillary sprouting and turns a non-vascularized hyperplasia into an expanding vascularized tumor[77].

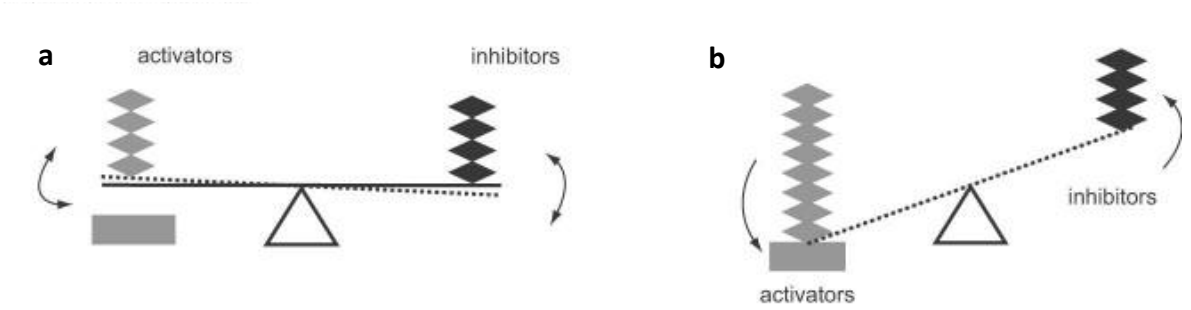

Figure 4. Angiogenic Switch. a) Regular balance between pro- and anti-angiogenic factors in physiological angiogenesis. b) Angiogenic switch in tumoral angiogenesis with up-regulation of pro-angiogenic factors. (Adapted from Nishida, N. et al., Angiogenesis in cancer.)

However, tumoral cells are not the only responsible cells for the angiogenic switch and subsequently tumoral angiogenesis. The tumor-associated host cells and elements of the ECM are also implicated in this switch. For instance, several cells of the tumor microenvironment like pericytes, cancer associated fibroblasts, and cells of the immune system, are of utmost importance for tumor progression. It appears that the recruitment of cells of the innate immune system to secrete pro-angiogenic factors and to trans-differentiate into ECs, integrating the new blood vessels, contributes to tumor angiogenesis. Tumor associated macrophages (TAM), for instance, act in tumor progression by paracrine action. They are attracted trough chemoattractants secreted by tumor cells including pro-angiogenic factors (VEGF), cytokines and chemokines. They may exhibit pro- or anti-tumor properties, depending on the stimuli present on the tumor microenvironment[75]. Moreover, the ECM composition (collagen type I, collagen type IV, fibronectin, laminin), physical properties (rigidity, porosity, topography) and biomechanical properties (elasticity) are frequently deregulated in tumors[78].

When the "angiogenic switch" occurs, besides angiogenic sprouting, many others angiogenic processes can take place in the tumor and support tumoral angiogenesis, such as intussusceptive angiogenesis, recruitment of endothelial progenitor cells (EPCs), vessel co-option, vasculogenic mimicry and lymphangiogenesis[79].

Intussusceptive angiogenesis was first observed in postnatal remodeling of capillaries in the lung where in the third week of rat life and during the first 2 years in humans, the volume of

the lungs increases by more than 20 times. This developmental process is characterized by preexisting vessels that split in two new vessels by the formation of a transvascular tissue pillar into the lumen of the vessel. New blood vessels formation by this process is faster because it doesn't need endothelial cell proliferation, they are remodeled into larger and thinner cells. In solid tumor environment, such as colon adenocarcinoma, intussusceptive angiogenesis has been demonstrated to be an important mechanism for tumoral angiogenesis since it is much faster, economical from an energetic and metabolical perspective and has better vascular transpermeability than angiogenic sprouting[80,81].

Recruitment of EPCs is also accepted as a mechanism supporting tumoral angiogenesis. The mobilization of EPCs for tumor growth is promoted by several growth factors, cytokines and chemokines. EPCs detach from the bone marrow niche, move to the vascular zone and will be released in the circulation[82]. Their integration implicates a complex multistep process which involves chemoattraction, active arrest and homing within angiogenic vasculature, transmigration to the interstitial space, incorporation into microvasculature and differentiation into ECs. In studies with breast cancer patients a higher level of EPCs was detected in peripheral blood and was suggested as a prognostic marker in tumor patients[79].

Vessel Co-option is a mechanism by which tumors require blood vessels from the host organism to obtain oxygen and nutrients supply. Evidently, surrounding blood vessels are not only used as a supply mechanism but also as channels for tumor cells migration. Currently, many studies try to identify markers for tumors that are independent of angiogenic sprouting, however it is difficult to distinguish newly formed vessels from mature vessels co-opted by the tumor[83].

Vasculogenic mimicry is a completely different process because it relies on tumoral cells, which differentiate to an endothelial phenotype and make tube-like structures. This mechanism, firstly observed in melanoma, gives the tumor a secondary circulation system of vasculogenic structures formed by tumoral cells[84].

Regarding the aforementioned alternative angiogenic processes, these can be the motive why typical angiogenic therapies – which use drugs that mainly target angiogenic sprouting – do not achieve the expected results[79].

Metastasis

The ability of cancer cells to cross tissue boundaries and to invade neighboring areas is what confers them malignancy.

Metastasis which is considered the major cause of cancer-related deaths, are the end product of a multi-step cell-biological process called metastasis cascade (Figure 5). Its major goal is to disseminate cancer cells to anatomically distant organ sites, concluding the process with their adaptation to foreign tissue microenvironments[85].

The metastatic process includes 7 major steps; it begins with local invasion where cancer cells residing within a well-confined primary tumor invade the ECM of surrounding tissues. Afterwards intravasation occurs, where locally invasive carcinoma cells enter into the lumina of lymphatic or blood vessels. Once cancer cells reach lumina of blood vessels they circulate and disseminate widely through the venous and arterial circulation. In the previous step drives cancer cells to distant organs where they arrest and extravasate initiating intraluminal growth and a microcolony that eventually ruptures the walls of surrounding vessels. Lastly, cancer cells start survival in a foreign microenvironment and form micrometastasis leading to a final metastatic colonization[86].

It has been demonstrated that the number of metastatic colonies arising at distant organs is dependent on the number of tumor cells shed into circulation and, for that reason, the appearance of metastasis is highly correlated with tumoral angiogenic activity. Numerous experiments revealed that decreasing primary tumor vascularity with angiogenic inhibitors is associated with decrease of metastatic colonies[85]. *Weidner et al.* showed a direct correlation between the vascular density and the likelihood of metastasis in human breast cancer patients[87]. However, as already discussed in the VEGF family sub-chapter, the success of the anti-VEGF therapy in metastasis development remain debated.

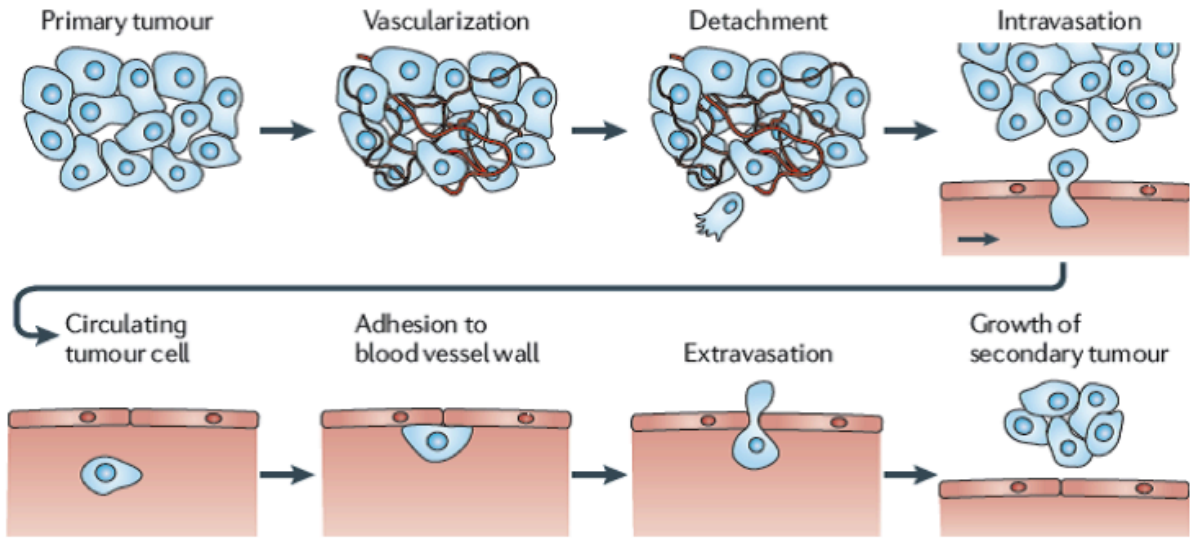

Figure 5. Metastatic Process. In this multi-step process, cells detach from a primary and vascularized tumor. Penetrate the surrounding tissue, enter nearby blood vessels (intravasation) and circulate in the vascular system. Some of these cells eventually adhere to blood vessels walls and are able to extravasate and migrate into the local tissue, where they can form a secondary tumor. (Adapted from Wirtz et al. The physics of cancer: the role of physical interactions and mechanical forces in metastasis).

Radiotherapy

Radiotherapy (RT) is a widely used local treatment for malignant tumors, which are characterized by uncontrolled growth and the ability of invading adjacent tissues and metastasize[88]. Almost 50% of all cancer patients will experiment radiotherapy at least once during their treatment, although radiotherapy importance as a main treatment is highly dependent on the type of cancer. The main goal of radiotherapy is to maximize the radiation dose to cancer cells, minimizing simultaneously the exposure of the surrounding normal tissues. Radiotherapy uses ionizing radiation with a curative intent or associated with chemotherapy and/or surgery performed before or after RT[89].

A better therapeutic ratio is normally achieved when radiation treatment is fractionated i.e, when IR is delivered to the patient over a period of weeks, rather than a single session. The fractionation schedule is used depending on the treatment goal and the characteristics of the tumor. The standard radiotherapy scheme involves the delivery of 2Gy per fraction per day, over five days a week, during several weeks. However, other schemes make use of dose fraction larger than 2 Gy or smaller than 1.8 – 2 Gy involving respectively hypofractionation or hyperfractionation than the conventional scheme. However, details of the previous procedures depend on national guidelines[90].

Radiotherapy for Rectal Cancer

Although surgery remains the mainstay of rectal treatment, adjuvant treatments, like radio- and chemotherapy improve survival and reduce local recurrence by treating any residual microscopic disease.

Radiation could be delivered pre-operatively (neoadjuvant) since it is considered advantageous since it improves local tumor control by reducing tumor volume and facilitates resection and enhanced sphincter-preservation after the reduction of tumor volume. The radiotherapy regimens currently available for rectal cancer treatment include short or long course pre-operative or long post-operative RT. The short course pre-operative radiotherapy (without chemotherapy) involves 5 Gy during 5 days per fraction per day (hypofractionated schedule)[91]. This type of therapeutic is developed in the northern Europe (mainly in Sweden) and it is generally followed by surgery one week later. The long course pre-operative radiotherapy is administered during 25-28 daily fractions with 1.8-2 Gy. Chemotherapy is frequently delivered concurrently with radiation in order to sensitize tumor to radiation, being thus termed as radiosensitizer chemotherapy. It is developed in the United States and some Europe countries. The surgery is performed 6-10 weeks after treatment. Finally, the long course post-operative treatment involves 1.8-2 Gy during 25-28 daily fractions. Radiosensitizer chemotherapy is also administered and it is known that this type of radiotherapy regimen induces additional morbidity. It is recommended when the risk of loco-regional recurrence is still high[92].

The choice of the radiotherapy regime is mostly based on clinical factors and anatomic imaging. More efforts are required to further individualize the treatment and to discover novel radiosensitizers aiming to improve cure rates and reducing long-term toxicity. However, attention should be paid to the interaction of cancer cells with other cell populations present in the tumors which may contribute to cancer progression and response to therapy[92].

Local recurrence seems to be no longer the main problem in rectal cancer, contrarily to distant metastases, which constitute the main cause of treatment failure.

The irradiated tumor volume

Therapeutic doses of IR induce a wide range of DNA lesions like i) mutations; ii) base damage or loss; iii) cross-linking (DNA-DNA or DNA -protein) and iv) single and double-strand breaks. For a typical therapeutic dose of around 2 Gy/fraction of sparsely ionizing radiation, about 3000 DNA lesions are produced per cell exposed[93]. Although mostly centered in DNA, ionizing radiation-induced damage also affects other macromolecules, namely lipids, resulting in lipid peroxidation and changes in membrane viscosity and dynamics, and proteins, by inducing aminoacids conversions, inter and intra-strand cross linking, cleavage, oxidation and carbonylation[94].

Although water radiolysis-generated free radicals have a very short life span, persistent oxidative stress, which can last for several hours or days after radiation exposure, is likely to be generated by mitochondria[95]. ROS production and progressive damage induce lipid peroxidation and protein inactivation, with major consequences for signal transduction. Generally, sphingomyelinase pathway is associated with apoptosis induction, while other signalling pathways, like those mediated by receptor tyrosine kinase (RTK), such as MAPK and PI3K/AKT or NF-κB, may be involved in cell survival upon irradiation, being frequently associated with cancer cell radioresistance.

Both tumor and normal cells can be affected by therapeutic doses of IR. The rationale for using radiation against cancer cells relied on their high proliferative rate and defects in DNA repair machinery, what makes them more sensitive to radiation-induced DNA damage than normal cells and thus excellent targets for pharmacological modulation.

However, the non-cancer cells such as ECs, fibroblasts and immune cells are also affected by ionizing radiation and it is crucial to understand their response since it could modulate the tumoral microenvironment and contribute to cancer progression upon irradiation.

Regarding the ECs, it is consistently described that ionizing radiation progressively reduces endothelial cell viability and proliferation, in a dose and time-dependent manner, leading to apoptosis and decreasing the formation of in vitro capillary-like structures[96,97]. Moreover, it was shown that growing vascular ECs exhibit a senescence-like phenotype after their exposure to 8 Gy of ionzing radiation[98]. This is accompanied by a significant reduction of cell

cycle progression and DNA replication, as well as suppression of in vitro invasion and migration activities (x5). The radiation-induced endothelial cell apoptosis, which mainly involves the activation of the sphingomyelinase pathway[99], is a key event of both early (inflammatory) and delayed (fibroproliferative) radiation toxicity[100,101].

The radiation-induced vascular lesions mostly affect microvessels (capillaries, sinusoids), leading to capillary rupture or thrombosis, but also has negative consequences for medium-size vessels and arteries[102], with irradiated muscular arteries of radiotherapy patients exhibiting increased thickness[103]. This radiation sensitive phenotype of ECs is particular relevant for normal tissue toxicity in dose hypofractionation schemes, as they involve the delivery of higher single doses per fraction[99].

It is generally assumed that tumor progression towards metastasis during or after radiotherapy is due to the appearance of resistant tumor cells through a combination of therapy-induced genetic instability, mutations and subsequent clonal selection of the most fitted cell. However, it was shown that radiotherapy also rapidly alters the tumor microenvironment and that anti-angiogenic approaches can enhance IR-induced tumor growth inhibition[20,104-106]. This data is not contradictory with the concept that the anti-proliferative and cytotoxic effects of radiation on ECs contribute in anti-tumoral treatment, as previous reported[100], but suggest that at certain doses and time frames, IR enhances the build of new vessels, supporting invasion and metastasis.

The concept that IR itself induces the production of pro-angiogenic molecules by the tumors, such as TGFb, FGF, IL-1Ra, IL-10, IL-3, L-4 and IL-5[107], that may activate the microenvironment, including the vasculature, brings the need to new approaches in order to avoid tumor re-growth and metastasis enhancement after radiotherapy. Clinically, while adjuvant radiotherapy significantly improves local tumor control, recurrences within a pre-irradiated field are associated with higher risk of local invasion and metastasis and poor prognosis when compared to recurrences outside the irradiated area[108-110]. Since angiogenesis is crucial for tumor re-growth and metastasis and since IR may stimulate as well as inhibit angiogenesis, many works have been developed to investigate the effects of the therapeutic doses of IR in the tumoral area and to prevent the putative pro-metastatic effect of radiotherapy.

The irradiated peri-tumoral tissue

Many works have been developed to investigate the effects of the therapeutic doses of IR in the tumoral volume and to prevent the putative pro-metastatic effect of radiotherapy. However, we must take into consideration that, during the radiation treatment, not only the tumoral area is exposed to a fractionated IR dose, but also the tissues surrounding the tumoral area, are exposed to doses lower than the tumor area dose. The molecular and biological effects of these LDIR on the healthy tissue surrounding the tumor area, and in particular on the vasculature, remain to be determined.

Recently, it was shown that sub-therapeutic doses of IR, lower than 0.8 Gy, present in the vicinity of the tumor volume target, enhance angiogenesis by activating the receptor 2 of VEGF in ECs[88]. In both Zebrafish and mice models, it was demonstrated that low-dose IR accelerates embryonic and adult angiogenesis[88]. This is strongly supported by the data obtained in a microarray study where several pro-angiogenic targets in ECs showed an increased fold change upon low-dose IR delivery (unpublished data from S. Constantino's lab- data not shown). Moreover, it was found that in a tumoral context these LDIR promote tumor growth and metastasis in a VEGFR dependent manner[88]. The data obtained in mice models were not validated in human so far.

Moreover, it was found that in contrast to inflammatory responses resulting from exposures to high doses, doses lower than 1 Gy decrease leukocyte adhesion to ECs *via* decreased liberation of E-selectin[111] and stimulated release of transforming growth factor-β (TGFβ)[112]. One study has also shown that whole body doses lower than 0.5 Gy actually decreased the number and size of atherosclerotic lesions in hypercholesterolaemic mice[113].

Aim of Study

Aim of Study

Our lab demonstrated that LDIR enhance angiogenesis and consequently promote tumor growth and metastasis[88]. That work was focused, in an innovative way, in the vasculature that surrounds the tumor and is exposed to LDIR. The findings are relevant in a tumoral context, since the vascular activation in these areas may contribute to tumour re-growth and metastasis after radiotherapy.

In this work we are interested in validating these findings in a human model. With this objective, material from patients with rectal cancer that received neoadjuvant radiotherapy was used. A strong network between bench researchers, physicists and clinicians was crucial to ensure a clinical protocol. In rectal cancer, radiosensitizer substances must be administrated simultaneously to potentiate the radiotherapy at the target tissue. According to previous results[88], the radiosensitizer substances do not inhibit the effect of LDIR in promoting angiogenesis and therefore we do not expect that it will interfere with the interpretation of our results.

From each patient, two distinct biopsies of parietal peritoneum were surgically removed:

1. An irradiated specimen exposed to doses from 5 to 30% of the therapeutic dose (100%), located in peritumoral tissue.

2. A non-irradiated specimen that will be used as an internal calibrator for each patient (paired control sample).

Both samples were analyzed with two different goals: i) to evaluate the activation state of ECs and ii) to measure the microvessel density.

In order to achieve our first goal, peritoneal biopsies were snap frozen, sectioned and immunostained for CD31 (EC marker) and a Laser Capture Microdissection (LCM) microscope was used to isolate these cells, followed by RNA extraction, cDNA synthesis and quantitative RT-PCR analysis. The level of activation of several pro-angiogenic targets (such as *VEGFR1*, *VEGFR2*, *ANG2*, *TGFB2*, *vWF*, *FGF2*, *HGF*, and *PDGF*) was analysed.

In order to accomplish the second goal of the work, immunohistochemistry for vWF was performed and microvessel density measured.

Material and Methods

Material and Methods

Patients

The samples used in this work were collected from patients with rectal cancer whom received neoadjuvant radiotherapy. Patients between 35 and 65 years old with a locally advanced rectal cancer in stage T2N+, T3N+, and T4bN1 without metastasis that received a cumulative dose of 50.4Gy participated in the present study with written informed consent (Summarized in Table 1).

Table 1. Summary of inclusion criteria of patients for the study.

Pathological Diagnosis	Cumulative Dose	Weeks between end of Radiotherapy and Surgery	Position of the patient during RT
Locally Advanced Rectal Cancer	50.4 Gy	8 weeks	Dorsal Decubitus

Patients (n=18) included in this study were mainly men (70,60%) with an average age of 53 years old, the oldest man being 65 years old, and the youngest one 38 years old. Women have an average age of 47 years old and represent 29,40% of the patients, the oldest woman being 59 years old, and the youngest one 40 years old. The men's group has patients in 3 stages of tumor whereas women only have patients in 2 stages of tumor. Overall, the most common tumor stage is T3, which represents a large tumor in a scale from T0 to T4 (Summarized in Table 2).

Table 2. General demographic characteristics and tumor staging of patients included in this study.

	Men	Women	Total
Gender	70,6%	29,4%	100%
Average Age (years)	52 (38 - 65)	47 (40 - 59)	51 (38 - 65)
Tumor Stage			
T2	N+ 1	N0 1	N+ 1
			N0 1
T2/3	-	N1 1	N1 1
T3	N+ 6	N+ 2	N+ 8
	N1 4	N1 1	N1 5
T4	N1 1	-	N1 1

This study was approved by the Ethics Committee of the Hospital de Santa Maria.

Radiotherapy

Radiotherapy was performed using an accelerator to produce an x-ray photon beam, operating at a dose rate of 300 MU/min. The treatment plan involved neoadjuvant radiotherapy in 28 fractions of 1.8 Gy, the cumulative dose being 50.4 Gy, in combination with chemotherapy (capecitabine – 5-fluorouracil (FU)). A dosimetric plan was set for each patient (Figure 6).

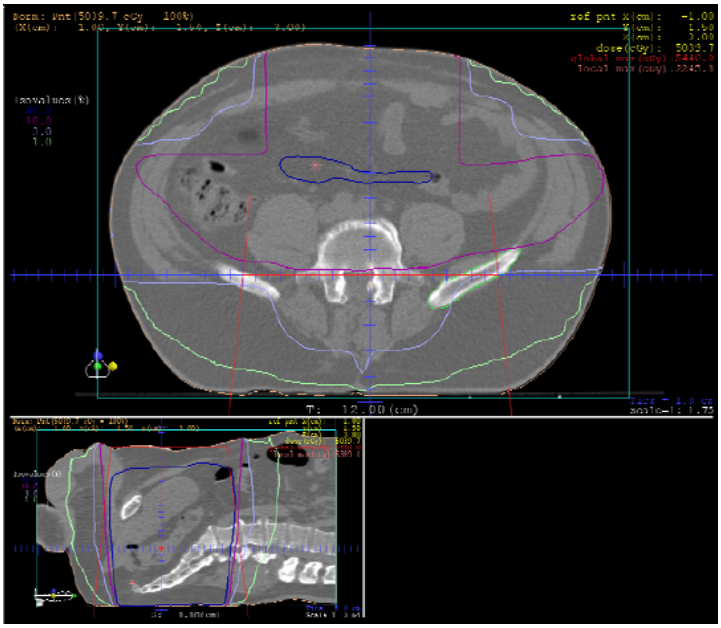

Figure 6. Dosimetric Plan/Isodose curves on a pelvic axial slice. The isodose curves represent the dose of IR delivered to the different tissues during radiotherapy. The blue line delimits the region that will receive therapeutic doses of radiation (including tumor). The dark and light purple lines delimit the region that will be irradiated with 5-30% of the therapeutic dose (low dose). The non-irradiated tissue will be removed outside of the green line.

To achieve a precise resection of both specimens at the moment of surgery, the dosimetric plan was evaluated using an anatomic reference (the right iliac crest) and tattoo marks (done by the clinicians before radiotherapy). Surgery was performed 8 weeks after the end of radiotherapy.

This methodology was performed in a close collaboration with the Department of Radiotherapy and Surgery of the Hospital de Santa Maria.

Clinical Samples

According to the dosimetric plan represented in Figure 6, two biopsies of parietal peritoneum were removed during surgery: unirradiated specimen (NIR) and irradiated specimen with 5-30% of therapeutic dose (IRLD).

Samples were placed in a cryomold, embedded in OCT (cryoprotective embedding medium), quickly frozen in cold isopentane and stored at -80ºC.

Immunohistochemistry for CD31

Using a cryostat, frozen samples were sliced in serial sections of 12µm, mounted in pre-cooled RNAse-free glass microscope slides (Carl Zeiss Microimaging) and stored at -80ºC until usage.

After thawed, samples were washed in ice cold RNAse-free water for 5 minutes, fixed in RNAse-free 70% ethanol for 5 minutes and washed again in ice cold RNAse-free water for another 5 minutes. Then samples were incubated with a primary antibody against CD31 (mouse anti-human; BD BioSciences), diluted 1:1500 in 2M NaCl phosphate buffered saline (PBS) solution, for 45 minutes at 4ºC. Next samples were washed twice with an ice cold 2M NaCl PBS solution and incubated with a secondary antibody (biotinylated anti-mouse; Vector Laboratories), diluted 1:400 in a 2M NaCl PBS solution, for 30 minutes at 4ºC. After this, samples were washed twice again with an ice cold 2M NaCl PBS solution and incubated with an Avidin-Biotin complex (Vectastain® Elite ABC; Vector Laboratories) for 20 minutes at room temperature. Finally, the color development was performed by using diaminobenzidine (DAB+; DAKO). After washing with an ice cold 2M NaCl PBS solution, the

sections were dehydrated by their immersion in increasing concentrations of ethanol (90-100%).

Laser Capture Microdissection

The PALM Microbeam 4.2 microscope (Carl Zeiss MicroImaging) was used to collect the ECs previously labeled with the CD31 antibody 1. After EC's selection, the microscope laser cuts and catapults the cells to a tube with an adhesive cap. For each sample, an area of 1 500 000 μm^2 corresponding to the endothelium was collected.

RNA Extraction

Total RNA was extracted from ECs collected with LCM using the RNeasy® Micro Kit (QIAGEN), following the protocol provided by the manufacturer.

cDNA synthesis and Pre Amplifications

RNA was concentrated with Speed Vacuum prior to cDNA synthesis. Using the RT^2 Nano PreAmp™ cDNA synthesis Kit (SABiosciences, QIAGEN), RNA was reverse transcribed into complementary DNA (cDNA) with the First Strand cDNA synthesis, followed by two rounds of pre-amplifications. cDNA was concentrated with speed vacuum before each round of pre-amplification. Pre-amplifications were performed for the following targets: *VEGFR1*, *VEGFR2*, *ANG2*, *TGFB2*, *VWF*, *FGF2*, *PDGFC*, *HGF* and *r18S*. The sequences of primers used are shown in Table 3.

Table 3. Primers Sequence

Molecular Target	Primers Sequence
VEGFR1	**Fw**: 5'-CCCTCGCCGGAAGTTGTAT-3'
	Rev: 5'-GTCAAATAGCGAGCAGATTTCTCA-3'
VEGFR2	**Fw:** 5'-ATTCCTCCCCCGCATCA-3'
	Rev: 5'-GCTCGTTGGCGCACTCTT-3'
ANG-2	**Fw**: 5'-AGGACACACCACGAATGGCATCTA-3'
	Rev: 5'-TGAATAATTGTCCACCCGCCTCCT-3'
TGFB2	**Fw**: 5'-GCTTTGGATGCGGCCTATTGCTTT-3'
	Rev: 5'-CTCCAGCACAGAAGTTGGCATTGT-3'
vWF	**Fw**: 5'- GTACAGCTTTGCGGGATACT-3'
	Rev: 5'- GCTCACTCTCTTGCCATTCT-3'
FGF-2	**Fw:** 5'- GCAGTGGCTCATGCCTATATT-3'
	Rev: 5'- GGTTTCACCAGGTTGGTCTT-3'
PDGF	**Fw**: 5'- AGGTCTTCAATCGTGGAAAGAA-3'
	Rev: 5'-CAGAACCCAGCTAGTGGAATAC-3'
HGF	**Fw:** 5'- GGTAAAGGACGCAGCTACAA-3'
	Rev: 5'- AGCTGTGTTCGTGTGGTATC-3'
18s	**Fw**: 5' GCCCTATCAACTTTCGATGGTAGT-3'
	Rev: 5'-CCGGAATCGAACCCTGATT-3'

Quantitative Real Time PCR

The mRNA expression of the targets referred above was analyzed by quantitative RT-PCR, which was performed using the Power SYBR® Green system (Invitrogen), following the manufacturer's protocol and an Applied Byosystems® RT-PCR 7500 Fast. The sequences of primers used were the same referred to in table 1. The Real Time PCR run method consisted of one holding stage of 2 minutes at 50ºC and 10 minutes at 95ºC, followed by 50 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. 18S was used as a housekeeping gene to normalize the quantification. The relative quantification was performed according to the comparative method ($2^{-\Delta\Delta C}{}_T$; Applied Biosystems User Bulletin no. 2P/N4303859), with the non-irradiated sample as internal calibrator. The formula used is $2^{-\Delta\Delta C}{}_T = 2^{-[\Delta C}{}_T^{(sample)-\Delta C}{}_T^{(calibrator)]}$, where $\Delta C_T(sample)=C_T(sample) - C_T(reference\ gene)$. For the internal calibrator, $\Delta\Delta C_T=0$ and $2^{-\Delta\Delta C}{}_T = 1$. For the remaining samples, the value of $2^{-\Delta\Delta C}{}_T$ indicates the fold change in gene expression relatively to the calibrator. ΔC_T value for each sample is the average of triplicates.

Immunohistochemistry for vWF

a. From frozen material

Using a cryostat, frozen samples were sliced in sections of 10µm, mounted in slides and stored at -20ºC until usage.

Slides were washed in ultra-pure water for 5 minutes and placed in a 0.2M NaCl PBS solution for another 5 minutes. Next, endogenous peroxidase were blocked with 0.3% H_2O_2 for 30 min in the dark and washed twice with a 0.2M NaCl PBS solution for 5 min. Afterwards, samples were incubated with a primary antibody (MO616) against vWF (mouse anti-human; Dako), diluted 1:200 in a 0.2M NaCl PBS solution/BSA, for 1 hour at room temperature. Next samples were washed three times with a 0.2M NaCl PBS solution and incubated with a secondary antibody (BA-9200) (biotinylated anti-mouse; Vector Laboratories), diluted 1:200 in a 0.2M NaCl PBS solution, for 30 minutes at room temperature. After this, samples were washed three times again with a 0.2M NaCl PBS solution and incubated with an Avidin-Biotin complex (Vectastain® Elite ABC; Vector Laboratories) for 30 minutes at room temperature. Then, samples were washed three times again with a 0.2M NaCl PBS solution. Finally, the color development was performed by using diaminobenzidine (DAB+; DAKO), for 5 minutes at room temperature. After washing with a 0.2M NaCl PBS solution, samples were washed with dH_2O for 3 min and stained with haematoxylin for 10 sec. Next, samples were washed in running water for 5 min and dehydrated in Ethanol (70% - 95% - 100%) for 10sec each. Finally, samples were placed in Xylol for 1 min and mounted with a few drops of mounting media.

b. From paraffin material

Biopsies were defrosted in PBS 1x overnight, included in paraffin, and sliced in sections of 3µm.

For depparafination slides were placed in an adequate rack and placed in deparaffinization xylene1 for 10 min, then placed in deparaffinization xylene2 for 10 min, and finally hydrated in an ethanol battery of 100% Ethanol, 95% Ethanol, and 70% Ethanol for 5 min each. Slides were washed for 5 min in distilled water.

Slides were incubated to 500 ml of Dako Target Retrieval Solution for 20 min near to boiling point. After incubation slides were cooled at room temperature for 15 min, washed with distilled water and placed in a 0.2M NaCl PBS solution till staining.

After epitope retrieval samples were washed in ultra-pure water for 5 minutes and placed in a 0.2M NaCl PBS solution for another 5 minutes. Next, endogenous peroxidase were blocked with 0.3% H_2O_2 for 30 min in the dark and washed twice with a 0.2M NaCl PBS solution for 5 min. Afterwards, samples were incubated with a primary antibody (MO616) against vWF (mouse anti-human; Dako), diluted 1:200 in a 0.2M NaCl PBS solution/BSA, for 1 hour at room temperature. Next samples were washed three times with a 0.2M NaCl PBS solution and incubated with a secondary antibody (BA-9200) (biotinylated anti-mouse; Vector Laboratories), diluted 1:200 in a 0.2M NaCl PBS solution, for 30 minutes at room temperature. After this, samples were washed three times again with a 0.2M NaCl PBS solution and incubated with an Avidin-Biotin complex (Vectastain® Elite ABC; Vector Laboratories) for 30 minutes at room temperature. Then, samples were washed three times again with a 0.2M NaCl PBS solution. Finally, the color development was performed by using diaminobenzidine (DAB+; DAKO), for 5 minutes at room temperature. After washing with a 0.2M NaCl PBS solution, samples were washed with dH_2O for 3 min and stained with haematoxylin for 10 sec. Next, samples were washed in running water for 5 min and dehydrated in Ethanol (70% - 95% - 100%) for 30sec each. Finally, samples were placed in Xylol for 1 min and mounted with a few drops of mounting media.

Microvessel Density Quantification

Analysis of the tissue samples was conducted using NanoZoomer SQ. After acquisition, images were opened with ImageJ software and microvessel density, i.e. number of capillaries (vWF positive cells) per area was determined.

Statistical Analysis

For statistical analysis, data were analyzed using SPPS software (v.20).

Normality was determined for all numeric data by Shapiro-Wilk test.

For *VWF* and *VEGFR2* expression analysis and microvessel density, the Student's T-test for independent samples was used to identify differences between experimental conditions, as data followed a normal distribution.

The Mann-Whitney test for independent samples was used to assess the expression of *VEGFR1*, *ANGPT2*, *TGFB2*, *FGF2*, *PDGFC* and *HGF* as normality could not be assumed. P-Values lower than 0,05 were considered statistically significant. The effect size and power were determined by using the G-Power software.

Results

Results

Low doses of ionizing radiation modulate the peritumoral tissue

In this last year, in order to understand if LDIR modulate the peritumoral tissue, we used material from 7 patients with rectal cancer that received neoadjuvant radiotherapy 8 weeks before tumor resection. There are two aspects that are important to take into account in this Results section: i) from each patient, parietal peritoneum exposed and not exposed to LDIR was used ii) the data obtained in both analysis (activation state of EC or microvessel density) were obtained using the respective non-irradiated sample as an internal calibrator. Since our objective was to evaluate the effect of LDIR in the vasculature, the activation state of ECs exposed to LDIR was evaluated. Therefore, the expression level of several pro-angiogenic factors was evaluated exclusively in ECs. Simultaneously, the microvessel density was also analysed in order to investigate whether tissues exposed to LDIR are significantly more vascularized when compared to non-irradiated ones.

In order to statistically analyze the results, the data obtained from 6 of these 7 patients were evaluated together with the data already obtained in our lab from 12 other patients. From a total of 18 patients analyzed, 2 were excluded from the study since no difference (in the EC activation state and microvessel density) was observed between the material irradiated or not with LDIR. The data obtained for the 16 patients included in the study and for the 2 excluded patients will be presented.

Knowing that age could affect the responsiveness of ECs to an angiogenic stimulus we will present the results obtained in both analyses, for the oldest and younger patients.

Finally, we will present data obtained separately for some patients in order to compare the results obtained both in EC activation and microvessel density.

The EC activation state is modulated by low doses of ionizing radiation

With the objective of analyzing the activation state of ECs, the expression level of several pro-angiogenic factors was analyzed in ECs isolated from parietal peritoneum removed from 7 patients with rectal cancer that received neoadjuvant radiotherapy, 8 weeks before tumor resection. With this objective, immunohistochemistry for CD31 was performed (Figure 7 A)

and ECs were selected using a software tool associated with LCM microscope (Figure 7 B). Finally, ECs were catapulted for a special tube with an adhesive cap.

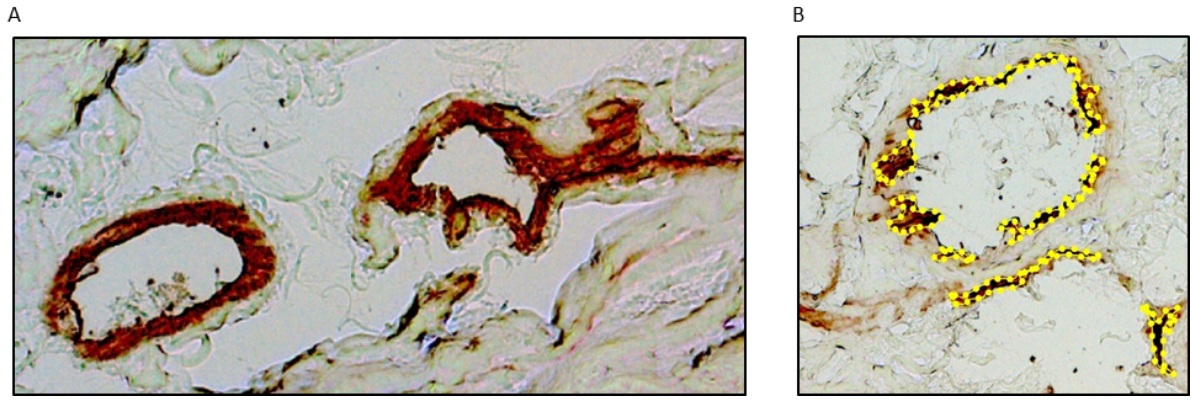

Figure 7. Representative images of ECs selection and isolation. ECs were stained by Immunohistochemistry using an antibody anti-CD31^{+}.(BD BioSciences) Stained ECs present a brownish-red color (A). Colored tissue was selected using a PALM RoboSoftware tool associated with PALM Microbeam 4.2 microscope (Carl Zeiss MicroImaging GmbH) (B). Selected tissues were catapulted to a eppendorf with an adhesive cap (AdhesiveCap®, Carl Zeiss MicroImaging GmbH).

The mRNA was extracted from human ECs exposed to LDIR or non-irradiated followed by cDNA synthesis. Quantitative RT-PCR was performed for the following targets: *VEGFR1*, *VEGFR2*, *ANG2*, *TGFB2*, *VWF*, *FGF2*, *PDGF*C, and *HGF*. Since one of the 7 patients did not present any difference between LDIR and NIR endothelial samples, it was excluded from the study. Note that the same patient does not present microvessel change after LDIR exposure. Figure 8 illustrates the data obtained for the 6 patients analyzed in this last year.

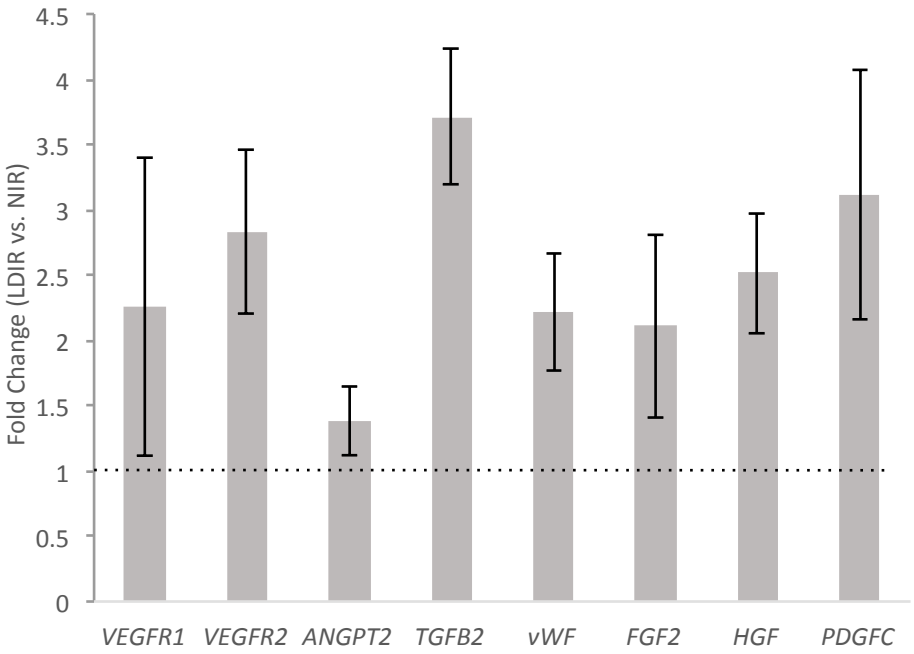

Figure 8. LDIR modulate the expression of pro-angiogenic factors in ECs. The mRNA expression of VEGFR1, VEGFR2, ANGPT2, TGFb2, vWF, FGF2, HGF and PDGF was quantified by RT-PCR in ECs isolated from an unirradiated specimen and a specimen irradiated with LDIR. Values were normalized for 18S to obtain relative expression levels. Results are expressed in fold change between irradiated (LDIR) and unirradiated (NIR) specimens. Each bar represents the relative gene expression for 6 patients. The error bar represents the standard error of the mean.

However, and to confer statistical analysis to our findings, the data obtained for these 6 patients were evaluated together with the data already obtained in our lab for other 10 patients. According to our results, the transcripts for all these genes were significantly up-regulated in ECs isolated from parietal peritoneum exposed to LDIR, comparing with the ECs from the non-irradiated one (Figure 9).

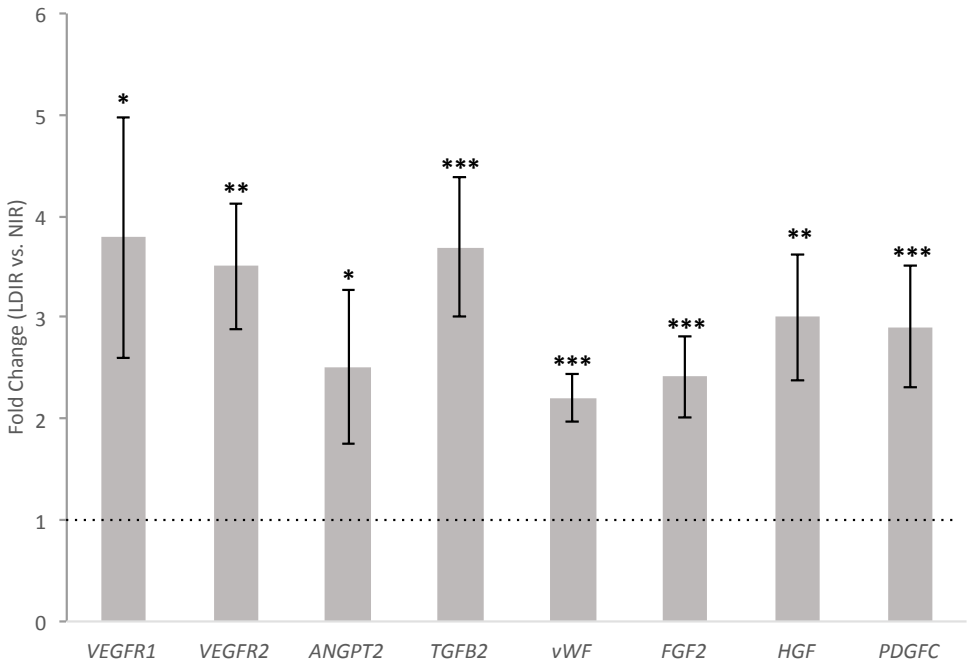

Figure 9. LDIR promote up-regulation of pro-angiogenic factors in ECs. The mRNA expression of VEGFR1, VEGFR2, ANGPT2, TGFb2, vWF, FGF2, HGF and PDGF was quantified by RT-PCR. ECs irradiated or not with LDIR were isolated and the unirradiated specimen were used as an internal calibrator. Values were normalized to 18S to obtain relative expression levels. Results represent the fold change between irradiated (LDIR) and unirradiated (NIR) specimens. Each bar represents the relative gene expression for 16 patients. The error bar represents the standard error of the mean. For fold change Mann-Whitney was performed, as the data does not follow a normal distribution, except for vWF and VEGFR2. For data that follows a normal distribution, Student's T-Test for independent samples was performed. For all targets, Mann-Whitney or Student's T-Test were significant (* p<0,05; ** p<0,01; ** p<0,001).

The microvessel density is modulated by low doses of ionizing radiation

In order to evaluate if the tissues exposed to LDIR are significantly more vascularized when compared to the non-irradiated ones, the same parietal peritoneum biopsies were used. With this objective, an immunohistochemistry for vWF was performed and the number of capillaries was evaluated in both IRLD and NIR samples. Since our biopsies have adipose tissue in their constitution, frozen sections are difficult to obtain and provide only moderate morphology (Figure 10 A). For that reason, it was not possible to analyze 2 from the 16 patients included in the study. In order to obtain best morphologic detail of sections, the material was simultaneously fixed and processed into paraffin (Figure 10 B). The representative images (Figure 10 A and 10 B) show that the objective was achieved and as expected a best morphology is obtained in paraffin sections; however, it is extremely difficult to visualize the capillaries, since the paraffin sections are much thinner (3 μm) than the frozen ones (12 μm). For that reason, we decided not to compromise the quantification

of the capillaries and frozen material was used, even though presenting moderate morphology.

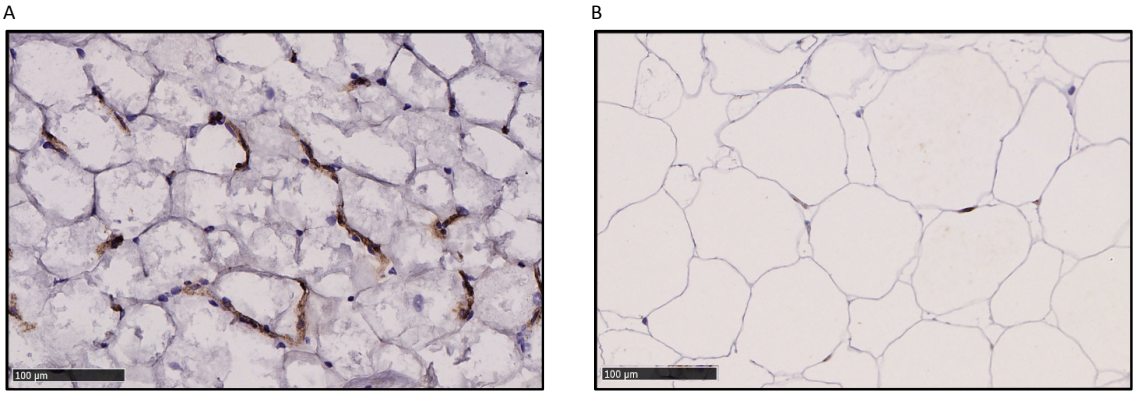

Figure 10. Representative images from human peritumoral tissue sections. Microvessels (brown) and nuclei (light blue) were identified by immunohistochemistry using anti-vWF (Dako) and counterstained with Haematoxylin, respectively. Images are representative of an immunohistochemistry performed in sections from frozen (A) and paraffin-embedded tissue (B).

According to our results, a significant increase in vascular density is obtained for the parietal peritoneum exposed to LDIR when compared to the non-irradiated one (Figure 11).

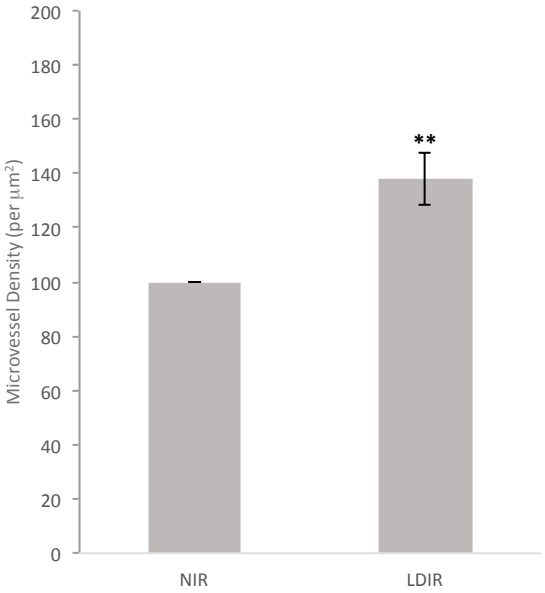

Figure 11. LDIR significantly increase microvessel density in peritumoral tissue. Microvessel density (number of microvessels/μm^2) was assessed by immunohistochemistry, using an antibody anti-vWF, for 14 patients. Results are shown in percentage of microvessel density per μm^2. For microvessel density, Student's T-Test for independent samples was performed, as the data follow a normal distribution. ** $p<0,01$.

Two excluded patients: no modulation by low doses of ionizing radiation

From a total of 18 patients, 2 patients 46 and 50 years old did not present EC activation state nor microvessel density from LDIR modulation. For that reason, they were excluded from the study (Figure 12).

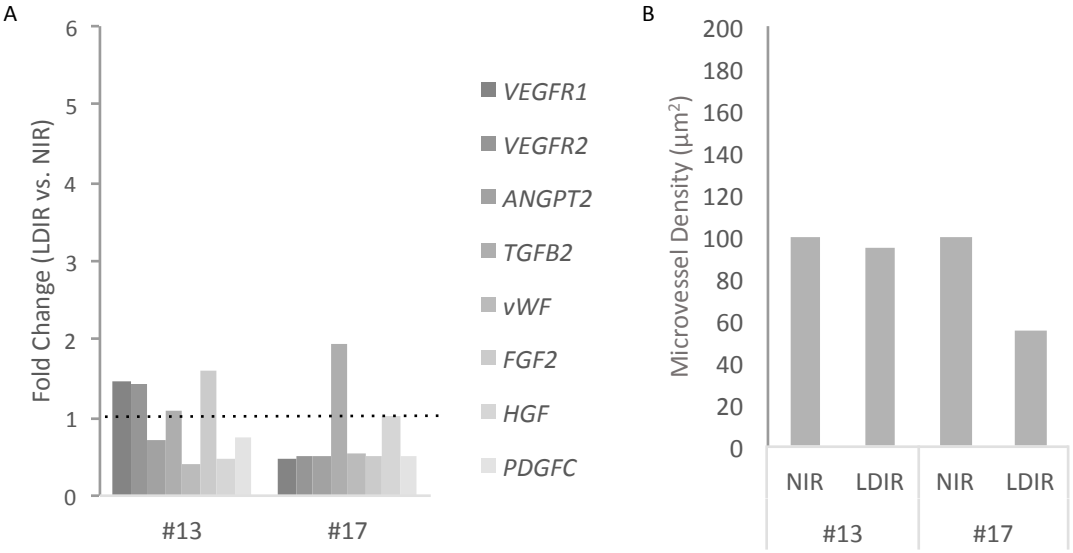

Figure 12. Two patients were excluded due to lack of response in endothelial activation and microvessel density. The mRNA expression of VEGFR1, VEGFR2, ANGPT2, TGFb2, vWF, FGF2, HGF and PDGF was quantified by RT-PCR in ECs isolated from an unirradiated specimen and a specimen irradiated with LDIR. Values were normalized to 18S to obtain relative expression levels. ECs expression levels (A) are expressed in fold change between irradiated (LDIR) and unirradiated (NIR) specimens, for patients 13 and 17 and,microvessel density (number of capillaries/µm^2) (B) was assessed by immunohistochemistry, using an antibody anti-vWF).

Low doses of ionizing radiation modulate the vascular response even in the oldest patients of the study

Knowing that age could reduce the responsiveness of ECs to an angiogenic stimulus and since it is our goal to evaluate if LDIR enhance an angiogenic response in human, patients more than 65 years old were excluded from this study.

However, we decided to evaluate separately the data obtained for the 60 years old or more patients in order to compare their response with younger patients and evaluate if the oldest patients are the ones that present a weakest response.

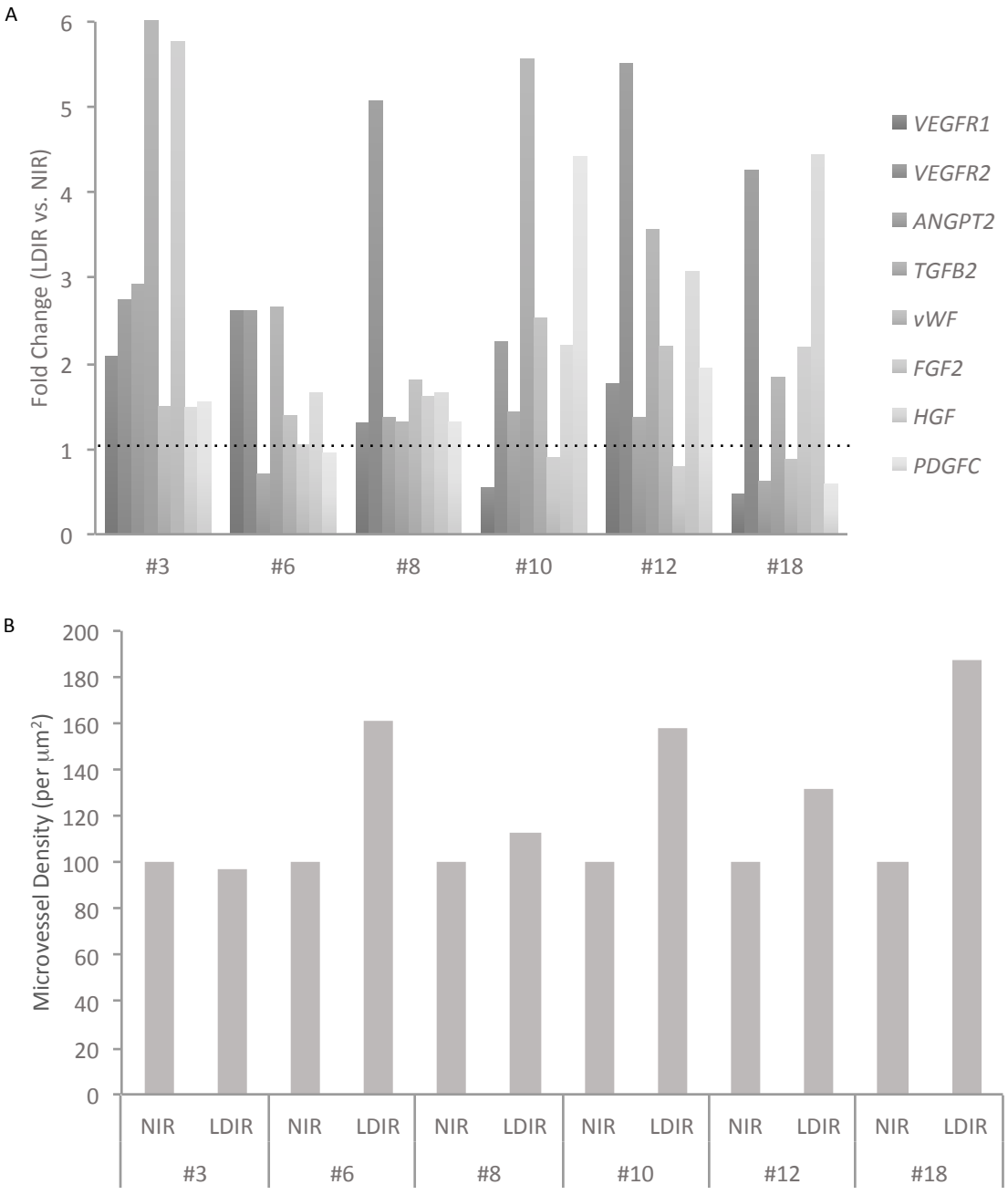

Figure 13. LDIR modulate vascular response in the oldest patients. The mRNA expression of VEGFR1, VEGFR2, ANGPT2, TGFb2, vWF, FGF2, HGF and PDGF was quantified by RT-PCR. ECs from specimens irradiated with LDIR and from unirradiated specimens were isolated by laser microdissection. Values were normalized to 18S to obtain relative expression levels. Results represent the fold change between irradiated (LDIR) and unirradiated (NIR) specimens, for the oldest patients (A). Microvessel density (number of capillaries/μm^2) was assessed by immunohistochemistry, using an antibody anti-vWF, for the oldest patients (B). Patients 3, 6, 8, 10, 12 and 18, are between 60 and 65 years old.

Interestingly, the 6 patients with ages between 60 and 65 years old present high levels of expression in ECs for at least 4 or 5 pro-angiogenic factors, simultaneously, after LDIR exposure (Figure 13 A) and the microvessel density is also augmented by LDIR in 5 from the 6 patients (Figure 13 B). Any of these older patients has a best response to LDIR than

patients with ages between 46 and 50 years old, younger but excluded from the study due to their absence of response (Figure 12).

EC activation state versus microvessel density

In order to understand if i) the patients that present an EC activation state after LDIR exposure also present an increase of microvessel density; ii) the patients that present the higher EC activation state are the same that present the higher microvessel density increase in response to LDIR or iii) there are patients that only present EC activation or microvessel density; we decided to evaluate the patients separately. According to our results we found that there are representative patients for almost all the hypothesis that were made. There are patients that present ECs with high levels of expression of several (4/8) proangiogenic targets and also higher levels of microvessel density after LDIR exposure (Figure 14 A and B). However, there are 2 patients that present the highest expression levels for all the pro-angiogenic targets without an increase of microvessel density (Figure 14 C and D). Finally, it is also interesting to observe that the patient that presents the highest increase in microvessel density in response to LDIR does not have any expression level change for any of the pro-angiogenic factors analyzed (Figure 14 E and F).

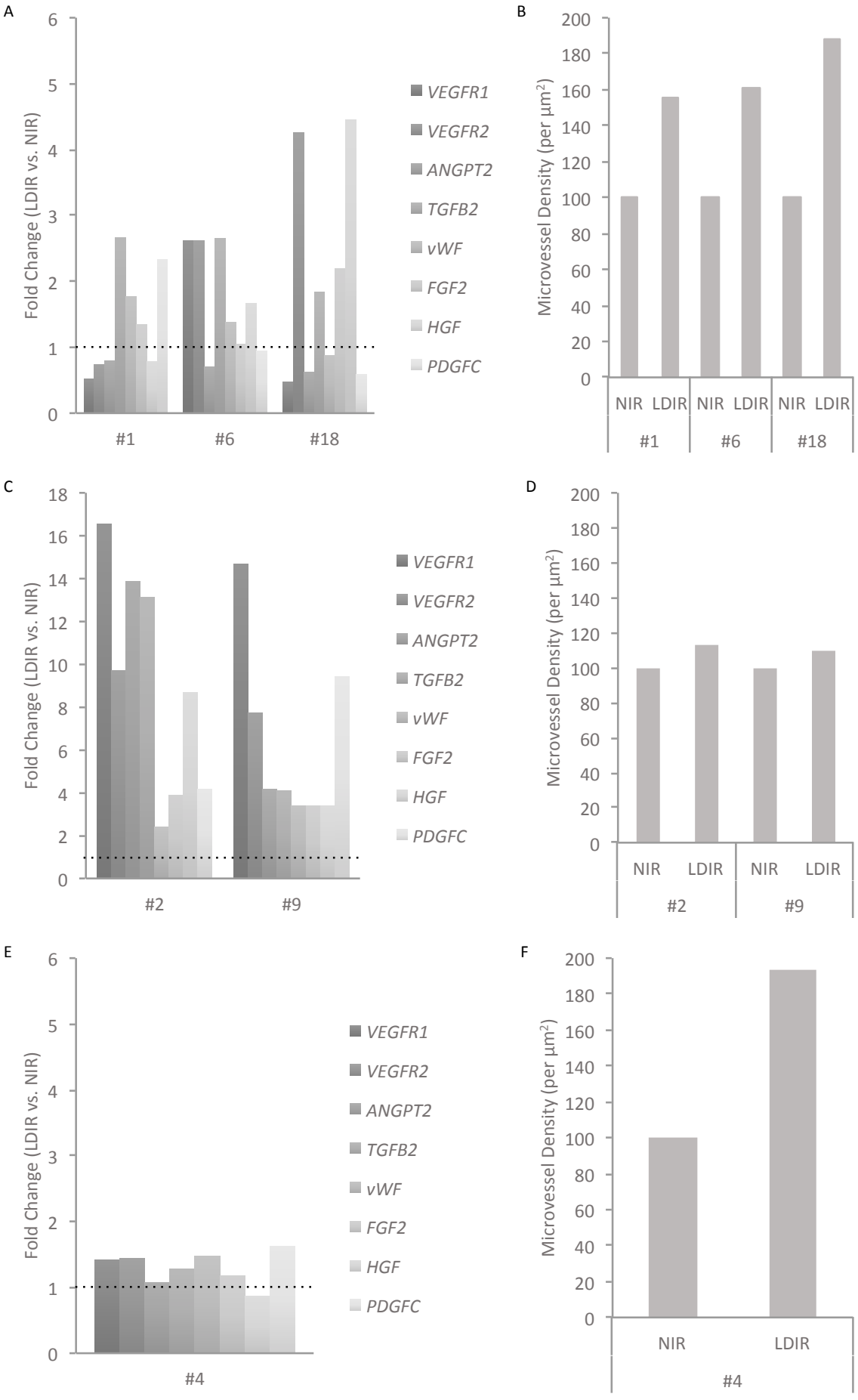
A
Fold Change (LDIR vs. NIR)
0
1
2
3
4
5
6
#1
#6
#18
VEGFR1
VEGFR2
ANGPT2
TGFB2
vWF
FGF2
HGF
PDGFC
B
Microvessel Density (per µm2)
0
20
40
60
80
100
120
140
160
180
200
NIR LDIR NIR LDIR NIR LDIR
#1
#6
#18
C
Fold Change (LDIR vs. NIR)
0
2
4
6
8
10
12
14
16
18
#2
#9
VEGFR1
VEGFR2
ANGPT2
TGFB2
vWF
FGF2
HGF
PDGFC
D
Microvessel Density (per µm2)
0
20
40
60
80
100
120
140
160
180
200
NIR LDIR NIR LDIR
#2
#9
E
Fold Change (LDIR vs. NIR)
0
1
2
3
4
5
6
#4
VEGFR1
VEGFR2
ANGPT2
TGFB2
vWF
FGF2
HGF
PDGFC
F
Microvessel Density (per µm2)
0
20
40
60
80
100
120
140
160
180
200
NIR LDIR
#4

Figure 14. ECs activation state vs. Microvessel Density. The mRNA expression of VEGFR1, VEGFR2, ANGPT2, TGFb2, vWF, FGF2, HGF and PDGF was quantified by RT-PCR in ECs isolated from an unirradiated specimen and a specimen irradiated with LDIR. Values were normalized to 18S to obtain relative expression levels. Results are expressed in fold change between irradiated (LDIR) and unirradiated (NIR) specimens. Microvessel density (number of capillaries/μm^2) was assessed by immunohistochemistry, using an antibody anti-vWF. Patients 1, 6 and 18 present both high levels of expression of several pro-angiogenic targets and microvessel density (A and B). Patients 2 and 9 present high levels of expression for all pro-angiogenic targets without increase of microvessel density (C and D). Patient 4 present the highest increase in microvessel density in response to LDIR without any expression level change for any of the pro-angiogenic factors (E and F).

Discussion and Conclusion

Discussion and Conclusion

Angiogenesis is defined as the growth of new blood vessels from a pre-existing vascular network, a fundamental process during embryonic development, wound healing and for normal homeostasis in adulthood. It is through the vascular network that the nutrients and oxygen are delivered to tissues. Under physiological conditions, this process is tightly regulated by a fine-tuned balance between pro- and anti-angiogenic factors However, pathological conditions as cancer can disrupt this balance leading to an "angiogenic switch".[1] Pro-angiogenic factors become upregulated inducing new blood vessels in the context of a tumoral angiogenesis, favoring oxygen and nutrient supply for the tumor, which enhance tumor growth and metastasis.

Radiotherapy uses ionizing radiation to damage and destroy tumor cells. However, clinical and experimental observations indicate that ionizing radiation might promote a metastatic behavior of cancer cells and that the irradiated host microenvironment might exert tumor-promoting effects. Therefore, a careful analysis of the putative tumor-promoting and pro-metastatic effect of ionizing radiation is imperative, as radiotherapy is an essential part of cancer treatment. Different studies show the mechanisms by which ionizing radiation activates cellular targets potentially contributing to invasion and metastasis [107,114-116]. Doses of ionizing radiation causing such stimulating effects are classically delivered inside the tumor target volume in daily small fractions, in order to limit the damage to healthy tissues and until a potentially curative dose has accumulated inside the tumor volume. Furthermore, the delivery in small fractions and the isodose distribution of external beam radiotherapy result in even lower doses of ionizing radiation outside the tumor target volume. The molecular and biological effects of these LDIR in the peritumoral area remain to be determined.

Sofia Vala et al. showed that *in vitro* LDIR activate the receptor 2 of VEGF on ECs and induces migration. *In vivo,* LDIR accelerate embryonic angiogenic sprouting during zebrafish development and promote adult angiogenesis during zebrafish fin regeneration[88]. Additionally, using mice models of leukemia and orthotopic breast cancer, it was demonstrated that LDIR increase tumor growth and induce metastasis[88] and these effects

were prevented by the administration of a VEGF receptor tyrosine kinase inhibitor immediately before ionizing radiation exposure.

Taking the aforementioned findings into account, the goal of this study was essentially to validate these data in humans. With this objective, samples from patients with rectal cancer that received neoadjuvant radiotherapy were used. These patients performed radiotherapy and a dosimetric plan was done for each patient before starting treatment, allowing the identification/localization of the tissues exposed to 5-30% of the therapeutic dose as well as the localization of non-irradiated tissues. However, there is a great deal of uncertainty in accurately defining the position of targets during the delivery of fractionated radiotherapy, both during a given fraction and between successive fractions. Targets may move during treatment due to respiratory or peristaltic movements and for that reason, parietal peritoneum was selected as the tissue to be removed, since this effect is expected to be minimized here. In a straight collaboration with the radiotherapy and the surgery services of CHLN, patients 35 to 65 years old were recruited to participate in this study. The distinct parietal peritoneum resection specimens were collected during resection surgery and frozen: i) a specimen exposed to doses from 5 to 30% of the therapeutic dose (100%), located in the vicinity of the tumor area and ii) an unirradiated specimen that will be used as an internal calibrator for each patient. This process of calibration is fundamental to compare patients with different aging, secondary diseases, and genetic and environmental background. Using LCM, ECs were isolated followed by RNA extraction procedure in order to perform quantitative RT-PCR analysis. Problematically, RNA is not stable under the labeling conditions usually needed to identify the cells of interest for microdissection. Recently a new immunolabeling method was described where RNA is preserved, and this preservation is compatible with standard microdissection procedures[117]. The same procedure[117], using a high salt buffer to stabilize RNA during prolonged antibody incubations in CD31 imunolabeling was used. To minimize RNase activity, we used AdhesiveCap from LCM.

Analysing a total of 18 patients, it was decided to exclude 2 patients since no response was detected for both EC activation state and microvessel density. We may hypothesize that there are some patients whose tissues do not respond to LDIR, even if they constitute a minor percentage in our study. We can also propose to use a radio-opaque marker in the future to optimize the localization and removal of the selected irradiated tissues.

Interestingly, in the other 16 patients a significant increase of EC activation state was observed after LDIR exposure by measuring the simultaneous expression level of several pro-angiogenic genes (*VEGFR1*, *VEGFR2*, *ANG2*, *TGFB2*, *VWF*, *FGF2*, *PDGFC*, and *HGF)*. The levels of expression in ECs exposed to LDIR were compared with those obtained from ECs removed from human non-irradiated tissues. The eight molecular targets that were included in our study, were selected from a global gene expression performed in HUVEC cells upon LDIR exposure. In this procedure, we did not differentiate between vascular and lymphatic ECs.

We also investigated whether tissues exposed to LDIR are significantly more vascularized when compared to non-irradiated ones. As described, an increase of microvessel density is significantly correlated with several prognostic factors, including lymph node metastasis[118]. There are several immunohistochemical markers that can identify ECs including antibodies that recognize epitopes on CD31 and vWF. Moreover, it was suggested that the anti-CD31 antibody stained the small vessels with immature endothelium; vWF antibody was shown to stain mainly the large to medium-size vessels and it was the antibody that we selected in our analysis[119]. With this objective, immunohistochemistry for vWF was conducted from frozen material since the thickness is greater when compared to paraffin embedded material and does not compromise the staining; even although the morphology in paraffin slides is better when compared to frozen ones. From 16 patients, the analysis was performed only in 14 due to technical problems. Interestingly, we found a significant increase in microvessel density in tissues exposed to LDIR when compared to non-irradiated ones. These results can be corroborated in the future by using the anti-CD31 antibody. Moreover, we can use a lymphatic marker in order to distinguish vascular from lymphatic vessels.

In this study patients older than 65 years were excluded since there is evidence that angiogenesis is reduced with age and consequently aging could interfere with the modulation of the angiogenic balance conferred by LDIR. It was described that VEGF-induced angiogenesis in aged rats[120] and rabbits[121] is attenuated and even angiogenesis-dependent tumor growth is retarded with age[122]. Several reports have demonstrated that wound healing is delayed in aged subjects and this is to some extent attributed to an impaired angiogenic process[123,124].

Even so, we confirmed that LDIR modulate the vascular response even in the oldest patients included in this study. Accordingly, 6 patients with 60 years or more years of age present a best response to LDIR (measured by EC activation state or microvessel density) than patients with ages between 46 and 50 years, younger but excluded from the study due to their absence of response.

By evaluating the patients separately, we also verified that there are patients that: i) present ECs with high levels of expression of several proangiogenic targets and also higher levels of microvessel density after LDIR exposure; ii) present the highest expression levels for all the pro-angiogenic targets without an increase of microvessel density; iii) present the highest increase in microvessel density in response to LDIR but do not have any expression level change for any of the pro-angiogenic factors analyzed. These findings are very curious and let us hypothesize that the effect of LDIR could be dynamic and temporally different in different patients. We would like to draw your attention to the fact that all the measurements were performed in material removed 8 weeks after the end of radiotherapy.

According to the clinical guidelines, the surgery in our hospital is done at the 8^{th} week preventing us from obtaining biopsies sooner. We consider this a limitative aspect since it is important to investigate if the expression of molecular targets could be modulated by the time interval between the end of the radiotherapy treatment and surgery. Therefore, we may hypothesize that an increase of microvessel density is achieved when i) active ECs are present in the peritumoral tissue or ii) active ECs were present in the peritumoral tissue. The difference could be that in some patients the ECs are still active after 8 weeks of the end of radiotherapy and in other patients the ECs may be active before and 8 weeks after the end of radiotherapy their activation level could have been restored. We can also hypothesize that ECs are active but the increase in microvessel density has not yet occurred. To address this question, the use of an experimental model could be useful to understand how the expression of these targets is modulated overtime after exposure to LDIR.

This truly translational study will surely provide for novel insights into the cellular effects of LDIR and be of use in the improvement of the current radiation oncology protocols.

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