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Role of VEGFC C-terminal fragment in colorectal cancer with metastatic capacity.

Tesis Doctoral para optar al grado de Doctora, presentada por:

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ABBREVIATIONS

A498= Human renal cell carcinoma

aa= amino acids

ACF= Aberrant cript foci

ADMTS3= A disintegrin and metalloproteinase with thrombospondin motifs 3

AJCC =American Joint Committee on Cancer

AKT= Serine/Threonine Kinase

AMPK= AMP-activated protein kinase

ANG = Angiogenin

Ang= angiopoietins

APAF1= Apoptotic protease-activating factor 1

APC= polyposis coli genes

APLN= Apelin

APLNR = G protein-coupled receptor APJ

ASCL1 = Achaete-scute homolog 1

BAX = Bcl-2 Associated X

Bex4 = Brain Expressed X-Linked 4

BLM=Bloom syndrome protein

BM =bone marrow

BMP=Bone Morphogenetic Proteins called

BMPR1=Bone Morphogenetic Protein Receptor Type 1A

BRAF = v-Raf murine sarcoma viral oncogene homolog B

C- terminal= COOH- terminus

C12orf50 = Chromosome 12 open reading frame 50

C14ORF6 =Ribonuclease, RNase A family, 11 (non-active);

CAF = Cancer-associated fibroblast

CASP 9 = Caspase-9

CASP3= Caspase-3

CASP7= Caspase-7

CatD= cathepsin D

CCBE1= collagen and calcium binding EGF domains 1

CCL11= Eotaxin

CD300A=CMRF35-like molecule 8

CD31= platelet endothelial cell adhesion molecule-1

CDC4 = cell division control protein 4

CIMP= CpG island methylator phenotype

CIN= chromosomal instability

COAD =colon adenocarcinoma

COX2= cyclooxygenase 2

CRC= Colorectal cancer

CSC=cancer steam cells

CT26 = Mouse colorectal adenocarcinoma

CTC= circulating tumour cells

D3= VEGFR-1 immunoglobulin homology domain 3

DALY =disability-adjusted life-year

DC = Dendritic cells

DEFA4 = Neutrophil defensin 4

DIABLO = diablo

DMEM = Dulbecco's Modified Eagle Medium

DMH = D minor homology

DNA= Deoxyribonucleic Acid

Ear1 =Eosinophil-associated, ribonuclease a family, member 1

Ear10 =Eosinophil-associated ribonuclease 10

Ear2 =Eosinophil cationic protein 2

Ear6= Eosinophil-associated, ribonuclease A family, member 6

EC= Endothelial cells

ECGM MV2= Endothelial Cell Growth Medium MV2

ECM = extracellular matrix

ECM= endothelial cell medium

EGF= Epidermal Growth Factor

EGF= epidermal growth factor

EGFR = Epidermal Growth Factor Receptor

EGFR=epidermal growth factor receptor

ELANE =Elastase

EMEM= Eagle´s Minimum Essential Medium

eNOS = endothelial nitric oxide synthase

ENSP00000476537=Ribonuclease A family member 11

EPX = Eosinophil peroxidase

Epx =Eosinophil peroxidase

ERK = extracellular signal-regulated kinase

FAK =focal adhesion kinas

FBS = Fetal bovine serum

FCER1A =High affinity immunoglobulin epsilon receptor subunit alpha

GF= growth factor

Gpx =glutathione peroxidase

Grb2 =Growth factor receptor-bound protein 2

HBD= Heparin binding domain

HDI= Human development Index

HEK 293 = Human embryonic kidney

HGF= Hepatocyte Growth Factor

HRAS1=harvey rat sarcoma viral oncogene

HS = Heparin Surfate

HSPG= heparan sulfate proteoglycans

HT29= Homo sapiens colorectal adenocarcinoma

HTRA2 = Serine protease HTRA2

IgD =immunoglobulin-like domains

IGF= insulin-like growth factor

IGFR1=insulin-like growth factor receptor 1

IL= Interleukins

IL5 =Interleukin-5

ISM2=Isthmin-2

ITG = integrin

KDR = kinase insert domain containing receptor

KLK3= kallikrein-3

KRAS = Kirsten rat sarcoma 2 viral oncogene homolog

LEC= Human Lymphatic Endothelial Cells

L-glu= L-glutamine

LKB1= liver kinase B1

LOH = loss of heterozygosity

LSEC = Liver sinusoidal endothelial cell

Ly6g6c =Lymphocyte Antigen 6 Family Member G6C

LY6H = Lymphocyte Antigen 6 Family Member H

Ly6i =Lymphocyte antigen 6I

LYVE-1= Lymphatic vessel endothelial hyaluronan receptor

Lyz2 =Lysozyme C-2

MAPK = mitogen-activated protein kinase

MC38= C57BL6 Murine Colon adenocarcinoma

MED28 =Mediator of RNA polymerase II transcription subunit 28

MEK= Mitogen-Activated Protein Kinase Kinase 1

MET= Mesenchymal Epithelial Transition

MMP= metalloprotease

MMR = mismatch repair

MPO =Myeloperoxidase

MPPED1= Metallophosphoesterase domain-containing protein 1

MPPED2= Metallophosphoesterase domain-containing protein 2

Ms4a3 =Membrane-spanning 4-domains subfamily A member 3

MSI= microsatellite instability

mTOR= mammalian target of rapamycin

MYO9B= Unconventional Myosin-IXb

NF-κB =nuclear factor kappa light chain enhancer of activated B cells

NK = Natural killer cells

NO =nitric oxide

NR2F2=Nuclear Receptor Subfamily 2 Group F Member 2

NR2F6 =Nuclear Receptor Subfamily 2 Group F Member

NRP-1=neuropilin-1

NSAIDs =Nonsteroidal anti-inflammatory drugs

N-terminal = NH_2 terminus

Ntrk1= High affinity nerve growth factor receptor

O.C.T= Optimal cutting temperature

P/S= penicillin/streptomycin solution

PBS= Phosphate buffer saline

PC= proprotein convertases

PDGF= platelet derived growth factor

PDPN =podoplanin

PFA= Paraformaldehyde

PHOX2A= Paired mesoderm homeobox protein 2A

PHOX2B= Paired mesoderm homeobox protein 2B

PI= Propidium Iodide

PI3K = Phosphoinositide 3-kinases

PlC-γ= Phospholipase C-γ

PPARγ =Peroxisome proliferator-activated receptor gamma

Prdxs =peroxiredoxins

PRG2 = Bone marrow proteoglycan

PRG2 =Bone marrow proteoglycan

prox-1 =Prospero homeobox 1 protein

PSA = prostate-specific antigen

PTN= pleiotrophin

R.T= room temperature

RAF= rapidly accelerated fibrosarcoma

RASD1= Dexamethasone-induced Ras-related protein 1

READ= rectum adenocarcinoma

RIPK2= Receptor-interacting serine/threonine-protein kinase 2,

RNA seq= RNA sequencing

RNA= Ribonucleic acid

RNASE2 = Ribonuclease A Family Member 2

RNASE2= Ribonuclease a family member 2

RNASE3 = Ribonuclease A Family Member 3

RNASE3 = Ribonuclease a family member 3

RNH1= Ribonuclease/angiogenin inhibitor 1

RPMI 1640= Roswell Park Memorial Institute 1640 Medium

scRNA-Seq = Single-cell RNA sequencing

SEPT4= Septin-4 .

Shc1=SHC-transforming protein 1

SHD= silk homology domain

SMAD4= Mothers against decapentaplegic homolog 4

Snord 116= Small Nucleolar RNA, C/D Box 116-1

SOD =superoxide dismutase

SPT1 =Serine Palmitoyltransferase Long Chain Base Subunit 1

SPTLC1= Serine Palmitoyltransferase Long Chain Base Subunit 1

SRC= sarcoma oncogene/tyrosine kinase

STRING = Search Tool for the Retrieval of Interacting Genes/Proteins

sVEGFR = soluble vascular endothelial growth factor receptor

SW480 = Primary human colon adenocarcinoma derivate cells

TAB1= TGF-beta-activated kinase 1;

TAM = Tumor-associated macrophages

TAN = tumor-associated neutrophils

TCGA= The Cancer Genome Atlas

TEK= Endothelial-specific receptor tyrosine kinase

TFG = Trafficking From endoplasmic reticulum to Golgi Regulator

TFG- β= transforming growth factor beta

TGFBR2= Transforming Growth Factor Beta Receptor 2 (TGFBR2),

TISCH =Tumor Immune Single-cell Hub

TLR2 =Toll-like receptor 2

Tlr2= Toll-like receptor 2

TMA =Tissue MicroArray

TME= tumor microenvironment

TNM = tumor, node, metastasis

Txnrd =thioredoxin reductasef

UICC= International Union against Cancer

VEGF=Vascular endothelial growth factor

VEGFR= Vascular endothelial growth factor receptor

VHD= VEGF homology domain

VNTR =variable number of tandem repeats

WHO = World Health Organization

XAF1= XIAP-associated factor 1;

XIAP =X-linked inhibitor of apoptosis

Y= tyrosine

Zmynd17= Zinc finger MYND domain-containing protein 17

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1.1 SUMMARY

Cancer has become the second leading cause of death worldwide in the 21st century (ref 121). It is the consequence of cell mutations, leading them to grow and divide uncontrollably as well as spread into nearby tissues, and hence developing metastasis. Moreover, cancer is a multi-step process, which can be defined by its cell´s hallmarks (ref 140, ref141, ref 191).

This present work has focused on colorectal cancer (CRC), being the second main cause of cancer death worldwide (ref 1). Genetic risk and lifestyle are the main reasons for CRC development (ref 3,ref 6, ref 10, ref 11). Colorectal cancer can be divided into initiation, promotion, progression and metastasis stages (ref 3, ref 16). Furthermore, the genetic alterations that occurs are encompassed in three main genetic and epigenetic aberrations, which are chromosomal instability (CIN), CpG island methylator phenotype (CIMP) and microsatellite instability (MSI) (ref 17). Moreover, these alterations emerge from three different carcinogenic pathways, such as adenoma carcinoma sequence, serrated pathway and inflammatory pathway (ref3). As a powerful tool for proper patient management and meaningful clinical research The American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) have create an unified an worldwide language tumour, node, metastasis (TNM) cancer alphanumeric staging system, which consist in classify solid tumours.

It has been demonstrated that there are autocrine loops in CRC, which play an important role in the initiation and progression of cancer (ref 208). Growth factors are involved in this autocrine stimulation, including vascular endothelial growth factor family (VEGF) members, although at first it was believed that their function was limited to vascular permeability and angiogenesis in a paracrine fashion (ref 141).

In humans, five different genes encode VEGF family members: VEGFA, VEGFB, VEGFC, VEGFD, and PlGF (ref 39). We have focused on the study of VEGF-C, since it has been reported its significant implication in CRC tumours microenvironment. In fact, VEGF-C

autocrine signalling is attributed to tumorigeneses, cancer progression and metastasis. It supplies tumour cells sustaining autonomy, promoting their survival, growth, migration and invasion. Moreover, it can alter the function of immune cells of tumour microenvironment, thus affecting the host response to tumours (Ref 140).

In addition to studying cancer, our group also studies proprotein convertases (PC). They are a protease family composed of 9 members, including PC1/PC3, PC2, Furin, PC4, PC5, PACE4, PC7, SKI-1, NARC-1. PC are implicated in the activation or inactivation of a wide spectrum of immature precursor proteins such as VEGFC. VEGF-C is newly synthesized as an inactive preproprotein, in need of two proteolytic cleavages to be biologically activated. This first cleavage is made by PC5, furin or PC7 convertases (Ref 39), concretely they cleave between the C- terminal SHD and the VHD, detaching and realising the C-terminal tail.

To this day, it has been believed that the realised C-terminal tail had not function at all, but we have dedicated our effort to elucidate its role in CRC; being this the aim of the present thesis. For this purpose we have lead our research in characterize the pro-tumour phenotype of C terminal-expressing stable cells *in vitro* either in an autocrine and paracrine fashion, as well as characterize C-terminal-expressing cells promotion of tumour growth *in vivo* mouse model. Beside, we have aimed to determinate the mechanism of action by which C-terminal and/or VEGF-C enhance tumour growth.

First, we have shown that C terminal expression is higher in tumour cancer tissue than in healthy tissue by an immunohistochemistry staining in colorectal cancer human tissue microarray (TMA). Seen that there is a significant difference in C terminal expression in tumour and healthy tissue, we have generated stable CT26 and MC38 mouse colon carcinoma cells, as well as HEK 293 embryonic kidney cells. Stable cells expressed empty vector as control, C terminal and the full VEGFC protein, in order to characterize the pro-tumour phenotype of C terminal based on the aforementioned cancer hallmarks. With these stable cells, we have performed an incuCyte proliferation and boyden chamber and wound healing migration assays, where it has been seen that Cterminal expressing cells are the ones which proliferate and migrate the most. Continuing with the

characterization, we have measured the viability and apoptosis via calcein AM staining and flow cytometry respectively. Results have shown that C terminal expressing cells have the highest viability and in apoptotic conditions dead the least.On the other hand, we have also analysed the *in vivo* paracrine signalling of C terminal by incubating human colorectal SW620 and liver sinusoidal endothelial cell LSEC with conditioned medium of transfected cells. We have perforemed an XTT assay in order to measure cells proliferation and the analyses have shown that cells incubated with Cterminal medium growth the most.

Following with C terminal characterization, we have performed an *in vivo* tumorigenesis assay in mouse with stable transfected CT26 and MC38 cells. Cterminal expressing cells generated tumours were the biggest and the weightiest. Moreover, we analysed these tumours vascularization by an immunofluorescence staining of angiogenesis (CD31) and lymphangiogenesis (LYVE1) markers. Immunostainings have demonstrated that bigger and weightier tumours tent to present more angiogenesis and lymphangionesis.

With the aim of elucidate Cteminal mechanism of action we have performed a RNAseq in stable transfected CT26 generated tumours. Then, we have validate by qPCR the genes, which have proved to be upregulated in Cterminal generated tumours. From these up regulated genes, first we have focused in EAR2. Since, Ear2 is overexpressed in colorectal cancer and it regulates cell survivability through XIAP (ref 150). After western blot, qPCR and in silico analysis, we have discarded EAR2, NR2F6 (the orthologous human protein of EAR2) and XIAP as mediators of C terminal signalling. At that time, we came back to our RNAseq , and after in silico analyses we have proposed RNASE2 as the best candidate for C terminal mechanism of action.

Therefore, the stable cells characterization have demonstrated that C terminal have features of cell hallmarks. Moreover, the in vivo assay has enabled us to study the tumour microenvironment, and even thought the RNAseq has not given us conclusive results, it has helped us to elucidate the possible Cterminal mechanism of action.

Summarising, the present work underlines the relevance of the released C-terminal fragment generated when pro-VEGF-C is activated by the action of convertases.

1.2 RÉSUMÉ

Le cancer est devenu la deuxième cause de décès dans le monde au 21e siècle (réf. 121). C'est la conséquence de mutations cellulaires, les conduisant à se développer et à se diviser de manière incontrôlable ainsi qu'à se propager dans les tissus voisins, et donc à développer des métastases. De plus, le cancer est un processus en plusieurs étapes, qui peut être défini par ses caractéristiques cellulaires (réf 140, réf141, réf 191).

Ce présent travail s'est concentré sur le cancer colorectal (CCR), qui est la deuxième principale cause de décès par cancer dans le monde (réf 1). Le risque génétique et le mode de vie sont les principales raisons du développement du CCR (réf 3, réf 6, réf 10, réf 11). Le cancer colorectal peut être divisé en stades d'initiation, de promotion, de progression et de métastase (réf 3, réf 16). De plus, les altérations génétiques qui se produisent sont englobées dans trois principales aberrations génétiques et épigénétiques, qui sont l'instabilité chromosomique (CIN), le phénotype de méthylation de l'île CpG (CIMP) et l'instabilité microsatellite (MSI) (réf 17). De plus, ces altérations émergent de trois voies cancérigènes différentes, telles que la séquence du carcinome de l'adénome, la voie dentelée et la voie inflammatoire (ref3). En tant qu'outil puissant pour une gestion appropriée des patients et une recherche clinique significative, le Comité mixte américain sur le cancer (AJCC) et l'Union internationale contre le cancer (UICC) ont créé un système de stadification alphanumérique du cancer unifié et mondial pour les tumeurs, les ganglions et les métastases (TNM), qui consistent à classer les tumeurs solides.

Il a été démontré qu'il existe des boucles autocrines dans le CCR, qui jouent un rôle important dans l'initiation et la progression du cancer (réf 208). Les facteurs de croissance sont impliqués dans cette stimulation autocrine, y compris les membres de la famille des facteurs de croissance de l'endothélium vasculaire (VEGF), bien qu'au début on ait cru que leur fonction était limitée à la perméabilité vasculaire et à l'angiogenèse de manière paracrine (réf 141).

Chez l'homme, cinq gènes différents codent pour des membres de la famille VEGF : VEGFA, VEGFB, VEGFC, VEGFD et PlGF (réf 39). Nous nous sommes concentrés sur l'étude du VEGF-C, car il a été rapporté son implication significative dans le microenvironnement des tumeurs CCR. En fait, la signalisation autocrine du VEGF-C est attribuée aux tumeurs, à la progression du cancer et aux métastases. Il alimente les cellules tumorales qui maintiennent leur autonomie, favorisant leur survie, leur croissance, leur migration et leur invasion. De plus, il peut altérer la fonction des cellules immunitaires du microenvironnement tumoral, affectant ainsi la réponse de l'hôte aux tumeurs (Réf 140).

En plus d'étudier le cancer, notre groupe étudie également les proprotéines convertases (PC). Il s'agit d'une famille de protéases composée de 9 membres, dont PC1/PC3, PC2, Furin, PC4, PC5, PACE4, PC7, SKI-1, NARC-1. Les PC sont impliquées dans l'activation ou l'inactivation d'un large spectre de protéines précurseurs immatures telles que le VEGFC. Le VEGF-C est nouvellement synthétisé sous la forme d'une préproprotéine inactive, nécessitant deux clivages protéolytiques pour être biologiquement activé. Ce premier clivage est réalisé par les convertases PC5, furine ou PC7 (Ref 39), concrètement elles clivent entre le SHD C-terminal et le VHD, détachant et réalisant la queue C-terminale.

À ce jour, on a cru que la queue C-terminale réalisée n'avait pas fonctionné du tout, mais nous avons consacré nos efforts à élucider son rôle dans le CRC ; étant cela le but de la présente thèse. Dans ce but, nous avons mené nos recherches pour caractériser le phénotype pro-tumoral des cellules stables exprimant le C-terminal in vitro soit de manière autocrine et paracrine, ainsi que pour caractériser la promotion des cellules exprimant le C-terminal de la croissance tumorale dans un modèle murin vivo. . De plus, nous avons cherché à déterminer le mécanisme d'action par lequel le C-terminal et/ou le VEGF-C stimulent la croissance tumorale.

Tout d'abord, nous avons montré que l'expression du C-terminal est plus élevée dans les tissus cancéreux tumoraux que dans les tissus sains par une coloration immunohistochimique dans le microréseau de tissus humains (TMA) du cancer

colorectal. Vu qu'il existe une différence significative dans l'expression du terminal C dans la tumeur et les tissus sains, nous avons généré des cellules stables de carcinome du côlon de souris CT26 et MC38, ainsi que des cellules rénales embryonnaires HEK 293. Les cellules stables ont exprimé le vecteur vide comme témoin, le C terminal et la protéine VEGFC complète, afin de caractériser le phénotype pro-tumoral du C terminal sur la base des caractéristiques du cancer susmentionnées. Avec ces cellules stables, nous avons effectué des tests de prolifération incuCyte et de chambre de Boyden et de migration de cicatrisation, où il a été constaté que les cellules exprimant Cterminal sont celles qui prolifèrent et migrent le plus. Poursuivant la caractérisation, nous avons mesuré la viabilité et l'apoptose via la coloration à la calcéine AM et la cytométrie en flux respectivement. Les résultats ont montré que les cellules exprimant le terminal C ont la viabilité la plus élevée et, dans des conditions apoptotiques, la mort la moins importante. D'autre part, nous avons également analysé la signalisation paracrine in vivo du terminal C en incubant le SW620 colorectal humain et la cellule endothéliale sinusoïdale du foie LSEC avec conditionné milieu de cellules transfectées. Nous avons effectué une Test XTT afin de mesurer la prolifération cellulaire et les analyses ont montré que les cellules incubées avec le milieu Cterminal croissent le plus.

Suite à la caractérisation C-terminale, nous avons effectué un test de tumorigenèse in vivo chez la souris avec des cellules CT26 et MC38 transfectées stables. Les tumeurs générées par les cellules d'expression Cterminale étaient les plus grosses et les plus lourdes. De plus, nous avons analysé la vascularisation de ces tumeurs par une coloration par immunofluorescence des marqueurs de l'angiogenèse (CD31) et de la lymphangiogenèse (LYVE1). Les immunomarquages ont démontré que les tumeurs plus grosses et plus lourdes tentent de présenter plus d'angiogenèse et de lymphangionèse.

Dans le but d'élucider le mécanisme d'action de Cteminal, nous avons réalisé un RNAseq dans des tumeurs transfectées stables générées par CT26. Ensuite, nous avons validé par qPCR les gènes, qui se sont révélés être régulés positivement dans les tumeurs générées par Cterminal. A partir de ces gènes régulés, nous nous sommes d'abord concentrés sur EAR2. Depuis, Ear2 est surexprimé dans le cancer colorectal et régule la capacité de survie des cellules via XIAP (réf 150). Après western blot, qPCR et analyse in

silico, nous avons écarté EAR2, NR2F6 (la protéine humaine orthologue de EAR2) et XIAP comme médiateurs de la signalisation C-terminale. A cette époque, nous sommes revenus à notre RNAseq, et après des analyses in silico nous avons proposé la RNASE2 comme meilleur candidat pour le mécanisme d'action C-terminal.

Par conséquent, la caractérisation des cellules stables a démontré que le terminal C présente des caractéristiques de caractéristiques cellulaires. De plus, le test in vivo nous a permis d'étudier le microenvironnement tumoral, et même si le RNAseq ne nous a pas donné de résultats concluants, il nous a aidés à élucider l'éventuel mécanisme d'action Cterminal.

En résumé, le présent travail souligne la pertinence du fragment C-terminal libéré généré lorsque le pro-VEGF-C est activé par l'action des convertases.

1.3 RESUMEN

El cáncer se ha convertido en la segunda causa de muerte en todo el mundo en el siglo XXI (ref. 121). Esta patología que puede tener origen tanto en el colon como en el recto es consecuencia de varias mutaciones celulares, que hacen que las células crezcan y se dividan sin control, así como que se propaguen a los tejidos cercanos y desarrollen metástasis. El cáncer es un proceso de varios pasos, que puede definirse por ciertas características celulares bien definidas las llamadas " cancer hallmarks" (Hanana 2022) (ref 140, ref141, ref 191 donde cobran especial relevancia: señalización proliferativa constante, evasión de supresión de crecimiento, reprogramación epigenética no-mutacional, inmunosupresión, inmortalidad replicacional, inflamación pro-tumoral, polimorfismos del microbioma, invasión y metástasis, inducción o acceso a vasculatura, senescencia, inestabilidad genómica y mutacional, resistencia a muerte celular, alteraciones metabólicas y plasticidad fenotípica.

Este trabajo se ha centrado en el cáncer colorrectal (CCR), siendo la segunda causa principal de muerte por cáncer en todo el mundo (ref 1). El riesgo genético y el estilo de vida son las principales razones para el desarrollo de CCR (ref 3, ref 6, ref 10, ref 11). El cáncer colorrectal se puede dividir en las etapas de iniciación, promoción, progresión y metástasis (ref 3, ref 16). Además, las alteraciones genéticas que se producen se engloban en tres aberraciones genéticas y epigenéticas principales, que son inestabilidad cromosómica (CIN), fenotipo metilador de islas CpG (CIMP) e inestabilidad de microsatélites (MSI) (ref 17). Asimismo , estas alteraciones surgen de tres vías cancerígenas diferentes, como la secuencia del carcinoma de adenoma, la vía serrada y la vía inflamatoria (ref3). Como una herramienta poderosa para el manejo adecuado del paciente y la investigación clínica significativa, The American Joint Committee on Cancer (AJCC) y the International Union Against Cancer (UICC) han creado un sistema alfanumérico de estadificación del cáncer de tumores, ganglios y metástasis (TNM) unificado y de lenguaje mundial, que consisten en clasificar los tumores sólidos.

La gestión de los pacientes presenta un reto para los sistemas sanitarios de todo el mundo, donde la carga socioeconómica del CRC es significativa. Actualmente los esfuerzos se centran en el cribado masivo y una detección temprana de la patología, lo

que permite la resección quirúrgica de la masa tumoral como tratamiento a la enfermedad. No obstante, en ocasiones la inviabilidad de este procedimiento supone el uso de terapias sistémicas basadas en distintos fármacos para revertir el crecimiento tumoral: estatinas, agonistas de PPARγ, metformina, suplenetación con calcio como adyuvante y fármacos no esteroideos como la aspirina. En este contexto, las terapias dirigidas son una nueva estrategia que han demostrado extender y mejorar el prognóstico de los pacientes de CRC. Estos compuestos inhiben directamente la proliferación, diferenciación y migración de las células cancerosas al dirigirse a distintas vías de señalización implicadas: Wnt/β-Catenina, Notch, Hedgehog, TGF-β/SMAD, EGF, IGF… No obstante, el estudio de las distintas vías implicadas todavía presenta grandes incógnitas, donde cobran especial importancia no solo las vías de señalización que inducen la proliferación y desarrollo del tumor, sino de los procesos biológicos que promueven la creación de un entorno nutricional favorable y la migración de la masa tumoral a otros órganos formando metástasis.

Se ha demostrado que existen bucles autocrinos en el CRC, que juegan un papel importante en la iniciación y progresión del cáncer (ref 208). Los factores de crecimiento están involucrados en esta estimulación autocrina, entre ellos cabe destacar, los miembros de la familia del factor de crecimiento endotelial vascular (VEGF), aunque al principio se creía que su función se limitaba a la permeabilidad vascular y la angiogénesis de manera paracrina (ref. 141). En humanos, cinco genes diferentes codifican los miembros de la familia VEGF: VEGF-A, VEGF-B, VEGF-C, VEGF-D y PlGF (ref. 39).

El presente trabajo se ha centrado en el estudio de VEGF-C, ya que se ha demostrado su importante implicación en el microambiente tumoral del Cáncer colorrectal. De hecho, la señalización autocrina de VEGF-C se atribuye a la tumorogénesis, la progresión del cáncer y la metástasis. VEGFC aporta a las células tumorales el sustento que necesitan para mantener su autonomía aparte de favorecer su supervivencia, crecimiento, migración e invasión. Además, puede alterar la función de las células inmunitarias del microambiente tumoral, afectando así la respuesta inmune del huésped a los tumores (Ref 140).

VEGF-C se sintetiza como un profactor inactivado, donde las proproteínas convertasas (PC) catalizan su escisión para su maduración y activación. Las proteínas convertasas son una familia de proteasas compuesta por nueve miembros, incluidos PC1/PC3, PC2, Furin, PC4, PC5, PACE4, PC7, SKI-1, NARC-1. Las PCs están implicadas en la activación y/o desactivación de un amplio espectro de proteínas precursoras inmaduras como el mencionado VEGF-C. La primera escisión de VEGF-C la realizan las convertasas PC5, furina o PC7 (Ref 39), concretamente cortan entre el C-terminal silk homology domain (SHD) y el VEGF homology domain (VHD), desprendiendo el fragmento C-terminal.

Hasta la fecha, se creía que el fragmento C-terminal liberado durante la escisión pro-VEGF-C no tenía ninguna función, pero hemos dedicado nuestro esfuerzo a dilucidar su papel en el cáncer colorrectal ; siendo este el objetivo principal de la presente tesis. Para ello nos hemos centrado en la caracterización del fenotipo protumoral de células estables que expresan C-terminal in vitro tanto de forma autocrina como paracrina, así como caracterizar las células que expresan C-terminal y promover el crecimiento tumoral en un ensayo in vivo con ratones. Además, nos hemos propuesto determinar el mecanismo de acción por el cual C-terminal y/o VEGF-C potencian el crecimiento tumoral.

En primer lugar, mediante un análisis in silico hemos visto que la proteína VEGFC interactúa con otras proteínas que presentan características tumorales, además de observar que altas expresiones de VEGFC correlacionan negativamente con la supervivencia de pacientes tanto de cáncer de colon como de recto. Basándonos en estos resultados in silico y en investigaciones que muestran la clara implicación del VEGFC en el cáncer , hemos querido saber si el C terminal del VEGFC tiene algún rol en el CCR, para ello hemos realizado un ensayo de inmunohistoquímica empleando un anticuerpo específico contra la región C-terminal de la citoquina de interés. Hemos demostrado que la expresión C-terminal es mayor en el tejido canceroso tumoral que en el tejido sano mediante una tinción inmunohistoquímica en microarrays de tejido humano (TMA) de cáncer colorrectal. Dado que hay un aumento significativo en la expresión C-terminal en el tumor en comparación con el tejido sano, hemos generado células de carcinoma de colon de ratón CT26 y MC38 estables, así como células de riñón

embrionario HEK 293. Las células estables generadas expresan: i) el vector vacío como control, ii) el fragmento C-terminal de VEGF-C y iii) la proteína VEGF-C completa. Una vez demostrada la estabilidad de las líneas celulares generadas, se ha procedido a caracterizar el fenotipo protumoral del C-terminal en función de las características del cáncer antes mencionadas donde la proliferación celular, la supresión de la muerte celular, el desarrollo angiogénico y linfangiogénico y la capacidad de migración cobran especial relevancia en el CRC. Con estas células estables, hemos realizado ensayos de proliferación mediante las técnicas de incuCyte y XTT. Por otro lado, se han empleado las técnicas Boyden chamber y de cicatrización de heridas para caracterizar la capacidad de migración de las células generadas. Se ha visto que las células que expresan C-terminal son las que más proliferan y migran. Continuando con la caracterización, y en relación al hallmark característico de la supresión de muerte celular, la cual otorga de propiedades de quimioresistencia a aquellos cánceres con peor pronóstico, hemos medido la viabilidad y apoptosis mediante inmunofluorescencida de la tinción de calceína AM y citometría de flujo. Los resultados han demostrado que las células que expresan C-terminal tienen la viabilidad más alta y mueren menos en condiciones apoptóticas. Por otro lado, también hemos analizado la señalización paracrina in vitro de C-terminal mediante la incubación de SW620 y LSEC con medio acondicionado de células transfectadas. Hemos realizado un ensayo XTT para medir la proliferación celular y los análisis han demostrado que las células incubadas con medio Cterminal son las que más proliferan. De esta forma, el fragmento C-terminal promovería el desarrollo tumoral in vitro estimulando el crecimiento celular y la migración de manera autocrina además de promover el crecimiento de células externas de manera paracrina.

Siguiendo con la caracterización de C-terminal, hemos realizado un ensayo de tumorigénesis in vivo en ratón con células transfectadas estables CT26 y MC38. Los tumores generados por células de expresión C terminal fueron los más grandes y los más pesados. El modelo de ratón generado presenta una alternativa más similar a lo que ocurre en la biología, donde el crecimiento tumoral se realiza en un contexto más amplio que in vitro de manera que distintas poblaciones celulares y la respuesta inmune intervienen. Además, el desarrollo angiogénico tan relevante en el crecimiento de CRC también se puede estudiar. Cuando analizamos la vascularización de estos tumores

mediante una tinción de inmunofluorescencia de los marcadores de angiogénesis (CD31) y linfangiogénesis (LYVE1), las tinciones han demostrado que los tumores de mayor tamaño y peso tienden a presentar más angiogénesis y linfangiogenesis. De esta manera, aquellas células con una mayor expresión del fragmento C-terminal tendrían un mayor desarrollo tumoral in vivo además de in vitro donde, en este caso, la respuesta inmune sería insuficiente para la generación de una compensación.

Con el objetivo de dilucidar el mecanismo de acción de C-terminal en la promoción de la capacidad de proliferación y migración de las células, hemos realizado un RNAseq en tumores generados por CT26 transfectados estables. Luego, hemos validado por qPCR los genes, que han demostrado estar sobreexpresados en tumores generados por C-terminal. A partir de estos genes sobreexpresados, primero nos hemos centrado en Ear2, ya que este gen se sobreexpresa en el cáncer colorrectal y promueve la supervivencia celular a través de la señalización mediada por Xiap (ref. 150). Después de analizar los por western blot, qPCR y análisis in silico, hemos descartado EAR2, su ortólogo humano NR2F6 y XIAP como mediadores de la señalización mediada por C-terminal. Visto que no eran buenos candidatos, volvimos a nuestros resultados de RNAseq y, después de realizar un análisis in silico, planteamos la hipótesis de que RNASE2 es un objetivo aguas abajo de la señalización mediada por C-terminal. Esta diana se ha caracterizado en varios tipos de cáncer y está aumentada en aquellos pacientes con adenocarcinoma de colon, estando la expresión de RNASE2 correlacionada con la supervivencia de los pacientes. Además, al comparar la expresión de NR2F6, XIAP y RNASE2 en poblaciones de células inmunes, el último gen fue el único que parecía estar regulado en ciertas células del sistema inmune (monocitos). Hay que recordar que durante el desarrollo del cáncer se produce un inmunoagotamiento donde la respuesta no es suficiente para compensar el crecimiento tumoral. De hecho investigaciones actual están muy centradas en desentrañar el agotamiento inmunológico que se produce durante el desarrollo del cáncer (ref. 206, ref. 207), por lo que una posible inmunoterapia combinada junto con una terapia basada en el anti-C-terminal podría ser de interés debido a su potencial efecto sinérgico en la inhibición del desarrollo del cáncer colorrectal .

Por lo tanto, la caracterización de células estables ha demostrado que las células transfectadas estables que expresan C-terminal desarrollan un aumento de las características del cáncer, como un aumento de la proliferación, la supervivencia frente a estímulos apoptóticos y la migración. Además, el ensayo in vivo nos ha permitido estudiar el microambiente tumoral, y aunque el RNAseq no nos ha dado resultados concluyentes, nos ha ayudado a dilucidar el posible mecanismo de acción de C-terminal. En resumen, el presente trabajo subraya la relevancia del fragmento C-terminal liberado generado cuando el pro-VEGF-C es activado por la acción de las convertasas.

2- INTRODUCTION

2. INTRODUCTION

2.1 COLORECTAL CANCER

2.1.1 Cancer

Cancer is the second leading cause of death worldwide in the 21st century, being 1 out of 6 deaths due to cancer. Dates from World Health Organization (WHO) show that cancer burden rises to 19.29 million new cases and 9,95 million cancer deaths in 2020, where lung cancer is the most aggressive one followed by colorectal cancer and liver (ref 121).(Figure 2.2)

Numerous forces can cause mutations in cells that can end in cancer, increasing the number of cases being diagnosed and requiring treatment and care. Besides, drug resistance is still the main limiting factor for cancer patients to recover, thus even if tumours can quickly diminish due to treatments, they can develop drug resistance, concluding in disease relapse (ref 136).Although, thankfully, life expectancy in cancer patients is becoming higher during the last years due to earlier prognosis and new treatments.

As mentioned before, cancer is the result of cells mutations, allowing them to divide, grow uncontrollably and spread into surrounding tissues becoming invasive thus metastatic. It is just needed a single mutated cell or a small-mutated cell group to start it. It can be caused by inherited defects in particular genes, the processes of life inside the cell or epigenetic factors, such as exposure to outside factors in the environment (tobacco, chemicals, radiation or unhealthy habits. (ref 140, ref141, ref 191))

Development of cancer occurs in a multi-step process and it can be defined by its cell´s hallmarks: sustaining proliferative signalling, enabling replicative immortality, evading growth suppressors, tumour-promoting inflammation, genome instability and mutation, resisting cell death, inducing or accessing vasculature, activating invasion and metastasis, deregulation cellular metabolism and unlocking phenotypic plasticity , polymorphic microbiomes, nonmutational epigenetic reprogramming, senescent cells and evading immune destruction. (ref 140, ref141, ref 191) (figure 2.1)

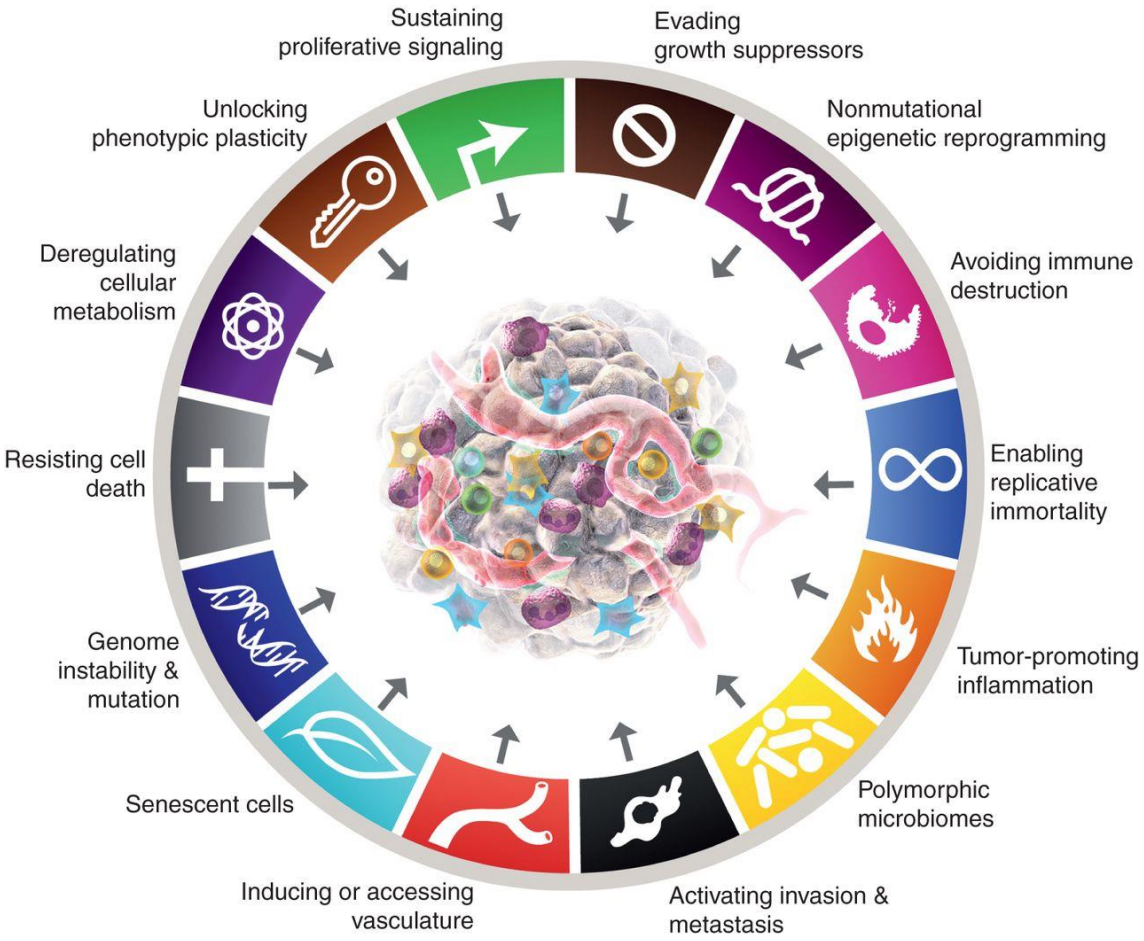


Figure 2.1: the Hallmarks of Cancer (Hanahan D. 2022)

2.1.2 Colorectal Cancer descriptive epidemiology

2.1.2.1 Incidence and trends

Colon and rectal cancer are grouped together in the term CRC, because they have many characteristics in common, so CRC can be originated either in the colon or in the rectum. Therefore, depending on where it is created it can also be called colon or rectal cancer. In this work we use the term CRC that covers both colon and rectal cancer.(ref 14)

CRC is the second most predominantly diagnosed cancer in women and the third in men, being the second main cause of cancer death worldwide (ref 1) (Figure 2.2).The CRC burden differs greatly between countries, and even between regions. Since incidence is related with socioeconomic development level, regions with high Human Development Index (HDI) present higher incidence compared with those with lower HDI, as shown in Figure 2.3.(Ref 2) . In other words, regions related with a combined westernized factors,

including physical inactivity, obesity, poor diets, smoking and alcohol drinking show higher CRC burden.

Apart from the abovementioned regional trends, there are age and sex trends related to CRC development. Taking in to account that cancer is an ageing disease, CRC development and death rates increase quickly among people aged 50-plus year-olds, occurring 90% of global cases and deaths after age 50 years. (Ref 3). Moreover, this age-adjusted rate varies between sexes, being men the ones who show higher incidence and death. For instance, men seem to be more affected by environmental factors than by genetic factors compared with women, being CRC heritability of 28% for men and 45% for women. Additionally, women benefit from the protective effect of endogenous oestrogen, of which men not. (ref 3)

From 2013 to 2017, each year burden rates decreased about 1%. This decreasing tend belongs mostly to older adults, masking the incidence rate increase of 2% every year among people younger than 50 and 1% in people from 50 to 64. In 2040, according to WHO, the CRC incidence will go up to 1, 7 in males and 1, 4 in females and it is expected to cause 880 000 deaths in men and 710 000 in women (figure 2.4) ref 121 GOBOCAN) (ref 194)

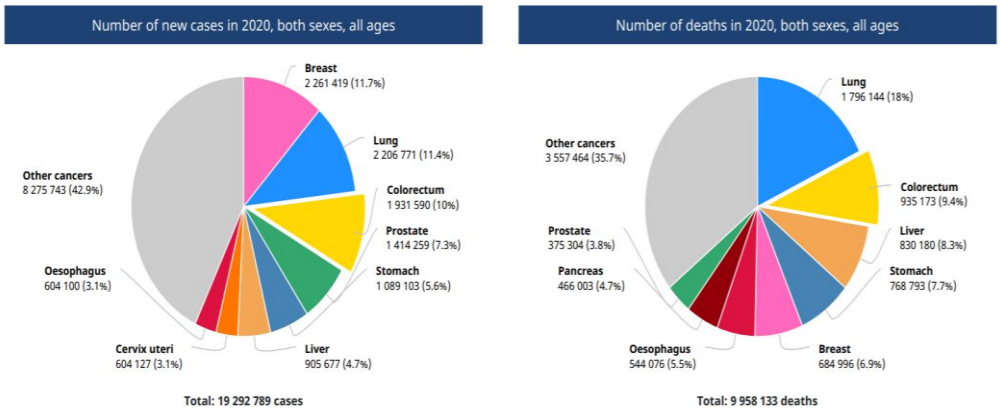


Figure 2.2: Number of new cases of different types of cancer in 2020 according to the World Health Organisation A. Number of new cases of different types of cancer in women**. B.** Number of new cases of different types of cancer in men

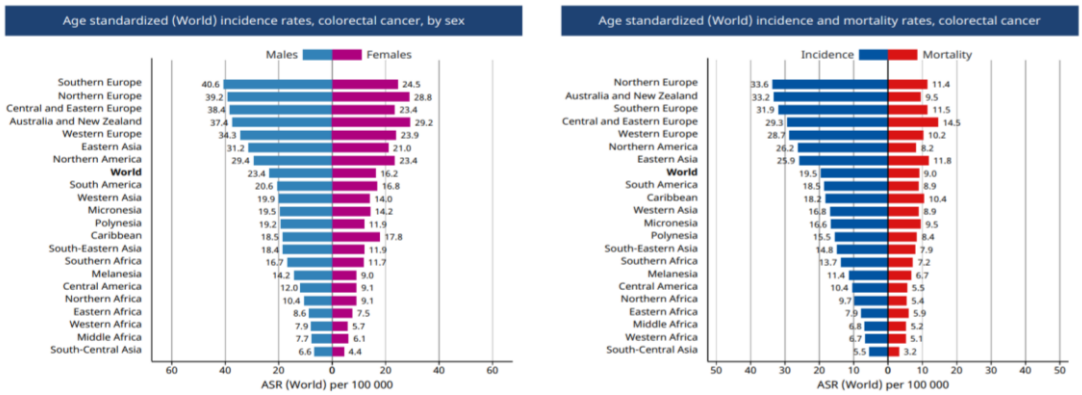


Figure 2.3: World incidence rate and mortality of colorectal cancer according to the World Health Organisation. A. Histogram showing the incidence rate of colorectal cancer by sex **B.** Histogram showing the incidence rate and mortality rate of colorectal cancer

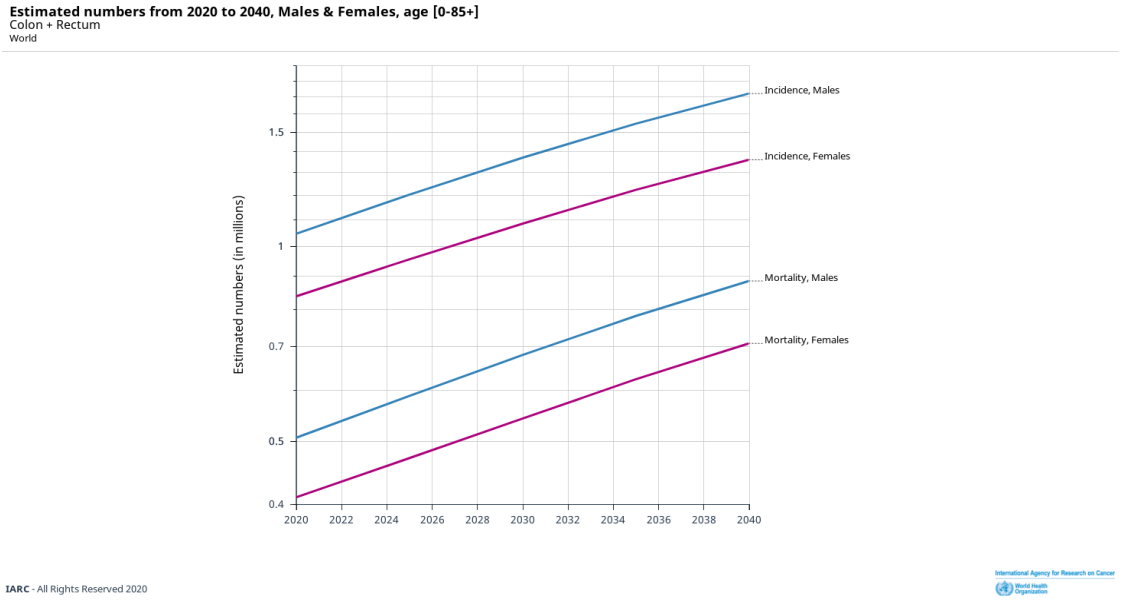


Figure 2.4: Estimated number of incidence in colon and rectum from 2020 to 2040 according to the World Health Organisation. The above lines correspond to colon cancer and the lines below correspond to rectum cancer.

2.1.2.2 Aetiological factors of colorectal cancer

Cancer is a genetic illness, provoked by somatic mutations, taking place in the affected tissue during carcinogenesis, or germline mutations becoming heritable. (ref 8)

Thus, several reasons are the responsible for the development of CRC, since there are multiple factors that can be the reason for mutations, thereby giving rise to it. These aetiological factors will be described hereunder:

- Genetic risk factors and predisposition

Even if most CRC (60-65%) are attributed to sporadic events though acquired somatic genomic alterations, 35-40% of cases are associated with inherited CRC susceptibility. This heritable components is comprised of 25% family history, 5% hereditary cancer syndromes, less than 1% known CRC low-penetrance genetic variations , and unknown inherited genomic aberrations. Concerning family history, heredo-collateral history of CRC or adenomatous polyposis increase the risk of suffer CRC in 50-old people, going from 1,8% to 3,4% if the person has at least one affected relative and to 6,9% in case of having two. (ref 6)Figure 2.5

The 5% of hereditary CRC are due to monogenic disorders like, familial adenomatous Polyposis (FAP), Lynch syndrome, the rare hamartomatous polyposis syndromes, and MYH-associated polyposis. (ref 7). This disorders show high-penetrance genes such as adenomatous polyposis coli *(APC*) alleles in FAP, mismatch repair (*MMR*) mutations in Lynch syndrome, liver kinase B1 (*LKB1*), mothers against decapentaplegic homolog 4 (*SMAD4*) and bone morphogenetic protein receptor type 1 (*BMPR1*) alleles in hamartomatous polyposis syndrome , and MYH alleles in MYH- associated polyposis. (ref 8).Less than 1% low penetrance genes concerns to transforming growth factor beta receptor 1 (*TGFβRI*) 6Ala, bloom syndrome protein (*BLM*) Ash, and Harvey rat sarcoma viral oncogene (*HRAS1*) variable number of tandem repeats (*VNTR*) among others. (ref 3)

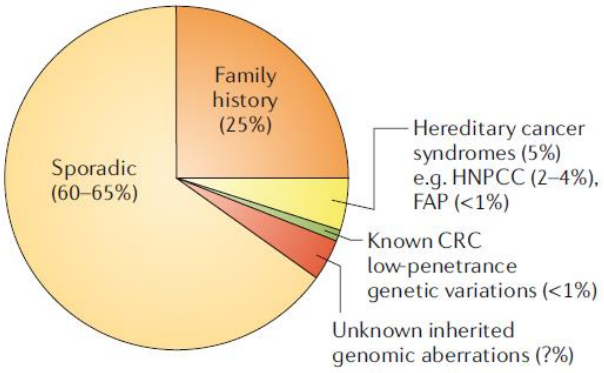


Figure 2.5: Sectors diagrams showing the percentages of develop colorectal cancer based on genetic risk factors and predisposition

- lifestyle and nutritional factors:

Besides from genetics contribution to individual CRC risk, population CRC incidence is heavily influenced by modifiable diet and lifestyle factors, such as obesity, physical activity and sedentary lifestyle, dietary patterns, alcohol, and smoking. (ref3)
Excess adiposity or obesity is an established risk factor, since it develops chronic low-grade systemic inflammation and insulin resistance, which may lead to colorectal carcinogenesis by decreasing apoptosis and increasing cell proliferation. The risk is higher in men than in women, due to, in women, after menopause, adipocytes become the main site for oestrogen production, which confer protection against CRC. (Ref 10, ref 3)

CRC is one of the few cancers, which can reduce its risk through physical activity, since its beneficial effects on immune system, inflammation, metabolic hormones and gut motility. (Ref 3).

Dietary patterns depending on their components combination, food groups and quantity can either increase or decrease CRC risk. (ref 3) . Consuming unhealthy diet characterized by high intake of red and processed meat, sugar-sweetened beverages, and refined desserts and grains increase CRC risk. In contrast, with the protective function of healthy dietary patterns based on high intake of fruits, vegetables, fibre, whole grains and low meat and sweets (ref 11)

Consumption of ethanol suppose a high CRC risk factor, due to its first metabolite; acetaldehyde. Acetaldehyde is toxic and may cause colorectal carcinogenesis by provoking DNA damage and intracellular folate destruction, required for proper DNA synthesis and methylation. (Ref 3)

Smoking increase the risk to develop CRC. The mixture of compounds of cigarettes can reach immediately and easily the colorectal mucosa via the circulatory system or by direct ingestion, inducing genetic and epigenetic aberrations giving rise to CRC. (Ref 3)

According to the American cancer society and taking in to account the above-mentioned aetiological factors, following is table 2.1 showing the relative risk for those established colorectal cancer risk factors (ref 139, table 2.1)

Table2.1: Relative risks for established colorectal cancer risk factors

Table 3. Relative Risks for Established Colorectal Cancer Risk Factors

	Relative risk*
Factors that increase risk:	
Heredity and medical history	
Family history[84]	
CRC	
1 or more first-degree relatives	2.2
1 or more first-degree relatives diagnosed before age 50	3.6
2 or more first-degree relatives	4.0
1 or more second-degree relatives	1.7
Adenoma	
1 or more first-degree relatives	2.0
Inflammatory bowel disease[115]	1.7
Type 2 diabetes[124]	
Male	1.4
Female	1.2†
Modifiable factors	
Heavy alcohol (daily average >3 drinks)[135]	1.3
Obesity (body mass index ≥30 kg/m²)[146]	1.3
Colon, male	1.5
Colon, female	1.1
Rectum, male	1.3
Rectum, female	1.0†
Red meat (100 g/day)[156]	1.1
Processed meat (50 g/day)[166]	1.2
Smoking[190]	
Current vs. never	1.5
Former vs. never	1.2
Factors that decrease risk:	
Physical activity[138]	0.7
Dairy (400 g/day)[166]	0.9

*Relative risk compares the risk of disease among people with a particular "exposure" to the risk among people without that exposure. Relative risk for dietary factors compares the highest with the lowest consumption. If the relative risk is more than 1.0, then risk is higher among exposed than unexposed persons. Relative risks less than 1.0 indicate a protective effect.
†Relative risk was not statistically significant.

2.1.3 Primary tumour and metastasis

Colorectal cancer is a multistage process of histopathologic and molecular changes that can start in either colon or rectum. Thus, it is therefore important to understand the normal structure and function of the colon and rectum. (ref 14)

2.1.3.1 The colon and rectum (location, histology and function)

The colon and the rectum form the large bowel, which is part of the digestive system. The colon is a U-shaped tube made of muscle found below the stomach and small intestine, which is connected to the rectum, a shorter tube. Anatomically, colon is divided into four parts in which the food travels through them. Starting from the part connected to the small intestine and finishing in the anus, the sections are named ascending, transverse, descending and sigmoid colon. (ref 14) . The colon absorbs water and electrolytes from the food matter that comes from the small bowel, forms and

transports the faeces, and it is in charge of the chemical digestion by gut microbes. The waste matter formed in the colon goes into the rectum, where it is store until it is released thought the anus.(ref 14) (figure 2.6).

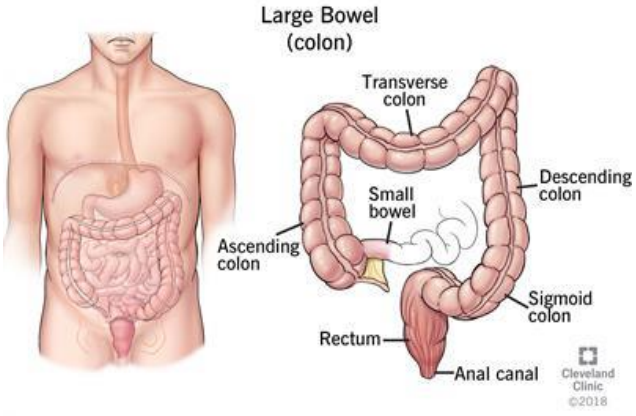


Figure 2.6: illustration of human colon and rectum

Histologically, as colon and rectum make up the large bowel they are form by the same four-tissue layers, which are not separated entirely one from another but are bin together by connective tissue and by vascular and neural elements. From the lumen to the outwards these four layer are: mucosa (epithelium, lamina propria, and muscularis mucosae), submucosa, muscularis propia (inner circular muscle layer, intermuscular space, and outer longitudinal muscle layer) and serosa or adventitia (ref 9) (figure 2.7)

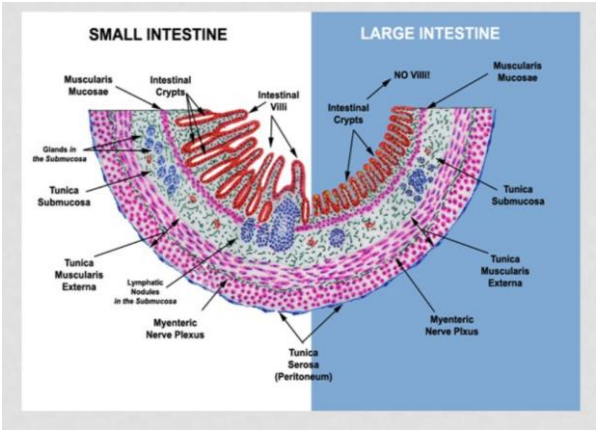


Figure 2.7: illustration of small and large intestine tissue parts

2.1.3.2 Mechanisms of colorectal cancer tumour initiation, promotion, progression and metastasis.

As all cancers, CRC can be divided into four main stages: initiation, promotion, progression and metastasis. Each stage duration becomes difficult to estimate since it can require decades for all stages to be completed, therefore involving a wide time range (ref 3)

In the initiation stage, normal intestinal epithelium cells undergo an irreversible genetic damage, predisposing the affected cells to subsequent neoplastic transformation. Then in promotion, these affected cells because of experience genetic and epigenetic alterations proliferate, giving rise to an abnormal tissue growth called neoplasia or polyps. In the following progression stage, benign tumour cells can transform into malignant cancer cells, acquire aggressive features, and develop metastatic potential. The final stage metastasis is characterized by the spread and invasion of these cancer cells, from the primary organ in this case the intestine, to both the adjacent or distant tissue and organs through the lymphatic system or the bloodstream (ref 3). Because of the colon and rectum venous drainages, the liver is the most typical organ of metastasis, (50% cases), follow by lungs and bones (Ref 16).

These genetic alterations are encompassed in three main genetic and epigenetic aberrations: chromosomal instability (CIN), CpG island methylator phenotype (CIMP) and microsatellite instability (MSI) (ref 17). CIN is marked by abnormalities in chromosomal structure and copy number, CIMP is characterized by hypermethylation at repetitive CG dinucleotides (CpG islands) in the tumor suppressor genes promoter regions, and MSI is defined by alterations in the length of microsatellite, the short nucleotide tandem repeats in DNA sequences throughout the entire genome.

2.1.3.3 Pathway of colorectal carcinogenesis

CRC emerge from three different carcinogenic pathways: adenoma carcinoma sequence, serrated pathway and inflammatory pathway (figure 2.8) (ref3)
Adenoma carcinoma sequence is the classic and most common pathway, which encompasses the majority of sporadic CRC (85-90%), through the sequential

accumulation of genetic mutations and CIN (ref 16). It starts with the development of benign tumor which arise in gland-like cells of the epithelial tissue called adenoma or polyps from normal cells, followed by adenocarcinomas, the malignant counterpart to adenoma which lead to cancer and finally to metastasis (ref 16).

Serrated pathway cover the 10-15% of sporadic CRC and is predominantly associated with the development of CIMP. Normal cells progress to hyperplastic polyps as the number of normal cells increase, to sessile serrated adenomas and, lastly to cancer and metastasis. (ref 3)

Inflammatory pathway is led by chronic inflammation. As the incidence of inflammatory intestine diseases is low it accounts less than the 2 % of all CRC. This model is characterized by the progression from normal cells to abnormal cells called indefinite dysplasia, to low grade dysplasia, to high-grade dysplasia, and finally to cancer and metastasis. (ref 3)

Furthermore, it should be also keep into account the important role and interaction of the tumor microenvironment (TME) in CRC progression and metastasis, since tumours are not only composed of normal colorectal cells, which have become cancerous via a series of mutations. Tumors are a complex mixture of highly interactive cells including other than cancer cells, stroma cells (fibroblasts, adipocytes and myoblasts), inflammatory cells (innate and adaptive immune cells), vascular cells (endothelial and mural cells) smooth muscle cells, and platelets. They trains by chaotic collection of cancer cells and they achieve new characteristics creating tumour microenvironment, which boost proliferation and survival of tumours. (ref. Sayfiyyah Ziyad 2011= ref 24).

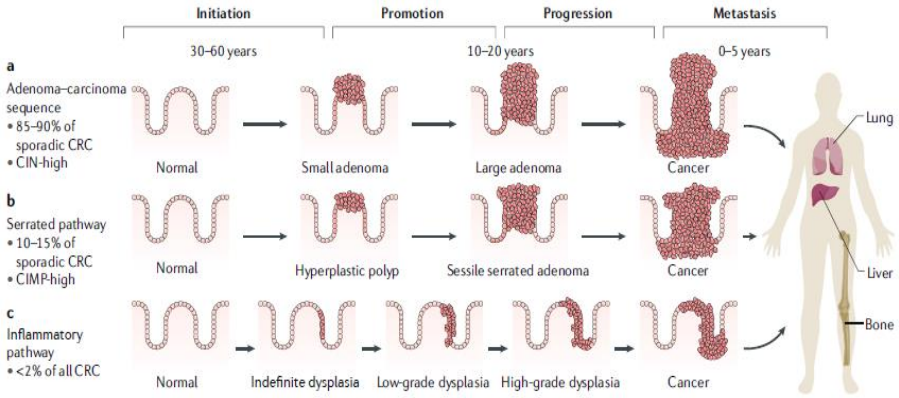


Figure 2.8: Pathways of colorectal carcinogenesis. A. Adenoma-carcinoma sequence **B.** Serrated pathway .**C** inflammatory pathway

As the adenoma carcinoma sequence is the most common and best-characterized pathway, it is interesting to know it in details, thus in figure 2.9 it is described. APC mutations due to environmental mutagens and/or spontaneous mutations in normal intestinal epithelium cells initiate it. These APC tumour suppressor gene mutations lead WNT-β-catenin deregulation resulting in aberrant crypt foci (ACF), which are clusters of abnormal tube-like glands in the wall of the rectum and colon. The ACF can form adenomas or polyps. Adenomas can experience more mutations, such as kirsten rat sarcoma 2 viral oncogene homolog (*KRAS*), v-Raf murine sarcoma viral oncogene homolog B *(BRAF*), loss of heterozygosity 18q (*LOH 18q),SMAD4*, cell division control protein 4 (*CDC4*), transforming growth factor beta receptor 2 (*TGFBR2*),trafficking from endoplasmic reticulum to Golgi regulator (*TFG*) and Bcl-2 Associated X (*BAX*) genes mutations. These mutations lead transforming growth factor beta (*TFG- β*) and, epidermal growth factor receptor (*EGFR*) deregulation, downstreaming to mitogen-activated protein kinase (*MAPK*) and Phosphoinositide 3-kinases (*PI3K*) signalling pathway. Consequently, cell´s survival, proliferation, apoptosis and differentiation is deregulated resulting in adenocarcinomas. Finally, these adenocarcinomas can progress to metastasis invading distal tissue and/or organs, including lungs and bones. (Ref 23)

As aforementioned, the TME is a pivotal key in CRC. In CRC are Natural killer cells (NK), Tumor-associated macrophages (TAM), tumor-associated neutrophils (TAN), cancer-associated fibroblast (CAF) and dendritic cells (DC) cells, which secrete pro-inflammatory cytokines, pro- angiogenic factors, extracellular matrix (ECM) degrading

factors, chemokines, growth factors and factors promoting the invasive motility of CRC cells.

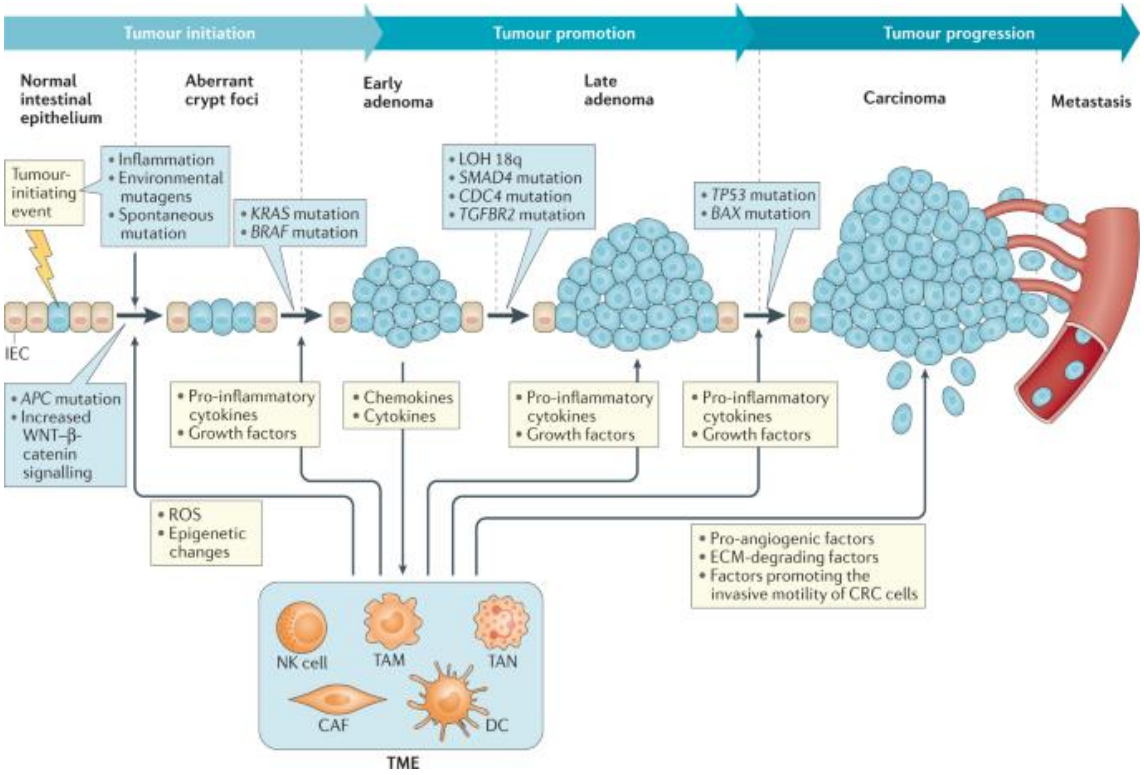


Figure 2.9: Illustration of the multi-stage process of colorectal progression, as well as all the involved cells and genes

2.1.3.4 TNM staging system

The American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) standardized the tumour, node, metastasis (TNM) cancer alphanumeric staging system, with the aim of create an unified and worldwide language of cancer staging in all disciplines, becoming a pivotal tool for proper patient management and meaningful clinical research(Ref 25,). It consist of classify solid tumours based on their size and extent, the spreading to local lymph nodes, and the existence of metastases. (Ref 26)

In TNM system is used “T” plus a letter or a number to describe the depth of the grown of primary tumor into the bowel lining, N represents the lymph nodes, M describes metastases and G stands for the number of cancer cells that appear healthy under a microscope. In table 2.2 is detailed each letter’s information. (ref 27)

Table 2.2 : Description of letters of TNM system

	Characteristics
TX	Primary tumor can not be evaluated.
T0	No evidence of cancer in the colon or rectum
Tis	Carcinoma in situ. Cancer cells are found only in the epithelium or lamina propria
T1	tumor has grown into the submucosa
T2	tumor has grown into the muscularis propria
T3	tumor has grown through the muscularis propria and into the subserosa, or it has grown into tissues surrounding the colon or rectum
T4a	tumor has grown into the surface of the visceral peritoneum
T4b	tumor has grown into other organs or structures

	Characteristics
NX	Regional lymph nodes can not be evaluated
N0	No spread to regional lymph nodes
N1a	tumor cells found in 1 regional lymph node
N1b	tumor cells found in 2 or 3 regional lymph nodes
N1c	nodules made up of tumor cells found in the structures near the colon that do not appear to be lymph nodes
N2a	tumor cells found in 4 to 6 regional lymph nodes
N2b	tumor cells found in 7 or more regional lymph nodes

	Characteirstics
GX	tumor grade can not be identified
G1	cells are more like healthy cells, called well differentiated
G2	cells are somewhat like healthy cells, called moderately differentiated
G3	cells look less like healthy cells, called poorly differentiated
G4	cells barely look like healthy cells, called undifferentiated

Combining T, N, and M information, clinicians classify tumours in different stages explained in table 2.3 and represent in figure 2.10

Table 2.3: Description of the stage of colorectal cancer

Stages	Features	Metastases	Code
Stage 0 (cancer in situ)	Cancer cells are only in the mucosa, or the inner lining, of the colon or rectum.	Not spread	
Stage I	Cancer invade the muscular layer of the colon or rectum	Not spread	T1 or T2, N0 M0
Stage II	A: Cancer has grown through the wall of the colon or rectum	Not spread	T3 N0 M0
	B: Cancer has grown through the layers of the muscle to the lining of the abdomen, called the visceral peritoneum	Not spread	T4a N0 M0
	C: Tumor has spread through the wall of the colon or rectum and has grown into nearby structures	Not spread	T4b N0 M0
Stage III	A: Cancer has grown through the inner lining or into the muscle layers of the intestine	spread to 1 to 3 lymph nodes it has not spread to other parts of the body	T1 or T2, N1 or N1c, M0; •
		spread to nodule of tumor cells in tissues that do not appear to be lymph nodes it has not spread to other parts of the body	• * T1, N2a, M0
	B: Cancer has grown through the bowel wall or to surrounding organs	Spread to lymph nodes or to a nodule of tumor in tissues that do not appear to be lymph nodes Not spread to other parts of the body	Three possible cases : • T3 or T4a, N1 or N1c, M0; • T2 or T3, N2a, M0; • T1 or T2, N2b, M0.
	C: Cancer has grown through the bowel wall or to surrounding organs	It has spread to 4 or more lymph nodes but not to other distant parts of the body	Three possible cases : • T4a, N2a, M0; • T3 or T4a, N2b, M0; • T4b, N1 or N2, M0
Stage IV	A: Cancer has spread to a single distant part of the body	metastasis	Any T, any N, M1a
	B: Cancer has spread to more than 1 part of the body	metastasis	Any T, any N, M1b
	C: Cancer has spread to the peritoneum	Metastasis	Any T, any N, M1c

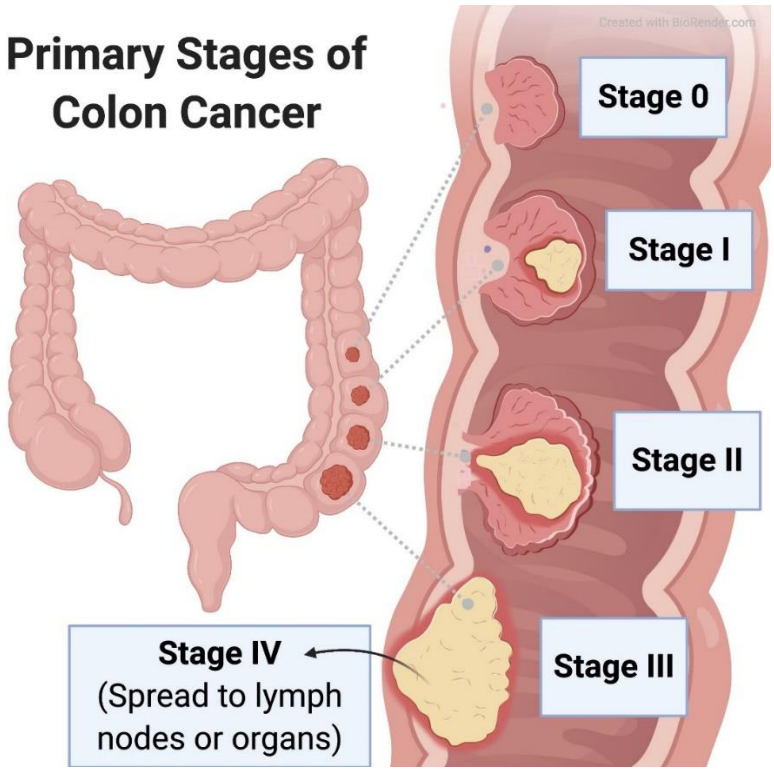


Figure 2.10: Illustration of the different stages of colon cancer

2.1.4 Prevention and treatments

In view of the fact that CRC is one of the most predominantly diagnosed cancer both in women and in men, the knowledge about its prevention, and treatment becomes important when it comes to battle it.

There are some chemopreventive potential drugs or substances against CRC, of which prophylactic features combined with an optimal diets and lifestyle, could exert an anticancer effect. Some of them are statins, peroxisome proliferator-activated receptor gamma (PPARγ) agonists, metformin, calcium, and non-steroidal anti-inflammatory drugs (NSAIDs) as aspirin. Aspirin, inhibit cyclooxygenase 2 (COX2), an enzyme that encourages tumour promoting inflammation and suppresses T cell- mediated antitumor immunity (ref 28)(ref 29; ref 30). NSAIDs, statins, PPARγ agonists, and metformin have cancer steam cells (CSC) suppressing effects by stem cell-regulating pathways, including Wnt, NOTCH, and bone morphogenetic proteins (BMP). As well as they regulate stem cell niche or TME through inflammatory nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and Wnt pathways, and they alter tumour metabolism via AMP-

activated protein kinase /mammalian target of rapamycin (AMPK/mTOR) pathway (ref 29) . Calcium also takes part against colorectal neoplasms, as it precipitates secondary bile acids, ionized fatty acids and haem iron in the colorectal lumen, resulting in a diminution of their carcinogenic effects on the colorectal mucosa. (Ref 3).

Even being aware of how to prevent it, cancer can be develop. The optimal CRC treatment is to reach a complete removal of the tumour and metastases, which generally need to be done by surgical intervention. Unfortunately, not all tumour are unresectable or patients tolerates the surgery, thus in these cases the goal is to diminish tumour sizes and suppress its spread and growth. For controlling cancer in such patients, radiotherapy and chemotherapy are the main strategies. Notable radiotherapy or chemotherapy can also be used before or / and after surgery as neoadjuvant or adjuvant treatment in order to shrink and stabilize the tumour. Furthermore, targeted therapies are novel optional strategies that have successfully extended survival for CRC patients. These avenues are small molecules that can directly inhibit cancerous cells proliferation, differentiation, and migration as they target various pathways. They mediate the initiation, progression and migration pathways of CRC via Wnt/β-catenin, Notch, Hedgehog, TGF-β /SMAD, vascular endothelial growth factor /vascular endothelial growth factor receptor (VEGF/VEGFR),epidermal growth factor /epidermal growth factor receptor(EGF/EGFR), hepatocyte growth factor / c- mesenchymal epithelial transition (HGF/c-MET), and insulin-like growth factor / insulin-like growth factor 1 receptor (IGF/IGF1R). As well as they activate signalling cascades pathways including PI3K/ Serine/Threonine Kinase (PI3K/AKT) or RAS/rapidly accelerated fibrosarcoma (RAF). Some of these targeted therapies are cetuximab, panitumumab (both anti- EGFR agents), bevacizumab, ziv-aflibercept, regorafenib, ramucirumab (the four of them are anti VEGF/VEGFR agents), pembrolizumab, nivolumab, and ipilimumab (the three of them are immune checkpoint inhibitors) (ref 31)

2.2. COLORECTAL CANCER, VASCULARISATION AND AUTOCRINE FACTORS.

2.2.1 VEGF family

Vascular endothelial growth factor (VEGF) was first discovered, isolated and cloned in 1989 (ref 140). Initially, it was identified as an endothelial cell-specific mitogen, which can induce both physiological and pathological angiogenesis (ref 141), but it has proven to be not limited to angiogenesis and vascular permeability (ref 141).

The vascular endothelial growth factors (VEGFs) family, currently are composed of seven disulphide- bonded homodimeric glycoproteins, including five mammalian factors VEGF-A, VEGF-B, VEGF-C, VEGF-D , and PlGF; one parapox virus family origin factor denoted VEGF- E, and several snake venom-derived proteins named VEGF-F (ref 40)(Ref 64)(ref 107). Although, there are naturally occurring heterodimers of VEGF-A and PlGF (ref 49).

The VEGF family members differ in their expression pattern, receptor specificity and biological functions (ref 140)

Each VEGF protein arise as assorted different variants either due to processing or due to alternative splicing. Every variant bind differently to both vascular endothelial growth factor receptors (VEGFRs) and to co-receptor, inducing different biological responses. Therefore, each isoform has a pivotal role in different vascularization contexts, spreading out from lymphatic to embryonic angiogenesis.

Specifically VEGF family members interacts with VEGF receptors (VEGFR), VEGFR-1, VEGFR-2 and VEGFR-3, and co-receptors heparin surfate (HS), non-tyrosine kinase receptors of the neuropilin (NRP) family, NRP-1 and NRP-2, and integrins, modulating signal-transductions (ref 3; ref 38).

Although, their vascularization features characterize them and they are considered as the chief regulators of angiogenesis during development and growth (ref Bonnie J Nieves 2009), their biological activities are not limited to the vascular system. They also play a crucial role in normal physiological functions including fatty acid uptake (ref 107), bone

formation, haematopoiesis, wound healing, and development (ref 44), as well as their role in different diseases for instance diabetes, macular degeneration and cancer among others (ref Bonnie J Nieves 2009).

In the case of cancer disease, VEGF- mediated signalling also takes place in tumour cells Despite, the expression of VEGF receptors was first thought to be limited to endothelial cells, now it is know that many cancer types also express them existing a correlation between their expression and clinical parameters (table 2.4)

Table 2.4: Expression of VEGFs and VEGFRs in human cancers

VEGF or receptor	Cancer
VEGFs	
VEGF	Bladder[129,130], brain[14,131,132], breast[‡] (REFS 38·133–135), colon[‡] (REFS 87·136), gastric[‡] (REF. 137), oral squamous[‡] (REFS 138·139), lung[‡] (REFS 140–143), mesothelioma[‡] (REF. 144), myeloid leukaemia[145], ovarian[146,147], pancreatic[91,148] and prostate[§] (REFS 149–151)
VEGFB	Breast[‖] (REFS 134,152) and lung[140]
VEGFC	Breast[155], cervical[‖] (REFS 153–155), colon[‡‖] (REFS 153·156·157), gastric[158], oral squamous[159], lung[‡] (REFS 140,153,160) and prostate[153]
VEGFD	Cervical[154], gastric[161] and lung[140]
PLGF	Breast[¶] (REF. 162), colon[‡] (REF. 136), gastric[‡§‖] (REF. 163) and hepatocellular[¶] (REF. 164)
VEGF receptors	
VEGFR1	Bladder[130], brain[131,132], breast[‡] (REFS 133–135·152·165), colon[18,92], head and neck[166], lung[‡] (REFS 140–142), melanoma[167], mesothelioma[144], myeloid leukaemia[145], oesophageal[168], ovarian[86,146,147], pancreatic (REFS 91,148) and prostate (REF. 169)
VEGFR2	Bladder[§] (REF. 129), brain[14,131,132,161,170,171], breast[‡] (REFS 133·135·172), cervical[173], colon[§‖] (REFS 87·174), endometrial[‡] (REF. 175), gastric[137], head and neck[166,176], hepatocellular[‡] (REF. 177), lung[‡] (REFS 140–142·178), melanoma[167], mesothelioma[144], multiple myeloma[179], myeloid leukaemia[145], oesophageal[168], ovarian[86,146,147], pancreatic[91,148], prostate[149,169], renal cell carcinoma[180], squamous[181] and thyroid[‖] (REF. 182)
VEGFR3	Breast[155], cervical[§] (REFS 153·154), colon[‡‖] (REFS 153·156), gastric[‡‖] (REFS 158·161), head and neck[159,166], lung[‡§] (REFS 140,153,160), oesophageal[168] and prostate[153]
NRP1	Brain[‖] (REFS 14·63·183·184), breast[‡] (REFS 135·185·186), colon[§] (REFS 83·187·188), lung[143,185,189], melanoma[167], ovarian[147,190,191], pancreatic[84,188,192–194] and prostate[§¶] (REFS 150,151,195)
NRP2	Bladder[196], breast[88,186,197,198], colon[113,197], lung[143,189,197], melanoma[197,199], ovarian[190], pancreatic[193,194], prostate[‡§] (REF. 15) and renal cell[§‖] (REF. 200)

NRP, neuropilin; PLGF, placental growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

*The data reported are primarily based on immunohistochemical analyses of tumours and indicate expression of VEGFs or VEGF receptors specifically in tumour cells.

‡Studies that showed a correlation between expression and poor survival or outcome.

§Studies that showed a correlation between expression and disease stage or progression.

‖Studies that showed a correlation between expression and metastasis.

¶Studies that showed a correlation between expression and recurrence.

In fact, VEGF-C has a significant influence in tumour microenvironment. For example, some immune cells, as cluster of differentiation 4 (CD4+) and forkhead box protein P3 (FOXP3) + regulatory T cells can express VEGF receptors. Consequently, VEGF can alter

the function of immune cells of tumour microenvironment, thus affecting the host response to tumours (Ref 140) (figure 2.11). Furthermore, in the hypoxic tumour microenvironment, macrophages secrete VEGF and in the tumour stroma are fibroblast, which secrete it.

Besides, the previously mentioned paracrine signalling, VEGF also acts via an autocrine mechanism. This autocrine signalling importantly provides tumour cells sustaining self-sufficiency and autonomy, promoting their growth, survival, migration and invasion, independently of angiogenesis. Indeed, VEGF autocrine signalling is attributed to aggressive cancers and poorly differentiated carcinomas.

Its autocrine mechanism turns VEGF in a key aspect of tumorigenesis. It promotes dedifferentiation, an epithelial-mesenchymal transition (EMT) phenotype, as well as, it regulates cancer steam cells 'pool size and self-renewal(ref 140). It should be noted the important role of EMT in colorectal cancer. EMT is the process by which cells lose their epithelial features (i.e. cell- cell contract and cell polarity) and acquire mesenchymal characteristics like increased motility (ref 145)

In addition to tumour cells, many other cell types, such as platelets, keratinocytes and renal mesangial cells, secrete them (ref 44).

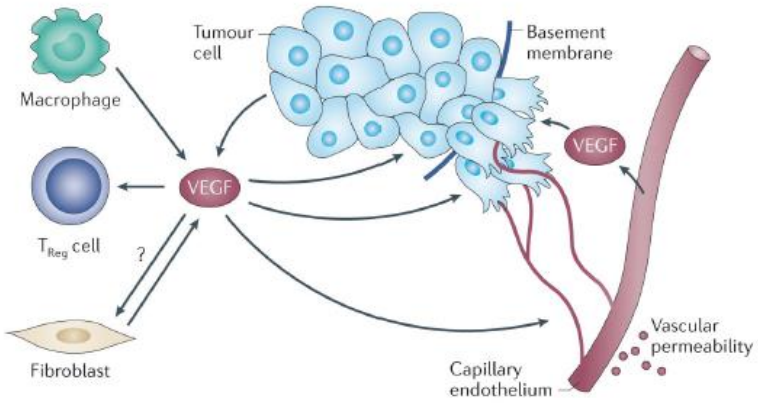


Figure 2.11: illustration of the influence of VEGF in tumour microenvironment

2.2.1.1 VEGF members

In humans, five different genes encode VEGF family members: VEGFA*, VEGFB, VEGFC, VEGFD*, and *PlGF* (ref 39). The human VEGF genes are identify by a strongly conserved seven exon structure, except for VEGF-A that has eight exons *(*Ref 41) Figura 2.12

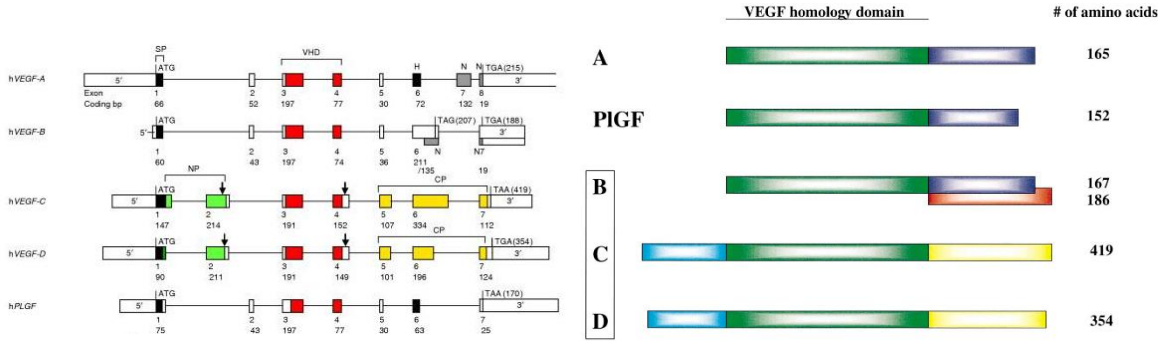


Figure 2.12: illustration of VEGF family member genes

All VEGF family members are identify by their central VEGF homology domain (VHD) also called PDGF/VEGF domain. (Ref EMBL-EBI, 2017). VHD displays a pattern of characteristically spaced cysteine residues giving rise to a cysteine knot motif, which consist of three intertwined disulfide bridges. These cysteine knots act as ligands for receptors and have a crucial role in extracellular signalling being determinants of protein folding and stability (ref 40). They are biologically active as homodimers or heterodimers and as said before they arbitrate their distinct biological functions by binding to three high affinity tyrosine kinase VEGFR (VEGFR1, VEGFR2 and VEGFR3) and co-receptors HS and NRP. Thereupon, the VEGF-C will be described in details:

2.2.1.1.1 VEGF-C

Vascular endothelial growth factor C (VEGF-C) was first discovered in 1996 as a factor capable of stimulate tyrosine phosphorylation of VEGFR-3(ref 68). It is abundantly expressed in spleen, lymph node, thymus, appendix, bone marrow, skeletal muscle, prostate, testis, placenta, heart, thyroid gland, ovary, colon and small intestine. In midgestation embryos is highly expressed in areas where the lymphatic vessels undergo sprouting from embryonic veins, including the perimetanephric, axillary, jugular areas and developing mesenterium, whereas in fetal tissue, expression occurs in liver, lung

and kidney (ref 69). Neither hypoxia nor oncogenes upregulate VEGF-C mRNA levels are rather IL-1 and TNF-α which increase it.

Both human and mouse VEGF-C genes span more than 40 Kb of DNA and are made up of seven exons. (ref 68). The VHD is encoded by exons 3 and 4, while exons 5 and 7 encode cysteine rich motifs. VEGF-C human gene is located on chromosome 4q34 and its cDNA encodes a protein of 419 aa residues (ref 68) (Figure 2.12)

VEGF-C present singular N- and C- terminal extensions flaking the VHD. The C- terminal domain of VECF-C, due to its cysteine –rich sequences (CX10CXCXC) evokes to protein component of silk , that´s why it has been termed as silk homology domain (SHD) (ref 87) . This SHD provides VEGF-C with most of its heparin affinity, as well as being a deciding factor for ECM sequestration (ref 39)

VEGF-C is newly synthesized as an inactive preproprotein (58 kDa) , requiring of two proteolytic cleavages to become biologically activated. These proteolytic processing occur in both the N- and C- terminal regions for biologically activation. These N- and C sequences are particularly long and act as propeptides that fold into their own domains (ref 39).

The first cleavage is executed by proprotein convertases (PC) like PC5, furin or PC7(Ref 39). They cleave between the C- terminal SHD and the VHD, concretely they cleave at the dibasic ArgArg 227 (HSIIRR227SL) (ref 89) , leading to a pro- VEGF-C peptide. Even if the cleavage is done, the SHD is not removed and stills covalently bound to the rest of pro- VEGF-C though cysteine bridges. Although the resulting pro- VEGF-C (29-31 kDa) is able to bind VEGFR-3, it cannot activate it (ref Jeltsch et al., 2014) Figure 2.13

The second proteolytic extracellular cleavage activates the protein and it happens after pro- VEGF-C secretion. It is made by many different enzymes as A disintegrin and metalloproteinase with thrombospondin motifs 3 (ADMTS3) (ref 39), plasmin (ref 88), thrombin, prostate-specific antigen / kallikrein-3 (PSA/KLK3) or cathepsin D (CatD),

resulting in non- covalent mature and active different five forms of VEGF-C. (ref 90)Figure 2.13.

In ADMTS3 cleavage (AHY^{114}NTE)(ref 90) , collagen and calcium binding EGF domains 1 (CCBE1) assistance is required ,since it enhances the VEGF-C activation by colocalizating VEGF-C and ADAMTS 3 on ECM and cell surface to create the trimeric activation complex and it increases the processivity of the ADMTS3 enzyme .From this cleavage result the named “major” form of mature VEGF-C. (ref 39). Figure 2.13

The called “minor” form is generated by plamin and thombin cleavage in LNSR ^{102} TEE aa, which is nine aa longer at its N- terminus compared with the “major “form (ref 39). The plasmin serin protease by itself can also cleaves pro- VEGF-C in EWR ^{127}KTQ aa , but in this case the produced form even if it is mature it is inactive.(ref 90) Figure 2.13

The PSA/KLKL3 serine protease complex cleaves in AHY^{114}NTEIL aa, resulting in maturated VEGF-C PSA/KLKL3 form of about 20 kDa. It has from N- terminal three aa residues less than the mature VEGF-C “major “form, but it stills binding VEGFR-2 and VEGFR-3. (ref 91) Figure 2.13

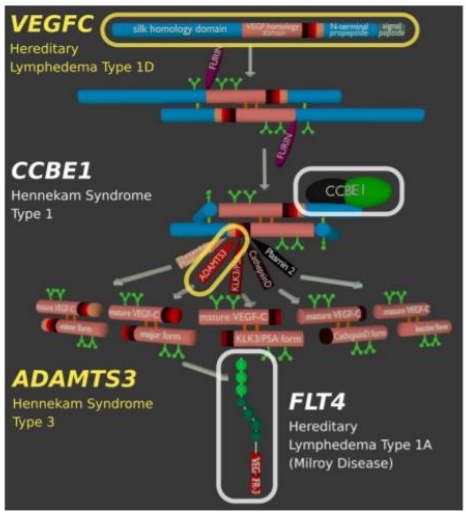
Cat D enzyme also contributes to VEGF-C maturation and activation via EIL^{119}KS aa cleavage, resulting in a VEGF-C form which resemble to the minor form of VEGF-D, thus it is called VEGF-C $_{DMH}$ (for D minor homology) (ref 90) Figure 2.13

All these VEGF-C mature form differ from each other at their N-terminus, since different proteases cleave at distinct positions between the NH_2 terminus (N-terminal) and the VHD (Ref 90)

However it should be mentioned that VEGF-C biosynthesis, enzymatic maturation and activation has been quite recently discover (ref Jeltsch et al., 2014; Roukens et al., 2015; Bui et al., 2016; Jha et al., 2017 ref 91). There are still some few points to clear up, so far most functions and receptors of VEGF-C are not distinguish between different VEGF-C

mature forms (ref 39). Hence, although the function of each different form will not be specified, herein a general explanation of the mature VEGF-C will be done.

Mature VEGF-C helped by NRP-1 or NRP-2 co-receptors but without heparin´s help (ref 40), binds either VEGFR-2 or VEGFR-3, which are characteristic for angiogenesis and lymphangiogenesis respectively, thus making VEGF-C a pivotal factor in these processes (ref 68). More concretely, it increases permeability, stimulates migration, and mitosis of endothelial cells, as well as, in embryogenesis it takes part in venous and lymphatic vascular systems formation, in addition to its role in adults' maintenance of differentiated lymphatic endothelium (ref 68). Beside these physiological processes, VEGF-C also intervenes in some pathologies, such as various cancers (CRC (ref 92), glioblastomas, haemangioblatomas (ref 93), lymphatic malformation 4 (ref 96), coronary arteria diseases (ref 95) , Milroy like primary lymphedema (ref 97) among others. Although much will not be specified now, since its role in cancer will be detailed later.



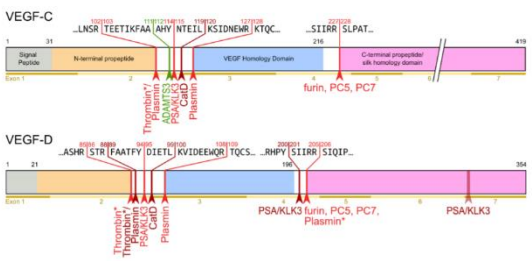


Figure 2.13:Ilustration of cleavage of VEGFC

2.2.2 VEGF receptors and co-receptors

All VEGF family members bind and are activated by one of the three homologous tyrosine kinase receptors: VEGFR-1, VEGFR-2 and VEGFR-3. (Ref 105) Figure 2.14. VEGF receptors have a similar structure; a seven tyrosine kinase immunoglobulin-like domains (IgD) in the extracellular domain , a single transmembrane helix, a juxtamembrane domain, a consensus tyrosine kinase sequence disrupted by a kinase insert domain and

a COOH-terminus (C terminal) tail (ref 105; ref 67, ref 49). Only VEGFR-3 present a distinct characteristic, it has a disulphide bridge within the fifth Ig-like loop, keeping the proteolytically cleaved N-terminal part tied to the C-terminal region(ref 108).

Furthermore, VEGFR are present as homo or heterodimers, specifically generated heterodimers are VEGFR1/VEGFR2 and VEGFR2/ VEGFR3.(Ref 108)

VEGFs bind to second and third IgD, activating the receptor *via* autophosphorylation of tyrosine residues , which either serve as binding site for downstream signalling molecules and permit full kinase activation. When VEGFs bind to their receptors conformational changes occur leading to dimers' rotation, which become crucial for kinase activation. Each ligand influence in a different fashion to receptor´s rotation degree affecting the activation level of the receptor (ref 108)

Moreover, there are co-receptors such as NRPs, HS and integrins that modulates VEGFR effect. These co-receptors are VEGF and VEGFR-binding cell –surface–expressed molecules (ref 49), which lack intrinsic catalytic activity (ref 107)

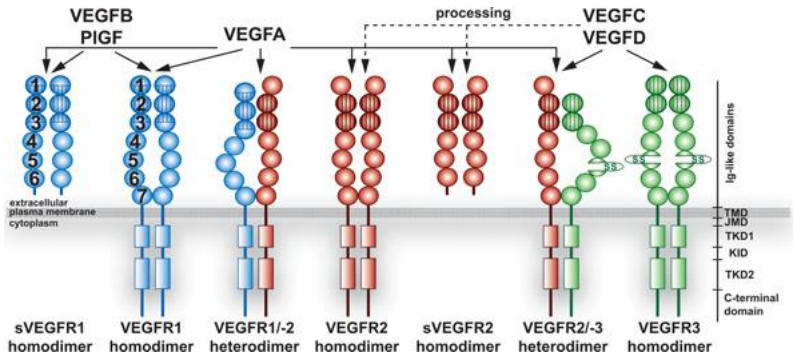


Figure 2.14: Illustration of VEGFs and their receptors

2.2.2.1 VEGFR-1

VEGFR-1 (also named Flt1 in the mouse) is a 180-185 kDa glycoprotein, which is activated when binding to VEGF-A, -B, and PlGF (ref 49) as well as the non-mammalian factor -F. Figure 2.14

Although mainly VEGFR-1 is relatively high expressed in vascular endothelial cells, both in development and in adulthood, there is as well an extensive range of non-endothelial cells, which express it, including monocytes, macrophages, human trophoblasts, renal mesangial cells, vascular smooth muscle cells, dendritic cells and different human tumour cell types (ref 49). In addition, VEGFR-1 expression is upregulated by hypoxia (ref 108).

Alternative splicing leads to the formation of soluble VEGFR-1 (sVEGFR-1), containing the N-terminal six extracellular IgD, which is high expressed in placenta figure 2.14. Furthermore, truncated intracellular VEGFR-1 variants also have been found.

VEGF-A, VEGF-B and PlGF ligands bind to receptor´s IgD 2, however IgD 1 and 3 are also need for high-affinity binding (ref 49). When ligands bind VEGFR-1, it can be phosphorylated on diverse tyrosine (Y) residues, such as Y1169, Y1213, Y1242, Y1309, Y1327, and Y1333. Both the phosphorylation pattern and tyrosine kinase activity are ligand dependent, giving rise to different interactions and downstream signalling.

Taking into account VEGFR-1 dependence on ligands and as mentioned before that it is presence in a broad range type of cells, it displays different biological functions in diverse cell types, and thus the exact function of this molecule is still under debate. (Ref 106)
In the case of VEGF-A, even if it binds with high affinity to VEGFR-1, the tyrosine kinase activity is weak (ref 108). VEGF-B does not interact with the VEGFR-1 immunoglobulin homology domain 3 (D3), therefore it does not effectively induce VEGFR -1 downstream signalling, but binding to VEGF-B promotes fatty-acid uptake in EC, which is crucial in organs with high metabolic stress as heart (ref 108, ref 49). On the other hand, binding of PlGF to VEGFR-1 implicates interaction with VEGFR-1 3D resulting in a strong tyrosine kinase activity. VEGFR-1/PlGF binding is pivotal for inflammation-associated angiogenesis in numerous diseases (ref 49). Although the signalling downstream of VEGFR-1 is still not completely understood, it has been proved to induce the ERK/MAPK and the PI3K/Akt-Rac1 pathways (ref 108, ref 52)

In ECs, VEGF-1 acts as a decoy receptor as its soluble form (sVEGFR-1) binds to VEGF-A, reducing VEGFR-2 signalling. Therefore, VEGFR-1 is considered as a negative angiogenesis regulator since it negatively regulates VEGFR-2 signalling. (Ref 108). However, it is associated with increased angiogenesis, by collecting bone marrow (BM) derived VEGFR-1 expressing macrophages, as well as it enhance hematopoietic cell migration and affect tumor metastasis. In fact, BM derived VEGFR-1 expressing macrophages supply a niche for metastasizing tumor cells (ref 108).

Furthermore, it shows to have a non-vascular role, as genetic deletion or systemic blocking of VEGFR-1 in peripheral sensory neurons seems to reduce tumor-induced nerve remodelling and cancer pain (ref 108)

Besides, previously mentioned sVEGFR-1´s decoy function, it is implicated in vascular maturation and in the maintenance of corneal avascularity, as well as, it regulates gestation since its overexpression is associated with pre-eclampsia. In addition, sVEGFR-1 is related with favourable prognosis cancer patients (ref 106). Moreover, VEGFR-1 can form a heterodimer with VEGFR-2 that has a strong mitogenic signal in ECs (ref 108)

2.2.2.2 VEGFR-2

Human VEGFR-2, also named kinase insert domain containing receptor (KDR) is the main receptor of VEGF (ref 45), as well as the best-characterized (ref 108). Its gene is positioned at chromosome locus 4q11-12. Mature VEGR-2 is a transmembrane glycoprotein with 230 kD molecular weight Figure 2.14. Despite there are other VEGFR-2 forms, only the mature glycosylated one can carry out the intracellular signal transduction (ref 45).

VEGFR-2 is mainly present in embryonic precursor cells, vascular EC, and lymphatic EC, circulating endothelial progenitor cells, pancreatic duct cells, retinal progenitor cells and megakaryocytes (ref 103) with a highest observed expression levels throughout physiological vasculogenesis and angiogenesis in addition to pathological angiogenic conditions such as cancer (ref 108)

VEGFR-2 is activated by binding to VEGF-A, - C, and -D, as well as binding to non-mammalian factors -E and –F. Moreover, neuropilin -1 (NRP-1) and NRP-2 enhances VEGFR-2 signalling, being crucial for some signalling pathway activation (ref 67, ref 108). The ligands bind to receptor´s IgD 2 and IgD3, this ligand –receptor interaction creates a VEGFR-2 homodimer or a VEGFR-2 heterodimer with either VEGFR-1 or VEGFR-3, provoking a phosphorylation of specific tyrosine residues, including Y801, Y951, Y1175 and Y1214 (Ref 45). Consequently , a diversity of signalling molecules bind to VEGFR-2 dimers activating downstream signalling pathways, which affect the physiological and pathological features of EC and the whole vascular environment (ref 45)

In particular, VEGFR-2 mediates angiogenesis, cardiovascular system, haematopoiesis, and embryonic development, as well as diseases such as rheumatoid arthritis, diabetic retinopathy, Alzheimer, coronary heart disease and several types of cancer.

The majority of these processes are arbitrated by diverse pathways, such as the ones mentioned bellow: PI3K/ AKT stimulates cell survival, endothelial nitric oxide synthase (eNOS) / nitric oxide (NO) and PI3K/ AKT activates cell permeability, mitogen-activated protein kinase kinase (MEK) /ERK encourages cell proliferation, and FAK/ Paxilin, PI3K/ Rac, and p38/ MAPK activated protein 2/3 pathways enhance cell migration (ref 45)

In addition, as in VEGFR-1, alternative splicing of VEGFR-2 gives rise to a soluble form (sVEGFR-2). In this case, sVEGFR-2 competes with VEGFR-3 for VEGF-C binding, resulting in an inhibition of lymphatic EC proliferation. (Ref 108)

2.2.2.3 VEGFR-3

VEGFR-3, alternatively named Fms related tyrosine kinase 4 (Flt4 in the mouse) is encoded by Flt4 gene, resulting in a 1363 aa protein (ref 108) Figure 2.14
VEGFR-3 is present in both lymphatic and blood vascular ECs during early embryogenesis and development, although during adulthood it is almost exclusively limited to lymphatic ECs. In addition, in post-partum it regulates the conversion of tip cells to stalk cells during angiogenic sprouting in the retina via Notch signalling. (ref 108)

VEGFR-3 is not only present in ECs, it is also expressed in non – endothelial cells, such as neural progenitor cells, macrophages, and osteoblast but its role is less understood (ref 108).

VEGFR-3 activates by binding to VEGF-C and –D ligands. When binding to unprocessed VEGF-C and –D ligands, VEGFR-3 forms homodimers resulting in an autophosphorylation of Y1230, Y1231, Y1265, Y1337, and Y1363 tyrosine residues activating ERK1/2 signalling pathway thus stimulating RAS activity and mitogenic signalling.

Being both VEGF-C and –D, VEGFR-2 and -3 ligands, it is noteworthy that VEGF- C shows higher affinity to VEGFR-3 than to VEGFR-2, while VEGF-D presents similar affinity for both receptors (ref 49)

However, if VEGFR-3 binds to the proteolytic processed forms of these ligands the affinity to both VEGFR-2 and -3 receptors increases inducing VEGFR-2 / VEGFR-3 heterodimers and phosphorylating Y1230, Y1231, and Y1265 among others . These tyrosine residues phosphorylation provokes PI3K/AKT activation, which is related with lymphendothelial proliferation, migration and survival (ref 49) thus it is pivotal for lymphatic development (ref 108)

As in VEGFR-1 and -2, there is also a soluble form of VEGFR-3 (sVEGFR-3), which inhibits VEGF-C/D binding thus impeding its signalling pathways (ref 108)

As mentioned before, VEGFR-3 is crucial for both angiogenesis and lymphangiogenesis processes, therefore a deregulation or mutation involves human diseases such as lymphedema or cancer. In fact, the axis VEGFR-3/VEGF-C has a crucial role in the TME since it promotes the formation of new lymphatic vessels from pre-existing ones, as well as lymphatic endothelial destabilization causing endothelial sprouting, leaking and enlargement of the vessels, which promotes the entrance of tumour cells into the lymphatics, followed by dissemination, and ending in metastasis. (Ref 106)

2.2.2.4 Neuropilins (NRPs)

Neuropilins (NRPs) are 130 kDa small cytoplasmic tailed transmembrane proteins .They were first identified as neuronal receptors for class 3 semaphorins that guide axon factors, which function in nervous system development (ref 140). NRPs mainly act as co-receptors for the reason that their lack of intrinsic catalytic function. (Ref 140)

There are two NRP homologues, NRP-1 and NRP-2, that show 44% homology at the amino acid level and which can form either homo- or hetero-multimers. (Ref 49)(Ref 140) figure 2.14. They are expressed in both endothelial and non-endothelial cells, including smooth muscle cells, neurons, epithelial cells and immune cells (Ref 106).

Concerning VEGF family members, NRP-1 binds VEGF-A, VEGF-B, PlGF and VEGF-E, while NRP-2 binds VEGF-A and VEGF-C. In addition, NRP-1 plays as a VEGFR-1 and VEGFR-2 co-receptor, while NRP-2 only acts as VEGFR-3 co-receptor (ref 108). NRP-1 is pivotal in VEGFR-2 signalling since it increases endothelial cells migration, survival, and permeability and enhance VEGFR-1/ VEGFR-2 heterodimer formation (ref 49) (ref 108). However, NRP-2 is crucial in lymphatic ECs (ref 103).

Furthermore, both NRPs have soluble forms, which sequester VEGF (ref 49). In fact, although NRPs have always been classified as co- factors, it has been shown that they can act as VEGF receptors and that they are expressed in tumour cells (Ref 140)

Indeed, an increased expression of NRP is associated with several cancers, such as leukaemia, lymphoma and with metastasis of solid tumours (Ref 106). Besides its role in cancer, NRPs are mainly involved in immunology, neural development and angiogenesis (ref 103)

2.2.2.5 Heparin sulfate (HS)

Heparin sulfate (HS) belongs to the glycosaminoglycan family of carbohydrates.
It modulates VEGF biology by binding to VEGFs, VEGFRs as well as co-receptors, including NRP-1 but not NRP-2 (ref 49, ref 106). Its interaction with VEGFR-2 increases its signalling amplitude and duration (ref 108). Moreover, it works as a reservoir for

growth factors, controlling their liberation causing growth factor gradients (Ref 499).HS may provoke tumour growth as it augments VEGF biology (ref 106)

2.2.2.6 Integrins

Integrins area a family of more than 20 transmembrane heterodimers. They are cell surface extracellular matrix (ECM) receptor (ref 140). They regulate cell-matrix adhesions though specific binding to extracellular matrix components, including fibronectin, vitronectin, collagen and laminin. Furthermore, they can bidirectionaly transmit signalling information. They are pivotal for some VEGFR axes activation (ref 49)

2.3. VEGF-C maturation and cancer

2.3.1 Convertases

The mamalian subtilisis/ kexin-like secretory proprotein convertases (PC) family are proteases implicated in the activation or inactivation of a wide spectrum of immature precursor proteins such as proteases, cytokines, neuropeptides, growth factors (GFs) , growth factor receptors (GFRs) , integrins, matrix metalloproteinases, enzymes, and even toxins and glycoproteins from infectious retroviruses. They cleave at single or paired basic residues, thus bioavailing these dormant molecules. (Ref geraldin 2020, hannu turpeinen 2011, nabil G. Seidah 2012 ; nabil G Seidah 2002, bradley k mccoll 2017).

As of today, this protease family is composed of nine PC: PC1/PC3, PC2, Furin, PC4, PC5, PACE4, PC7, subtilisin-kexin-like isozyme-1 (SKI-1) and neural apoptosis-regulated convertase-1 (NARC-1). They are classified according to their substrate cleavage site. PC1/PC3, PC2, furin, PC4, PC5, PACE4 and PC7, rather, seven of the nine PC are aa specific and they are structurally and biochemically similar to each other, and to the yeast and bacterial proteins from which they were probably derived (kexin and subtilisin respectively).[ref:geraldin 2020 [19], [20], [21]; Andrew w. Artenstein 2011). The remaining two SKI-1 also called site -1 protease (S1P) and NARC-1PC are different from the other seven due to their distinct domain structures and they are non-basic amino acid-specific convertases, (REF- geraldin 2020 n22; Andrew w. Artenstein 2011) Figure 2.15

The majority of PCs (PC1/PC3, PC2, furin, PC4, PC5, PACE4 and PC7) cleave immediately after the consensus sequence (K/R) -(X) n- (K/R) ↓, where X is any aa except Cys and n equals where n = 0, 2, 4 or 6 residues. SK1 cleaves in the consensus motif (R/K)-X-(L, I, V)-Z ↓, where Z is any aa except Pro, Cys, Glu, and Val. In what concerns NARC-1, up to the present it is only known that it cleaves itself once at the motif VFAQ↓SIP, with Val at P4, and it has not been identified yet any compound as its substrate (ref: N.G. Seidah, 2019),ref geraldine siegfried 2020 ,22;).figure 2.15

These proteases are involved in the regulation of a variety of cellular functions, such as, cellular growth, adhesion, differentiation, cell-to-cell communications and endocrine/ paracrine functions (ref; Nabil G Seidah 2002). In fact, most angiogenesis and lymphangiogenesis factors must be bioactivated by the cleavage of the PC, turning these proteins, essential in the initiation, promotion and progression of cancer (ref : PMID: 8334671).

Although, PC are not only involve in cancer. There are many other diseases where they are pivotal. Their pathogenicity covers hormones and endocrinopathies, infectious illnesses caused by bacterial toxins or viral infections, lipid disorders, cardiovascular diseases, osteoarthritis, atherosclerosis and neurodegerative diseases amongst the most noted. (Ref 49). However, here we are going to go in depth in PC role in cancer.

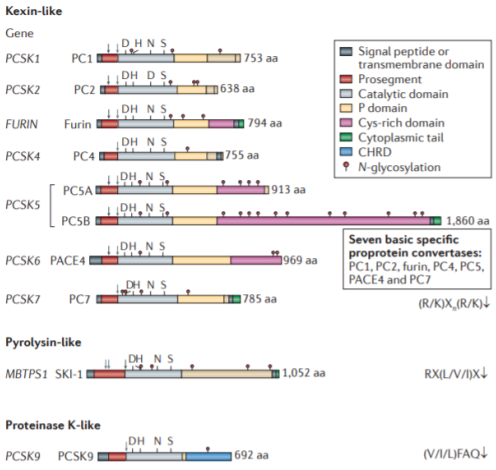


Figure 2.15: illustration of all converstases

2.3.2 PC inhibitors

In view the wide range of pathological processes where PC are involved, they may represent a therapeutic target, since they can be suppress by the use of PC inhibitors (ref 79). Potent inhibitors have been successfully tested, (ref 79, ref 84, ref 85, ref 86), giving rise to become promising treatments or adjuvant therapies for multiples diseases such as cancer (ref 80, ref 83). Assorted strategies have been studied and developed to inhibit PCs *in vivo* and *in vitro*, starting from small molecules to antibodies and proteins aimed against PC (ref 80).

2.3.2.1 Small molecules

Small molecules by competitive inhibition, block accessibility of substrates to enzyme´s catalytic cleft; however, they do not offer specificity towards a particular PC. Those inhibitors are chloromethylketones (for example decanoyl-R-V-K-R-chloromethylketone- CMK), poly-arginine derivatives, and streptamine derivatives (Ref 80)

2.3.2.2 Peptidomimetics

Peptidomimetics are compounds that mimic natural peptides, which possess the capacity to interact with the biological target and provoke the same biological result (ref 81). In this case, they mimic the PC recognition side, enabling interactions that are more specific. This include Alpha-1-antitrypsin and derivatives such as Alpha-1-antitrypsin Portland (PDX), which is a bioengineered variant containing PCs recognition sequence (ref 80).

2.3.2.3 Antibodies

Concerning antibodies, nano-bodies are variable domains of antibodies´ heavy chain-only (ref 82). They target the P domain of PC, which has been show in mutagenesis experiments to be important for the efficient cleavage of PC, in addition to their activity site (Ref 80).

2.3.3 Pc substrates in tumour growth and metastasis

Once seen the presence of PC in cancer, it becomes relevant to elucidate their substrates in cancer, whereby grow factors, metalloproteinases, adhesion molecules, chemokines amongst others are found.

2.3.3.1 Metalloproteinases

Metalloproteinases, highly homologous Zn (++)-endopeptidases (ref 73), intervene in a cascade of proteolytic events, which lead cell migration and ECM degradation, facilitating metastasis (ref 48). This cascade needs to be activated by PCs. Precisely PCs cleaves stromelysin group (str-1, str-2, str-3), membrane-type matrix metalloproteinases family (MT1-MMPs, MT2-MMPs, MT3-MMPs, MT4-MMPs, MT5-MMPs)(ref 63,ref 48), matrix metalloproteinase (MMP-1, MMP-2, MMP-8, MMP-9, MMP-13) and the adamalysin metalloproteinases (ADAM1, ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAMTS1, ADAMTS2, ADAMTS3, ADAMTS4, ADAMTS5, ADAMTS13).(ref 48).

2.3.3.2 Cell adhesion molecules

Cell adhesion molecules are cell surface proteins, which mediate cell- ECM, or cell-cell interactions. They are classified in four families: immunoglobulin-like adhesion molecules, integrins, cadherins and selectins (ref 70). Likewise, they mediate adhesion; they act as tumor suppressors since they restrict cell growth by contact inhibition (Ref 71). Thus, aberrant function can lead to development of cancer, even more if they are in charge of diversity functions as cell growth, signal transduction, site-specific gene expression, inflammation, differentiation, morphogenesis, immunologic function, wound healing, and cell motility (ref 72). Even though not many cell adhesion molecules are cleaved directly by PC, these molecules need directly or indirectly PC activity as they activate their above inductors, such as cytokines and growth factors (GF). Up to day, it is know that a total of integrin´s 9 of 18 known α-subunits are cleaved by PC. (ref 48, Ref 62).

2.3.3.3 Growth factors (GF)

GF are polypeptides that bind to cell membrane receptors stimulating cell proliferation (ref 74). They are pivotal for cell differentiation and cell division, since they mediate cell progression in the cell cycle (ref 48). They are classified in platelet-derived growth factors family (PDGF), vascular endothelial growth factor family (VEGF) (chapter 2.3.1), epidermal growth factor family (EGF), fibroblast growth factor family (FGF), insulin-like growth factors (IGF), hepatocyte growth factor, neurotrophin family, TGF, and Ang (ref 75). Many of this GF are synthetize as proproteins that must be activated by PC. For instance: PDGF are cleaved by PC5, PACE4, and PC7 (ref 56), VEGF is processed by furin, PC5 and PC7 (ref 53, ref 54), EGF is activated by PCS9 (ref 76), FGF is cleavage by PC5 , PACE4 and PC7 (ref 55). Also IGF are processed by furin and PC5 (ref 60), hepatocyte growth factor is activated by furin (ref 77) , PC5 is involved in neurotrophin family activation (ref 78), furin, PC5, PC6 are responsible of the cleavage of angiopoetins (ref 57), and TGF is activated by Furin (ref 61).

2.4 VEGF-C and convertases in cancer

Considering as aforementioned the crucial role of growth factors in cancer and since the need to be processed by convertases to be bioactivated, among all the previously named growth factors, this present work has been focused on the role of specifically VEGF-C and convertases in cancer, which will be better detailed below.

2.4.1 VEGF-C and cancer

As mentioned in chapter 2.3.1.3, due to VEGF-C involvement in the regulation of physiological and pathological angiogenesis and lymphangiogenesis, it is present in a large range of different human types of cancer. Some of those cancer are breast carcinoma, acute myeloid leukaemia, papillary thyroid carcinoma, non-small cell lung cancer, prostate cancer, cervical carcinoma, ovarian carcinoma, bladder cancer, as well as in gastrointestinal malignancy including oesophageal carcinoma and CRC among others. (Ref 110) (table 2.4)

In fact, VEGF-C promotes tumour-associated angiogenesis and lymphangiogenesis, immune tolerance and tumour cells proliferation, invasion and migration (ref 141). VEGF-C is pivotal in tumour lymphangiogenesis inducing the creation of additional lymphatic vessels that supply routes by which tumour cells enhance metastasis to distal tissue, as well as it induces angiogenesis sprouts.

In addition, it has been shown that in breast and gallbladder cancer besides VEGF-C´s paracrine mechanism, it also acts as an autocrine growth factor (ref 141) (ref 142) (ref 144). Moreover, it has been reported that as well as promoting leukemic cells´ proliferation, VEGF-C may promote leukemic cell survival via inhibition of apoptosis (ref 143)

Patients in advanced stages with lymph node metastasis, lymphatic invasion, distal metastasis, and poor prognosis, indeed, show high expression of VEGF-C (ref 110)(ref 144), while a downregulation of VEGF-C expression reduces tumour growth and metastasis (ref 113)

It has been demonstrated that downregulation of VEGF-C in cancer cells, which express its receptor VEGFR-3, attenuates paracrine regulation of lymphangiogenesis, diminishing tumour drainage and spread of cancer cells. In addition, this downregulation inhibits autocrine signal that is in charge of cancer cells proliferation and acquisition of fibroblast-like morphology, as well as it enhances CSC motility and characteristics, which increases tumour initiation ability and drug resistance (ref 113) (ref 140) (ref 141)
Specifically, in human CRC, VEGF-C is present in cytoplasm of CRC cells. It has been demonstrated to be highly expressed in the primary tumour of patients with either lymphatic involvement or lymph nodes, as well as it has been reported to not be involved in hematogenous metastasis , including liver metastasis. Moreover it exist a correlation between VEGF-C and tumor depth. (ref 115)

2.4.2 VEGF-C targeted anti-cancer therapies

Considering the crucial role of VEGF-C in tumorigeneses, cancer progression and metastasis, together with its high expression in a variety of malignancies, there are

VEGF-C targeted therapies, which might improve current cancer therapies. These therapies are monoclonal antibodies, IgG fusion proteins or soluble receptor protein, multi-kinase inhibitors, and RNA interference (ref 110).

- Monoclonal antibody:

This therapy consist in human monoclonal antibodies that display a high affinity and specificity to VEGF-C , its receptors and co-receptors, thus blocking their function and therefore stopping tumor progression and metastasis. In fact, it has been shown that a combined use of antibodies blocking both the receptors and the ligand improves therapeutic results (Ref 110).

- IgG fusion proteins or soluble receptor protein:

As mentioned before, there is a soluble VEGFR-3 (sVEGFR-3), which competes with VEGFR-3 to trapped VEGF-C avoiding VEGFR-3 signal pathway activation. On this basis, this therapy lies in a new receptor-immunoglobulin (Ig) fusion protein, which could bind VEGF-C reducing tumor growth and metastasis (ref 110).

- Multi-kinase inhibitors

When VEGF-C binds to VEGFRs, a tyrosine kinase phosphorylation cascade is activated giving rise to lymphangiogenesis and tumor metastasis among other processes. There are small molecules, named multi-kinase inhibitors, which block tyrosine kinases. Despite their adverse side effects, their ability to inhibit several kinases result in a decrease in tumor growth and metastasis (ref 110). There are multi-kinase inhibitors which block VEG-C receptor phosphorylation pathways, such as cediranib, E1080, sunitinib (ref 110), PKC412 (N-benzoylstaurosporine) (Ref 116) and regorafenib (ref 117). In fact, PKC412 and regorafenib have been used in pre-clinical trials in combination with other anti-colorectal cancer therapies with good results (ref 116, ref 117)

- RNA interference

RNA interference (RNAi) appears as an excellent tool to silence successfully specific genes, becoming a powerful promise concerning cancer therapy (ref 110). Researches

have shown that RNA-mediated knockdown of VEGF-C gives rise to a significant inhibition of cancer progression (ref 118)

2.4.3 VEGF-C and convertases

Should not be ignored, that as mentioned before VEGF-C is newly synthesized as an inactive preproprotein, in need of two proteolytic cleavages to be biologically activated. This first cleavage is made by PC5, furin or PC7 (Ref 39). They cleave between the C-terminal SHD and the VHD, concretely at the dibasic ArgArg 227 (HSIIRR227SL) (ref 89) , resulting in a pro- VEGF-C peptide. Therefore, this first cleavage becomes crucial and indispensable for VEGF-C activation.

In view of the role of convertases in VEGF-C activation, the above-mentioned PC inhibitors might be a useful and powerful tool in cancer therapies, at least in combination with other therapies or as adjuvant therapy.

Indeed, PC inhibitors have been the centre of numerous clinical trials, confirming their potential clinical application (Ref 123, ref 124, ref 125, ref 126). As concerns cancer, actually there are (ref 122) several terminated or complete clinical trial based on PC.
One of them lies in immunohistochemistry diagnostic test, which supports that PACE4-FL isoform is a good thyroid malignancy biomarker,(ref 102).The others involve a cellular vaccine formerly named FANG but now called VIGIL (ref 80). The vaccine consists in a bidirectional small hairpin RNA interference targeting furin, that alone (ref 105, ref 108) or combine with other drugs, such as Carboplatinum, (ref 103) Bevacizumab (104), Durvalumab, Temozolomide and Irinotecan (ref 106), or Pembrolizumab (ref 107) have obtained good results as a cancer therapy. Incredible as it may seem there are no clinical trials concerning anti-cancer therapies that involve convertases inhibitors and VEGF-C.
Through this work, it has been aimed to elucidate the role in colorectal cancer of the resulting C-terminal from the cleavage of VEGF-C by the convertases, since nobody has considered it and it could be the key of tumor progression and metastasis.

3- HIPOTHESIS AND OBJETIVES

Sujeto a confidencialidad por la autora

4- EXPERIMENTAL PROCEDURES

Sujeto a confidencialidad por la autora

5- RESULTS

Sujeto a confidencialidad por la autora

6- DISCUSSION

Sujeto a confidencialidad por la autora

7- CONCLUSION

Sujeto a confidencialidad por la autora

8- BIBLIOGRAPHY

8. BIBLIOGRAPHY

Abhinand, C. S., Raju, R., Soumya, S. J., Arya, P. S., & Sudhakaran, P. R. (2016). VEGF-A/VEGFR2 signaling network in endothelial cells relevant to angiogenesis. *Journal of Cell Communication and Signaling*, *10*(4), 347. https://doi.org/10.1007/S12079-016-0352-8

AJCC Cancer Staging Manual. (2017). *AJCC Cancer Staging Manual*. https://doi.org/10.1007/978-3-319-40618-3

Akagi, K., Ikeda, Y., Miyazaki, M., Abe, T., Kinoshita, J., Maehara, Y., & Sugimachi, K. (2000a). Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. *British Journal of Cancer*, *83*(7), 887–891. https://doi.org/10.1054/BJOC.2000.1396

Akagi, K., Ikeda, Y., Miyazaki, M., Abe, T., Kinoshita, J., Maehara, Y., & Sugimachi, K. (2000b). Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. *British Journal of Cancer*, *83*(7), 887–891. https://doi.org/10.1054/BJOC.2000.1396

Akwii, R. G., Sajib, M. S., Zahra, F. T., & Mikelis, C. M. (2019). Role of Angiopoietin-2 in Vascular Physiology and Pathophysiology. *Cells*, *8*(5), 471. https://doi.org/10.3390/CELLS8050471

Baeriswyl, V., & Christofori, G. (2009). The angiogenic switch in carcinogenesis. *Seminars in Cancer Biology*, *19*(5), 329–337. https://doi.org/10.1016/J.SEMCANCER.2009.05.003

Bafico, A., & Aaronson, S. A. (2003). *Classification of Growth Factors and Their Receptors*. https://www.ncbi.nlm.nih.gov/books/NBK12423/

Baldanzi, G., & Graziani, A. (2015). Physiological Signaling and Structure of the HGF Receptor MET. *Biomedicines*, *3*(1), 1. https://doi.org/10.3390/BIOMEDICINES3010001

Bartoschek, M., & Pietras, K. (2018). PDGF family function and prognostic value in tumor biology. *Biochemical and Biophysical Research Communications*, *503*(2), 984–990. https://doi.org/10.1016/J.BBRC.2018.06.106

Bassi, D. E., Fu, J., de Cicco, R. L., & Klein-Szanto, A. J. P. (2005). Proprotein convertases: "Master switches" in the regulation of tumor growth and progression. *Molecular Carcinogenesis*, *44*(3), 151–161. https://doi.org/10.1002/MC.20134

Bassi, D. E., Zhang, J., Cenna, J., Litwin, S., Cukierman, E., & Klein-Szanto, A. J. P. (2010). Proprotein convertase inhibition results in decreased skin cell proliferation, tumorigenesis, and metastasis. *Neoplasia (New York, N.Y.)*, *12*(7), 516–526. https://doi.org/10.1593/NEO.92030

Bergers, G., & Benjamin, L. E. (2003). Tumorigenesis and the angiogenic switch. *Nature Reviews Cancer 2003 3:6*, *3*(6), 401–410. https://doi.org/10.1038/nrc1093

Birkedal-Hansen, H., Moore, W. G. I., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A., & Engler, J. A. (1993). Matrix metalloproteinases: a review. *Critical Reviews in Oral Biology and Medicine : An Official Publication of the American Association of Oral Biologists*, *4*(2), 197–250. https://doi.org/10.1177/10454411930040020401

Blobe, G. C., Schiemann, W. P., & Lodish, H. F. (2009). Role of Transforming Growth Factor β in Human Disease. *Http://Dx.Doi.Org/10.1056/NEJM200005043421807*, *342*(18), 1350–1358. https://doi.org/10.1056/NEJM200005043421807

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *68*(6), 394–424. https://doi.org/10.3322/CAAC.21492

Chau, K., Hennessy, A., & Makris, A. (2017). *Placental growth factor and pre-eclampsia*. *31*. https://doi.org/10.1038/jhh.2017.61

Chen, J.-C., Chang, Y.-W., Hong, C.-C., Yu, Y.-H., & Su, J.-L. (2013). The Role of the VEGF-C/VEGFRs Axis in Tumor Progression and Therapy. *Int. J. Mol. Sci*, *14*, 88–107. https://doi.org/10.3390/ijms14010088

Chen, R., Lee, C., Lin, X., Zhao, C., & Li, X. (2019). Novel function of VEGF-B as an antioxidant and therapeutic implications. *Pharmacological Research*, *143*, 33–39. https://doi.org/10.1016/J.PHRS.2019.03.002

Chrétien, M., Seidah, N. G., Basak, A., & Mbikay, M. (2008). Proprotein convertases as therapeutic targets. *Http://Dx.Doi.Org/10.1517/14728222.12.10.1289*, *12*(10), 1289–1300. https://doi.org/10.1517/14728222.12.10.1289

Chubak, J., Kamineni, A., Buist, D. S. M., Anderson, M. L., & Whitlock, E. P. (2015). Aspirin Use for the Prevention of Colorectal Cancer. *Evidence Synthesis*, *133*, i–116. https://www.ncbi.nlm.nih.gov/books/NBK321661/

Claesson-Welsh, L. (2012). Signal transduction by vascular endothelial growth factor receptors. *Vascular Pharmacology*, *56*(5–6), 308. https://doi.org/10.1016/J.VPH.2011.08.009

Claesson-Welsh, L., & Welsh, M. (2013). VEGFA and tumour angiogenesis. *Journal of Internal Medicine*, *273*(2), 114–127. https://doi.org/10.1111/JOIM.12019

Colorectal Cancer Statistics | How Common Is Colorectal Cancer? (n.d.). Retrieved January 26, 2022, from https://www.cancer.org/cancer/colon-rectal-cancer/about/key-statistics.html

Compton, C. C., & Greene, F. L. (2004). The Staging of Colorectal Cancer: 2004 and Beyond. *CA: A Cancer Journal for Clinicians*, *54*(6), 295–308. https://doi.org/10.3322/CANJCLIN.54.6.295

de La Chapelle, A. (2004). Genetic predisposition to colorectal cancer. *Nature Reviews. Cancer*, *4*(10), 769–780. https://doi.org/10.1038/NRC1453

Denaro, N., Russi, E. G., & Merlano, M. C. (2018). Pros and Cons of the New Edition of TNM Classification of Head and Neck Squamous Cell Carcinoma. *Oncology*, *95*(4), 202–210. https://doi.org/10.1159/000490415

Dubois, C. M., Blanchette, F., Laprise, M. H., Leduc, R., Grondin, F., & Seidah, N. G. (2001). Evidence that Furin Is an Authentic Transforming Growth Factor-β1-Converting Enzyme. *The American Journal of Pathology*, *158*(1), 305. https://doi.org/10.1016/S0002-9440(10)63970-3

Duffy, A. M., Bouchier-Hayes, D. J., & Harmey, J. H. (2013). *Vascular Endothelial Growth Factor (VEGF) and Its Role in Non-Endothelial Cells: Autocrine Signalling by VEGF*. https://www.ncbi.nlm.nih.gov/books/NBK6482/

Elsir, T., Smits, A., Lindström, M. S., & Nistér, M. (2012). Transcription factor PROX1: its role in development and cancer. *Cancer and Metastasis Reviews 2012 31:3*, *31*(3), 793–805. https://doi.org/10.1007/S10555-012-9390-8

Fagiani, E., & Christofori, G. (2013). Angiopoietins in angiogenesis. *Cancer Letters*, *328*(1), 18–26. https://doi.org/10.1016/J.CANLET.2012.08.018

Feng, Y., Hu, J., Ma, J., Feng, K., Zhang, X., Yang, S., Wang, W., Zhang, J., & Zhang, Y. (2011). RNAi-mediated silencing of VEGF-C inhibits non-small cell lung cancer progression by simultaneously down-regulating the CXCR4, CCR7, VEGFR-2 and VEGFR-3-dependent axes-induced ERK, p38 and AKT signalling pathways. *European Journal of Cancer (Oxford, England : 1990)*, *47*(15), 2353–2363. https://doi.org/10.1016/J.EJCA.2011.05.006

Ferrara, N., Gerber, H. P., & LeCouter, J. (2003). The biology of VEGF and its receptors. *Nature Medicine*, *9*(6), 669–676. https://doi.org/10.1038/NM0603-669

Fradet, L., Temmar, R., Couture, F., Belzile, M., Fortier, P. H., & Day, R. (2018). Evaluation of PACE4 isoforms as biomarkers in thyroid cancer. *Journal of Otolaryngology - Head & Neck Surgery = Le Journal d'oto-Rhino-Laryngologie et de Chirurgie Cervico-Faciale*, *47*(1). https://doi.org/10.1186/S40463-018-0311-X

Gabrilovich, D. I., & Dikov, M. M. (2003). Vascular endothelial growth factor. *The Cytokine Handbook*, 1017–1034. https://doi.org/10.1016/B978-012689663-3/50048-X

Gastrointestinal tract 5: the anatomy and functions of the large intestine | Nursing Times. (n.d.). Retrieved January 26, 2022, from https://www.nursingtimes.net/clinical-archive/gastroenterology/gastrointestinal-tract-5-anatomy-functions-large-intestine-23-09-2019/

Gaudet, D., Langslet, G., Gidding, S. S., Luirink, I. K., Ruzza, A., Kurtz, C., Lu, C., Somaratne, R., Raal, F. J., & Wiegman, A. (2018). Efficacy, safety, and tolerability of evolocumab in pediatric patients with heterozygous familial hypercholesterolemia: Rationale and design of the HAUSER-RCT study. *Journal of Clinical Lipidology*, *12*(5), 1199–1207. https://doi.org/10.1016/J.JACL.2018.05.007

Global Cancer Observatory. (n.d.). Retrieved January 27, 2022, from https://gco.iarc.fr/

Gordon, K., Schulte, D., Brice, G., Simpson, M. A., Roukens, M. G., van Impel, A., Connell, F., Kalidas, K., Jeffery, S., Mortimer, P. S., Mansour, S., Schulte-Merker, S., & Ostergaard, P. (2013). Mutation in vascular endothelial growth factor-c, a ligand for vascular endothelial growth factor receptor-3, is associated with autosomal dominant milroy-like primary lymphedema. *Circulation Research*, *112*(6), 956–960. https://doi.org/10.1161/CIRCRESAHA.113.300350

Guérit, E., Arts, F., Dachy, G., Boulouadnine, B., & Demoulin, J. B. (2021). PDGF receptor mutations in human diseases. *Cellular and Molecular Life Sciences 2021 78:8*, *78*(8), 3867–3881. https://doi.org/10.1007/S00018-020-03753-Y

Guren, M. G. (2019a). The global challenge of colorectal cancer. *The Lancet. Gastroenterology & Hepatology*, *4*(12), 894–895. https://doi.org/10.1016/S2468-1253(19)30329-2

Guren, M. G. (2019b). The global challenge of colorectal cancer. *The Lancet. Gastroenterology & Hepatology*, *4*(12), 894–895. https://doi.org/10.1016/S2468-1253(19)30329-2

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(5), 646–674. https://doi.org/10.1016/J.CELL.2011.02.013/ATTACHMENT/3F528E16-8B3C-4D8D-8DE5-43E0C98D8475/MMC1.PDF

Hardy, R. G., Meltzer, S. J., & Jankowski, J. A. (2000). ABC of colorectal cancer: Molecular basis for risk factors. *BMJ : British Medical Journal*, *321*(7265), 886. https://doi.org/10.1136/BMJ.321.7265.886

Hayes Ryan, D., McCarthy, F. P., O'Donoghue, K., & Kenny, L. C. (2018). Placental growth factor: A review of literature and future applications. *Pregnancy Hypertension*, *14*, 260–264. https://doi.org/10.1016/J.PREGHY.2018.03.003

Hoeben, A., Landuyt, B., Highley, M. S., Wildiers, H., van Oosterom, A. T., & de Bruijn, E. A. (2004). Vascular Endothelial Growth Factor and Angiogenesis. *Pharmacological Reviews*, *56*(4), 549–580. https://doi.org/10.1124/PR.56.4.3

Holmes, D. I. R., & Zachary, I. (2005). The vascular endothelial growth factor (VEGF) family: angiogenic factors in health and disease. *Genome Biology*, *6*(2), 209. https://doi.org/10.1186/GB-2005-6-2-209

Iyer, S., & Acharya, K. R. (2011). Tying the knot: The cystine signature and molecular-recognition processes of the vascular endothelial growth factor family of angiogenic cytokines. *The Febs Journal*, *278*(22), 4304. https://doi.org/10.1111/J.1742-4658.2011.08350.X

Jenny, B., Harrison, J. A., Baetens, D., Tille, J. C., Burkhardt, K., Mottaz, H., Kiss, J. Z., Dietrich, P. Y., de Tribolet, N., Pizzolato, G. P., & Pepper, M. S. (2006). Expression

and localization of VEGF-C and VEGFR-3 in glioblastomas and haemangioblastomas. *The Journal of Pathology*, *209*(1), 34–43. https://doi.org/10.1002/PATH.1943

Jha, S. K., Rauniyar, K., Chronowska, E., Mattonet, K., Maina, E. W., Koistinen, H., Stenman, U. H., Alitalo, K., & Jeltsch, M. (2019). KLK3/PSA and cathepsin D activate VEGF-C and VEGF-D. *ELife*, *8*. https://doi.org/10.7554/ELIFE.44478

Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N., & Alitalo, K. (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO Journal*, *15*(2), 290–298. https://doi.org/10.1002/J.1460-2075.1996.TB00359.X

Kastrinos, F., & Syngal, S. (2011). Inherited Colorectal Cancer Syndromes. *Cancer Journal (Sudbury, Mass.)*, *17*(6), 405. https://doi.org/10.1097/PPO.0B013E318237E408

Keum, N. N., & Giovannucci, E. (2019a). Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. *Nature Reviews. Gastroenterology & Hepatology*, *16*(12), 713–732. https://doi.org/10.1038/S41575-019-0189-8

Keum, N. N., & Giovannucci, E. (2019b). Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. In *Nature Reviews Gastroenterology and Hepatology* (Vol. 16, Issue 12, pp. 713–732). Nature Research. https://doi.org/10.1038/s41575-019-0189-8

Khatib, A. M., Siegfried, G., Chrétien, M., Metrakos, P., & Seidah, N. G. (2002). Proprotein Convertases in Tumor Progression and Malignancy: Novel Targets in Cancer Therapy. *The American Journal of Pathology*, *160*(6), 1921–1935. https://doi.org/10.1016/S0002-9440(10)61140-6

Khromova, N., Kopnin, P., Rybko, V., & Kopnin, B. P. (2011). Downregulation of VEGF-C expression in lung and colon cancer cells decelerates tumor growth and inhibits metastasis via multiple mechanisms. *Oncogene 2012 31:11*, *31*(11), 1389–1397. https://doi.org/10.1038/onc.2011.330

Kim, D. H., Xu, W., Kamel-Reid, S., Liu, X., Jung, C. W., Kim, S., & Lipton, J. H. (2009). Clinical relevance of vascular endothelial growth factor (VEGFA) and VEGF receptor (VEGFR2) gene polymorphism on the treatment outcome following imatinib therapy. *Annals of Oncology*, *21*(6), 1179–1188. https://doi.org/10.1093/annonc/mdp452

Kim, T. il. (2014). Chemopreventive drugs: Mechanisms via inhibition of cancer stem cells in colorectal cancer. *World Journal of Gastroenterology : WJG*, *20*(14), 3835. https://doi.org/10.3748/WJG.V20.I14.3835

Klein-Szanto, A. J., & Bassi, D. E. (2017a). Proprotein convertase inhibition: Paralyzing the cell's master switches. *Biochemical Pharmacology*, *140*, 8–15. https://doi.org/10.1016/J.BCP.2017.04.027

Klein-Szanto, A. J., & Bassi, D. E. (2017b). Proprotein convertase inhibition: Paralyzing the cell's master switches. *Biochemical Pharmacology*, *140*, 8–15. https://doi.org/10.1016/J.BCP.2017.04.027

Koch, S., & Claesson-Welsh, L. (2012). Signal Transduction by Vascular Endothelial Growth Factor Receptors. *Cold Spring Harbor Perspectives in Medicine*, *2*(7). https://doi.org/10.1101/CSHPERSPECT.A006502

Koch, S., Tugues, S., Li, X., Gualandi, L., & Claesson-Welsh, L. (2011). Signal transduction by vascular endothelial growth factor receptors. *Biochemical Journal*, *437*(2), 169–183. https://doi.org/10.1042/BJ20110301

Kong, D., Zhou, H., Neelakantan, D., Hughes, C. J., Hsu, J. Y., Srinivasan, R. R., Lewis, M. T., & Ford, H. L. (2020). VEGF-C mediates tumor growth and metastasis through promoting EMT-epithelial breast cancer cell crosstalk. *Oncogene 2020 40:5*, *40*(5), 964–979. https://doi.org/10.1038/s41388-020-01539-x

Kubiczkova, L., Sedlarikova, L., Hajek, R., & Sevcikova, S. (2012a). TGF-β - an excellent servant but a bad master. *Journal of Translational Medicine*, *10*(1), 1–24. https://doi.org/10.1186/1479-5876-10-183/FIGURES/4

Kubiczkova, L., Sedlarikova, L., Hajek, R., & Sevcikova, S. (2012b). TGF-β - an excellent servant but a bad master. *Journal of Translational Medicine*, *10*(1). https://doi.org/10.1186/1479-5876-10-183

Künnapuu, J., Bokharaie, H., & Jeltsch, M. (2021). Proteolytic Cleavages in the VEGF Family: Generating Diversity among Angiogenic VEGFs, Essential for the Activation of Lymphangiogenic VEGFs. *Biology 2021, Vol. 10, Page 167*, *10*(2), 167. https://doi.org/10.3390/BIOLOGY10020167

Lal, N., Chiu, A. P. L., Wang, F., Zhang, D., Jia, J., Wan, A., Vlodavsky, I., Hussein, B., & Rodrigues, B. (2017). Loss of VEGFB and its signaling in the diabetic heart is associated with increased cell death signaling. *American Journal of Physiology -*

Heart and Circulatory Physiology, *312*(6), H1163–H1175. https://doi.org/10.1152/AJPHEART.00659.2016/ASSET/IMAGES/LARGE/ZH40051722230008.JPEG

Lal, N., Puri, K., & Rodrigues, B. (2018). Vascular Endothelial Growth Factor B and Its Signaling. *Frontiers in Cardiovascular Medicine*, *5*, 39. https://doi.org/10.3389/FCVM.2018.00039/BIBTEX

Lange, C., Storkebaum, E., de Almodóvar, C. R., Dewerchin, M., & Carmeliet, P. (2016). Vascular endothelial growth factor: a neurovascular target in neurological diseases. *Nature Reviews. Neurology*, *12*(8), 439–454. https://doi.org/10.1038/NRNEUROL.2016.88

Lee, J. Y., & Spicer, A. P. (2000). Hyaluronan: a multifunctional, megaDalton, stealth molecule. *Current Opinion in Cell Biology*, *12*(5), 581–586. https://doi.org/10.1016/S0955-0674(00)00135-6

Lee, S. N., Choi, I. S., Kim, H. J., Yang, E. J., Min, H. J., & Yoon, J. H. (2017). Proprotein convertase inhibition promotes ciliated cell differentiation - a potential mechanism for the inhibition of Notch1 signalling by decanoyl-RVKR-chloromethylketone. *Journal of Tissue Engineering and Regenerative Medicine*, *11*(9), 2667–2680. https://doi.org/10.1002/TERM.2240

Lei, X., Shi, F., Basu, D., Huq, A., Routhier, S., Day, R., & Jin, W. (2011). Proteolytic Processing of Angiopoietin-like Protein 4 by Proprotein Convertases Modulates Its Inhibitory Effects on Lipoprotein Lipase Activity. *The Journal of Biological Chemistry*, *286*(18), 15747. https://doi.org/10.1074/JBC.M110.217638

Leppänen, V. M., Jeltsch, M., Anisimov, A., Tvorogov, D., Aho, K., Kalkkinen, N., Toivanen, P., Ylä-Herttuala, S., Ballmer-Hofer, K., & Alitalo, K. (2011). Structural determinants of vascular endothelial growth factor-D receptor binding and specificity. *Blood*, *117*(5), 1507–1515. https://doi.org/10.1182/BLOOD-2010-08-301549

Levine, S. J. (2008). Molecular Mechanisms of Soluble Cytokine Receptor Generation. *The Journal of Biological Chemistry*, *283*(21), 14177. https://doi.org/10.1074/JBC.R700052200

Li, J. P., Huang, Z. J., Lu, X. S., Zhou, Y. C., Shao, Y., He, X. P., Chen, S. R., Wang, D. D., Qin, L. sen, & Sun, W. H. (2016). Pre-clinical characterization of PKC412, a multi-kinase

inhibitor, against colorectal cancer cells. *Oncotarget*, *7*(47), 77815. https://doi.org/10.18632/ONCOTARGET.12802

Li, X., & Eriksson, U. (2001). Novel VEGF family members: VEGF-B, VEGF-C and VEGF-D. *The International Journal of Biochemistry & Cell Biology*, *33*(4), 421–426. https://doi.org/10.1016/S1357-2725(01)00027-9

Lopez-Perez, E., Seidah, N. G., & Checler, F. (1999). Proprotein Convertase Activity Contributes to the Processing of the Alzheimer's β-Amyloid Precursor Protein in Human Cells: Evidence for a Role of the Prohormone Convertase PC7 in the Constitutive -Secretase Pathway. *Journal of Neurochemistry*, *73*(5), 2056–2072. https://doi.org/10.1046/J.1471-4159.1999.02056.X

Lugano, R., Huang, H., & Dimberg, A. (2018). Vascular Endothelial Growth Factor Receptor (VEGFR). *Encyclopedia of Signaling Molecules*, 5884–5892. https://doi.org/10.1007/978-3-319-67199-4_101914

Martinez-Corral, I., Zhang, Y., Petkova, M., Ortsäter, H., Sjöberg, S., Castillo, S. D., Brouillard, P., Libbrecht, L., Saur, D., Graupera, M., Alitalo, K., Boon, L., Vikkula, M., & Mäkinen, T. (2020). Blockade of VEGF-C signaling inhibits lymphatic malformations driven by oncogenic PIK3CA mutation. *Nature Communications 2020 11:1*, *11*(1), 1–14. https://doi.org/10.1038/s41467-020-16496-y

McColl, B. K., Baldwin, M. E., Roufail, S., Freeman, C., Moritz, R. L., Simpson, P. J., Alitalo, K., Stacker, S. A., & Achen, M. G. (2003). Plasmin Activates the Lymphangiogenic Growth Factors VEGF-C and VEGF-D. *The Journal of Experimental Medicine*, *198*(6), 863. https://doi.org/10.1084/JEM.20030361

McColl, B. K., Paavonen, K., Karnezis, T., Harris, N. C., Davydova, N., Rothacker, J., Nice, E. C., Harder, K. W., Roufail, S., Hibbs, M. L., Rogers, P. A. W., Alitalo, K., Stacker, S. A., & Achen, M. G. (2007). Proprotein convertases promote processing of VEGF-D, a critical step for binding the angiogenic receptor VEGFR-2. *The FASEB Journal*, *21*(4), 1088–1098. https://doi.org/10.1096/FJ.06-7060COM

McMahon, S., Grondin, F., McDonald, P. P., Richard, D. E., & Dubois, C. M. (2005). Hypoxia-enhanced Expression of the Proprotein Convertase Furin Is Mediated by Hypoxia-inducible Factor-1: IMPACT ON THE BIOACTIVATION OF PROPROTEINS. *Journal of Biological Chemistry*, *280*(8), 6561–6569. https://doi.org/10.1074/JBC.M413248200

Mei, C. M., & Shen, S. (2009). The roles of cell adhesion molecules in tumor suppression and cell migration: A new paradox. *Cell Adhesion & Migration*, *3*(4), 334. https://doi.org/10.4161/CAM.3.4.9246

Nojadeh, J. N., Sharif, S. B., & Sakhinia, E. (2018). Microsatellite instability in colorectal cancer. *EXCLI Journal*, *17*, 159. https://doi.org/10.17179/EXCLI2017-948

Obesity and Cancer Fact Sheet - National Cancer Institute. (n.d.). Retrieved January 26, 2022, from https://www.cancer.gov/about-cancer/causes-prevention/risk/obesity/obesity-fact-sheet

Okegawa, T., Pong, R.-C., Li, Y., & Hsieh, J.-T. (n.d.). *The role of cell adhesion molecule in cancer progression and its application in cancer therapy **.

Rao, J. N., & Wang, J.-Y. (2010). *Intestinal Architecture and Development*. https://www.ncbi.nlm.nih.gov/books/NBK54098/

Raskov, H., Pommergaard, H. C., Burcharth, J., & Rosenberg, J. (2014). Colorectal carcinogenesis-update and perspectives. *World Journal of Gastroenterology : WJG*, *20*(48), 18151. https://doi.org/10.3748/WJG.V20.I48.18151

Rauniyar, K., Jha, S. K., & Jeltsch, M. (2018). Biology of vascular endothelial growth factor C in the morphogenesis of lymphatic vessels. *Frontiers in Bioengineering and Biotechnology*, *6*(FEB), 7. https://doi.org/10.3389/FBIOE.2018.00007/BIBTEX

Ren, G., Roberts, A. I., & Shi, Y. (2011). Adhesion molecules: Key players in Mesenchymal stem cell-mediated immunosuppression. *Cell Adhesion & Migration*, *5*(1), 20. https://doi.org/10.4161/CAM.5.1.13491

Ribatti, D. (2011). From the discovery of Vascular Endothelial Growth Factor to the introduction of Avastin in clinical trials - an interview with Napoleone Ferrara. *International Journal of Developmental Biology*, *55*(4–5), 383–388. https://doi.org/10.1387/IJDB.103216DR

Rifai, O. al, Susan-Resiga, D., Essalmani, R., Creemers, J. W. M., Seidah, N. G., & Ferron, M. (2021). In vivo analysis of the contribution of proprotein convertases to the processing of FGF23. *BioRxiv*, 2021.01.15.426882. https://doi.org/10.1101/2021.01.15.426882

Roy, H., Bhardwaj, S., & Ylä-Herttuala, S. (2006). Biology of vascular endothelial growth factors. *FEBS Letters*, *580*(12), 2879–2887. https://doi.org/10.1016/J.FEBSLET.2006.03.087

Santos, R. D., Ruzza, A., Hovingh, G. K., Wiegman, A., Mach, F., Kurtz, C. E., Hamer, A., Bridges, I., Bartuli, A., Bergeron, J., Szamosi, T., Santra, S., Stefanutti, C., Descamps, O. S., Greber-Platzer, S., Luirink, I., Kastelein, J. J. P., & Gaudet, D. (2020). Evolocumab in Pediatric Heterozygous Familial Hypercholesterolemia. *The New England Journal of Medicine*, *383*(14), 1317–1327. https://doi.org/10.1056/NEJMOA2019910

Scamuffa, N., Sfaxi, F., Ma, J., Lalou, C., Seidah, N., Calvo, F., & Khatib, A. M. (2014). Prodomain of the proprotein convertase subtilisin/kexin Furin (ppFurin) protects from tumor progression and metastasis. *Carcinogenesis*, *35*(3), 528–536. https://doi.org/10.1093/CARCIN/BGT345

Schmidt, A. F., Carter, J. P. L., Pearce, L. S., Wilkins, J. T., Overington, J. P., Hingorani, A. D., & Casas, J. P. (2020). PCSK9 monoclonal antibodies for the primary and secondary prevention of cardiovascular disease. *The Cochrane Database of Systematic Reviews*, *10*(10). https://doi.org/10.1002/14651858.CD011748.PUB3

Schmitt, M., & Greten, F. R. (2021). The inflammatory pathogenesis of colorectal cancer. *Nature Reviews Immunology 2021 21:10*, *21*(10), 653–667. https://doi.org/10.1038/s41577-021-00534-x

Schultheis, B., Folprecht, G., Kuhlmann, J., Ehrenberg, R., Hacker, U. T., Köhne, C. H., Kornacker, M., Boix, O., Lettieri, J., Krauss, J., Fischer, R., Hamann, S., Strumberg, D., & Mross, K. B. (2013). Regorafenib in combination with FOLFOX or FOLFIRI as first- or second-line treatment of colorectal cancer: results of a multicenter, phase Ib study. *Annals of Oncology*, *24*(6), 1560–1567. https://doi.org/10.1093/ANNONC/MDT056

Senzer, N., Barve, M., Kuhn, J., Melnyk, A., Beitsch, P., Lazar, M., Lifshitz, S., Magee, M., Oh, J., Mill, S. W., Bedell, C., Higgs, C., Kumar, P., Yu, Y., Norvell, F., Phalon, C., Taquet, N., Rao, D. D., Wang, Z., … Nemunaitis, J. (2012). Phase I trial of "bi-shRNAi(furin)/GMCSF DNA/autologous tumor cell" vaccine (FANG) in advanced cancer. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *20*(3), 679–686. https://doi.org/10.1038/MT.2011.269

Siegfried, G., Basak, A., Cromlish, J. A., Benjannet, S., Marcinkiewicz, J., Chrétien, M., Seidah, N. G., & Khatib, A.-M. (2003a). The secretory proprotein convertases furin,

PC5, and PC7 activate VEGF-C to induce tumorigenesis. *The Journal of Clinical Investigation*, *111*(11), 1723–1732. https://doi.org/10.1172/JCI17220

Siegfried, G., Basak, A., Cromlish, J. A., Benjannet, S., Marcinkiewicz, J., Chrétien, M., Seidah, N. G., & Khatib, A. M. (2003b). The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. *Journal of Clinical Investigation*, *111*(11), 1723–1732. https://doi.org/10.1172/JCI200317220

Siegfried, G., Basak, A., Cromlish, J. A., Benjannet, S., Marcinkiewicz, J., Chrétien, M., Seidah, N. G., & Khatib, A.-M. (2003c). The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. *Journal of Clinical Investigation*, *111*(11), 1723. https://doi.org/10.1172/JCI17220

Simons, M. (2007). Silky, sticky chimeras-designer VEGFs display their wares. *Circulation Research*, *100*(10), 1402–1404. https://doi.org/10.1161/01.RES.0000269333.10849.9E

Soulet, F., Siegfried, G., & Khatib, A.-M. (n.d.). *Furin-cleaved ELA/Apela precursor displays tumor suppressor function in renal cell carcinoma through mTORC1 activation*. https://doi.org/10.1172/jci.insight.129070

Stacker, S. A., & Achen, M. G. (2018). Emerging Roles for VEGF-D in Human Disease. *Biomolecules*, *8*(1). https://doi.org/10.3390/BIOM8010001

Stawowy, P., Kallisch, H., Kilimnik, A., Margeta, C., Seidah, N. G., Chrétien, M., Fleck, E., & Graf, K. (2004). Proprotein convertases regulate insulin-like growth factor 1-induced membrane-type 1 matrix metalloproteinase in VSMCs via endoproteolytic activation of the insulin-like growth factor-1 receptor. *Biochemical and Biophysical Research Communications*, *321*(3), 531–538. https://doi.org/10.1016/J.BBRC.2004.07.001

Stawowy, P., Kallisch, H., Veinot, J. P., Kilimnik, A., Prichett, W., Goetze, S., Seidah, N. G., Chrétien, M., Fleck, E., & Graf, K. (2004). Endoproteolytic Activation of αv Integrin by Proprotein Convertase PC5 Is Required for Vascular Smooth Muscle Cell Adhesion to Vitronectin and Integrin-Dependent Signaling. *Circulation*, *109*(6), 770–776. https://doi.org/10.1161/01.CIR.0000112583.50762.DE

Stawowy, P., Meyborg, H., Stibenz, D., Stawowy, N. B. P., Roser, M., Thanabalasingam, U., Veinot, J. P., Chrétien, M., Seidah, N. G., Fleck, E., & Graf, K. (2005). Furin-like proprotein convertases are central regulators of the membrane type matrix

metalloproteinase-pro-matrix metalloproteinase-2 proteolytic cascade in atherosclerosis. *Circulation*, *111*(21), 2820–2827. https://doi.org/10.1161/CIRCULATIONAHA.104.502617

Tabung, F. K., Brown, L. S., & Fung, T. T. (2017). Dietary Patterns and Colorectal Cancer Risk: A Review of 17 Years of Evidence (2000–2016). *Current Colorectal Cancer Reports*, *13*(6), 440. https://doi.org/10.1007/S11888-017-0390-5

Testa, U., Pelosi, E., & Castelli, G. (2018a). *medical sciences Colorectal Cancer: Genetic Abnormalities, Tumor Progression, Tumor Heterogeneity, Clonal Evolution and Tumor-Initiating Cells*. https://doi.org/10.3390/medsci6020031

Testa, U., Pelosi, E., & Castelli, G. (2018b). Colorectal cancer: genetic abnormalities, tumor progression, tumor heterogeneity, clonal evolution and tumor-initiating cells. *Medical Sciences (Basel, Switzerland)*, *6*(2), 31. https://doi.org/10.3390/MEDSCI6020031

The Peptidergic Neuron. (1996). *The Peptidergic Neuron*. https://doi.org/10.1007/978-3-0348-9010-6

Toivanen, P. I., Nieminen, T., Laakkonen, J. P., Heikura, T., Kaikkonen, M. U., & Ylä-Herttuala, S. (2017). Snake venom VEGF Vammin induces a highly efficient angiogenic response in skeletal muscle via VEGFR-2/NRP specific signaling. *Scientific Reports 2017 7:1*, *7*(1), 1–12. https://doi.org/10.1038/s41598-017-05876-y

Toma, M., Beluşică, L., Stavarachi, M., Apostol, P., Spandole, S., Radu, I., & Cimponeriu, D. (2012). Rating the environmental and genetic risk factors for colorectal cancer. *Journal of Medicine and Life*, *5*(Spec Issue), 152. /pmc/articles/PMC6880217/

Tugues, S., Koch, S., Gualandi, L., Li, X., & Claesson-Welsh, L. (2011). Vascular endothelial growth factors and receptors: anti-angiogenic therapy in the treatment of cancer. *Molecular Aspects of Medicine*, *32*(2), 88–111. https://doi.org/10.1016/J.MAM.2011.04.004

Ugorski, M., Dziegiel, P., & Suchanski, J. (2016). Podoplanin - a small glycoprotein with many faces. *American Journal of Cancer Research*, *6*(2), 370. /pmc/articles/PMC4859667/

Vagner, J., Qu, H., & Hruby, V. J. (2008). Peptidomimetics, a synthetic tool of drug discovery. *Current Opinion in Chemical Biology*, *12*(3), 292. https://doi.org/10.1016/J.CBPA.2008.03.009

Wada, H., Suzuki, M., Matsuda, M., Ajiro, Y., Shinozaki, T., Sakagami, S., Yonezawa, K., Shimizu, M., Funada, J., Takenaka, T., Morita, Y., Nakamura, T., Fujimoto, K., Matsubara, H., Kato, T., Unoki, T., Takagi, D., Wada, K., Wada, M., … Hasegawa, K. (2020). Distinct characteristics of vegf-d and vegf-c to predict mortality in patients with suspected or known coronary artery disease. *Journal of the American Heart Association*, *9*(9), 15761. https://doi.org/10.1161/JAHA.119.015761

Wang, C. A., & Tsai, S. J. (2015). The non-canonical role of vascular endothelial growth factor-C axis in cancer progression: *Https://Doi.Org/10.1177/1535370215583802*, *240*(6), 718–724. https://doi.org/10.1177/1535370215583802

Wang, W., Yuan, J., & Jiang, C. (2021). Applications of nanobodies in plant science and biotechnology. *Plant Molecular Biology*, *105*(1–2), 43–53. https://doi.org/10.1007/S11103-020-01082-Z

Wang, X., Bove, A. M., Simone, G., & Ma, B. (2020). Molecular Bases of VEGFR-2-Mediated Physiological Function and Pathological Role. *Frontiers in Cell and Developmental Biology*, *8*, 1314. https://doi.org/10.3389/FCELL.2020.599281/BIBTEX

What Is Colorectal Cancer? | How Does Colorectal Cancer Start? (n.d.). Retrieved January 26, 2022, from https://www.cancer.org/cancer/colon-rectal-cancer/about/what-is-colorectal-cancer.html

Whayne, T. F. (2017). PCSK9 inhibitors in the current management of atherosclerosis. *Archivos de Cardiología de México*, *87*(1), 43–48. https://doi.org/10.1016/J.ACMX.2016.11.013

Wise, L. M., Inder, M. K., Real, N. C., Stuart, G. S., Fleming, S. B., & Mercer, A. A. (2012). The vascular endothelial growth factor (VEGF)-E encoded by orf virus regulates keratinocyte proliferation and migration and promotes epidermal regeneration. *Cellular Microbiology*, *14*(9), 1376–1390. https://doi.org/10.1111/J.1462-5822.2012.01802.X

Wu, N. Q., Li, S., & Li, J. J. (2015). Update of Clinical Trials of Anti-PCSK9 Antibodies. *Cardiovascular Drugs and Therapy*, *29*(2), 159–169. https://doi.org/10.1007/S10557-015-6582-9

Xie, Y. H., Chen, Y. X., & Fang, J. Y. (2020). Comprehensive review of targeted therapy for colorectal cancer. *Signal Transduction and Targeted Therapy 2020 5:1*, *5*(1), 1–30. https://doi.org/10.1038/s41392-020-0116-z

Yamazaki, Y., Matsunaga, Y., Tokunaga, Y., Obayashi, S., Saito, M., & Morita, T. (2009). Snake venom Vascular Endothelial Growth Factors (VEGF-Fs) exclusively vary their structures and functions among species. *The Journal of Biological Chemistry*, *284*(15), 9885–9891. https://doi.org/10.1074/JBC.M809071200

Yu, X., & Ye, F. (2020). Role of Angiopoietins in Development of Cancer and Neoplasia Associated with Viral Infection. *Cells*, *9*(2). https://doi.org/10.3390/CELLS9020457

Zelenay, S., van der Veen, A. G., Böttcher, J. P., Snelgrove, K. J., Rogers, N., Acton, S. E., Chakravarty, P., Girotti, M. R., Marais, R., Quezada, S. A., Sahai, E., & Reis E Sousa, C. (2015). Cyclooxygenase-Dependent Tumor Growth through Evasion of Immunity. *Cell*, *162*(6), 1257–1270. https://doi.org/10.1016/J.CELL.2015.08.015

Zhang, D. W., Lagace, T. A., Garuti, R., Zhao, Z., McDonald, M., Horton, J. D., Cohen, J. C., & Hobbs, H. H. (2007). Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. *Journal of Biological Chemistry*, *282*(25), 18602–18612. https://doi.org/10.1074/JBC.M702027200/ATTACHMENT/956CC431-76AE-41A2-AD91-356A5F13FC9D/MMC1.PDF

Ziyad, S., & Iruela-Arispe, M. L. (2011). Molecular Mechanisms of Tumor Angiogenesis. *Genes & Cancer*, *2*(12), 1085. https://doi.org/10.1177/1947601911432334