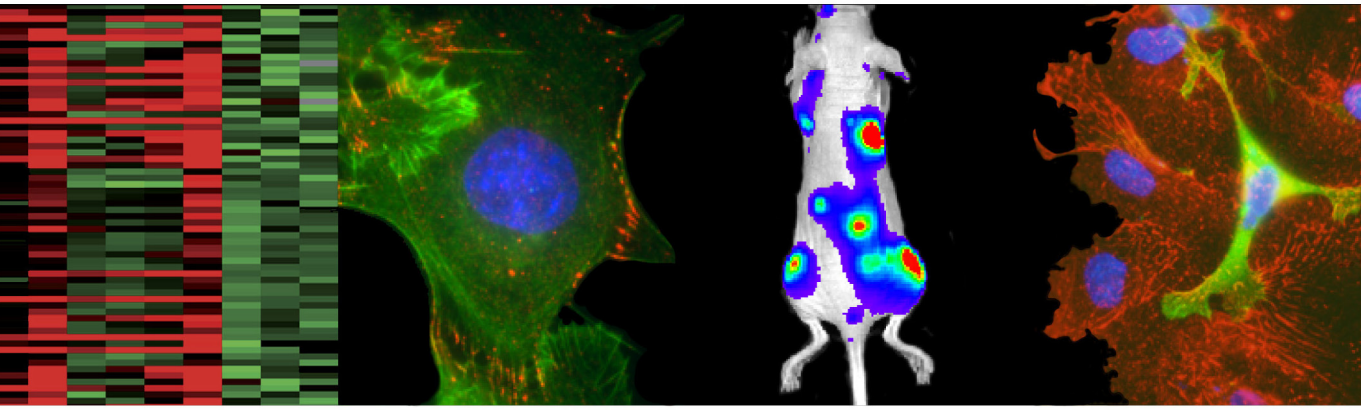
Circulating tumor cells in metastatic colorectal cancer

from basic understanding to clinical practice

PhD Thesis, 2014
Jorge Barbazán García



USC
UNIVERSIDADE
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Circulating tumor cells in metastatic colorectal cancer

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Memoria que presenta:
Jorge Barbazán García
para optar ao grao de Doutor en Bioloxía

Fdo: Jorge Barbazán García
Santiago de Compostela, Maio 2014.

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CERTIFICAN

Que a presente memoria titulada **"Circulating tumor cells in metastatic colorectal cancer: from basic understanding to clinical practice"** presentada por **Don Jorge Barbazán García** para optar ao **Grao de Doutor en Bioloxía,** foi realizada baixo a nosa dirección no laboratorio de Oncoloxía Médica Traslacional, vinculado ao Departamento de Medicina da Universidade de Santiago de Compostela. Asimesmo, consideran que dito traballo está en condicións de ser defendido como Tese de Doutoramento ante o tribunal correspondente.

E para que así conste firmamos a presente en Santiago de Compostela, a 2 de Maio de 2014.

Fdo: Dr Rafael López López

Fdo: Dr Miguel Abal Posada

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O verdadeiro heroísmo está en transformar os desexos en realidades, e as ideas en feitos

A.R. Castelao

Témosche un bo traballo feito!

María García “Maruja”

Vicky, vai por ti!

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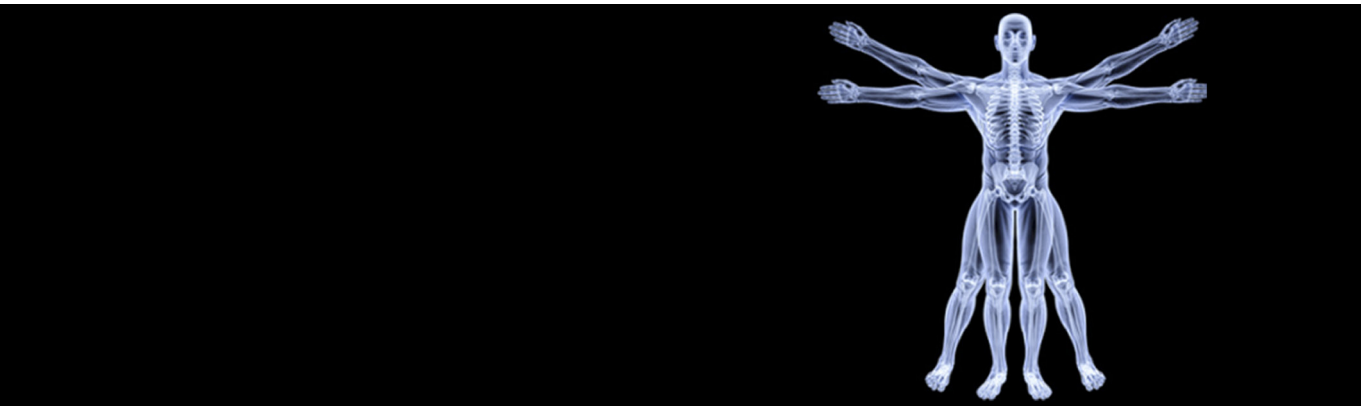
General Introduction and Objectives



General Introduction

1. Colorectal Cancer

1.1 Epidemiology

Colorectal Cancer (CRC) constitutes the third cancer type in terms of incidence worldwide, with more than 1.3 million new cases diagnosed in 2012 [1], which means that 2 new CRC cases are diagnosed every minute in the world. In terms of mortality, around 690.000 CRC-related deaths were reported in the same year, positioning CRC as the fourth cancer type, after lung, liver and stomach cancers [1] (Figure 1). Globally, CRC accounts for almost 9,7% of the total number of diagnosed cancers worldwide, and for 8,5% of deaths caused by cancer [1].

By sex, CRC represents the third most commonly diagnosed cancer in males after lung and prostate cancer, respectively, and it is the fourth cancer-related death cause, after lung, liver and stomach cancers. Regarding females, breast cancer leads the list of the most diagnosed cancer types, with CRC in the second place, accounting for more than 610.000 new estimated cases in 2012. After breast and lung cancers, CRC is the third most frequent cancer-related death cause in women [2] (Figure 1).

CRC incidence rates have notably increased in several areas historically at low risk, especially including countries in Eastern Asia and Europe [3,4]. These unfavorable trends are thought to reflect a combination of factors including changes in dietary patterns, obesity and an increased prevalence of smoking [3,5,6]. While CRC death rates have been decreasing in several Western countries, largely resulting from improved treatment and early detection, rates continue to increase in many developing countries, with more limited resources and health infrastructure, particularly in Central and South America and Eastern Europe [2].

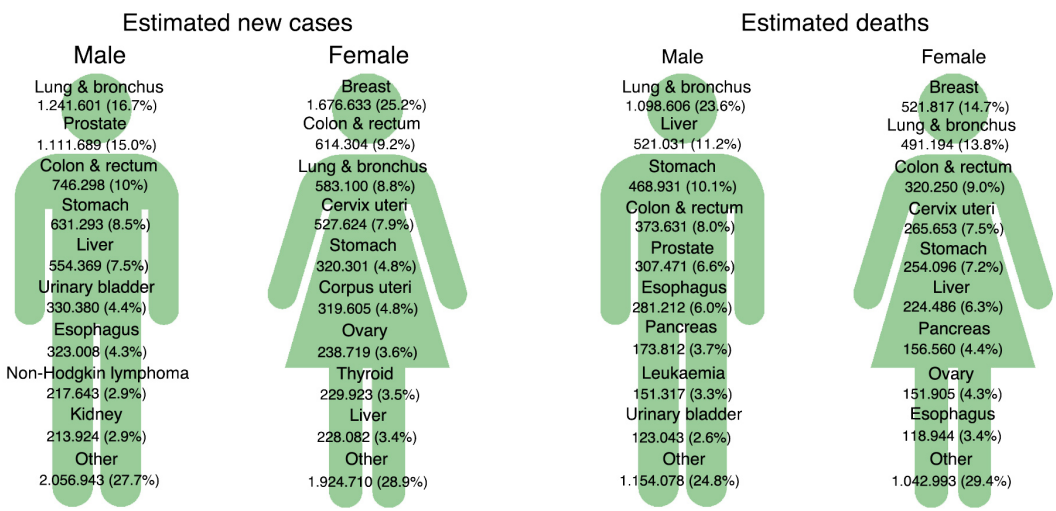


Figure 1: Estimated new cancer cases and deaths worldwide for leading cancer sites by sex.
Source: GLOBOCAN 2012.

Spain is considered one of the developed countries where CRC incidence rates have continued to rise during the last years, especially among men. 24,2 and 43,9 of every 100.000 women and men were, respectively, diagnosed of CRC in 2012 in Spain [1]. In the same year, mortality rates were 8,4 and 17,1 (of each 100.000 individuals) for women and men, respectively [1]. This places CRC as the most diagnosed cancer in Spain (taking both sexes into account), and the second in terms of mortality, only after lung cancer [1] (Figure 2). However, CRC mortality trends in Spain have been decreasing for men since 2001, with a mean of 0,5% per year. For women, a decrease of 1,2% per year is registered since the mid-90s [6]. Galicia is ranked the third Spanish region in terms of CRC mortality, only after Asturias and País Vasco, for both sexes, with death rates falling in the same ranges as Spanish ones [6].

Recent results from the EUROCARE-5 study, revealed that the European mean age-standardized 5-year survival for CRC patients was 57,0% in the period 2000-2007 (64,3% for the U.S, 2001-2007 [7]) with negligible differences between sexes. Central and Northern European countries displayed slightly higher survival rates, with Southern countries, including Spain, presenting rates similar to the European mean (57,1% for Spain) [8]. European CRC 5-year survival rates were continuously increasing in the last years, with 30,3% in 1991, and 44,7% in 2002 [9].

It is important to mention that CRC survival rates greatly vary depending on the stage on which the cancer is detected. When CRC is detected at a localized stage, the 5-year relative survival rate is 90,1%. After the cancer regionally spreads to involve adjacent organs or lymph nodes, survival rate drops to 69,2%. When the disease has spread to distant organs, 5-year survival rate is 11,7% (data from 2008, U.S) [7].

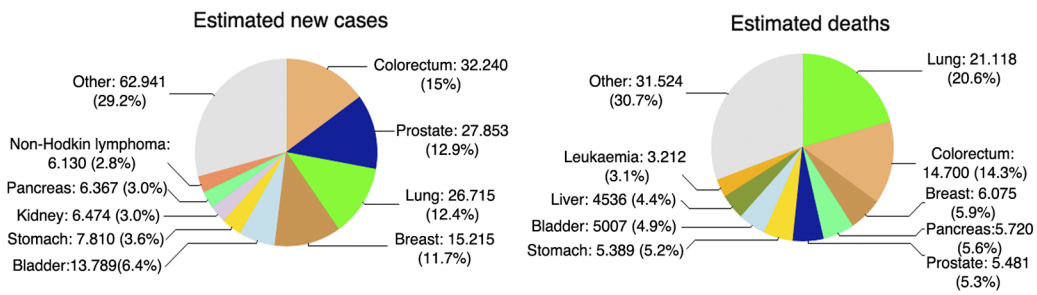


Figure 2: Estimated new cancer cases and deaths in Spain for leading cancer sites. Source: GLOBOCAN 2012

1.2 Risk factors and causes

1.2.1 Sporadic CRC

Most cases of CRC arise sporadically, and factors like age, previous colonic polyps or environmental factors, contribute to the development of CRC [10]. About 20% of all patients with CRC are supposed to have some component of familial risk, without strictly fulfilling criteria for hereditary CRC [10,11]. A detailed list of the main risk factors for sporadic CRC is shown in Table 1, upper section.

1.2.2 Hereditary CRC

Between 5-10% of CRC patients develop in the setting of defined hereditary cancer syndromes, with Hereditary Nonpolyposis Colorectal Cancer (HNPCC) and Familial Adenomatous Polyposis (FAP), as the two main forms of hereditary CRC [10,11]. An extended list of hereditary CRC syndromes is shown in Table 1, lower section.

1.2.2.1 Familial Adenomatous Polyposis (FAP)

FAP is an autosomal dominant disease caused, in 80% of affected individuals, by a germline mutation in the adenomatous polyposis coli (*APC*) gene [12]. Its prevalence is approximately 1:10.000 births. FAP patients can develop more than 100 colorectal adenomas (50% of patients by age 15 years, 95% by age 35 years), and it arises in almost 100% of patients by the age of 40 years if left untreated [11]. Disease severity is associated with proximity of mutations to the central region of *APC* gene, with mutations between codons 1445 and 1578 linked to an increased risk of desmoid tumors. A FAP variant is attenuated FAP, with patients developing from 10 to 100 adenomas. *APC* mutations in patients with attenuated FAP, are typically located at the 5' or 3' ends of the gene [12].

1.2.2.2 Hereditary Nonpolyposis Colorectal Cancer (HNPCC)

HNPCC, also known as Lynch syndrome, is the most common hereditary disorder associated with CRC. It displays an autosomal dominant inheritance pattern. The onset of the disease is at 45 years on average (versus 63 years for the general population) [10]. Lynch syndrome is associated with germline mutations in mismatch repair genes (most commonly *MLH1*, *MSH2*, or *MSH6*) [12,13]. Pathology of CRC on HNPCC affected individuals is often poorly differentiated, with an excess of mucoid and signet cell features, a Crohn's-like reaction, and an excess of infiltrating lymphocytes within the tumor [10].

Table 1. CRC Risk factors and causes

Sporadic CRC (88-94%)

Older age
Male sex
Cholecystectomy
Ureterocolic anastomosis
Hormonal factors: nulliparity, late age at first pregnancy, early menopause

Environmental factors

Diet rich in meat and fat, and poor in fiber, folate and calcium
Sedentary lifestyle
Obesity
Diabetes mellitus
Smoking
Previous irradiation
Occupational hazards (e.g. asbestos exposure)
High alcohol intake

Personal history of sporadic tumors

History of colorectal polyps
History of colorectal cancer
History of small bowel, endometrial, breast, or ovarian cancer

Familial CRC (20%)

First or second degree relatives with CRC, criteria for hereditary CRC not fulfilled:

- One affected first-degree relative increases risk 2-3-fold
- Two or more affected first-grade relatives increase risk 4-25-fold
- Index case <45 years increases risk 3-9-fold
- Familial history of colorectal adenoma increases risk 2-fold

Hereditary CRC (5-10%)

Polyposis-syndromes:

- Familial adenomatous polyposis (FAP)
- Gardner's syndrome
- Turcot's syndrome
- Attenuated adenomatous polyposis coli
- Flat adenoma syndrome

Hereditary non-polyposis colorectal cancer (HNPCC)(Lynch syndrome)

Hamartomatous polyposis syndromes:

- Peutz-Jeghers syndrome
- Juvenile polyposis syndrome
- Cowden syndrome

Adapted from Weitz et al [11]

1.3 Molecular carcinogenesis

CRC evolves through a stepwise accumulation of genetic and epigenetic events, which lead to the transformation of the normal colonic mucosa into invasive cancer. Most CRCs develop from preexisting adenomas that transform into malignant lesions in a process estimated to take about 10-15 years [13]. The course of the disease varies depending on polyp's characteristics. The presence of multiple adenomas (>3), large size adenomas (>1cm in diameter), adenomas with villous change, and adenomas with high grade dysplasia, predispose for high risk CRC [14]. Recently, a specific subtype of adenomas, the serrated adenomas were described, showing a distinct pattern of morphological and molecular changes in their transformation process (see section 1.3.1.4) [15].

1.3.1 Carcinogenesis pathways for CRC development

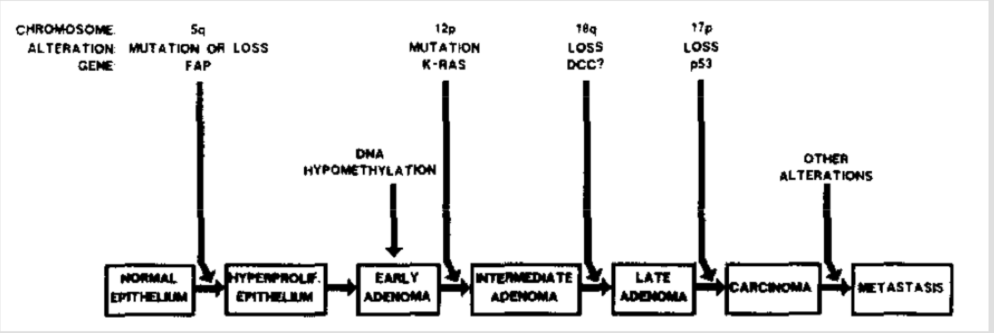
The first model for CRC carcinogenesis was proposed by Fearon and Vogelstein in 1990 [16] (Box 1). In this initial model, there are three important features: i) colorectal neoplasia arises from the sequential mutation of oncogenes, leading to their activation, coupled to an inactivating mutation of tumor suppressor genes, ii) mutation of at least 4-5 genes is necessary for cancer to develop and iii) the accumulation of molecular alterations rather than their order is responsible for determining the biologic behavior of the tumor. In the last two decades, other molecular pathways contributing to CRC carcinogenesis have been described (Box 1). The Chromosomal Instability (CIN), Microsatellite Instability (MSI), and the CpG Island Methylator (CIMP) pathways, represent the three main molecular pathways that have been recognized. More recently, the serrated pathway, a fourth mechanism for CRC carcinogenesis, has been described. Based on tumor features related to the previous mentioned pathways, a molecular classification of colorectal carcinomas has been proposed (Box 2).

1.3.1.1 Chromosomal instability pathway (CIN)

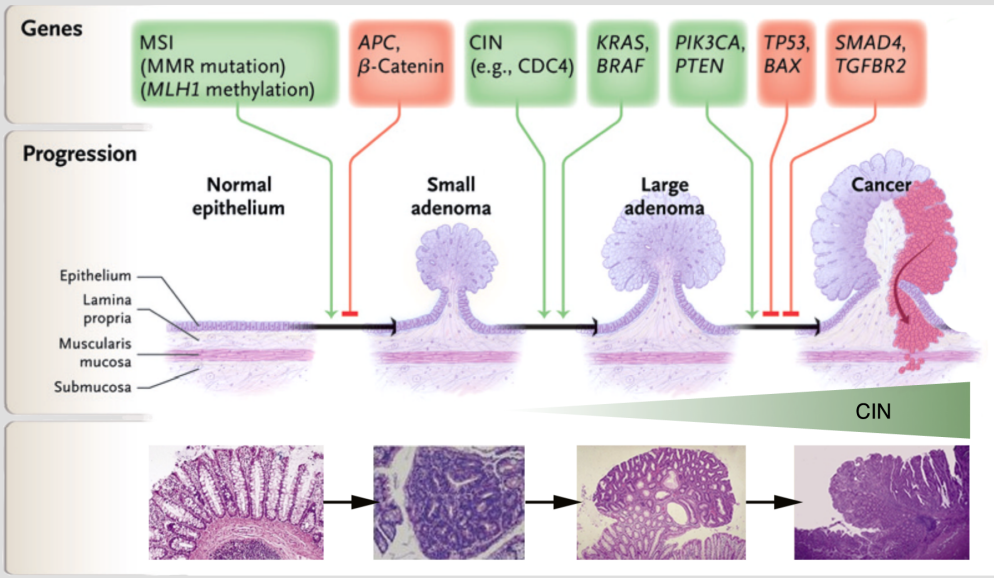
Cromosomal instability accounts for 65-70% of sporadic CRC. It is characterized by gain or loss of whole chromosomes or chromosomal regions harboring genes relevant for the process of colorectal carcinogenesis [13,17]. The main causes of CIN are defects in chromosomal segregation during mitosis, which finally originate aneuploidy (imbalance in chromosome number), telomere dysfunction, or defects in the mechanisms responsible for DNA damage response [18]. Chromosomes 1, 5, 8, 17 and 18 have the highest frequency of allele loss, and common altered cancer genes by chromosomal gains or losses are *VEGF*, *MYC*, *MET*, *LYN* or *PTEN* [19]. The most common single genetic alterations are mutations in the *APC* or *KRAS* genes [13].

BOX 1: Colorectal carcinogenesis models

Back in 1990, Fearon and Vogelstein proposed a model for colorectal carcinogenesis that comprised a stepwise sequence of events involving oncogenes and tumor suppressor genes (particularly those in chromosomes 5q, 17p and 18q). APC inactivating mutation drives the initial transformation of the normal intestinal epithelium into an hyperproliferative tissue (also termed Aberrant Crypt Foci or ACF). Progression lo later adenomas and early carcinomas requires the presence of activating mutations of the Kras proto-oncogene, and inactivation of p53. Loss of heterozygosity at chromosome 18q is also a prerequisite, involving genes such as Deleted in Colorectal Cancer (DCC).



More recently, additional pathways contributing to colorectal carcinogenesis have been added to this initial model. Chromosomal instability (CIN), Microsatellite instability (MSI) or CpG methylation (CIMP) have been included as new factors involved in the progressive acquisition of an aggressive phenotype. The figure below shows specific genetic alterations at specific stages of tumor development.



Abbreviations: MSI: Microsatellite Instability; MMR: Mismatch repair; CIN: Chromosomal Instability. Green arrows indicate genetic activation, whereas red lines represent inactivation events.

Adapted images from: Fearon and Vogelstein [16]; Markowitz and Bertagnolli [23].

1.3.1.1.1 *KRAS* (12p12)

The *KRAS* proto-oncogene encodes a GTP-binding protein and is mutated in 30-60% of CRC and large adenomas [20]. The *KRAS* product is involved in signal transduction and, when mutated, becomes constitutively activated, affecting cellular pathways such as cell growth, differentiation, survival or cell motility, among others.

1.3.1.1.2 *APC* (5q21)

APC is a tumor suppressor gene, which product has different cellular roles such as the control of cell division, cell-cell adhesion or cell migration. It belongs to the canonical Wnt pathway, where APC protein forms a complex with β-catenin, axin, and GSK3 (Glycogen synthase kinase 3). Loss of both gene alleles is a requisite for *APC* loss of function (Knudson's two hit hypothesis [21]), which leads to the deregulation (constitutive activation) of the Wnt pathway by progressive accumulation of cytoplasmic β-catenin, affecting cell proliferation, angiogenesis or apoptosis [22].

1.3.1.1.3 *P53* (17p13)

P53 is a tumor suppressor transcription factor that activates genes involved in cell cycle arrest, apoptosis, senescence, autophagy, and cellular metabolism. P53 also mediates cell adaptation to different kinds of stress or external insults. Loss of P53 protein function leads to a deregulation of DNA damage control mechanisms [17,23].

1.3.1.1.4 *SMAD2*, *SMAD4* and *DCC* (18q21.1)

SMAD2 and SMAD4 proteins are involved in the TGF-β pathway, key for the regulation of cellular growth as well as apoptosis [18]. Loss of SMAD proteins activation occurs in approximately 10% of CRCs [24]. *DCC* (Deleted in colorectal cancer) is a tumor suppressor gene that encodes for a transmembrane receptor of the IgG superfamily for netrins, molecules involved in axon guidance and developed of the nervous system [23]. DCC protein also induces apoptosis through Caspase-3 activation, in the absence of its ligand netrin-1, process that fails after *DCC* mutation (inactivation) [25,26].

1.3.1.2 Microsatellite Instability pathway (Mutator pathway)

Microsatellites are short repeat nucleotide sequences that are spread all over the genome and are susceptible to errors during the process of DNA replication. The DNA mismatch repair system (MMR) is the responsible for recognizing and repair errors during the process of replication, and its inability to perform this task efficiently, transduces into the appearance of microsatellite instability (MSI). Genes involved in the MMR system include *MSH2*, *MLH1*, *MSH6*, *PMS2*, *MLH3*, *MSH3* or *PMS1*. MSI is divided into MSI-high or MSI-low, depending on the number of genes altered from a total of five selected markers [27]. MSI-high is defined by instability of at least two genes, while MSI-low is considered when instability is detected in only one gene. When no instability is detected, tumors are considered microsatellite stable or MSS. MSI was first described linked to the presence of HNPCC. Sporadic CRC is frequently linked to the hypermethylation silencing of *MLH1* [28], and usually appears combined to the presence of MSI in at least one other MMR gene, and thus considered MSI-High [29,30]. MLH-1 loss of expression in sporadic CRC is usually detected in sessile serrated adenomas, reason why these tumor types are not often included in the microsatellite instability pathway (Box 2).

BOX 2: Molecular classification of colorectal carcinoma.

	Chromosomal instability pathway	Mismatch repair pathway	Serrated pathway	
	Hereditary and sporadic	Hereditary	Hereditary	Sporadic
CIMP status	Negative	Negative	High	--
MSI status	MSS	MSI-H	MSI-H	MSI-L
CIN status	+++	---	---	---
Kras mutation	+++	+/-	---	---
BRAF mutation	---	---	+++	+++
MLH1 status	Normal	Mutation	Methylated	Partial Methylation

Abbreviations: CIMP: CpG island methylator phenotype. MSS: Microsatellite stability. MSI: Microsatellite instability. MSI-H: High-level microsatellite instability. MSI-L: Low-level microsatellite instability. +++: present. +/-: might or might not be present. ---: absent.

Adapted from Cunningham et al [10]

1.3.1.3 CpG Island Methylator pathway (CIMP)

DNA methylation confers a gene expression regulation mechanism, which does not imply modifications in the DNA nucleotide sequence, termed epigenetics. Methylation of DNA occurs at cytosine bases when cytosine (C) and guanosine (G) are located in a dinucleotide pair, i.e. CpG. Repetitive series of CpG dinucleotides are often located together in particular regions of gene promoters, named CpG islands. Promoter methylation interrupts gene expression by directly inhibiting transcription factor binding, and also by influencing histone acetylation and thus accessibility of the gene to the necessary transcriptional machinery [31]. The expression of some genes involved in CRC carcinogenesis was found to be repressed by promoter hypermethylation (*APC*, *MCC*, *MLH-1* or *MGMT*) [13].

A panel of markers has been proposed to classify colorectal cancers into well-defined subsets, based on their gene methylation status. This panel includes five genes (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*) [32]. CpG Island Methylator phenotype (CIMP+ or high) is defined when at least three of the mentioned markers are methylated (CIMP- or low, when two or less methylated genes are present). CIMP high accounts for approximately 15-20% of sporadic CRC and usually display common features [13,17,23].

1.3.1.4 Serrated pathway

Until the late 1990s, colorectal polyps where generally divided into two major subtypes: hyperplastic and adenomatous polyps, with this last type proposed as the main precursor of colorectal cancer [Fearon and Vogelstein model [16] (Box 1)]. Hyperplastic polyps were considered non-malignant lesions with little clinical relevance [33]. However, after several clinical observations, hyperplastic polyps were progressively associated with an elevated risk for CRC development [34-36]. In 1990 Longacre and Fenoglio-Preiser first used the term “serrated adenoma” to describe a subset of hyperplastic polyps associated with colorectal carcinogenesis [37].

Serrated polyps form a heterogeneous group of colorectal lesions that includes Hyperplastic polyps (HP), sessile serrated adenomas (SSA) and traditional serrated adenomas (TSA) (Figure 3) [15]. Serrated polyps morphologically differ from colorectal adenomas in the presence of a “serrated” morphology: saw tooth-shaped infoldings of the surface and crypt epithelium, caused by hypermaturation of the gland epithelium due to low extent of cell loss by apoptosis (Figure 3). 10% of all CRCs are proposed to arise from serrated polyps [38].

Recent genetic analyses of serrated lesions have proposed the serrated pathway as a main route leading for CRC. However, the genetic alterations occurring in this pathway are not well clarified and there is great variability in the frequency of molecular changes described [15,39-41], although the most common genetic alterations involve *BRAF* and *KRAS* mutations (Box 2).

Currently, some ambitious projects are being carried out, with the aim of gaining a deeper understanding of cancer genomics to improve the global management of cancer patients. Of particular interest is The Cancer Genome Atlas (TCGA), driven by the National Human Genome Research Institute (U.S). Interestingly, recent results from this project have shown that a specific subset of 24 genes was significantly mutated in patients with CRC, proposing, among others, *ARID1A*, *SOX9* and *FAM132B/WTX* as potential driving genes for CRC [42].

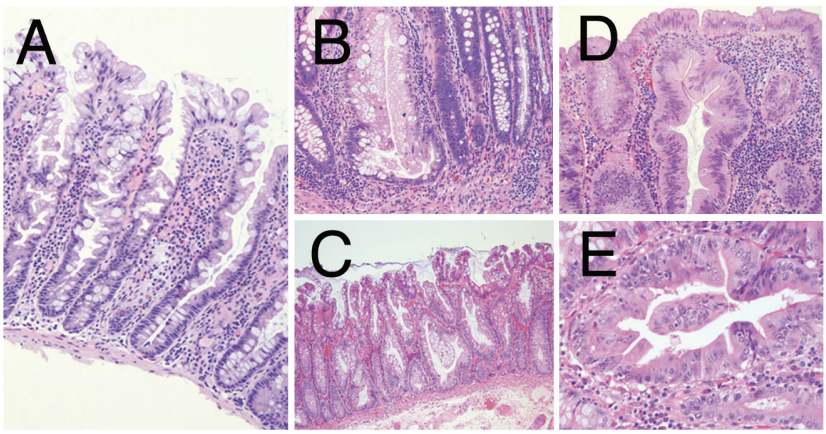


Figure 3: Representative hematoxilin & eosin stainings for different types of serrated polyps. A) An hyperplastic polyp with serrated glands limited only to the upper one-half of the crypt. **B and C)** Sessile serrated adenomas showing abnormal architectural features such as L-or-T-shaped crypts, and serrations that extend all the way to the crypt base. **D and E)** Traditional serrated adenomas showing a serrated appearance with crypt lumens lines by tall columnar cells with enlarged, crowded and hyperchromatic nuclei. Adapted from Noffsinger A.E. et al [30].

1.3.2. Mouse models for colorectal carcinogenesis

The development of experimental models that mimic the molecular pathways behind CRC carcinogenesis has been crucial for its better understanding. In this respect, mouse models closely emulate many aspects of the human disease counterpart, recapitulating some of the genetic lesions underlying sporadic or hereditary forms of CRC. Two main CRC mouse models have been developed to this regard, genetically engineered and chemically induced, which basically differ on the methodology used for the generation of colorectal tumors.

1.3.2.1 Genetically engineered CRC mouse models

Genetically Engineered Mouse Models (GEMMs) have been the basis for the study of hereditary CRC and, at the same time, for the elucidation of the role played by some of the genes involved in the development of sporadic CRC.

The first model for FAP, termed Min (multiple intestinal neoplasia) mouse, was developed

by random mutagenesis, and carried a truncation mutation at codon 850 of the *Apc* gene [43]. Min mice developed up to 100 polyps in the small intestine in addition to colon tumors. Later models specifically targeting the *Apc* gene by directed mutagenesis were developed. *Apc* truncation at codon 716 ($Apc^{\Delta 716}$) [44] or 1638 (Apc^{1638N}) [45], resulted in the formation of polyps in the small intestine. Apc^{1638N} mice displayed a long lifespan (a year or more), perhaps because of the fact that, on average, each mouse developed only 3-5 adenomas/carcinomas. However lifespan for $Apc^{\Delta 716}$ mice was much reduced because of the formation of a large number of gastrointestinal adenomas. Genetic models bearing mutation in additional genes (*Cdx2* [46] or *Smad4* [47]), in combination with $Apc^{\Delta 716}$, have shown to increase the rate of polyps and tumor formation as well as tumor invasiveness. Mutations in the beta-catenin gene, whose stability is regulated by Apc, caused the formation of up to 3000 polyps in the small intestine, reinforcing the role of the *Apc* gene in the development of FAP [48].

Regarding HNPCC, mouse models bearing alterations in the *Msh2* and *Mlh1* genes have been developed. *Msh2* deficient mice developed gastrointestinal adenomas and carcinomas. However, 50% of the animals died before the age of 6 months (when the formation of intestinal tumors can be observed) due to the development of T-cell lymphomas, suggesting the involvement of *Msh2* in lymphoblastic lymphomas [49]. *Mlh1* knockout mice displayed a reduced survival similar to *Msh2* -/- animals, also due to the presence of T-cell lymphomas and small intestinal tumors [50]. The combination of *Mlh1* and Apc^{1638N} mutant mice dramatically increased the number and invasiveness of intestinal tumors, consequently decreasing mice survival [50].

Other mouse models have been developed with mutations in additional genes, not directly implied in hereditary CRC. Mice bearing mutations in the *Tgfβ1* gene dramatically accelerated the progression of colorectal adenocarcinomas [51]. Mutated *K-ras* transgenic mice represent one of the most important models for sporadic colorectal cancer. Colon-specific expression of activated mutant *K-ras* ($K\text{-}ras^{G12D}$) resulted in the development of single or multiple lesions recapitulating the stages of tumor progression [52,53].

1.3.2.2 Chemically induced CRC models

As the spontaneous incidence of CRC in mice is low (1-4%), many chemicals have been used to induce CRC. Some of this carcinogens include dimethyhydrazine (DMH), azoxymethane (AOM), dextran sulfate sodium (DSS), 2-amino-1-methyl-6-phenylimidazol, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or 3,2'-dimethyl-4-aminobiphenyl (DMBA). Dosage and treatment duration, as well as the route of administration, determine the effectiveness of these drugs for the formation of colorectal tumors. Moreover, it has been shown that the genetic background of the mice plays a significant role in the development of tumors when using this approach [54].

1.4 Screening, diagnosis and staging

1.4.1 Screening

Colorectal cancer constitutes a disease characterized by a long preclinical stage, with the progression from early adenoma to invasive cancer taking years [55]. Moreover, and despite advances in treatment, 40-50% of patients presenting with symptomatic CRC eventually die of metastatic disease [56]. These characteristics of CRC make it more suitable for population screening than any other malignancy.

1.4.1.1 Conventional CRC screening tests

Current CRC screening tests can be broadly differentiated as early detection tools or cancer-prevention tools, depending on their modes of action [57]. Cancer prevention tools constitute tests that can find both colorectal polyps and lesions that have progressed to a more advanced stage. Early detection tools constitute a series of methodologies that are only able to detect CRC, but they are not suitable for the detection of possible pre-malignant lesions. Table 2, summarizes conventional CRC screening tests.

Fecal occult blood test (FOBT) and Fecal immunochemical test (FIT) are the two main early detection tools available [58]. FOBTs can be used to quantitatively detect microscopic amounts of haem in the stool. FITs detect human globin by means of an antibody-based assay, detecting also microscopic amounts of material in the stool, as FOBTs [57-59]. Advantages and disadvantages of early detection tools are summarized in Table 2, lower section. These techniques provide fast and inexpensive results, however, they are being replaced by imaging techniques, as a positive test does not prove a definitive diagnosis. Cancer prevention tools mainly include imaging techniques that allow visualization of the inner part of the intestine for the detection of polyps or more advanced lesions (Figure 4). In some cases, these techniques allow the removal of existing lesions in the same procedure [57]. Advantages and disadvantages of the main cancer-prevention tools are summarized in Table 2, upper section.

Related to the early detection of CRC, the local government of Galicia has recently implemented a screening program for population considered at risk (women and men between 50 and 69 years), based on the use of FOBT. This type of strategies are of key importance for the reduction of CRC-related mortality rates, especially in regions like this, where factors including dietary habits and the presence of an ageing population, increase the risk for CRC development.

Table 2. Conventional CRC screening tests

Screening test	Advantages	Disadvantages
Cancer-prevention tools		
Flexible sigmoidoscopy	-Fairly quick and safe -Sedation not required -Does not require a specialist -Done every 5 years	-Images only 1/3 of the colon -Can miss small polyps -Can't remove all polyps -Small risk of bleeding, infection of bowel tear -Colonoscopy will be needed if abnormal
Colonoscopy	-View of the entire colon -Can biopsy and remove polyps -Done every 10 years -Can diagnose other diseases	-Can miss small polyps -Full bowel preparation needed -Sedation needed -Small risk of bleeding, infection of bowel tear
Double-contrast barium enema (DCBE)	-View of the entire colon -Relatively safe -Done every 5 years -No sedation needed	-Can miss small polyps -Full bowel preparation needed -Cannot remove polyps during testing -Colonoscopy will be needed if abnormal
CT-colonography (virtual colonoscopy)	-Fairly quick and safe -View of the entire colon -Done every 5 years -No sedation needed	-Can miss small polyps -Full bowel preparation needed -Cannot remove polyps during testing -Colonoscopy will be needed if abnormal
Early detection tools		
Fecal occult blood test (FOBT)	-No direct risk to the colon -No bowel preparation -Sampling done at patient's home -Inexpensive	-May produce false-positive results -Should be done every year -Colonoscopy will be needed if abnormal
Fecal immunochemical test (FIT)	-No direct risk to the colon -No bowel preparation -Sampling done at patient's home -Fairly inexpensive	-May miss many polyps and some cancers -May produce false-positive results -Should be done every year -Colonoscopy will be needed if abnormal

Data from Kuipers et al [57]

1.4.1.2 Other screening modalities

1.4.1.2.1 Imaging techniques

CRC screening methodologies are continuously improving, with the aim of lowering patient burden and enhancing neoplasia detection. In the last years, methods that include modifications of conventional endoscopes to improve visualization have been developed [60,61]. An interesting recent technique is the use of a colon-capsule endoscopy [62,63]. This system consists in an indigestible capsule with a 172-degree video imager at each end. The capsule is moved through the colon by peristalsis and transmits images to an external data recorder carried by the screenee. This type of imaging requires performing certain previous clinical procedures to maximize the quality of the final images, and its current costs represent a limitation for the widespread of the capsule in screening programs [64].

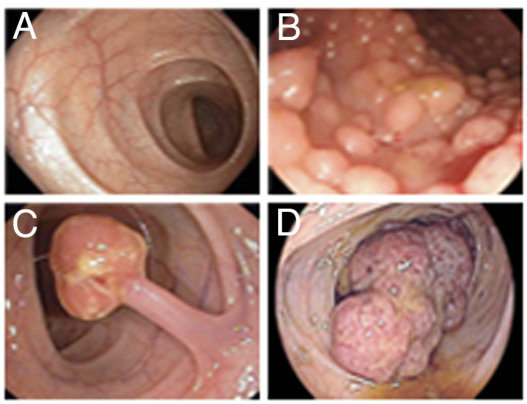


Figure 4: Representative colonoscopy images. A) Normal intestinal wall. **B)** Numerous polyps characteristic of HNPCC. **C)** A pedunculated adenoma with signs of minimal bleeding at the base of the stalk. **D)** Advanced colorectal carcinoma.

1.4.1.2.2 Molecular markers

Apart from the direct bowel observation by different imaging procedures, several indirect molecular markers have been explored as screen-detection tools for CRC and precursors of cancer. These markers can be measured in serum, tissue or stools, and they mainly reflect the mechanism of exfoliation of neoplastic cells and secretion of mucus containing abnormal glycoproteins in CRC [57]. Serum markers include, for example, the analysis of the methylation status of septin-9 (*SEPT9*) because its ability to discriminate between normal and cancerous tissues [65]. The expression analysis of MMP7 (Matrix metalloproteinase-7) and PTGS2 (prostaglandin G/H synthase 2) from RNA has also been assessed [66]. Some proteins have been analyzed in the stool (calprotectin or carcinoembrionic antigen, CEA) [67]. However, evidences from all the previous mentioned markers are still in an initial step, and larger validation studies should be conducted to delineate its value as screening markers.

1.4.2 Signs and symptoms

Most colorectal cancers are diagnosed after the onset of symptoms which mainly include [68]:

- A change in bowel habits, such as diarrhea and constipation, that lasts for more than a few days.
- Rectal bleeding, dark stools or blood in the stool.
- Abdominal pain
- Weakness and fatigue.
- Unintended weight loss.

1.4.3 Diagnosis and staging

CRC is definitively diagnosed on the basis of the results of colonoscopy or sigmoidoscopy, followed by tumor biopsy examination [10]. A newly diagnosed case of CRC undergoes a complete check, in order to determine cancer extent. Physical examination and a complete colonoscopy (to rule out metachronous tumors) are performed, and a computed tomography (CT) of the chest, abdomen, and pelvis are carried out to identify metastatic disease (Figure 5). Ultrasounds and Magnetic Resonance Imaging (MRI) can also be used to determine the extent of disease. Positron Emission Tomography (PET) with the 18-fluoro-2deoxy-D-glucose (FDG-PET) can be used to rule out extrahepatic spread (Figure 5). Based on all the obtained parameters, using the above-mentioned methodologies, staging is described according to the TNM (tumor, node, metastases) system [69] (Table 3) (Figure 6). An accurate tumor staging results critical for the management of disease, as the choice of an appropriate treatment is based on it.

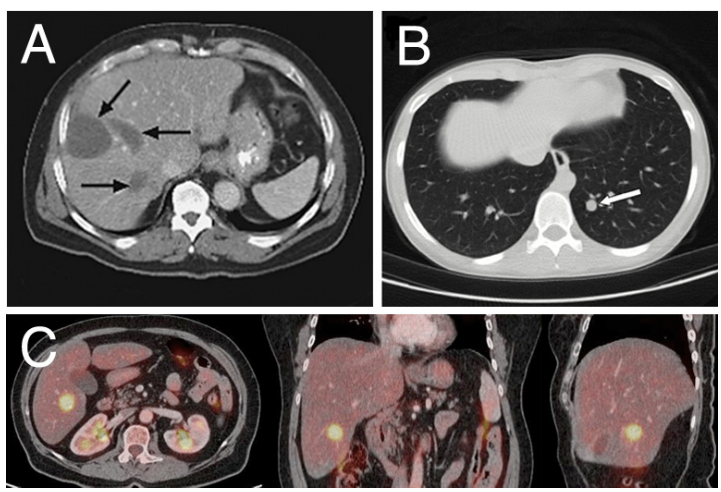


Figure 5: Detection of metastatic lesions in CRC by imaging techniques. **A)** Liver and **B)** lung lesions detected by computed tomography. **C)** Metastatic liver nodes detected by FDG-PET

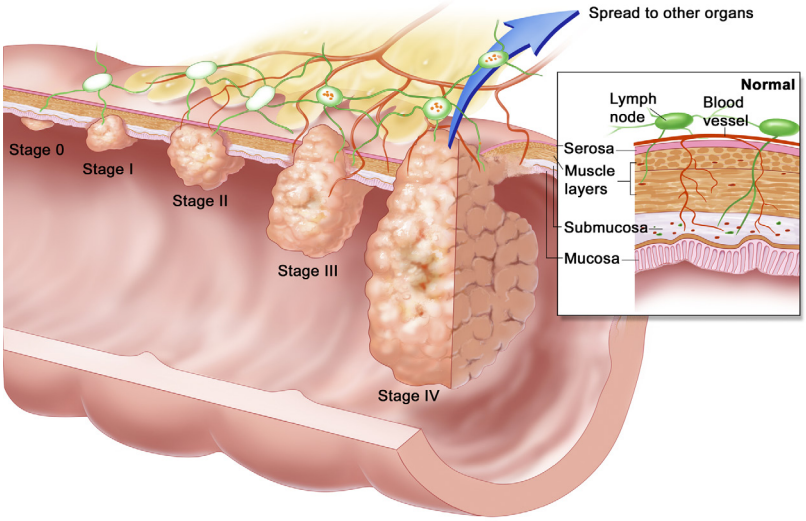


Figure 6: Colorectal cancer stages. As colorectal cancer progresses from stage 0 "carcinoma in situ" to stage IV, cancer cells sequentially grow through the layers of the rectum wall, and spread to lymph nodes and distant organs in the most advanced stages.

Table 3. TNM classification of colon cancer

T=primary tumor

TX: primary tumor cannot be assessed.
T0: no evidence of primary tumor.
Tis: carcinoma in situ: intraepithelial or invasion of lamina propria
T1: tumor invades submucosa
T2: tumor invades muscularis propria
T3: tumor invades through the muscularis propria into subserosa or into peritonealised pericolic or perirectal tissues
T4a: tumor penetrates the surface of the visceral peritoneum
T4b: tumor directly invades or is histologically adherent to other organs or structures

N=regional lymph nodes

NX: regional lymph
N0: no regional lymph node metastasis
N1a: metastasis in one regional lymph node
N1b: metastasis in two to three regional lymph nodes
N2a: metastasis in four to six regional lymph nodes
N2b: metastasis in seven or more regional lymph nodes

M=distant metastasis

MX: distant metastasis cannot be assessed
M0: no distant metastasis
M1a: distant metastasis to one site
M1b: distant metastasis to more than one site

Staging

Stage I	(T1/T2, N0, M0)	**Stage IIIA**	(T1/T2, N1, M0)
Stage IIA	(T3, N0, M0)	**Stage IIIB**	(T3/T4, N1, M0)
Stage IIB	(T4a, N0, M0)	**Stage IIIC**	(Any T, N2a/N2b, M0)
Stage IIC	(T4b, N0, M0)	**Stage IVA**	(Any T, Any N, M1a)
		Stage IVB	(Any T, Any N, M1b)

Adapted from Cunningham et al [10]

1.4.4 Prognostic factors and biomarkers

A prognostic factor is defined as a measurement of a disease-related parameter, through a direct or indirect observation, that is associated with the clinical outcome of the patient in the absence of therapy or with the application of a standard therapy that patients are likely to receive [70]. The American Joint Committee on Cancer (AJCC) has established a colorectal working group, which has evaluated the role of biologic, genetic, molecular and other nonanatomic factors with prognostic value in CRC [71]. Prognostic factors are classified into five different categories (I, IIA, IIB, III and IV) (Table 4) based on the existence of conclusive evidences about their effectiveness, with category I including the best characterized factors, currently used in routine clinical patient management, and category IV including factors that have been studied but didn't show prognostic significance. Examples of prognostic factors included in each category, are summarized in Table 4, right panel.

To date, a limited number of factors have shown the necessary prognostic evidences to be considered as clinically relevant, and only tumor extent related features are considered of strong prognosis value. The presence of carcinoma *in situ*, a malignancy that has not yet penetrated the basement membrane of the epithelium to invade the underlying lamina propria, is considered a major good prognosis factor. Moreover, the presence of distant metastasis or cancer spread to regional lymph nodes constitute two important factors that determine patient outcome [72]. The evaluation of serum CEA is the most accepted and frequently used tumor prognostic biomarker in CRC, partly due to its relatively inexpensive and easy detection. The main use of CEA in CRC is in surveillance following curative resection for primary cancer [73,74], where CEA levels higher than 5.0ng/ml have an adverse impact on patient prognosis, independent of tumor stage [75].

In the last years, alternative biomarkers have been proposed in order to evaluate patient prognosis more accurately. In this regard, the analysis of Circulating Tumor Cells (CTCs), tumor cells that have shed into the circulation from the primary tumor, has become of a great interest. CTCs are proposed as a major vehicle for tumor dissemination and metastasis formation, so it is reasonable to think that their presence in the blood could provide information about disease status, separating patients with different prognosis depending on the existence of CTCs or not (and if yes, their number) in patient's blood. In fact, several works have confirmed the validity of CTC count as an independent prognostic factor, mainly in metastatic CRC (mCRC) [76-81]. Another promising biomarker to evaluate patient outcome is the analysis of circulating tumor DNA (ctDNA), DNA fragments mainly originated from apoptotic or necrotic tumor cells that discharge their DNA into the blood circulation [82]. Elevated concentrations of cell-free ctDNA fragments have been found to be associated with unfavorable outcome in some clinical studies [82,83]. Moreover, ctDNA sequencing offers the possibility to assess tumor-specific mutations without the need of

tissue biopsy, making easier the evaluation of tumor characteristics [83]. Both CTCs and ctDNA analysis have been proposed as new promising biomarkers for patient prognosis in CRC, providing valuable information from a simple and non invasive blood sample, reason why their use has led to the occurrence of the term "liquid biopsy". Detailed information of the clinical value of CTCs can be found in introduction section 3.

Table 4. Classification of prognostic markers in CRC.

Category	Definition	Prognostic factors included
Category I	Well supported by literature, generally used in patient management, and of sufficient importance to modify TNM stage groups	-Pathological assessment of tumor extent (pT) -Regional lymph node metastases (pN) -Presence/absence of blood/lymphatic vessel invasion -Residual tumors -Elevated serum CEA
Category IIA	Extensively studied biologically and/or clinically. Sufficient to be noted in a pathology report	-Presence of residual tumor after resection -Radial margins -Histologic grade -Tumor border configuration
Category IIB	Well studied but not sufficiently established	-Lymphocytic infiltration of tumor or peritumoral tissue -Histologic types -Tumor tissue molecular markers -18q/DCC loss (LOH) -mutated *KRAS* -MSI -Thymidylate synthase (TS) high -p27 low -Bcl-2 loss -p53 inactivation
Category III	Less well studied and not yet sufficiently established	Perineural invasion, microvessel density, tumor cell associated proteins or carbohydrates, peritumoral fibrosis, peritumoral inflammatory response, focal neuroendocrine differentiation, nuclear organizing regions, proliferation indices.
Category IV	Studied and shown to have no consistent prognostic significance	Tumor size and histologic tumor type

Data from López-Gómez et al [72]

1.5 Treatment

CRC treatment encompasses different strategies that are applied or not mainly depending of the grade of tumor extent, reason why an adequate staging is crucial for the selection of the most suitable therapy.

1.5.1 Surgery

Surgery represents the only curative modality for localized CRC and also provides a potentially curative option for selected patients with limited metastatic disease in liver and/or lung. In many cases, even in those who cannot be cured by surgery, this procedure benefit them through palliation of symptoms such as obstruction and bleeding from the primary tumor [84]. When CRC is found at a very early stage, usually *in situ* carcinomas, local excision of the primary tumor can be applied. Total resection of the tumor should be done in cases of larger tumor extent.

1.5.2 Adjuvant CRC therapy

Despite improvements in CRC surgery, many patients still have a high risk of tumor recurrence. To reduce those risks, adjuvant treatment is used in a subset of patients suffering from non-disseminated disease. Adjuvant therapy corresponds to any given treatment that is administered to the patient after the main therapy, which is normally surgery is this kind of patients.

For stage III patients, adjuvant therapies are mainly based in the use of 5-Fluorouracil (5-FU), alone or in combination with other agents [85]. For example, 5-FU plus Leucovorin (LV) has demonstrated an improvement in patient outcome [86]. Adjuvant therapy is also used for stage II CRC patients.

In the last years, the combination of targeted therapies with classic chemotherapeutic agents has gained importance. The introduction of monoclonal antibodies that target the Epidermal Growth Factor Receptor (EGFR) (Cetuximab or Panitumumab) or Vascular Endothelial Growth Factor (VEGF) (Bevacizumab), has expanded the agents available for use in adjuvant setting [85]. However, these agents have not demonstrated enough clinical utility when applied in the adjuvant setting so far, and their main field of application is in metastatic CRC (see section 1.5.4).

1.5.3 Neoadjuvant CRC therapy

A neoadjuvant therapy is a treatment given as a first step, to shrink the tumor before the main treatment, usually surgery, is given. Neoadjuvancy is not considered for non-metastatic stages of CRC, as surgery is usually the first treatment, and is in rectal cancer where this kind of therapies are generally applied [87]. However, in resectable metastatic CRC disease, similar therapeutic regimes to those used for adjuvancy in non-metastatic CRC are being used. FOLFIRI scheme (Irinotecan + Leucovorin + 5-FU), and combinations with targeted therapies are also used in this setting.

1.5.4 Metastatic CRC treatment

The majority of patients with metastatic colorectal cancer (mCRC) cannot be cured, although a subset with hepatic and/or lung metastases are potentially curable with surgery. For the rest, treatment is palliative and generally consists of systemic therapy, which has demonstrated to improve both progression-free survival (PFS) and overall-survival (OS) when compared to patients receiving only supportive care [88,89].

For advanced colorectal cancer (stage IV), first line chemotherapy is mainly based in the use of fluoropyrimidines (5-FU or capecitabine), which represent the basis of current common treatments. Other drugs, such as Irinotecan (a topoisomerase I inhibitor) or oxaliplatin (a second generation platinum derivative), have demonstrated significant activity in mCRC [89-91]. However, they are not commonly used as single agents, as their combination with 5-FU or capecitabine significantly improve their effectiveness [89,92,93].

The election of more or less aggressive therapies is normally guided by patient's performance status (PS). PS is categorized using an scale defined by the Eastern Cooperative Oncology Group (ECOG), that attempts to quantify patient's general well-being [94] (Table 5). First-line refractory patients (with good performance status) are generally proposed for a second line therapy based of a different drug combination.

Current therapeutic approaches generally include the combination of classic chemotherapies with modern biological agents that target specific signaling pathways commonly altered in CRC. The heterogeneous nature of cancer means that personalized medicine (i.e. tailoring therapy to an individual patient) is a promising approach for maximizing efficacy and minimizing the toxicity of treatment. This kind of therapeutic strategies also reduce therapy-related costs, as treatment is only given to those patients likely to benefit.

Table 5. ECOG performance status scale.

Grade	Characteristics
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry work of a light sedentary nature.
2	Ambulatory and capable of all selfcare but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	Capable of only limited selfcare, confined to bed or chair more than 50% of waking hours.
4	Completely disabled. Cannot carry on any selfcare. Totally confined to bed or chair.
5	Dead

Data from Oken et al [94]

Two main signaling pathways are currently being targeted using biological agents: Vascular Endothelial Growth Factor (VEGF) and Epidermal Growth Factor Receptor (EGFR).

1.5.4.1 Agents targeting VEGF: Bevacizumab

Bevacizumab (Genentech, San Francisco, U.S) is a recombinant, humanized, monoclonal antibody that blocks the activity of VEGF, a central mediator of tumor-related angiogenesis [95]. When used in combination, Bevacizumab improves PFS compared with chemotherapy alone [96,97], preferably when applied with irinotecan-based regimens [98]. However, and despite much research, is still poorly understood which patients/tumors characteristics are best treated with anti-angionenics, and no validated predictive markers (markers that indicate which patients will respond to a given therapy) are currently available [99].

1.5.4.2 Agents targeting EGFR: Cetuximab and Panitumumab

EGFR is overexpressed in about 70% of patients with mCRC and has been considered a poor prognostic factor [100]. Panitumumab (Amgen, Thousand Oaks, U.S) and Cetuximab (ImClone, Branchburg, U.S) are two monoclonal antibodies targeting active EGFR, normally administered in combination with classical chemotherapy drugs, showing improved efficacy [101]. Molecular events such as overexpression or mutation of genes involved in the EGFR signaling pathway (for example *KRAS*, *NRAS* or *BRAF*) identify patients that are likely to respond to anti-EGFR drugs [95,102] (see section 1.5.5).

1.5.4.3 New therapeutic agents

Considerable advances in the development of new mCRC therapeutic approaches have been achieved in the last years, as evidenced by the increasing number of molecules (usually monoclonal antibodies or small inhibitors) available for mCRC patient's treatment [89]. Some of the new therapeutic agents, currently used in the clinics, or under testing in clinical trials are summarized in Table 6.

Table 6. Summary of new therapeutic agents for mCRC treatment

Molecule	Type	Target
Agents being used in the clinical setting		
Aflibercept	Recombinant protein	VEGF
Regorafenib	TKI	RTKs
Agents under clinical testing		
Cediranib	TKI	VEGF
Mapatumumab	MoAb	TRAIL-receptor
IGFR Inhibitors	MoAb and TKI	IGFR
AMG102	MoAb	HGF
Vismodegib	Small-Inhibitor	Hedgehog Signaling
Vemurafenib	Small-Inhibitor	Mutated BRAF
AZD6244	Small-Inhibitor	MEK
Olaparib	Small-inhibitor	PARP
Dasatinib	TKI	SRC

Abbreviations: TKI: Tyrosine Kinase Inhibitor; MoAb: Monoclonal Antibody; RTKs: Tyrosine Kinase Receptors
Data from López-Gómez et al [89]

1.5.5 Predictive markers

One of the main problems of CRC therapies is the existence of toxicity-related side effects, which are sometimes reason enough to stop a the administration of a therapy. Moreover, actual targeted therapies are sometimes directed to a very specific molecule. All this makes necessary the existence of predictive markers that allow the identification of subpopulations of patients who are more likely to respond to a given therapy, avoiding unnecessary therapy-related toxicities in patients with low response probabilities.

In CRC one of the best-studied examples of a predictive biomarker is the *KRAS* gene mutational status for patients treated with anti-EGFR antibodies. *KRAS* mutation originates a constitutively active GTPase, which will transmit signals to its downstream effectors irrespective of the presence of EGFR ligands. Lievre et al showed for the first time that *KRAS* mutations in mCRC patients are a predictor of resistance for Cetuximab therapies and are associated with worse prognosis [103]. After this first study, the association of *KRAS* mutations and resistance to anti-EGFR treatment, either Cetuximab or Panitumumab,

was confirmed in large, retrospectively evaluated phase III studies [104,105]. More recently, activating mutations in *RAS* (either *KRAS* or *NRAS*) have been suggested as negative predictive biomarkers for anti-EGFR therapy [102]. These clinical data is supported by the biological similarities existing between *KRAS* and *NRAS*, together with the fact that mutations in both genes results in increased levels of guanosine triphosphate-bound RAS proteins [106]. However, *HRAS*, the third member of the RAS family, is rarely mutated in CTC, which discards its association with this type of targeted therapies.

Other molecular markers have been identified and validated as predictive markers for a specific therapy, indicating the probability of tumor response, but also giving information about other therapy-related features, like toxicity. Table 7 shows an overview of current predictive molecular markers in CRC. Not included in the table, but of an increasing interest is the analysis of CTCs, especially for patients with disseminated disease. Until now, CTCs have been used mainly as an indicator of patient prognosis, based on the CTC number at baseline, e.g. before therapy. Several studies have focused now on the potential role of CTCs as a predictive biomarker. CTC number changes along treatment were found to be related with therapy effectiveness in CRC, mostly in metastatic disease. [76,77,108-112]. Moreover, CTCs offer the possibility of analyzing particular gene or protein alterations, usually related to therapy response when assessed in the primary tumor, which provides an opportunity for non-invasive and real-time monitoring of treatments [113,114]. Detailed information about the utility of CTCs as therapy response predictive markers can be found in introduction section 3.

Table 7. Predictive biomarkers in CRC

Marker	Alteration	Predictive for:
Thymidilate-Syntase (TS)	Increased expression	Decreased 5-FU response
Dihydropyrimidine-dehydrogenase (DPD)	Decreased DPD activity	Increased 5-FU toxicity Increased 5-FU response
UDP-glucuronosyl transferase 1A1 (UGT1A1)	Decreased UGT1A1 activity	Increased Irinotecan-induced neutropenia
ERCC1	Decreased ERCC1 expression	Increased oxaliplatin response and survival
RAS (*KRAS* and *NRAS*)	Constitutive GTPase activation	Resistance to anti-RGFP MoAb's therapy
BRAF	Constitutive activation	Decreased survival
COX2	Reduced promoter activity	Increased Cetuximab PFS
IL-8 VEGF	Increased VEGF expression	Increased recurrences

Abbreviations: 5-FU: 5-Fluorouracil; MoAb's: Monoclonal Antibodies;
Adapted from Winder et al [107]

2. Cancer metastasis. Focus on colorectal cancer

2.1 The metastatic cascade

Whereas surgical resection and adjuvant therapy can cure well-confined primary CRC tumors, metastatic disease is largely incurable because of its systemic nature and the resistance to current therapeutic agents. This explains why more than 90% of mortality from cancer is attributable to the existence of metastatic disease [115,116].

Cancer metastasis has been a focus of innumerable research works for many years. However, and although considerable understanding of the process of cancer spreading has been achieved, much is still unknown, due to its complexity. Cancer metastases are formed following the completion of a succession of cell-biological events (also termed the metastatic cascade [121,117]) by which epithelial cells in primary tumors are able to invade the surrounding stroma, enter the circulation and finally reach a target organ to form a distant tumor mass [116] (Figure 7). Many of these events are orchestrated by molecular pathways operating within carcinoma cells. Moreover, their interaction with non-tumor stromal cells also plays a pivotal role throughout the whole process of metastasis.

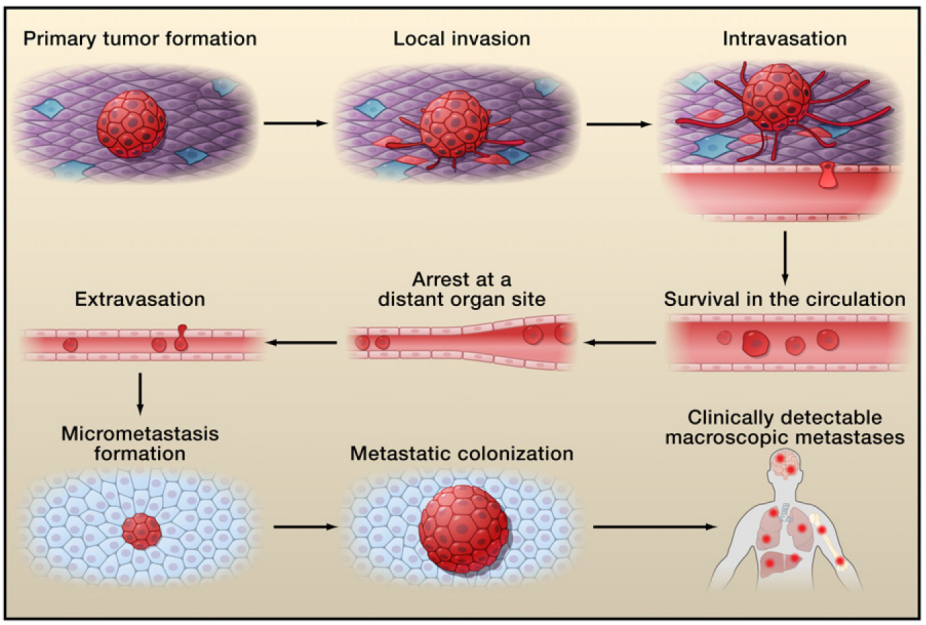


Figure 7: The invasion-Metastasis cascade. During metastatic progression, tumor cells exit their primary sites of growth by a process of local invasion and subsequent intravasation into nearby blood vessels. After their translocation into the bloodstream, they must survival in the circulation and arrest at distant sites for to further escape the circulatory system by extravasation. Micrometastasis formation depends on the ability of cancer cells to adapt and thrive in the foreign microenvironments of distant tissues. Carcinoma cells are depicted in red. Image from Valastyan et al [121].

2.1.1 Local invasion

After primary tumor formation and in order to invade the tumor surrounding stroma, cancer cells must first breach the basement membrane (BM), a specialized extracellular matrix (ECM) that separates the epithelial tumor compartment from the neighbor stromal cells [116,118,119]. In a non-tumor situation, BM plays a crucial in the maintenance of the epithelial architecture, by providing a structural support for the correct alignment and polarization of epithelial cells, a key process for the development of a highly organized epithelial tissue [119,120].

Depending on the tumor type and the surrounding tissue, various patters of invasion can be observed [121]. Most carcinomas can invade as cohesive multicellular units through a process termed “collective invasion”. Alternatively, tumor cells can invade as single cells, invasion mechanism evidenced by the presence of isolated and dispersed tumor cells in the tumor-adjacent tissues [121,122].

2.1.1.1 Collective invasion

Collective invasion is characterized by the mainteinance of cell-cell adhesions between invading cells, by the continuous expression of adhesion molecules and other components of the adhesive apparatus (cadherins, tight junction proteins, adhesion receptors of the immunoglobulin superfamily and gap junctions) [121] (Figure 8). To finally penetrate the BM, cancer cells must develop the ability of degrading and remodel the ECM, process that allows them to break through the surrounding extracellular components. Collectively migrating colon cancer cells engage and cluster integrins in anterior protrusions toward the ECM and shown elevated expression of specific matrix-metalloproteases (MT1-MMP, uPA/uPAR or MMP2) for remodeling the ECM and move towards adjacent tissues [123]. The morphological organization of collectively invading cells can vary from strands of few cells to broad cellular masses, that can migrate as isolated cellular clusters or as invading tip-like structures, characterized by the presence of one or more leading cells (with actin-rich invading structures and elevated metalloprotease activity), that guide the invasion of the whole group [122]. All these invading structures are characterized by the presence of well-established cell-cell junctions, supracellular contractility and a high rate of cell-matrix adhesions turnover (Figure 8).

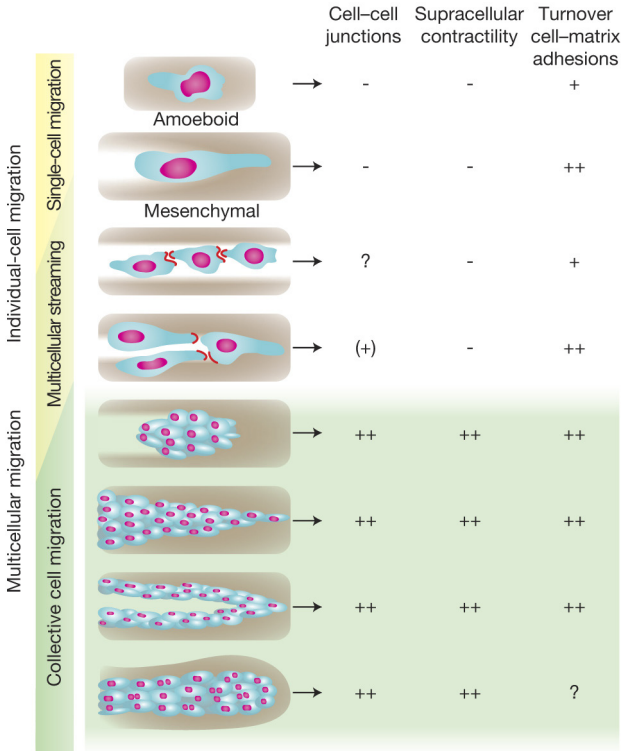


Figure 8: Cancer cell invasion modalities. Cancer cell invasion patterns include amoeboid or mesenchymal single-cell migration; multicellular streaming with or without weak junctional contacts or collective cell migration. Although each pattern can be described as a different process, overlapping cell behavior can result in mixed or unstable phenotypes. Intensity range of proposed properties (from absent to strong) is indicated by minus and plus signs.
Adapted from Friedl et al [127].

2.1.1.2 Single cell invasion

One of the main traits that characterized single cell invasion/migration is the complete or partial loss of cell-cell contacts. Individual tumor cells may invade via two distinct programs: the mesenchymal invasion (protease, stress fiber and integrin-dependent), or the amoeboid invasion (integrin-independent, Rho/ROCK-dependent) [124]. Mesenchymal invading cells frequently display many hallmarks of the Epithelial to Mesenchymal Transition (EMT) process (see Box 3), and usually display high levels of cell-matrix adhesion and proteolysis. On the contrary, amoeboid invading cells are largely independent of these requirements but rely on cortical actomyosin contractility [116,121,125-127].

A range of intermediate phenotypes were described between single and collective cell migration. Single cells can migrate individually, but close enough one from each other, and following similar paths, normally imposed by the presence of a chemical gradient or by the physical characteristics of the ECM, in an invasion modality termed “multicellular streaming” [128]. Very weak or no cell-cell contacts were detected in this specific situation (Figure 8). Multicellular streaming has been observed in ortothopic breast cancer and melanoma models, but not in CRC [122,124,129].

Once invading cells degrade the component of the basement membrane, and migrate through it, they reach the stromal compartment where they must coexist with several

different tumor-associated cell types (fibroblasts, endothelial cells, adipocytes and bone-marrow derived cells [130,131]). As primary tumor progression proceeds, the stroma becomes increasingly "reactive" acquiring typical characteristics of the stroma observed in wound healing or inflammation, and these features further enhance the aggressive behavior of carcinoma cells [116]. For example, stroma fibroblasts (also termed Cancer Associated Fibroblasts, or CAFs) can remodel the ECM through enzymes, rendering it more supportive of tumor cell invasion [132,133] (Figure 9). Altogether, the specific characteristics of the stromal cells contribute to the migration and invasion of cancer cells through the stromal compartment towards nearby lymph and blood vessels.

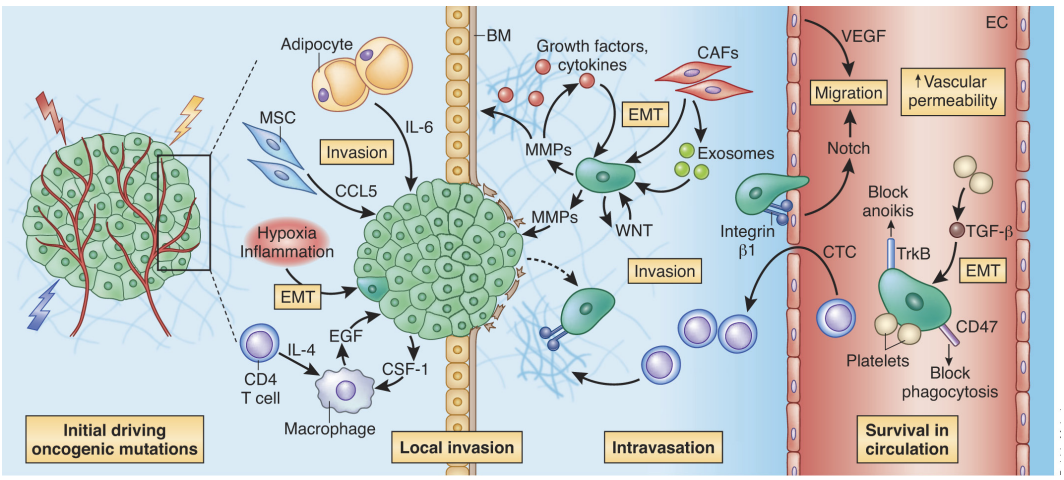


Figure 9: The early steps of metastasis: tumor invasion, dissemination and survival in the circulation. In order to disseminate, carcinoma cells need to acquire the capabilities to break down the basement membrane, invade into the stroma, enter the bloodstream and manage to survive before they can arrest at distant sites and grow into clinically detectable metastases. Examples of tumor-stroma crosstalk are illustrated. EC: endothelial cells. Image from Wan et al [138].

2.1.2 Intravasation

The term intravasation refers to the process by which invasive carcinoma cells enter into the lumina of tumor associated lymphatic or blood vessels. Although lymphatic spread of CRC is routinely observed in human tumors and represents an important prognostic marker for disease progression, dissemination via the hematogenous circulation appears to represent the major mechanism by which metastatic cells disperse [115]. Growing tumors need to develop neo-vasculature in order to grow beyond the diffusion limit of preexisting blood vessels [134], process that facilitates the escaping of tumor cells from the primary tumors through the circulation [115]. Moreover, newly formed blood vessels within the tumor are characterized by its leaky structure, with not well-established cell-cell contacts between endothelial cells, and under continuous reconfiguration, characteristics that are likely to facilitate intravasation [135]. The molecular mechanisms controlling intravasation remain to be well defined. In colon cancer, it has been recently shown that Notch signaling in cancer cells can be stimulated by coexisting stromal cells, further enhancing intravasation and thus, promotion of metastasis [136] (Figure 9).

2.1.3 Survival in the circulation

Once malignant cancer cells have invaded the circulatory compartment, they attain ready access to virtually all organs of the body. These circulating tumor cells (CTCs), represent carcinoma cells that are en route between primary tumors and sites of dissemination and therefore may represent “metastatic intermediates” [116].

CTCs must be able to survive several stresses, including physical damage from hemodynamic shear forces, and immune mediate-killing [115]. Carcinoma cells seem to overcome these threats through the formation of relative large associations with blood platelets, that protect them both from shear forces and facilitates evasion of immune detection [130] (Figure 9). Moreover, the lack of integrin-dependent adhesion to ECM components, that is normally essential for cell survival, makes CTCs more susceptible of undergoing anoikis (apoptosis triggered by loss of anchorage to substratum) [137,138]. To this regard, it has been shown that brain-derived neurotrophic factor (Bdnf) receptor (trkB) conferred resistance to anoikis in rat intestinal epithelial cells [139] (Figure 9). Moreover, in a prostate cancer model, Talin1, a key component of the focal adhesion complex, regulated anoikis resistance further promoting metastasis formation [140] (see introduction section 4 for further Talin1 information). However, the relevance of anoikis in the process of metastasis remains uncertain, as it make take mere minutes for a malignant cell departing from a primary tumor to encounter a capillary bed and adhere to the vascular wall [115]. Due to the large diameter of CTCs (20-30μm), they are likely to become trapped in narrow capillaries (≈8 μm) in their first pass through the circulation [116] (Figure 10), and if the time that CTCs spend devoid of adhesion is so short, anoikis may not be a very significant impediment during metastasis progression.

2.1.4 Arrest at distant sites and extravasation

Having invaded and endured the circulation, metastatic cells must at some point escape the bloodstream to finally colonize a target organ, in a process termed extravasation [115]. Despite the theoretical ability of CTCs to disseminate to a wide variety of secondary loci, clinicians have long noted that individual carcinoma types form metastasis in only a limited subset of target organs [141]. Particularly in CRC, liver is largely the preferential site for metastasis formation, with lung as a frequent, but secondary site. In fact, CRC metastatic dissemination seems to follow a stepwise manner in which lung metastasis develops only after the formation of considerable liver metastases. This pattern can be explained by the anatomical layout of the vasculature, which drain most CTCs through mesenteric circulation into the liver [133]. This model for CRC dissemination is partially supported by a study showing lower number of viable CTCs in the peripheral blood, compared to the mesenteric blood in patients with CRC [142]. Moreover, carcinoma cells arriving in the liver encounter fenestrated sinusoids that are highly permeable even in their normal state and consequently seem to pose only minor obstacles to extravasating tumor cells [143].

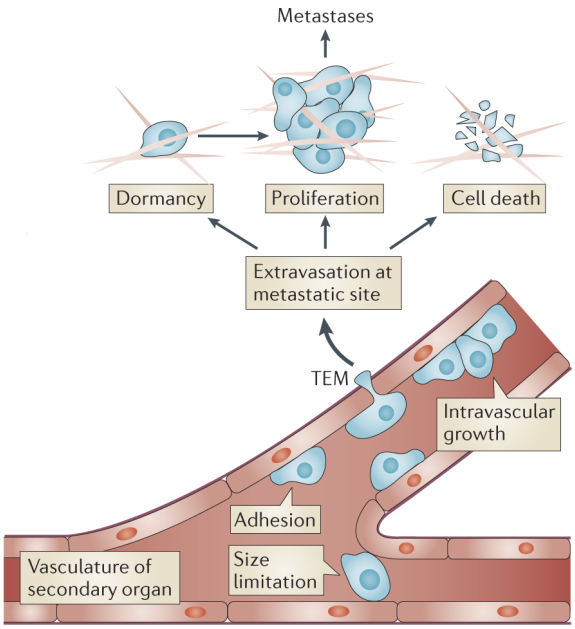


Figure 10: The late steps of metastasis: arrest in the circulation, extravasation and distant metastasis formation. After survival in the circulation, cancer cells leave the circulation in a process called extravasation at potentially secondary sites. Cancer cells can be retained at capillary sites due to size restrictions, or by the specific interaction with adhesion molecules expressed by endothelial cells. After adhesion, cancer cells transmigrate through the endothelial layer (transendothelial migration or TEM), and after this they invade the endothelial basement membrane. Extravasated cells can then enter a state of dormancy or proliferate within this new microenvironment, were a few of them will give raise to metastases.
Adapted from Reymond et al [125]

However, tissue-specific arrest of CTCs might not be only a physical process, by which CTCs are retained in capillary beds due to size restrictions. Some cells may elude this rapid trapping because of their unusual plasticity, enabling then to be lodged in the microvessels of more distal organs [116]. An alternative hypothesis is that CTCs have predetermined predilections to lodge in certain tissues. In fact, entry of CRC cells into the hepatic vasculature can initiate a proinflamatory cascade that results in Kupffer cells being triggered to secrete chemokines that upregulate vascular adhesion receptors, enabling adhesion of CTCs to the microvasculature of the liver [144]. Another hypothesis is that tumor cells, either at primary sites or in the circulation, can release soluble factors of microvesicles to convert incipient metastatic sites into compatible "premetastatic niches" [145,146]. For example, melanoma-derived exosomes have been show to promote metastasis by education of bone marrow cells [147], and Tenascin C, a component of the extracellular matrix, produced by breast cancer cells in primary tumors, can remodel secondary sites promoting a more permissive niche for CTCs implantation [148].

Metastatic cells can either escape the circulation individually, or considerable growth within the intravascular space may occur before target organ colonization [149] (Figure 10). To escape the circulation, CTCs must first interact with endothelial cells lying in the luminal side of blood vessels. Although the process of tumor cell extravasation remains to be well defined, several similitudes to the normal extravasation process of leucocytes at inflammatory sites have been identified [120,150]. Initial attachment of CTCs to endothelial cells is mediated by interaction of endothelial selectins (E-selectin) and its ligands in cancer cells [tetrasaccharide sialyl Lewis x (sLex) or sialyl Lewis a (sLe^a), MUC1 or CD44[120,151]] (Figure 11, step 1).

After initial attachment to ECs, CTCs must establish more stable adhesions in which integrins are mainly involved (Figure 11, step 2). In the context of extravasation, several integrins on cancer cells have been implicated in their attachment to the endothelium and transmigration across it [152]. Particularly, β1, β4 and αVβ3 integrins contributed to the strong adhesion between cancer and endothelial cells [120]. Together with integrins, MUC1 and CD44 have also been implicated in this type of adhesion [153-155]. Chemokine production by cancer cells also contributes to extravasation. For example, CCL2A (CC-chemokine ligand 2) produced by colon cancer cells interacts with its receptor (CCR2) on ECs to increase extravasation and metastasis into the lungs [156]. After firm adhesion to ECs, cancer cells must migrate through them in a process termed transendothelial migration (TEM) (Figure 11, step 3). Two main forms of TEM have been described, mostly on the basis of work using leukocytes: Paracellular and transcellular TEM. Paracellular TEM is defined by the migration of cancer cells through EC junctions after their disruption by cancer cells. Transcellular TEM is defined as the migration of cancer cells directly through the EC body [120]. So far, it is not clear which route is used by cancer cells in vivo, or whether the route depends on the vascular bed or on the cancer type.

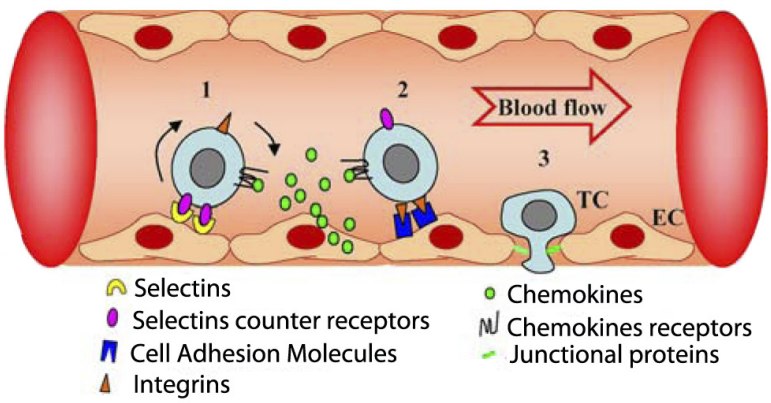


Figure 11: Cancer cell extravasation. Cancer cells first interact with blood vessel endothelial cells in a transient adhesion process, which involves endothelial selectins and their counterpart receptors on cancer cells (1). The second step (2) consists in the establishment of firmer adhesions and is mediated through chemokines and cell adhesion molecules on the endothelium and integrins on cancer cells. The last step (3) is characterized by the extravasation of cancer cells through endothelial cells junctions. EC: endothelial cells; TC: tumor cells. Adapted from Gout et al [156]

2.1.5 Micrometastasis formation and target organ colonization

In order to form micrometastases, extravasated carcinoma cells must survive in the foreign microenvironment that they encounter in the parenchyma of distant tissues, which greatly differs from the one in the primary tumor. Due to this, most cancer cells will die by apoptosis initiated by an adverse environment [157] (Figure 10). To overcome this adverse scenario, two main strategies have been proposed for cancer cells to successfully establish a micrometastasis: adaptation of cancer cells to the new microenvironment or preparation of the metastatic niche prior to the arrival of cancer cells from the primary tumor, or both [116]. One example for cancer cell adaptation involves activation of Src tyrosine kinase signaling, which increases the capacity of breast carcinoma cells to persist in bone without influencing their initial homing to this tissue. The metastatic niche preparation hypothesis, also termed the “premetastatic niche”, implied the release of systemic signals by primary tumors that induce changes in ECM proteins at distant sites [145]. As mentioned before, the production of molecules such as lysyl oxidases (LOXs) [158], Tenascin C [148], or the release of vesicular structures (exosomes) [147] have been related to the formation of a premetastatic niche.

Successful micrometastasis formation by cancer cells does not imply further proliferation for the formation of large macroscopic metastases. In fact, it seems that the vast majority of tumor cells suffer either slow attrition over periods of weeks and months or persist as microcolonies in a state of apparent long-term dormancy, retaining viability in the absence of any net gain or loss in overall cell number [159] (Figure 12). Factors like the ability of extravasated cancer cells to induce angionenesis, or their self-renewal capacity, have shown to be key steps for the outgrowth of micrometastases. In fact, it has been proposed that only a subpopulation of neoplastic cells, termed “tumor initiating cells, or TICs”, possess enough self-renewal ability to effectively give raise to macrometastases [160]. EMT transcription factors such as Snail, Twist or ZEB1 (see Box 3) have been proposed as possible molecules facilitating the acquisition of a TIC phenotype [161]. This, together with other factors such as microRNAs, or the coexistence with stromal cells at distant sites, finally enable that cancer cells complete an intricate, multistep, cell-biological process that culminates in the formation of macroscopic, life-threatening growths at distant organ sites [116].

2.2 Metastasis formation: a matter of number and time

Despite the implications that the appearance of metastasis has in patient survival, the metastatic process, as a whole, is extraordinarily inefficient. Animal modeling suggests that less than 0,02% of tumor cells that are shed into the circulation can survive and form metastasis [159]. Cancer spreading constitutes a process limited by multiple factors from its very early steps. Cancer cell survival at the invasive areas of primary tumors is restricted by the presence of an inhospitable surrounding stromal environment, together with the ineffective oxygen and nutrient input, due to the lack of well-established tumor vasculature. Data from animal models indicate that survival in the circulation, arrest at distant sites and extravasation seem to be the more effective processes in terms of cell survival. More than 80% of cells intravenously implanted were found to succeed in extravasating [157]. In contrast, once tumor cells exit the microvasculature into the parenchyma of foreign tissues, very low rates of cell survival are observed (<3% of intravenously implanted cells finally form micrometastases, and less than 0,02% form macrometastases [157]) (Figure 12). Consistent with this experimental observation is the fact that large numbers of CTCs can be detected within the bloodstream of carcinoma patients, including those who develop few, if any, over metastases [162]. Moreover, the appearance of micrometastases into the bone marrow of breast cancer patients, was not closely related with the development of macroscopic metastases, with only 50% of the patients developing tumors after 10 years [163], further supporting the idea of the metastatic spreading as a highly inefficient process.

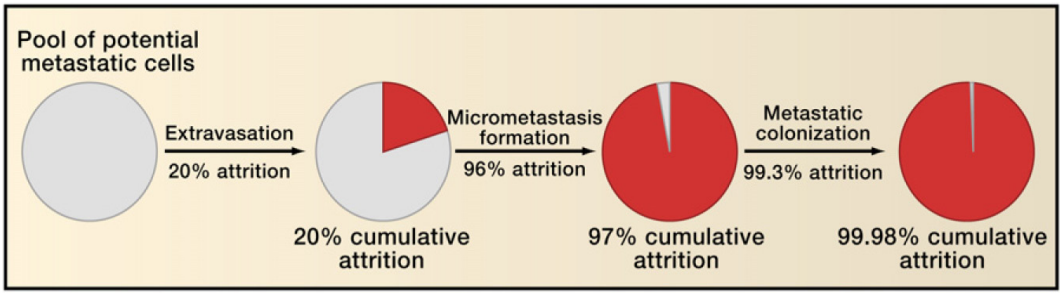


Figure 12: The inefficiency of the metastatic cascade. Data from experimental models has revealed inefficiency of the metastasis formation process, with a rate of attrition that often exceeds 99%. Here is depicted the approximate fraction of intravenously implanted tumor cells that have died after passage through the indicated steps of the invasion-metastasis cascade. Image from Valastyan et al [121].

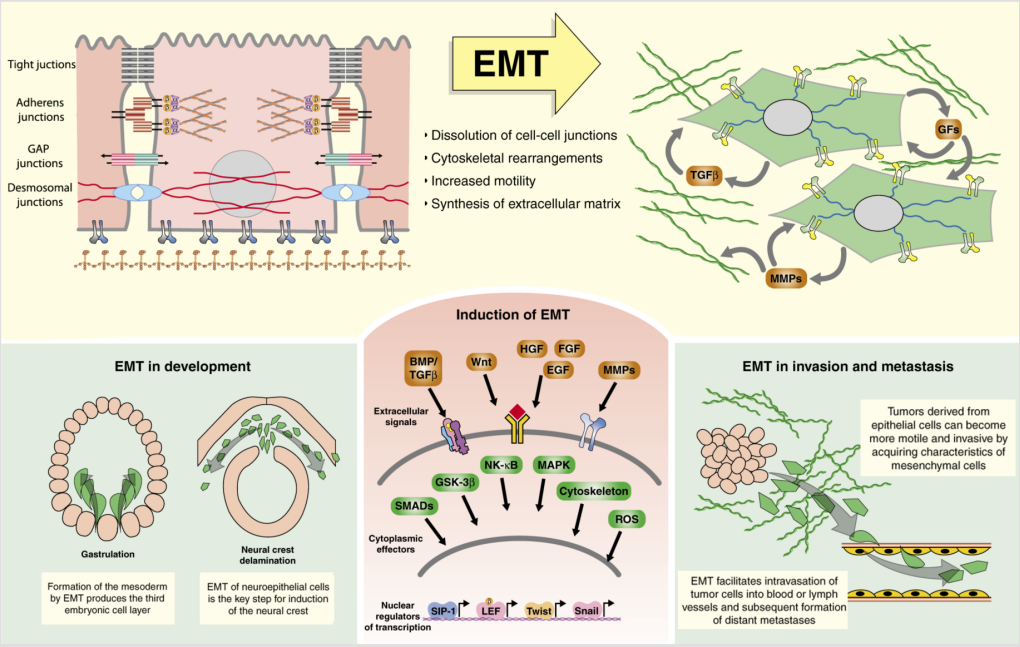
In contrast to the traditional notion that metastasis is a late event in tumor progression, increasing evidence suggests that tumor cells can disseminate from the earliest preneoplastic lesions, sometimes even before the formation of overt primary tumors [164]. In small cell lung cancer and pancreatic cancer, metastatic disease often appears at the time of or shortly after the initial diagnosis, whereas many patients with breast, prostate and skin cancers show metastasis after a prolonged period of dormancy [165]. In the case of CRC, progression

from adenomas to invasive carcinomas is a relatively slow process that may take years. However, from invasive carcinomas, tumor cell dissemination and colonization of distant organs (e.g. the liver or lungs) constitutes a fast event [143]. It is estimated that most genetic alterations for CRC metastasis are acquired during progression to the invasive carcinoma stage, and few, if any, additional genetic events are required for the formation of distant metastasis [166], which may, al least in part, explain temporal dynamics of CRC progression. Despite the high rates of attrition that accompany certain steps of the invasion-metastasis cascade, overt metastasis do eventually rise in many CRC (and other carcinoma) patients, where they almost invariably represent the source of terminal disease.

BOX 3: The epithelial to mesenchymal transition (EMT) at a glance

What is EMT?

The EMT process, essential for embryonic development, is an orchestrated series of events in which cell-cell (tight junctions, adherens, GAP and desmosomal junctions) and cell-matrix (ECM) (integrin-based) interactions are altered due to reorganization of the cytoskeleton of epithelial cells, causing a transition to a motile mesenchymal phenotype. Inappropriate utilization of EMT mechanisms is an integral component of the progression of many epithelial-tissue tumors



EMT inducers

A variety of related EMT inducers have been described (see Figure), some closely related with cancer. Growth factors (HGF, FGF, EGF, VEGF, HIF, PDGF, BMP's, IGF or TGF-β), chemokines (IL-6, TNF-α) and external factors like nicotine or UV light are potential factors triggering EMT.

EMT transcription factors (EMT-TFs)

Upon EMT induction, expression of a series of EMT-TFs is activated, ultimately resulting in the acquisition of EMT-like cellular features (see Figure). Snail, Slug, ZEB1, ZEB2, E47 or Twist are TFs typically related with EMT, responsible, among others, for EMT-related E-cadherin repression.

EMT in cancer

The EMT process is closely related with tumor invasion and metastasis (see figure). Moreover, resistance to cell death and senescence, resistance to chemotherapy, and the acquisition of a stem cell phenotype (Tumor Initiating Cells, TICs) have been also directly linked to EMT.

Adapted from Radisky et al [167]

3. Circulating tumor cells

Circulating Tumor Cells (CTCs) are cells that circulate in the blood of cancer patients, originating either from primary tumors or metastases. The presence of CTCs in patients with cancer was first reported in 1869 by the Australian physician Thomas Ashworth [168]. Since their discovery, CTCs have been regarded as a very promising field of research due to opportunities that lie in the easy and relatively inexpensive collection of peripheral blood samples and thus, of tumor cells. Serial monitoring of CTCs could provide an estimate of circulating disease burden over time and shed light on some of the fundamental processes that lead to an aggressive phenotype, metastasis and resistance to current therapies. Taking into account that CTCs in the bloodstream are potentially coming from primary tumors, bone marrow and distant metastases [117,169,170], it is expectable that CTCs represent tumor heterogeneity as well as or better than any single biopsy [171]. It is also important to note that, at least a subpopulation of CTCs, possibly hold all the necessary properties for the initiation of a metastatic tumor at a distant site, as recently reported in breast cancer [172]. Altogether, these CTC characteristics make them a key component of the metastatic cascade where to focus on, due to their potential as prognosis and predictive biomarkers, but also as interesting targets for the development of new therapeutic strategies aiming the eradication of specific CTC populations responsible for metastasis formation and thus, possibly modifying the course of cancer disease.

3.1 Methods for circulating tumor cell isolation and quantification

One main limitation for the determination of circulating tumor burden in cancer patients is their very rare frequency of appearance, estimated as one CTC per $\approx 10^7$ white blood cells (WBCs) per milliliter of blood [173,174]. The scarcity of CTCs and the abundance of leucocytes amongst they are present, demand extreme sensitive and specific CTC isolation and quantification methods. During the last years, an important number of technologies for CTC detection were developed, based on several properties that make CTCs differentiable from non-tumor cells also present within the blood. The expression of tumor-specific markers, the larger size of CTCs compared with WBCs, or some functional abilities of CTCs (e.g. invasiveness), have been exploited for their isolation, quantification and further molecular characterization (Table 8).

Enrichment of CTCs by immunomagnetic capture has been the most successful and widely used approach to date. The CellSearch® platform (Janssen Diagnostics, Raritan, NJ, U.S), enriches CTCs using ferromagnetic beads coated with an antibody towards EpCAM, an adhesion molecule highly expressed in epithelial cells and absent in leucocytes. In this system, after enrichment, positive expression of cytokeratins 8, 18 and 19, and absence of

the leucocyte marker CD45, are used to identify CTCs [76,175-177] (Figure 13). However, CTC numbers in the circulation, especially for the analysis of non-metastatic patients, where CTC burden is even lower, confer a limitation to the usefulness of this system.

To this regard, other technologies have been recently developed in order to analyze larger volumes of patient's blood, allowing for the capture significant more CTCs. An interesting recent technology, based on the use of antibody-mediated CTC capture, is the GILUPI cell collector (GILUPI Nanomedizin, Germany) [178]. This device consists in an anti EpCAM antibody-coated functionalized medical wire, which is directly placed into the antecubital vein of the patient for 30 min. This allows the isolation of CTCs from very large volumes of blood and which could increase the final CTC number. Other recent approach is the use of the leukapheresis (LA) technique [179]. LA is a standard clinical method that is frequently used to isolate mononuclear cells (MNCs) from blood for various applications, including stem cell harvest. LA enables the extracorporeal continuous density-based cell separation of MNCs with a density of 1,055-1,08 g/ml from several liters of total processed blood. Taking into account that tumor epithelial cells fall in the optimal density for LA separation, and that the whole patient's blood is processed almost three times, makes LA an optimal (although not easy to perform and of relative risk for the patient) technique for the recovery of large numbers of CTCs.

It is also important to consider that tumors and thus probably also CTCs, are heterogeneous within and between patients [180,181]. This, together with the fact that tumor cells may undergo epithelial to mesenchymal transition (EMT) during some of the steps of the metastatic cascade [182], implies that methodologies such as CellSearch® could be loosing specific tumor cell populations. CTC isolation methods based of physical properties (e.g, size, density, electric charges or deformability) have the advantage of being marker independent, consequently allowing the isolation of virtually all tumor cell types present in the blood. Filtration through special filters (ISET [183] and ScreenCell [184]), separation of tumor cells based on size through centrifugal forces (Dean Flow Fractionation [185]) or cell separation based on differential membrane capacitance (Dielectrophoretic field-flow fractionation [186]) (Table 8) are some of the recently developed techniques for CTC isolation, independently of the expression of surface markers. Although not yet tested in large clinical trials, those methodologies are proposed as promising new strategies for better patient outcome prediction (due to the analysis of all CTC subtypes), but more importantly for the better understanding of the biology of CTCs at the molecular level.

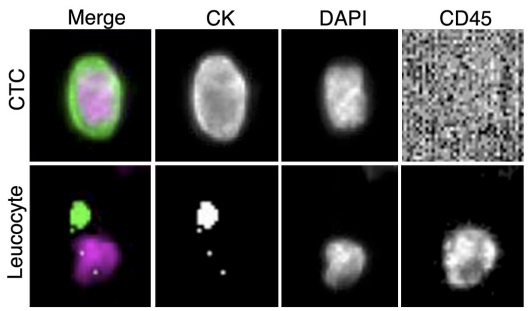


Figure 13: Examples of circulating tumor cells and leucocytes isolated with the CellSearch® system. After EpCAM enrichment, CTCs are identified by their cytokeratin/DAPI positive and CD45 negative staining. Contaminating leucocytes in the sample are characterized by their negative expression for cytokeratins, and positive for DAPI and CD45.

Table 8. Selected technologies for CTC detection

Technology	Enrichment method	Detection method	Notes
Antibody-based CTC capture assays			
CellSearch [76,175-177]	Anti-EpCAM-coated magnetic beads	ICC for CKs, CD45 and DAPI	FDA approved for advanced breast, prostate an colorectal cancer
MagSweeper [187]	Anti-EpCAM-coated magnetic beads	Microscopic visualization	Live cells can be isolated
GILUPI cell collector [178]	Functionalized EpCAM-coated medical wire	ICC for EpCAM, CKs and DAPI	*In vivo* collection
CTC iChip [188]	Magnetic bead capture combined with microfluidic inertial focusing	ICC or RT-PCR	Positive selection by EpCAM or negative by CD45 gives a broad population of enriched cells
CTC chip [162] and Herringbone chip [189]	Anti-EpCAM-coated microposts and chip surface	ICC for CKs, CD45 and DAPI with/without tumor type specific markers.	Microfluidic microchip technology
Ephesia CTC-chip [190]	Functionalized magnetic beads combined with microfluidics	ICC for cell surface and nuclear markers	Flexibility with capture antibody
AdnaTest [191,192]	Anti MUC1/EpCAM coated magnetic beads.	Multiplex RT-PCR for a panel of genes (MUC1, HER2 or EpCAM)	Direct CTC quantification not possible due to cell lysis.
IsoFlux [193]	Anti-EpCAM-coated magnetic beads combined with microfluidics.	ICC for CKs, CD45 and Hoechst.	Anti-mouse IgG-coated beads available for user-defined antibody capture
Physical characteristic-based assays			
ISET [183] and Screencell [184]	Filtration based on size	ICC or FISH	Nonepithelial cells can be isolated
Dean Flow Fractionation [185]	Size-based selection using centrifugal forces	ICC for CKs, EpCAM, CD45 and Hoechst	Nonepithelial cells can be isolated
Dielectrophoretic field-flow fractionation [186]	Membrane capacitance	ICC	Selected CTCs are viable
EPISPOT [194]	CD45 depletion and short-term culture in plates coated in antibody against MUC1, PSA or CK-19	IF	Detection of only viable CTCs
CAM [195,196]	Density gradient centrifugation and cells applied to CAM for short-term culture	ICC for cell surface markers	Detection of only viable CTCs
Other assays			
ImageStream [197]	Pre-enrichment by any method of choice	Flow-cytometry-based imaging using multimarker IF	Cells can be imaged for up to 10 cell-surface or intracellular markers
High throughput fluorescent scanning [198]	Red cell lysis and density gradient centrifugation	ICC of cell surface and nuclear markers	Nonepithelial cells can be isolated on a slide
DEPArray [186]	Pre-enrichment by any method of choice	Fluorescence imaging that enables movement of cells within chip by electric field changes	Isolation of purified single cells for downstream analysis

Abbreviations: CAM: collagen adhesion matrix, CTC: circulating tumor cell; CKs: citokeratins; DAPI: 4',6-diamino-2-phenylindole; EpCAM: epithelial cell adhesion molecule; FISH: fluorescence in situ hybridization; HER2: human epidermal growth factor receptor 2; ICC: immunocytochemistry; MUC1: mucin 1; PSA: prostate-specific antigen; RT-PCR: real-time PCR.

3.2 Clinical applications

3.2.1 Baseline CTC enumeration

The CellSearch® technology is currently the only FDA approved technology for CTC quantification in metastatic breast, colorectal and prostate cancers [76,199,200]. Specifically for metastatic CRC (mCRC), the presence of three or more CTCs per 7,5ml of blood at baseline (e.g. before treatment), predicted poor patient outcome (both for PFS and OS), compared to patients with less than 3 CTCs, who displayed longer survival times [76,77,177]. Similar studies in metastatic breast [175,199,201], prostate [200,202] and lung [203] cancers, have shown that the baseline epithelial CTC counts were prognostic for patient survival. In non-metastatic cancers, CTCs are even more rare than in the metastatic setting. However, the subgroup of patients with detectable CTCs, usually comprising 10-20% of the patients, does have a considerable worse prognosis than those without CTCs [204,205].

3.2.2 Circulating tumor cells as predictive markers

As previously mentioned, changes in the number of CTCs along a particular treatment were found to be an effective predictor of therapy response in patients suffering from different cancer types, and particularly in CRC. Usually enumerated using the CellSearch® platform, several studies were carried out in patients with mCRC, showing that reduction of the number of CTCs along treatment (at different times from baseline, depending on the study) had an impact in patient survival, with these patients showing better response rates and longer survival times, compared with patients with no CTC reduction or with an increase in number along treatment [108,109,111,112]. Recently, results from a meta-analysis in metastatic breast cancer (mBC), show, in a cohort of more than 650 mBC patients, a significant correlation between the changes in the number of CTCs during treatment (after 5 weeks from baseline) and patient prognosis (both for PFS and OS) [206]. CTC analysis along treatment of cancer patients would ideally provide information to help clinical decisions regarding the possible application of second-line chemotherapies, in order to improve tumor response rates and avoid unnecessary therapy-derived toxicities. To this regard, in the SWOG0500 trial [206], 120 patients with mBC with no early CTC decrease in first-line chemotherapy were randomized to continue the same chemotherapy line or switch early to the second-line. However, results showed no significant improvement in overall survival between both groups of patients, highlighting the clear necessity of more effective alternative therapies to rescue patients non-responding to first-line treatments. Despite the failure of treatment change based of CTC dynamics, CTC enumeration for predictive purposes continues to be attractive because it is less costly and relatively less invasive than radiologic imaging, and may provide a more rapid assessment of treatment related changes than radiographs, which typically require a longer period of treatment (generally

2-3 cycles of systemic therapy) before tumor regression becomes readily apparent.

Ideally, CTCs will be used as a liquid biopsy to select a specific personalized therapy on the basis of the molecular features of a patient's tumor. *KRAS* and *BRAF* mutational status in CTCs have been assessed in mCRC patients candidates for anti-EGFR targeted therapies, showing discrepancies with gene mutations found in primary tumor tissue [207], probably due to primary tumor heterogeneity. In non small-cell lung cancer (NSCLC), several studies have demonstrated the utility of the analysis of *EGFR* mutations or EML4-ALK rearrangements in CTCs, for the prediction of targeted therapies effectiveness, or for the identification of resistance biomarkers [208-210]. However, determination of specific mutations in CTCs remains challenging, due to the low number of CTCs, the lack of amplification of genes of interest or to the presence of an elevated number of leucocytes in the sample even after CTC enrichment.

3.2.3 Global molecular profiling of CTCs

Large scale molecular profiling of CTCs is even more challenging than the mere enumeration or the evaluation of the molecular status of single genes by different methodologies.

Gene-expression profiling of CTCs provides a very interesting insight into their biology and heterogeneity, allowing the identification of molecular CTC-markers related with different steps of the metastatic cascade, at the same time that identifies potential therapeutic CTC-based targets [211-213]. However, RNA degradation and the presence of an elevated amount of biological "noise" in CTC enriched samples, remain significant hurdles for effective CTC transcriptomic analysis. Transcriptional profiling of single CTCs, isolated by micromanipulation, provides a smart solution for the understanding of molecular CTC characteristics, avoiding the negative influence of the presence of contaminating leucocytes in the sample [214,215]. Although these techniques remain in their infancy, the contribution that single-cell profiling of CTCs could make to our understanding of cancer heterogeneity, disease evolution, and tumor biology is clearly evident.

Global genetic alterations can be also assessed in CTCs at a genomic level, which provides essential information about how representative are CTCs from the total heterogeneity present in the primary tumor or in metastatic tissues. For example, in CRC, array-comparative genome hybridization (CGH) and next generation sequencing (NGS) of a panel of CRC-associated genes of isolated CTCs, compared with matched primary tumors and metastases, showed the presence of CTC exclusive mutations or genetic aberrations, not detected in tumor tissues. Interestingly, most of CTC-specific alterations were subsequently detected in primary tumors at a subclonal level after additional sequencing, indicating that CTCs could better provide a global overview of tumor genomics and have the potential of be more informative than analysis of single biopsy samples [216].

4. Integrin signaling and Talin

Regulating adhesion and de-adhesion between cells and their surrounding extracellular matrix (ECM) is critical for cell migration, for example during embryonic development, immune responses, cardiovascular function, angiogenesis and tumor invasion and metastasis, as well as for other cell features like survival and proliferation [217,218]. In the last part of this thesis we described Talin1, a key component for the establishment of cell-ECM interactions at focal adhesion (FA) sites, as a relevant molecule for CTC extravasation from blood and subsequent metastasis formation.

FAs are large multiprotein complexes that assemble around the integrin family of cell adhesion molecules (heterodimers of noncovalently associated α and β subunits) that are typically linked to the actin cytoskeleton [219]. Talin [220,221], together with filamin [222] and α-actinin [223,224], are known to be the key players in this linkage, which can bind directly and simultaneously to both actin and integrin cytoplasmic tails.

The effects of Talin on integrin function are broad. It transduces signals across integrins in both the inside-out and outside-in directions and it also influences the organization of the actin network and the composition of focal adhesions [225-227]. Talin was discovered in 1983 by the group of Barridge and coworkers, as a protein highly enriched at cell adhesion sites [228,229]. Two different isoforms of the Talin gene (*Tln*) have been described in mammals: *Tln1* and *Tln2*. Both isoforms share 74% identity. However, their expression pattern is different, with *Tln1* gene expressed almost ubiquitously and *Tln2* expressed mainly in heart and brain [230]. Moreover, and despite the high identity rate between both isoforms, they do not compensate each other. When *Tln1* is inactivated, *Tln2* levels do not increase and existing levels do not compensate for *Tln1*, and vice versa [231-233]. Moreover, *Tln1* knockout mice are embryonically lethal, due to defects during the process of gastrulation, while *Tln2* mice are viable and fertile [233].

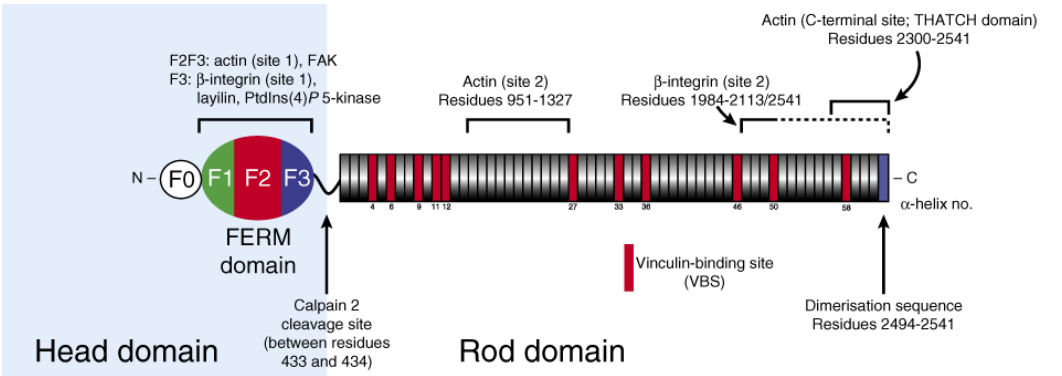


Figure 14: Domain structure and binding partners of Talin. Talin comprises a globular N-terminal head domain, and a flexible rod domain. Binding sites for β-integrins, Actin, Vinculin, FAK, laylin and phosphatidylinositol (4)-phosphate 5-kinase, as well as cleavage site for Calpain 2 are represented. Adapted from Critchley et al [233].

The human Talin is a 270KDa protein composed of 2541 amino acids (aa) and consists of an N-terminal head (433aa) (Talin-H) followed by a much larger rod domain (Talin-R) (Figure 14). Talin-H contains four subdomains (F0, F1, F2 and F3) with F1-F3 being homologous to a typical FERM domain (F for 4.1 band protein, E for Ezrin, R for Radixin and M for Moesin), which is implicated in the localization of proteins to the plasma membrane [234], as it has binding sites for the cytoplasmic domains β-integrins and laylin (a hyaluronan receptor) as well as for filamentous actin (f-actin). The head domain also binds to two signaling molecules that regulate the dynamics of FAs, namely PIPK1γ90 (a splice variant of phosphatidylinositol (4)-phosphate 5-kinase type Iγ) and focal adhesion kinase (FAK), although it is not clear whether binding of FAK is direct [219]. Talin-R consists of a series of helical bundles which contain multiple protein binding sites for Vinculin [235], RIAM [236], integrin [237], and a dimerization sequence at the C-terminus [238] (Figure 14).

In resting state cells, Talin distribution is random and diffuse and is autoinhibited from binding to integrins and membrane. Talin-R plays a major role to structurally restrain the Talin-H binding to the plasma membrane and integrins [227]. Upon agonist stimulation, Talin is rapidly localized to the plasma membrane [239]. Protein Kinase C alpha (PKCα), Rap1A, Rap1 effector RIAM, and phosphatidylinositol 4,5 biphosphate (PIP2) have been shown to play important roles in the Talin membrane localization and activation of its integrin binding function [227,240,241] (Figure 15). Calpain 2-mediated cleavage of Talin also facilitate the binding of Talin1 to integrins, but cleavage appears not to be required for integrin activation in cells [242].

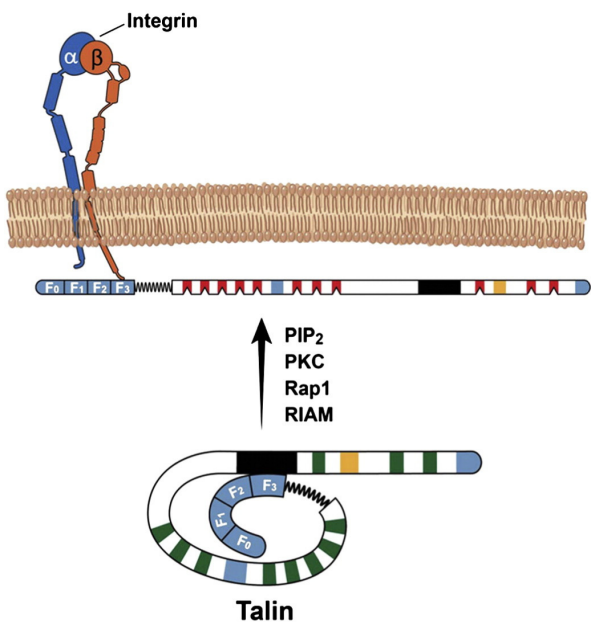


Figure 15: Mechanism of Talin activation. In resting cells, Talin is in a autoinhibitory folded conformation, with vinculin-binding sites dormant (in green). Upon activation, mediated by RIAM, Rap1, PKC or PIP2, Talin conformation changes into its unfolded version, and Vinculin binding sites become activated (in red), as well as F3 head domains, promoting Talin binding to β-integrins cytoplasmic tails. The secondary integrin binding sites are represented in orange. Image from Das et al [241].

Once Talin is activated, it is recruited to focal adhesion complexes to functionally interact with integrin cytoplasmic tails. Since Talin contains two β integrin binding sites, one within the FERM and the other within the rod domain, Talin homodimers have up to four integrin-binding sites, thus providing a biochemical base to Talin's ability to act as an integrin crosslinker, promoting clustering of integrins at specific focal adhesion sites [243].

Binding of Talin to integrin cytoplasmic tails has been widely studied, finding higher affinity rates to β1 and β3 integrin subunits [219,244]. Following the interaction of Talin1 with the β-integrin tail, conformational changes are propagated across the membrane to the extracellular domains of integrins, increasing their affinity for ligands. After binding of individual integrins to their ECM ligands, a Talin-mediated connection is formed between the ECM and the cytoskeleton, giving raise to the formation of nascent adhesions. Forces transmitted through these immature adhesions contribute to the reinforcement of the ECM-cytoskeleton link and to the recruitment of additional cytoskeletal and signaling proteins [245]. As adhesions mature, multiprotein complexes assemble at the cytoplasmic face of clustered, ligand-bound integrins, forming mature focal adhesions and finally transmitting signals into the cell, regulating processes such as cell spreading, adhesion or migration [227,246]. Proteins recruited to focal adhesion complexes include focal adhesion kinase (FAK), Src-family kinases, integrin-linked kinase (ILK), paxillin or vinculin, among others, and together orchestrate the whole array of cellular functions regulated through the interaction of cell-ECM at focal adhesion sites [246].

Objectives

The main objectives of this thesis have been delineated in order to cover some of the major current limitations in the field of CTC research, with the final goal of improving patient clinical management. By focusing on mCRC patients, this thesis comprises four principal objectives: the improvement of CTC detection methods sensitivity, the molecular characterization of CTCs for the deepening in their functional biology, the optimization of new prognostic and predictive CTC-related biomarkers and finally, the discovery of CTC-related molecules critical for the efficiency of metastatic dissemination.

1. **The development of an improved method for CTC isolation and quantification from patients with mCRC.**
    a. The design of an improved qPCR-based CTC detection procedure, in order to maximize detection rates.
    b. The search for potential candidate genes for CTC detection, based on their expression pattern in epithelial and intestinal tissues. Their expression analysis in CTC-enriched samples from mCRC patients, using healthy donor's blood as a background indicator.
    c. The development of a mathematical model for the combination of selected CTC-markers, and its validation as a prognostic factor for the prediction of mCRC patient's survival.

2. **The molecular characterization of CTCs in mCRC patients, in terms of their gene expression profile.**
    a. The optimization of a methodology to explore the genetic characteristics of CTCs from very low cellularity samples, based on the linear amplification of the genetic material obtained using the technique proposed on objective 1, and its hybridization onto gene expression microarrays.
    b. The determination of the specific gene expression profile of CTC-enriched samples from mCRC patients, using negative control samples from healthy volunteers as background indicators.
    c. A bioinformatic analysis on the main biological pathways and processes underlying CTC gene-expression profile.
    d. The validation of microarray data by testing the expression of a set of selected CTC-specific genes by qPCR in an independent cohort, and in primary and metastatic tumor tissues.

3. **The evaluation of a panel of CTC markers as a prognostic and predictive tool for the analysis of therapy response in mCRC patients.**
    a. The selection of CTC-markers from objectives 1 and 2 for the improved CTC quantification using the general methodology described in objective 1.
    b. The gene expression assessment of a set of EMT markers in a group of CTC-isolated samples from mCRC patients, and its comparison with samples from healthy volunteers. The selection of CTC-related EMT markers differentially expressed between patients and controls.
    c. The evaluation of the prognostic value of the selected CTC-marker panel for the prediction of patient survival at baseline (before treatment).
    d. The classification of mCRC patients in therapy responders o non-responders based on the variations of CTC-markers along treatment and the analysis of their predictive value.
    e. The direct comparison of CTC-markers and conventional imaging techniques for the early assessment of therapy response.

4. **The characterization of the role of Talin1 in the process of metastatic dissemination mediated by CTCs, and the analysis of Talin1 as a new potential therapeutic target in mCRC.**
    a. The search for candidate CTC-genes from objective 2 and the validation of Talin1 as a molecule expressed in CTCs from mCRC patients.
    b. The study of the effect of Talin1 depletion in HCT116 and CT26 colorectal cancer cell lines on *in vitro* assays mimicking the process of CTC extravasation from the bloodstream, by interaction with endothelial cells and subsequent basement membrane invasion.
    c. A study of the importance of Talin1 for metastasis formation from CTCs, modeled using HCT116 cell line, in an *in vivo* mouse model for hematogenous cancer dissemination.
    d. The assessment of Talin1 expression levels in CTCs from mCRC patients as a prognostic and predictive biomarker, as well as its expression pattern in primary colorectal carcinomas and metastatic tissues.

Chapter II

A logistic model for the detection of circulating tumor cells in human metastatic colorectal cancer

Jorge Barbazán, María Vieito, Alicia Abalo, Lorena Alonso-Alconada, Laura Muinelo-Romay, Marta Alonso-Nocelo, Luís León, Sonia Candamio, Elena Gallardo, Urbano Anido, Andreas Doll , María de los Ángeles Casares, Antonio Gómez-Tato, Miguel Abal & Rafael López-López

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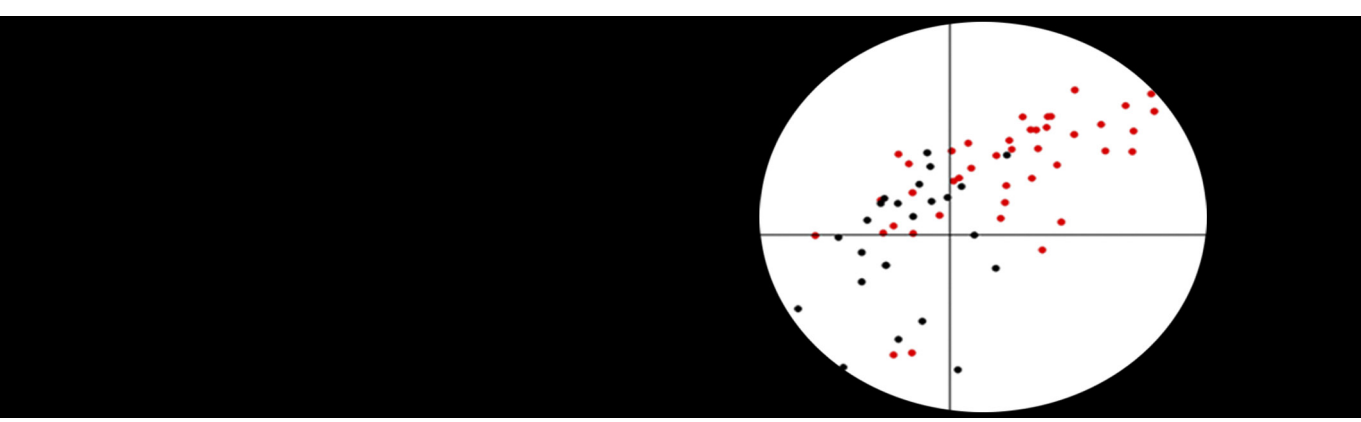
http://onlinelibrary.wiley.com/doi/10.1111/j.1582-4934.2012.01544.x/abstract;jsessionid=483295FD67D56D715307464294B0A1FA.f02t01



Chapter III

Molecular characterization of circulating tumor cells in human metastatic colorectal cancer

Jorge Barbazán, Lorena Alonso-Alconada, Laura Muinelo-Romay, María Vieito, Alicia Abalo, Marta Alonso-Nocelo, Sonia Candamio, Elena Gallardo, Beatriz Fernández, Ihab Abdulkader, María de los Ángeles Casares, Antonio Gómez-Tato, Rafael López-López & Miguel Abal

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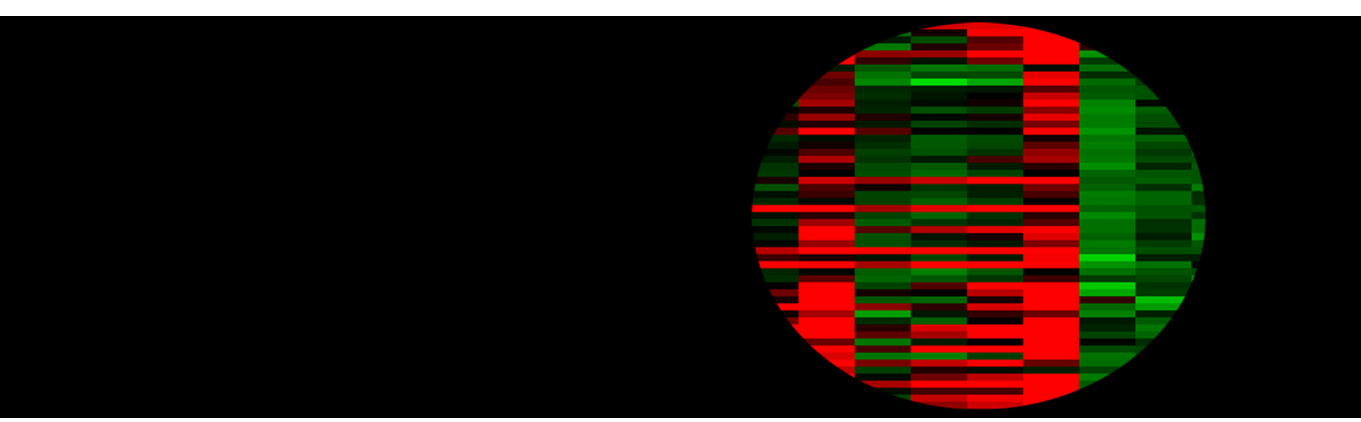

Chapter IV

A multimarker panel for circulating tumor cells detection predicts patient outcome and therapy response in metastatic colorectal cancer

Jorge Barbazán, Laura Muinelo-Romay, María Vieito, Sonia Candamio, Antonio Díaz-López, Amparo Cano, María de los Ángeles Casares, Antonio Gómez-Tato, Rafael López-López & Miguel Abal

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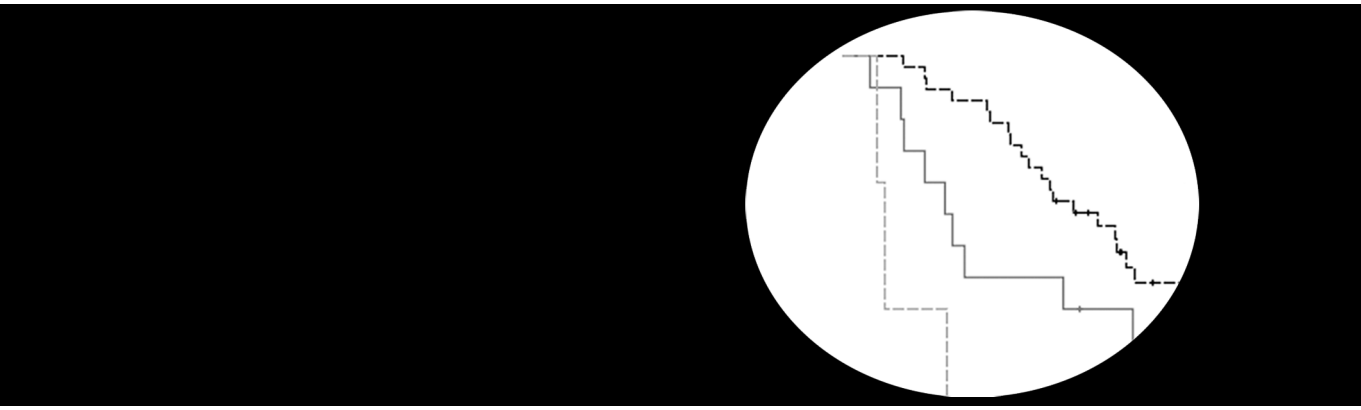
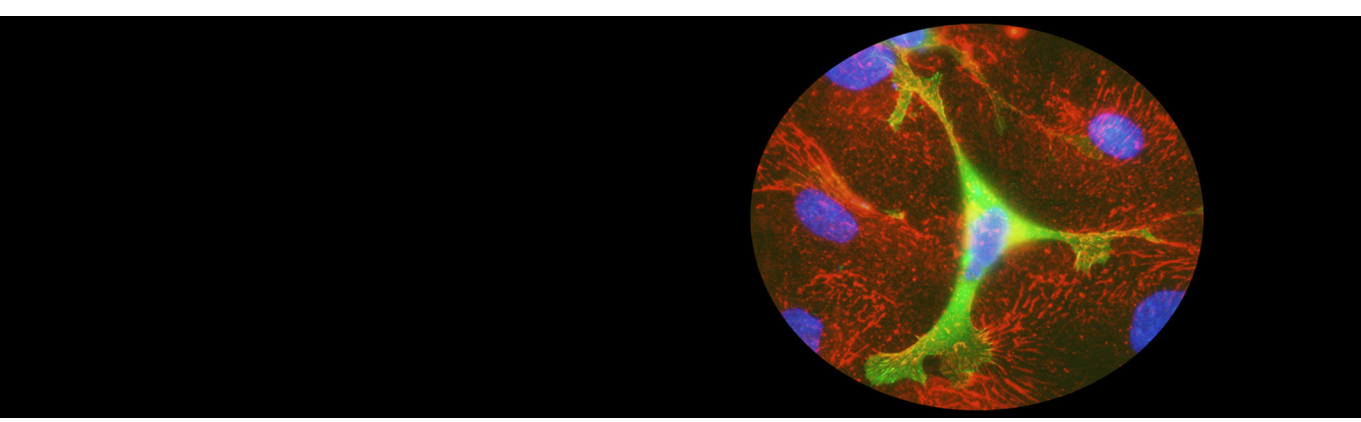


Chapter V

Talin1 enhances colorectal cancer dissemination through circulating tumor cells by promoting cancer cell extravasation and invasion

Jorge Barbazán, Nadia Elkhatib, Sara Geraldo, Lorena Alonso-Alconada, Vasily Gurchenkov, Alexandros Glentis, Beatriz Fernández, Tomás García-Caballero, Rafael López-López, Danijela Vignjevic & Miguel Abal

Submitted



Talin1 enhances colorectal cancer dissemination through circulating tumor cells by promoting cancer cell extravasation and invasion

Jorge Barbazán[1], Nadia ElKhatib[2], Sara Geraldo[2], Lorena Alonso-Alconada[1], Vasily Gurchenkov[2], Alexandros Glentis[2], Beatriz Fernández[3], Tomás García-Caballero[3], Rafael López-López[1], Danijela Vignjevic[2] and Miguel Abal[1,*]

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The appearance of cancer metastasis constitutes the main factor that determines patient outcome, causing a dramatic drop in survival rates. During tumor dissemination, circulating tumor cells (CTCs) must interact with blood-vessels endothelial cells to effectively extravasate from the circulation and possibly give raise to the formation of distant metastasis. We have previously reported a gene expression signature of CTCs in metastatic colorectal cancer (mCRC) that highlighted the importance of cell adhesion and migration abilities for CTC biology. In this work, among these genes described as CTC-specific, we have identified Talin1 as a potential molecule driving CTC extravasation. Talin1 depletion in CRC cell lines suppressed their ability to adhere and endothelial monolayer and further transendothelial migration, partially due to their inability to establish focal contacts with fibronectin fibers at the surface of endothelial cells, a novel cell-cell interaction mechanism described here. Talin1 also influenced later extravasation steps by regulating basement membrane invasion. These in *in vitro* findings were further confirmed in an in vivo model for cancer hematogenous dissemination. Finally, Talin1 expression in CTCs from mCRC patients was found to correlate with patient prognosis as well as with therapy response. Altogether, these findings suggest: i) a principal role for Talin1 in the process of CTC escape from the circulation and subsequent implantation prior to metastasis establishment and ii) a potential value for Talin1 as a therapeutic target against cancer dissemination and as a prognosis and predictive tool, key points to improve patient's management at the clinical setting.

Introduction

Colorectal Cancer (CRC) constitutes the third cancer type worldwide in terms of incidence [2]. The appearance of metastasis, a stepwise sequence of events by which primary tumors spread to distant sites, represents the main cause for CRC related deaths [306]. To effectively establish secondary tumors, cancer cells must overcome a large number of physical and biological barriers before colonizing distant sites. After breaching of the basement membrane (BM) at the primary site, intravasation into pre-existing and newly formed blood and lymph vessels, and survival in the circulation, tumor cells must extravasate from the bloodstream at the target organ before its

Key words: Circulating Tumor Cells, Talin1, Extravasation, Metastasis, Endothelial cells, Basement membrane invasion
Running title: Talin1 promotes CTC dissemination in colorectal cancer
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History: Submitted

colonization [138,143]. Despite the low efficiency of the metastasis process (only 0,01% of the tumor cells reaching the circulation might develop into distant tumors [157]), metastatic disease continue to be the main challenge in oncology.

Circulating Tumor Cells (CTCs), tumor cells that escape the primary tumor and reach the bloodstream, are considered to be one of the main responsible for cancer dissemination [143,307]. During the last years, several methodologies have been developed for the effective isolation, quantification and characterization of CTCs from patients with different malignancies [308], widely evidencing their prognostic value [83]. Moreover, it has been recently shown that CTCs can initiate metastasis in a xenograft model [172], reinforcing their role as metastatic cells and positioning CTCs as interesting targets for the treatment of disseminated disease.

Recently, our group reported a global gene expression profile of CTCs coming from metastatic CRC (mCRC) patients, in which we described a set of CTC-specific genes that could serve as detection and prognosis markers, but also as potential therapeutic targets due to their expression in CTCs [213]. Among them, we found Talin1, a protein encoded by the *TLN1* gene, which belongs to the integrin-signaling pathway. Together with a large number of others, Talin1 is involved in the formation of focal adhesions (FAs) at cell-extracellular matrix (ECM) contact sites. Talin1 links the cytoplasmic domains of β1 and β3-Integrins to actin filaments in the cytoplasm [219,309], playing a key role in the process of integrin inside-out activation, but also as a transducer molecule for outside-in signaling upon integrin activation and interaction with the ECM [227,310]. Regarding the role of Talin1 in cancer, it has been recently shown that Talin1 expression was related with cancer progression to metastasis in prostate and in oral squamous cell carcinomas [140,311], by promoting increased tumor invasiveness and anoikis resistance.

Cancer hematogenous dissemination through CTCs requires the attachment of cancer cells in the bloodstream to the endothelial layer of blood vessels, as a first step in extravasation [312], process in which several adhesion molecules, including integrins, are involved [312,313]. Elevated Talin1 expression in CTCs from mCRC patients, lead us to hypothesize a role for this molecule in the process of CTCs escape from the circulation and thus, for metastasis formation. In this work we evaluated how Talin1 is involved in different steps of tumor cell extravasation, including attachment to endothelial monolayers, transendothelial migration (TEM) and BM invasion. We also demonstrate that Talin1 influences metastasis formation in an *in vivo* model for cancer dissemination. Moreover, Talin1 expression levels in CTCs from mCRC patients were found to be an effective marker for prognosis and therapy response effectiveness. Altogether, our results suggest Talin1 both as an interesting marker for patient outcome and as a potential therapeutic target against cancer dissemination through CTCs.

Materials and methods

Cell culture

Human adenocarcinoma HCT116 and mouse carcinoma CT26 colon cancer cell lines were obtained from American Type Culture Collection (ATCC) and cultured in DMEM medium (Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS) (Life Technologies) and 1% penicillin/streptomycin (Life Technologies). Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from Life Technologies and cultured in endothelial basal medium EBM-2 (Lonza). For HUVECs, culture vessels were pretreated with 0,2% gelatin diluted in PBS for 30min at 37ºC. Human Embryonic Kidney 293 (HEK293) cells were purchased from ATCC and cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin.

Plasmids, shRNA, antibodies and proteins

EGFP tagged Paxillin was kindly provided by D Vignjevic (Institut Curie, Paris). Luciferase reporter pLenti CMV V5-LUC Blast (w567-1) and lentiviral packaging and envelope vectors (psPAX2 and pmD2.G, respectively) were purchased from Addgene. Lentiviral transduction particles bearing ShRNA against Talin1 (human: 5'-CCGGCCCAGAGTATTAACGCTCCAACT

CGAGTTGGAGCGTTAATACTCTGGGTTTT TG-3' and 5'-CCGGGCCTCAGATAATCTGGTG AAACTCGAGTTTCACCAGATTATCTGAGG CTTTTT-3', mouse: 5'-CCGGTGGTGAAGACGATGCAATTTGC TCGAGCAAATTGCATCGTCTTCACCATTT TTG-3' and 5'-CCGGCGCTCCAAGAGTATTA TTAATCTCGAGATTAATAATACTCTTGGAG CGTTTTTG-3' and Non Target Control (NTC) shRNA (5'-CCGGCAACAAGATGAAGAGCACCAA CTCGAGTTGGTGCTCTTCATCTTGTTGTT TTT-3') that targets not known mammalian genes, were purchased from Sigma Aldrich (MISSION Transduction Particles). All experiments were performed using two independent ShRNAs for each cell line, obtaining similar results for both. Only one is shown in the results section for simplification.

Mouse anti-α-Tubulin (Clone DM 1A) and rabbit anti-Fibronectin were from Sigma-Aldrich. Goat anti-Talin (C20) was from Santa Cruz Biotechnology. Mouse anti-Vinculin was obtained from Dr Vignjevic (Institut Curie, Paris). Labelled-phalloidin, DAPI and all secondary antibodies were purchased from Molecular Probes. Human fibronectin was purchased from Life Technologies and used at 20µg/ml in PBS for surface coating (1h at room temperature or overnight at 4ºC).

Lentiviral infections

For Talin1 knockdown, $2x10^4$ HCT116 or CT26 cells were seeded on a 24-well plate and incubated 24 hours at 37ºC. Next day, culture medium was replaced and Hexadimethrine Bromide (HDMB) (Sigma Aldrich) was added at a final concentration of 8µg/mL. Lentiviral infection was carried out following manufacturer's instructions using a multiplicity of infection (MOI) of 10 (previously optimized using MISSION TurboGFP Control Transduction Particles (Sigma Aldrich)). Puromycin (Life Technologies) was used as selection antibiotic at a concentration of 1µg/mL (for HCT116) and 6µg/mL (for CT26).

For the generation of luciferase expressing cells, viral particles were generated in HEK293 cells by co-transfection of packaging and envelope vectors (psPAX2 and pMD2.G, respectively) together with pLenti CMV V5-LUC Blast (w567-1) using Lipofectamine 2000 (Life Technologies), according manufacturer's instructions. 48 hours after transfection, culture medium containing lentiviral particles was collected, filtered and added to HCT116 and CT26 cells (shControl and shTalin1) for 24 hours. Hexadimethrine Bromide (HDMB) (Sigma Aldrich) was added at a final concentration of 8µg/mL. Cell lines stably expressing luciferase reporter gene were selected and maintained with Blasticidine HCL (Life Technologies) at 10µg/mL.

Western blot

Cells were washed with PBS, lysed in lysis buffer (1% triton, 50mM Tris pH7.5,1mM EDTA, 150mM NaCl and protease inhibitors cocktail (Sigma Aldrich). Protein was quantified using the Bicinchoninic Acid assay (BCA) (Sigma Aldrich) and boiled in 2x Laemmli buffer. The samples were separated by SDS-PAGE on 10% gels, transferred to nitrocellulose membranes, and blocked in 5% non-fat dried milk for 60 min. The membranes were incubated with primary antibodies overnight at 4ºC followed by incubation with peroxidase-conjugated secondary antibodies for 1h at RT. Immunoreactive bands were detected using an ECL kit (Thermo Scientific).

Real time quantitative gene expression

To check Talin1 knockdown at mRNA level, cancer cells were washed once with PBS and RNA was extracted with the High Pure RNA isolation kit (Roche) following manufacturer's instructions. cDNA was synthesized (MuLV reverse transcriptase, Life Technologies), and gene expression was evaluated using hydrolysis probes (Life Technologies). Data was represented as fold change relative to the expression in the non-target controls. GAPDH was used as loading control.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde in PBS for 20 min, followed by permeabilization with extraction buffer for 30 secs [0.5% Triton X-100 and 4% PEG (MW 40 000) in cytoskeleton PEM buffer (100 mM Pipes, pH 6.9, 1 mM MgCl2,

and 1 mM EGTA)] containing 2μM phalloidin. After permeabilization, cells were briefly washed twice with PEM buffer and once with PBS. Primary antibodies were diluted in PBS and incubated at 1:100 dilution (except for mouse anti-vinculin, 1:2), for 1h at room temperature in a humid chamber. Secondary antibodies were diluted 1:200 in PBS and incubated together with labelled phalloidin (1:200) and DAPI (1:1000) for 1h at room temperature in the dark. Cells were then washed with PBS and mounted onto glass slides with Prolong Gold (Life Technologies).

Quantification of FAs size and number

CT26 and HCT116 cells (Control and Talin1 Knockdown) were seeded onto fibronectin-coated coverslips. After 12-16h (for CT26) or 48h (for HCT116) cells were fixed and stained for Vinculin, Talin, Phalloidin and DAPI. Cells were imaged with a 100x objective of a wide-field microscope (DM6000 B/M; Leica) equipped with a CCD camera (CoolSNAP HQ;Photometrics). The lengths of FAs were measured using the MetaMorph Imaging software. 10 cells were quantified for each different cell line and condition.

Quantification of FAs dynamics

CT26 (shControl and shTalin1) cells were transfected with EGFP-tagged Paxillin using a Nucleofector (Lonza) according to the manufacturer's instructions and seeded onto fibronectin-coated glass dishes. Turnover of FAs was analyzed 24h later using 63x NA 1.4 objective of an inverted microscope (TE2000; Nikon) equipped with a spinning dish head (CSU22; Yokogawa) and a charge coupled device camera (CoolSNAP HQ2; Photometrics). Images were acquired every 2 minutes for during 3-4 hours. FA turnover was defined as elapsed time between the first and the last frames in which FA was observed. At least 150 FAs were quantified for each condition in a minimum of 10 different cells. Lamellipodia lifetime was defined as the elapsed time between the first frame where one single lamellipodia protrusion started to form, until the frame where it completely retracted. A minimum of 10 different cells was analyzed. Mann-Whitney test was used to evaluate significant differences between conditions.

Cell proliferation assay

For the analysis of cell proliferation dynamics, 10^4 cancer cells were seeded per well on 96-well culture plates and incubated at 37ºC for 24, 48 and 72h. After incubation, the number of viable cells was determined by using AlamarBlue (Life Technologies) following manufacturer's recommendations.

Adhesion assays

For evaluation of tumor cell adhesion to HUVECs, $3x10^4$ endothelial cells were seeded in gelatin-coated 96-plate wells and incubated overnight for cell monolayer formation. Cancer cells were stained with Calcein AM (Life Technologies) and plated at a concentration of $2,5x10^5$ cells/well over HUVEC monolayers in triplicates. Basal fluorescence emission was measured immediately after cell seeding in a microplate luminometer (LumiStar Optima, BMG LabTech) to evaluate possible differences in cell number. Cells were then incubated at 37ºC for the specified time. After incubation, wells were washed three times to remove non-adherent cells, and fluorescence intensity was measured. Adhesion was represented as the percentage of adherent cells per condition, normalized to the initial cell number per well. All experiments were run at least three independent times.Adhesion of tumor cells to different ECMs was tested using the ECM Cell Adhesion Array plates (Millipore), following the previous Calcein AM labeling protocol.

Flow-adhesion assays

For the evaluation of tumor cell adhesiveness under flow conditions, we used a setup composed by a culture chamber (PocMini-2, PeCon GmbH) connected to a syringe pump (NE-1000, New Era Pump Systems Inc) The culture chamber was placed on a thermostated platform to maintain the temperature at 37ºC during the experiment. The whole setup was placed in an inverted microscope (Zeiss Axio Vert A1) to allow cell visualization. Tumor cells were stained with Calcein AM and resuspended at a concentration of 10^5 cells/mL in complete growth medium. Cells were then pumped through the culture chamber

at 500µl/min during 1 hour, followed by a 10min wash with complete medium. After this time, the bottom glass from the culture chamber was removed and gently washed with culture medium to eliminate non-attached cells. Tumor cells attached to the glass surface, were trypsinized, centrifuged and resuspended in 100µl of PBS. Attached cells were indirectly quantified by fluorescence emission measurement in a luminometer. To evaluate cell adhesiveness under flow conditions, the bottom glass of the culture chamber was either coated with fibronectin, or seeded with $5x10^5$ HUVECs in EBM-2 medium and incubated at 37ºC overnight for monolayer formation. All experiments were performed in three independent runs.

2D and transwell migration assays

For the evaluation of cell migration ability in two-dimensional surfaces, CT26 cells (ShControl and shTalin1) were seeded onto fibronectin-coated glass bottom dishes. Cells were incubated 15 minutes at 37ºC for initial cell attachment, and then migration was visualized in clear field under an inverted videomicroscope (Nikon Eclipse Ti) using a 10x objective. Images were acquired every 3 minutes for 8 hours and 10 random fields were imaged for each condition. Migration speed and path length was quantified using the manual tracking plugin implemented on Fiji (ImageJ) in 50-100 cells per condition. Experiments were performed three independent times.

For transwell migration assays, 10^5 CT26/HCT116 cells (ShControl and shTalin1) were seeded on the upper chamber of 24-well hanging cell culture inserts (8,0 μm pore size polycarbonate membrane; Millicell, Millipore) in 100 μl of DMEM medium containing 5% FBS. The lower chamber was filled with DMEM supplemented with 20% FBS to create an FBS chemotactic gradient. Cells were incubated for 24h (CT26) or 72h (HCT116) at 37ºC. Cells that migrated through the membrane were trypsinized, collected and stained with Calcein AM (5 μM) according to manufacturer's recommendations. After staining, cells were centrifuged, resuspended in 100 μl of PBS and fluorescence intensity was quantified using a luminometer. Three independent assays were run for each experimental condition. Results were represented as the relative number of shTalin1 migrating cells compared to control cells, defined as 100%, for each cell line.

Transendothelial (TEM) migration assay

The effect of Talin1 knockdown on migration through an endothelial monolayer was evaluated using transendothelial migration assays. 10^5 HUVECs were seeded on the upper chamber of a gelatin-coated 24-well hanging cell culture insert (Millicell, Millipore) in EBM-2 complete medium, and incubated 4-6h at 37ºC to form a monolayer. Monolayer integrity was assessed in a control insert by addition of high-molecular-weight FITC-dextran (Sigma Aldrich) to the upper chamber (1mg/mL). 600 μl of EBM-2 medium were added to the lower chamber. Every 30 min, 50 μl samples were collected from the lower chamber and diluted to 1ml with PBS. 100 μl of diluted sample were transferred to a 96-well black plate and fluorescent content was quantified at 492/520 nm absorption/emission wavelengths. No fluorescence intensity is detected in the lower chamber once the HUVEC monolayer reaches confluency.

$5x10^5$ CT26 cells (control and shTalin1) were stained with Calcein AM (Life Technologies) as previously described, and added to the upper chamber of the cell culture insert once the HUVEC monolayer was established, in 10%FBS DMEM medium. 20%FBS DMEM medium was added to the lower chamber to create a chemotactic gradient. Cells were allowed to migrate through endothelial cells for 24h at 37ºC. Transmigrated cells were trypsinized, collected, centrifuged, resuspended in 100 μl of PBS and fluorescence emission was measured in a luminometer. At least three independent assays were run for each experimental condition. Results were represented as the relative shTalin1 CT26 transmigrated cells compared to control CT26 cells, defined as 100%.

HUVEC monolayer integrity assay

Disruption of HUVE cells monolayer integrity by cancer cells was analyzed using the XCELLigence system (Roche Applied Sciences) as previously described [327] This method enables

real-time monitoring of cellular events by using impedance measurements across interdigitated microelectrodes integrated on the bottom of tissue culture microtiter plates (E-plates). Impedance changes occur if cells attach to E-plates or change their size, shape and number. Briefly, 2,5x10^4 HUVECs were seeded on gelatin-coated E-plates and incubated 20-24 hours for monolayer establishment. Impedance readings were performed every 10 minutes and monolayer formation was evidenced by a cell index flattening. After this time, 2x10^4 HCT116 or CT26 cells (ShControl and ShTalin1) were added on top of the HUVE cells and impedance was monitored again for 1-4 hours. Invasion of cancer cells was measured by their ability to trigger endothelial cells retraction, which results in a cell index drop. Results were normalized to the time of addition of tumor cells, and invasion was quantified as the slope variation along the analysis time for each condition. Experiments were performed in duplicates and repeated three independent times.

Tumor cell-HUVEC interaction assay

For the evaluation of fibronectin production by HUVECs, 10^5 endothelial cells were seeded on gelatin-coated 12mm glass coverslips and incubated overnight at 37ºC. Cells were then fixated with 4% PFA for 20min at room temperature, stained for fibronectin, phalloidin and DAPI, and visualized on a Leica SP5 confocal microscope. Images analysis and three-dimensional reconstructions were performed with Fiji (ImageJ). For analysis of cancer cell-endothelial cell interaction, 4x10^5 CT26 control and shTalin1 cells were seeded on 12-well plates and transfected to transiently express EGFP-Paxillin using Lipofectamine LTX (Life Technologies) according to manufacturer's protocol. 10^5 HUVECs were seeded on a gelatin-coated 12mm glass coverslip and incubated overnight to form a monolayer. After 24 hours of transfection, cancer cells were trypsinized and seeded on top of the HUVEC monolayer in 10%FBS DMEM medium. Cocultures were incubated for 2 hours at 37ºC to allow tumor cell-HUVEC contact establishment. Cells were then fixated with 4% Paraformaldehyde (PFA) (Sigma Aldrich) for 20min at room temperature, washed with PBS and stained for fibronectin, phalloidin and DAPI. Cell interactions were visualized with a 100x objective of a wide-field microscope (DM6000 B/M; Leica) equipped with a CCD camera (CoolSNAP HQ;Photometrics).

Native basement membrane migration/ invasion assay

The influence of Talin1 on migration/invasion on a physiologic system was evaluated as previously described by Schumacher et al [314]. The peritoneal basement membrane (BM) was isolated from BALBc mice, and mounted on 6,5mm diameter Transwell (BD Biosciences) from which the polycarbonate membrane was cut out, using tissue adhesive (Vetbound, 3M). Mesothelial cells were eliminated by treating the membrane with ammonium hydroxide (1M) (Sigma Aldrich) for 1h at room temperature. After PBS washing, 10^5 tumor cells were plated on the top of the BM in DMEM medium supplemented with 2% FBS. DMEM supplemented with 10% FBS was used as a chemoattractant in the lower chamber. After 4 days of incubation at 37ºC, the samples were washed with PBS, fixed with 4% paraformaldehyde 40min at room temperature, and stained at both membrane sides with phalloidin and DAPI for two hours. After staining, the whole insert was mounted on a glass bottom dish. Migrating/invading cells were imaged with a 20x objective of a laser-scanning confocal microscope (Leica SP8.) 3 to 5 random areas were imaged per sample and experiments were performed in three independent runs. Quantification of cell invasion was performed by counting the total number of nuclei/actin protrusions existing in the lower side of the BM, and they were expressed as a ratio with respect to the total number of nuclei in each area, indicator of cell number for each experiment. Images, z-sections, 3D reconstructions, and quantifications were performed with Fiji (ImageJ).

Experimental metastasis assays

HCT116 control or shTalin1 cells (5x10^5 in 0.1 ml of sterile PBS) stably expressing luciferase reporter were injected into the left ventricle of the heart of female nude mice, aged 7 weeks (nu/nu, Charles River) under 2,5% isoflurane/air anesthesia.

Only mice with evidence of a satisfactory intracardiac injection, indicated on day 0 by systemic bioluminescence distributed throughout the animal, continued in the experiment. Metastasis formation was monitored every week by bioluminescent imaging with a Xenogen IVIS (IVISR Lumina II) system coupled to Living Imaging software 4.2 (Xenogen Corporation). Luciferin (150 mg/kg in PBS; Firefly Luciferin, Caliper Lifescience Corp, Hopkinton, MA, U.S) was injected intraperitoneally as the substrate for the luciferase. Mice were sacrificed after four weeks and metastasis were macroscopically visualized and confirmed by luminescence emission. All tumor-bearing tissues were collected, fixed in 4% formalin, and embedded in paraffin. Haematoxilin-Eosin staining was performed to determine the presence of metastasis. Mice were housed and maintained under specific pathogen-free conditions and used in accordance with institutional guidelines and approved by the Committee for Animal Care from the Universidade de Santiago de Compostela (USC).

CellSearch CTC quantification in mice

To further explore distant dissemination, a 50μl of peripheral blood sample was extracted before mice sacrifice and tested for the presence of CTCs with the CellCaptureTM CTC Mouse Kit (Veridex LLC, Janssen Diagnostics). Briefly, mice blood was mixed with 25μl of Anti-EpCAM ferrofluids and incubated for 15 minutes. After immunomagnetic capture and enrichment, CTC were immunofluorescent staining using Anti-Cytokeratins 8, 18 and 19 and DAPI. Analysis and enumeration of CTC was performed by an experienced user using the CellTracks Analyzer II® (Veridex LLC, Janssen Diagnostics).

CTCs immunoisolation and qPCR quantification in mCRC patients

Circulating tumor cells were indirectly quantified in blood from 50 mCRC patients as previously described [273]. Blood from 20 healthy volunteers was extracted and analyzed as control samples. Briefly, CTCs were immunoisolated from 7,5ml of peripheral blood using magnetic beads coated with an antibody towards EpCAM (CELLection Epithelial Enrich, Life Technologies) following manufacturer's instructions. Total CTC RNA was extracted with the QIAmp viral RNA mini kit (Qiagen) and cDNA was synthesized using SuperScriptIII (Life Technologies). To further optimize the sensibility of detection, a preamplification step was performed using TaqMan PreAmp Master Mix kit (Life Technologies). Talin1 and Protein Tyrosine Phosphatase Receptor Type C (PTPRC or CD45) expression was quantified by real time quantitative PCR using hydrolysis probes (Life Technologies) in a StepOne Plus thermocycler (Life Technologies). Every sample was run in duplicate for each gene and appropriate negative controls were included in each qPCR reaction plate. Cq values (defined as the cycle number at which the fluorescence reached a fixed threshold value) for each transcript were normalized to 40 (maximum number of cycles), and this value to the 40-Cq value for CD45 [(40-Cq target)-(Cq CD45)], used as a reference gene as it detects hematopoietic cells unspecifically isolated. We previously reported the validity of CD45 as a reference gene [273] as its expression levels are equal in patient and healthy donor samples.

Gene expression analysis in primary tumors and metastatic tissue

Primary colorectal carcinomas (n=8) and metastasis (liver metastasis, n=7; lung metastases, n=7) were processed by the Pathology Department of the Complexo Hospitalario Universitario of Santiago de Compostela. The superficial non-invasive zone and the deep invasive area of the primary tumors were macroscopically dissected, ensuring similar tumor percentages. RNA was purified (TRIZOL reagent, Invitrogen; RNeasy kit, Qiagen), cDNA was synthesized (MuLV reverse transcriptase, Life Technologies), and gene expression was evaluated using hydrolysis probes (Life Technologies). Data was represented as fold change relative to the expression in the superficial non-invasive area. *GAPDH*, *ACTB* and *RLPLO* housekeeping genes were used as loading controls.

Data analysis

Differences between groups were assessed by applying Mann-Whitney non-parametric t-tests. In the cases were data were normalized to the relative values of ShControl cells, Wilcoxon-signed rank test. For survival analysis, prognostic groups were set based on Talin1 CTC expression levels. Single patients were included into low or high-Talin1-CTC groups if Talin1 levels were, respectively, below or above cutoff, defined as the 75% percentile. Kaplan-Meier (KM) and univariate COX regression survival analysis were used to study associations between Talin1 expression levels and PFS/OS. All statistic tests were performed with SPSSv20.0 and GraphPad prism v5 software and considered significant when p≤0.05.

Results

Talin1 is expressed in CTCs from mCRC patients and mediates tumor cell adhesion to endothelial cells and transendothelial migration

Previously, we showed that, among other genes, Talin1 is expressed in CTCs coming from CRC patients bearing metastasis. Here we validate those results in a bigger cohort (50 mCRC patients and 20 healthy donors), and we confirmed that Talin1 was specifically expressed in CTCs coming from mCRC patient's blood (Supplementary Figure S1A) (p<0,01). Talin1 expression effectively discriminated mCRC patients from healthy controls, as evidenced by ROC curve analysis (AUC: 0,816, p=0,0017) (Supplementary Figure S1B).

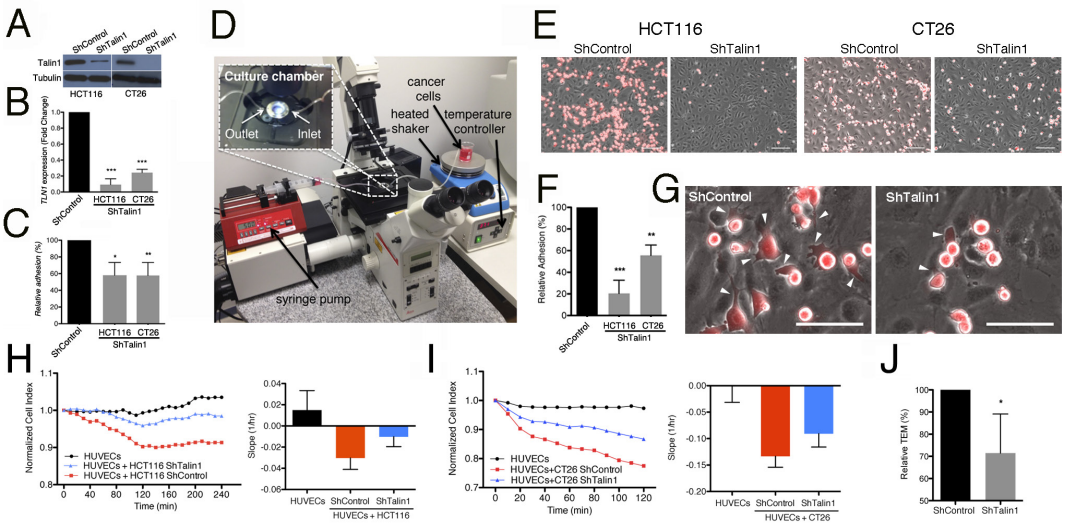


Figure 1. Talin1 influences cancer cells extravasation-related abilities. A) Western blot showing Talin1 protein expression in ShControl and ShTalin1 HCT116 and CT26 cells. A-Tubulin was used as loading control. **B)** Relative Talin1 gene expression levels of ShControl cells compared to ShTalin1 (HCT116 and CT26) measured by qPCR. GAPDH was used as loading control. **C)** HCT116 and CT26 ShTalin1 cells adhesion to a HUVEC monolayer, normalized to adhesion of ShControl cells. **D)** Flow adhesion assay setup. Cells were maintained at 37ºC under agitation and forced to flow through a closed culture chamber system, over a HUVEC monolayer using a syringe pump. **E)** Representative images of HCT116 and CT26 cells (Control and Talin1 depleted) adhering to HUVECs under flow conditions. Cancer cells were labelled with Calcein AM, and represented on red. HUVE cells were imaged in bright field. Bars 100µm **F)** Tumor cell-HUVEC adhesion quantification for HCT116 and CT26 cell lines, from three independent assays. Adhesion of ShTalin1 cells is represented normalized to ShControl cells adhesion for both cell lines. **G).** Representative images of membrane protrusions formation in CT26 cells (ShControl and ShTalin1) after 1 hour of flow adhesion. Bars: 50µm. (H, I) HUVEC monolayer integrity assays. Endothelial cell retraction was measured in the XCELLigence system under the presence of ShControl or ShTalin1 HCT116 **H)** or CT26 **I**) cells, based on Cell Index reduction over time. Quantification of endothelial retraction (Cell Index slope during analyzed time) is represented in right panels for each cell line. **J)** Transendothelial migration of CT26 cells (ShControl and ShTalin1) after 24 hours of coculture with HUVECs.
(*): p<0,05, (**): p<0,01, (***): p<0,001

The first barrier that a CTC must overcome to escape the circulation and possibly establish a metastasis in a distant organ is the luminal endothelial layer of blood vessels. As Talin1 was found to be expressed in CTCs, we sought to evaluate its potential role in the steps involved in the process of CTC extravasation from the circulation. For that, Talin1 expression was stably knocked-down in two different cell lines (HCT116 and CT26) by shRNA lentiviral delivery (Figure 1A, B). Moreover, and as a model of endothelial cells, Human Umbilical Vein Endothelial Cells (HUVECs) were used.

After Talin1 depletion, both HCT116 and CT26 cells, showed a significantly decreased ability to attach to a preformed HUVEC monolayer, in a static adhesion assay, when compared with control cells (Figure 1C). In order to mimic the physiological conditions of a CTC being in the bloodstream, we analyzed the adhesion capacities of Talin1 depleted cancer cells in a flow adhesion assay. Tumor cells were flowed over an endothelial monolayer during a determined time period, and the final percentage of cells adhering the HUVEC layer was quantified. Using this setup (Figure 1D) we found that both HCT116 and CT26 Talin1-depleted cells failed to attach to the endothelial cell monolayer under flow conditions, when compared with control cells (Figure 1E, quantification in 1F). Moreover, and specially for the highly invasive CT26 cells, ShControl cells were able to attach and formed membrane protrusions, that could be helping in the following steps of transmigration through the endothelial layer. However, in the case of Talin1 depletion, even though some cells succeed to attach to the HUVEC layer, they formed fewer membrane protrusions (Figure 1G).

An important step in the process of tumor cell extravasation from the circulation, after attachment to the vascular endothelium, is the retraction of endothelial cells to enable cancer cell migration between them before reaching the underlying basement membrane. To check whether Talin1 could influence endothelial retraction, we performed an endothelial monolayer integrity assay. A monolayer of HUVECs was formed in the wells of an E-plate (XCELLigence system, Roche), and then $2x10^4$ cancer cells were added and cocultured with endothelial cells. Figures 1H and I represent the normalized impedance signal obtained for each cell line (HCT116 and CT26, respectively) from the moment of cancer cell addition. When no cells were added to the monolayer of HUVECs, cell index (a direct indicator of the total percentage of the plate occupied by cells) was maintained over time, or even slightly increased (black lines). After the addition of cancer cells, cell index rapidly decreased, possibly due to the disruption of endothelial junctions and retraction of the endothelial monolayer (Figures 1H and I, red lines), as quantified by the slope decrement of each line over the analysis time (Figures 1H and I, right panels). Both for HCT116 and CT26 cells, Talin1 depletion lead to a reduction in endothelial monolayer retraction (Figures 1H and I, blue lines, quantification in right panels). These results, suggest that Talin1 could be relevant for the initial steps of CTC transmigration through the endothelial barrier. Finally, we found that ShTalin1 CT26 cells were significantly less efficient in migrating through a HUVEC monolayer, when compared with control cells, in a TEM assay (Figure 1J).

Together these results indicate that Talin1 plays a role in the extravasation step of CTCs from the circulation by influencing both the adhesion and transmigration of cancer cells to an endothelial monolayer.

Cancer cell adhesion to endothelial cells partially depends on FAs-endothelial fibrillary fibronectin contacts

Several cell adhesion molecules (CAMs) mediate the connection between cancer cells and endothelial cells [312]. For example, VCAM, ICAM or L1 molecules, expressed by the vascular endothelium, interact with α4β1 integrin, CD44 or αvβ3 receptors present on different cancer cells. Talin1 is involved in formation of cell-matrix adhesions, however, little is known about its role in cell-cell attachment. Here we hypothesize that, al least in part, adhesion of cancer cells to the endothelial layer could be mediated through the interaction of cancer cell FAs with the ECM produced by endothelial cells at their surface. To this regard, we found a large amount of

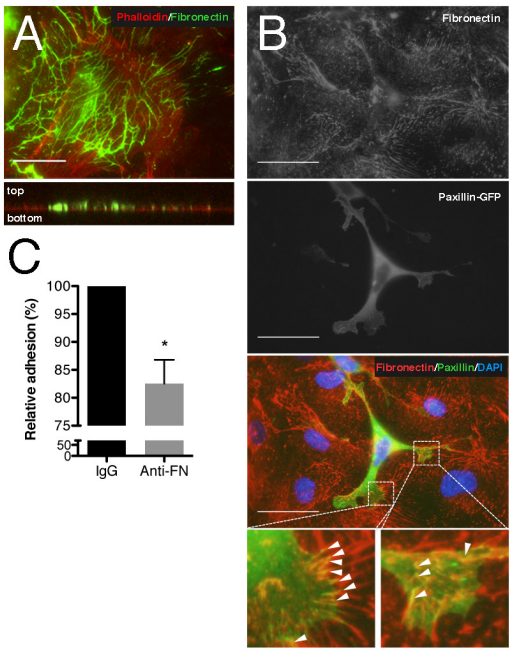


Figure 2. CRC CT26 cells adhere to HUVECs by FA interaction with fibronectin fibers produced by endothelial cells. A) Confocal HUVECs images stained for fibronectin (green) and phalloidin (F-Actin) (red). Lower panel represents a Z section of a single HUVE cell showing fibrillary fibronectin production on the top surface of the cell. Bars: 20µm. **B)** Immunofluorescence of CT26 ShControl and HUVECs cocultures. Cancer cells were transfected to express Paxillin-GFP (green). HUVECs cells were stained for fibronectin (red). Cell nuclei were DAPI stained (blue). Colocalization of paxillin-GFP labelled FAs with HUVECs fibronectin fibers was found (white arrows, lower panel blowup). Bars: 20µm **C)** CT26 ShControl cells adhesion to HUVECs after antibody blocking of fibronectin. Results are represented relative to IgG treatment. (*): p<0,05.

fibrillary fibronectin deposited on the surface of endothelial cells (Figure 2A), that could serve as docking points for cancer cells attachment. In fact, when CT26 (ShControl and ShTalin1) expressing Paxillin-GFP, another FA-complex protein, were seeded on top of the endothelial monolayer, we found that control cells formed numerous focal contacts with fibers present on the HUVE cells (Figure 2B). Moreover, antibody blocking of endothelial fibronectin lead to a reduction of CT26 adhesion to the endothelial monolayer (Figure 2C). Most of the Talin1 depleted cells, were washed out during the immunofluorescence process, with only few rounded cells remaining attached to the monolayer, suggesting that were not able to establish firm contacts with endothelial cells (data not shown).

Together, these results indicate that attachment of tumor cells to the endothelium is partially dependent on FAs-fibronectin interaction, and could represent an additional mechanism for CTC adhesion to endothelial cells prior to transendothelial migration, with Talin1 as a key component for the correct formation of those contacts.

Talin1 knockdown alters FA number and dynamics, adhesion to ECM and migration of cancer cells

To gain a mechanistic insight into the role of Talin1 in cancer cell extravasation, we investigated the effect of Talin1 depletion on FA formation and its relation with cell adhesion and migration abilities. Talin1-depleted cells showed a significant reduction in the number of FAs formed when plated onto fibronectin-coated surfaces, as evidenced by Talin1 and Vinculin staining (Figure 3A, quantification in 3B), which directly implies a defect in cell spreading (Figure 3A, phalloidin staining). No changes in FA size were found, although HCT116 ShTalin1 cells tended to form bigger adhesions (Figure 3C). Talin1/Vinculin positive aggregates were still observed after knockdown of Talin1, which suggests a non-complete depletion of Talin1 (Figure 3A).

To investigate whether the reduction of Talin1 expression levels in CRC cell lines could influence FA turnover, the dynamics of CT26 ShControl and ShTalin1 Paxillin-GFP cells were followed over time by spinning disk microscopy. In ShControl CT26 cells, FAs formed and disassembled in 25 minutes on average. However, in Talin1 depleted cells, FAs displayed a significantly shorter turnover time (≈12min) (Figure 3D, quantification in 3E). This alteration of FA turnover in Talin1-depleted cells had a direct implication on stabilization of membrane protrusions (Figure 3F). ShControl CT26 cells generated broad and stable protrusions, which protruded and retracted in 70-80min on average. In Talin1-depleted cells, protrusions were less stable, with significantly shorter lifetime, about ≈25min (Figure 3G) (Supplementary Movie S1).

Functionally, Talin1 knockdown significantly decreased cell adhesion to fibronectin-coated

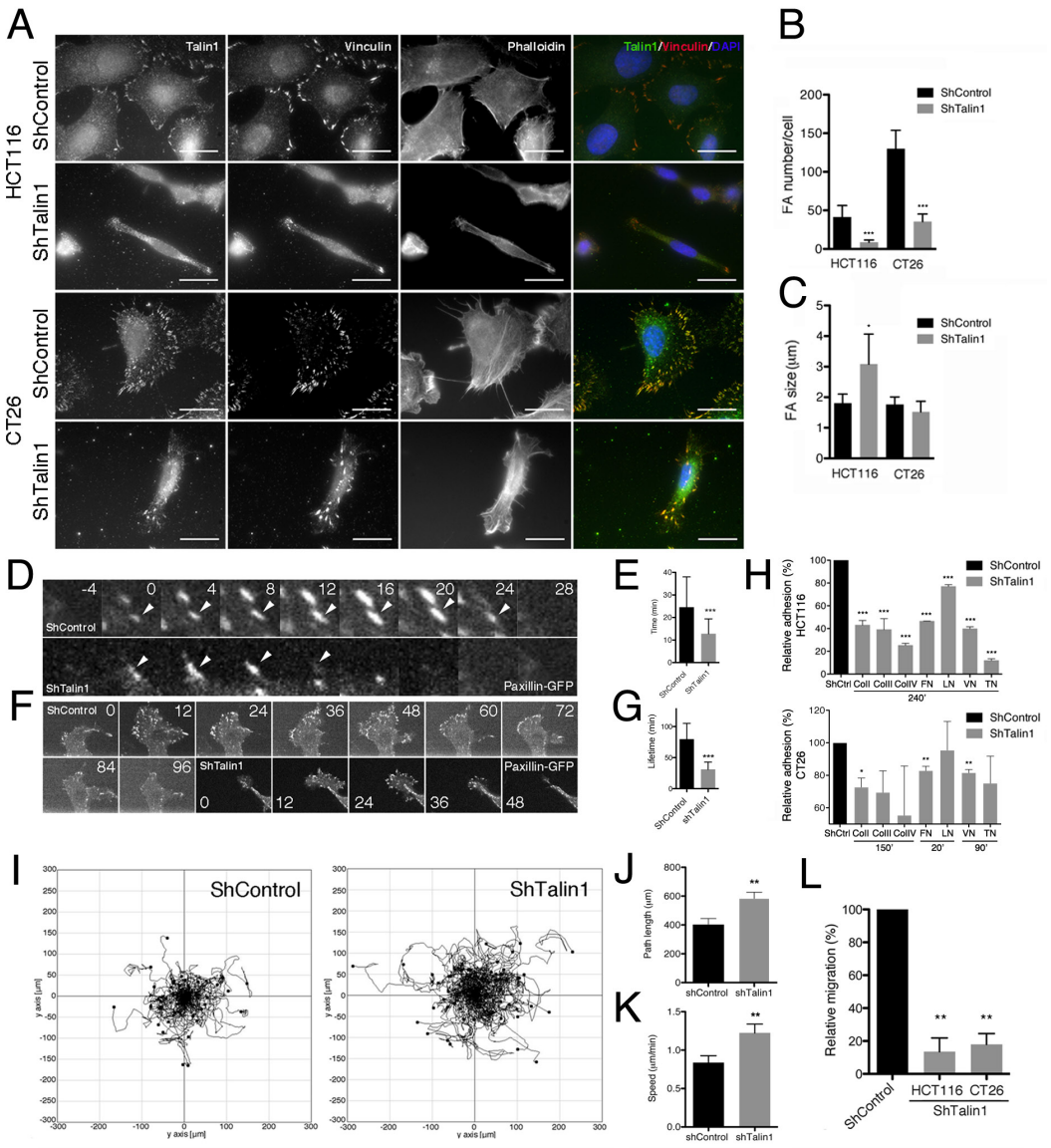


Figure 3. Talin1 regulates FA number, turnover, cell adhesion and migration. A) Focal Adhesions (FAs) immunofluorescence (Talin1 and Vinculin) in HCT116 and CT26 control and Talin1-depleted cells. Phalloidin staining was used to image F-Actin, and DAPI as nuclear labeling. Bars: 20µm **B)** FA number and size **C)** quantification based on Vinculin staining in HCT116 and CT26 control and Talin1-depleted cells. **D)** FA turnover in CT26 ShControl and ShTalin1 cells, expressing Paxillin-GFPTime in minutes. **E)** Quantification of FA turnover time. **F)** Lifetime of cellular protrusions in CT26 ShControl and ShTalin1 cells. **G)** Quantification of membrane protrusions lifetime. **H)** HCT116 and CT26 (control and ShTalin1) adhesion to different ECM substrates at indicated incubation times. **I)** Migration paths of CT26 (control and Talin1-depleted) on fibronectin-coated 2D surfaces. Representative migration diagram of a single experiment. Quantification of path length **J)** and cell velocity **K)** from three independent experiments. **L)** Transwell migration of HCT116 and CT26 cells (24 and 72 hours of migration, respectively)
(*): p<0,05; (**): p<0,01; (***): p<0,001

surfaces, and to different other extracellular matrices including Collagen (I, II and IV), Laminin, Tenascin and Vitronectin (Figure 3H), as a direct consequence of the reduced FA formation. To assess the effect of Talin1 depletion on cell migration, we first performed 2D migration assays in fibronectin-coated culture surfaces (Figure 3I). After 8 hours, ShControl CT26 cells displayed migration paths of approximately 400µm and an average speed of 0,8µm/min, while Talin1-depleted cells migrated significantly faster (≈1,2 µm/min) and at longer distances

(≈600 µm) (Figures 3J and K) (Supplementary Movie S2). Thus, more dynamic FAs and protrusions in Talin1-depleted cells support their faster migration. No differences were found in cell persistence between control and Talin1 depleted cells (data not shown). Interestingly, using a transwell assay, we found that, ShTalin1 cells were significantly less effective in migrating through the porous membrane of the transwell insert compared to control cells (after 24 and 72 hours, respectively) (Figure 3L).

Together, these results show that Talin1 influences FA formation and turnover, which consequently regulates cell adhesion to the ECM and the maintenance of cellular protrusions modifying cell migration ability.

Talin1 depletion decreases cancer cell invasion in a native basement membrane in vitro model

After transendothelial migration, cancer cells must be able to invade the extracellular matrix underlying the endothelial layer. To further investigate the role of Talin1 on cancer cell invasion, we performed a native BM invasion assay. Experiments were performed as previously described by Schumacher et al [314] (scheme in Figure 4A), replacing the porous membrane of a transwell insert with the peritoneal BM obtained from mice, which is structured in two well-defined laminin layers, surrounding an intermediate layer reach in collagen. CT26 ShControl or ShTalin1 cells were seeded on top of the membrane and incubated for 4 days. After this time, we found that Talin1 depleted cells were less effective in terms of BM invasion when compared with control cells (Figure 4B) as both the number of actin protrusions breaking through the BM structure and the number of traversing cell nuclei were significantly reduced in Talin1 depleted cells (Figure 4C and D) (Supplementary Movie S3). Together, these results suggest that Talin1 could be implicated in cell features regulating invasiveness, reinforcing its role in later extravasation steps.

Talin1 depletion reduces metastasis formation in an in vivo tumor dissemination model

To investigate how the expression of Talin1 could be modulating the metastatic potential of CTCs, we injected in the left heart ventricle of nude mice, HCT116 control and Talin1-depleted cells, mimicking the conditions of a CTC while in the bloodstream. HCT116 ShControl cells

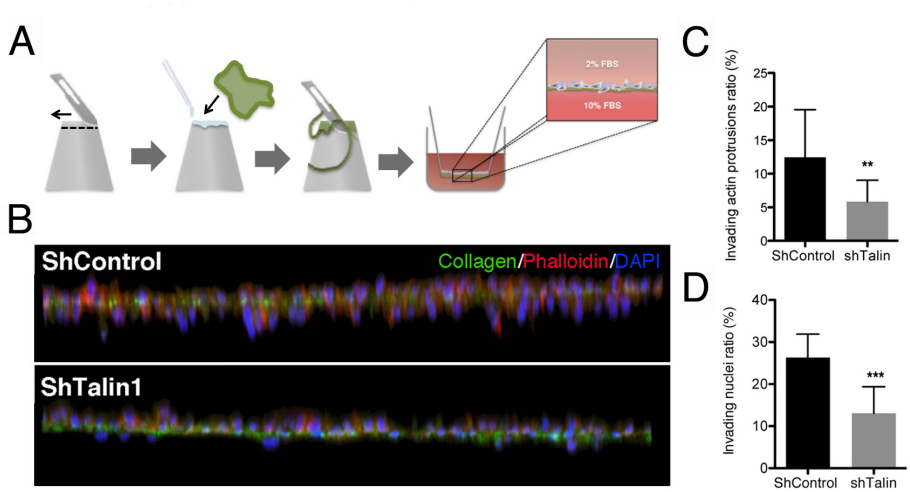


Figure 4. Talin1 knockdown reduces native basement membrane invasion ability of cancer cells. A) Schematic representation of native basement membrane invasion experiments setup. **B)** Representative confocal Z sections of a native basement membrane CT26 ShControl and ShTalin1 cells. Basement membrane was visualized by reflectance (green). Cell nuclei were DAPI stained (blue), and F-Actin was stained with Phalloidin (red). Quantification of invading CT26 cells from three independent assays. **C)** Invading actin protrusions of ShTalin1 cells compared to ShControl. **D)** Relative invading nuclei of ShTalin1 cells compared to ShControl
(**): p<0,01; (***): p<0,001

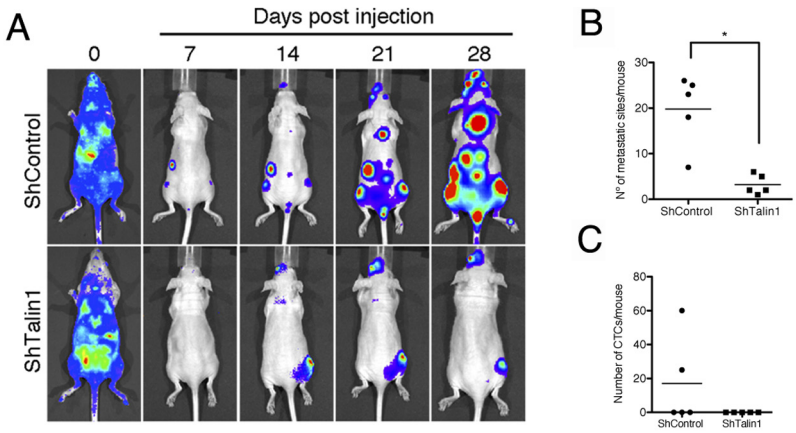


Figure 5: Talin1 knockdown reduces metastasis formation of human CRC cells in a tumor dissemination mouse model. A) Representative images of HCT116 ShControl and ShTalin1 cells, expressing a luciferase reporter gene, injected intracardiacally in Nude mice. Injection control (day 0) is represented in top left panel. Right panels represent metastasis formation for both cell lines overtime. **B)** Quantification of number of metastatic foci per mouse for ShControl and ShTalin1 cells. (*): p<0,05 **C)** CellSearch (Veridex) CTC quantification in injected mice at sacrifice day (28 days).

effectively survived in the circulation and rapidly formed tumors, mainly in bone tissue, adrenal glands and ovaries (Figure 5A, upper panels). HCT116 ShTalin1 failed to form metastasis when compared with control cells (Figure 5A, lower panels), with significantly less affected organs (Figure 5B). This effect was not due to proliferation differences between control and Talin1-depleted cells (Supplementary Figure S2). Interestingly, at the moment of sacrifice (28 days post injection) no CTCs were detected in ShTalin1 injected mice, while 2 out of 5 mice (40%) were positive for CTC count. Similar results were obtained with an independent ShTalin1 clone (data not shown). Together these results show that Talin1 is an essential molecule to allow organ colonization by tumor cells from the bloodstream and thus, relevant in terms of CTC biology.

Talin1 expression correlates with patient prognosis, therapy response and tumor progression in mCRC

To finally validate our hypothesis of Talin1 as a CTC marker and a potential therapeutic target against cancer dissemination in mCRC, we analyzed the expression levels of Talin1 in a set of 50 samples of immunoisolated CTCs from blood of mCRC patients at baseline, before the onset of a given chemotherapy. High or low Talin1 CTC expression levels were defined based on the 75% percentile of the global Talin1 expression data from the whole patient set. Kaplan-Meier analysis revealed that patients with High-CTC-Talin1 had a mean Progression Free Survival (PFS) time of 6,9 months, and Overall Survival (OS) of 10,5 months, compared to 12,3 (PFS) or 24,6 (OS) months for patients classified as Low-CTC-Talin1 (Log-Rank test: PFS: p=0,004; OS: p=2x10-5) (Figures 6A and B). Hazard Ratio (HR) for High-CTC-Talin1 patients was 2,63 for PFS and 4,47 for OS, compared with Low-CTC-Talin1 patients, as calculated by Cox regression analysis (PFS: p=0,010; OS: p=0,0003). Together these results validate the utility of Talin1 expression levels in CTCs as a prognosis marker in mCRC.

To investigate the role of Talin1 as a predictive marker for therapy effectiveness monitoring in mCRC patients, we evaluated its expression in CTCs in the previous set of patients after the administration of a first-line chemotherapy. All patients were analyzed after 4 weeks after therapy onset (one chemotherapy cycle). When required, a third sample was collected before cycle 5 (≈week 16). Patients were considered responders (R) when classified as CTC-Talin1-Low both at baseline and 4 weeks. Patients at the High-CTC-Talin1 group at both time points were classified as non-responders (NR), together with those moving from the Low-CTC-Talin1 group at baseline to the High-CTC-Talin1 one at 4 weeks. Finally, patients changing from the High-CTC-Talin1 group at baseline to the Low-CTC-Talin1

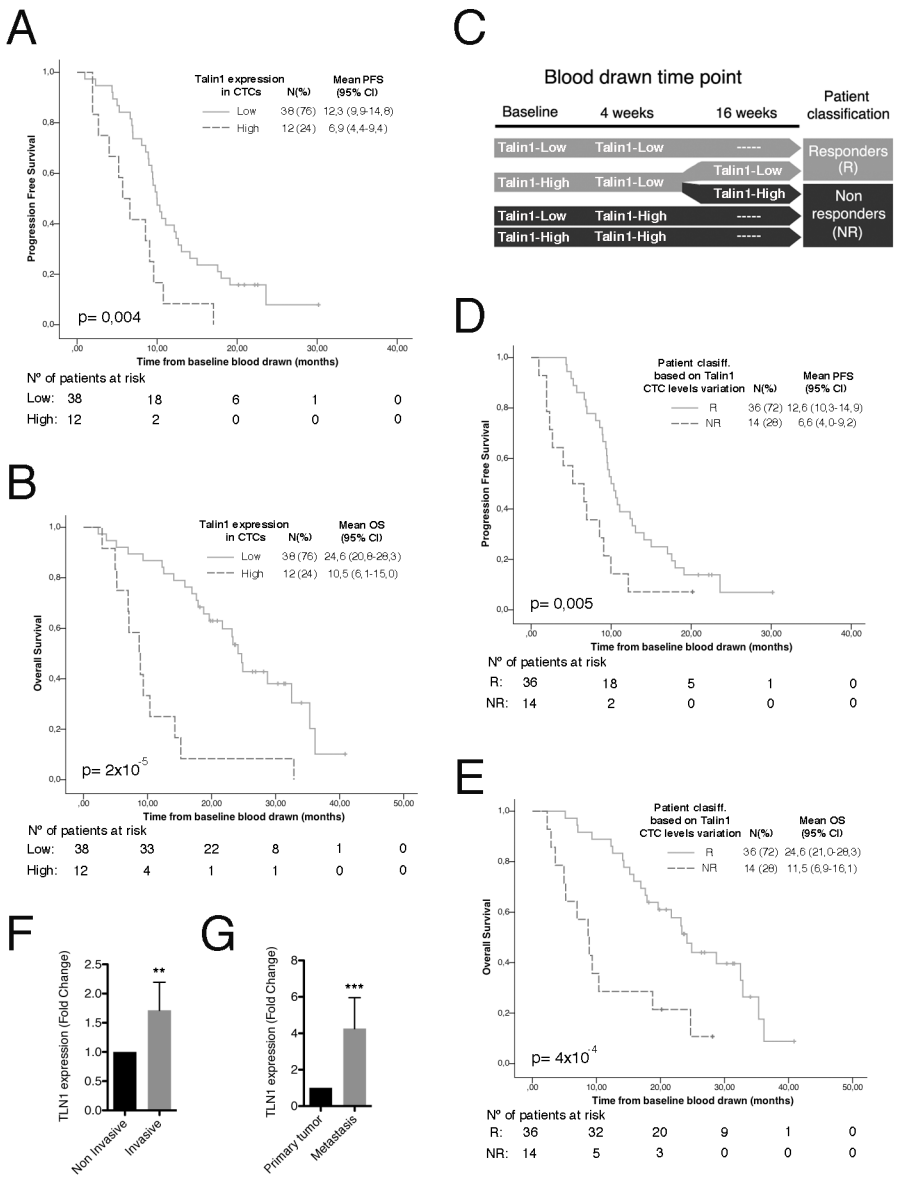


Figure 6. Talin1 expression in CTCs is a prognosis and predictive marker in mCRC patients. Kaplan-Meier survival plots for Progression Free Survival (PFS) A) and Overall Survival (OS) **B)** for mCRC patients with High or Low Talin1 expression in CTCs at baseline. **C)** Schematic representation of mCRC patient's classification in therapy responders (R) or non-responders (NR) based on Talin1 expression in CTCs during treatment. Kaplan-Meier survival plots for PFS **D)** and OS **E)** for patients classification into therapy responders (R) or non-responders (NR) based on the variation of Talin1 expression in CTCs along treatment. **F)** Talin1 gene expression in the invasive front of CRC primary tumors, relative to the non-invasive area. **G)** Talin1 gene expression in CRC metastatic lesions (lung and liver) from primary CRC carcinomas, relative to its expression in the non-invasive tumor area of primary tumors.
(**): p<0,01; (***): p<0,001

one at 4 weeks were preventively included in the group of responders and a confirmatory sample was drawn before cycle 5 of chemotherapy. If those patients were corroborated as Low-CTC-Talin1, they were classified as responders; on the contrary, they were considered as non-responders if the third sample resumed to the High-CTC-Talin1 group (scheme in Figure 6C).

Kaplan-Meier survival analysis showed that patients classified as responders based on the expression levels of CTC Talin1 along treatment,

had a mean PFS of 12,6 months (24,6 months for OS), compared with 6,6 months for the group of non-responders (11,5 months for OS) (Log-Rank: PFS: p=0,005, OS: p=4x10-4) (Figures 6 D and E). Hazard Ratio (HR) for the group of non-responders was 2,47 for PFS and 3,47 for OS, compared with Low-CTC-Talin1 patients, as calculated by Cox regression analysis (PFS: p=0,012; OS: p=0,002). Together these results highlight the value of Talin1 expression levels in CTCs as a predictive marker for therapy response evaluation in mCRC patients.

Finally, and to analyze Talin1 expression correlation with tumor progression, we evaluated Talin1 gene expression levels in the invasive and non-invasive areas of a series of 7 primary CRC tumors, and in 14 liver and lung metastasis of independent patients sets. The expression of *GAPDH*, *ACTB* and *RPLPO* housekeeping genes, was also analyzed as loading controls. We found a significant 1,7-fold Talin1 upregulation (p<0,01) in the invasive area of primary colorectal carcinomas, relative to the non-invasive area of the same tumor (Figure 6F). A mean 4-fold Talin1 upregulation (p<0,001) was found in liver and lung metastatic tissue when compared with the non-invasive area of the primary tumors set (Figure 6G). As a whole, these results indicate a possible gradual increment of Talin1 expression in tumor cells as tumor progression moves forward.

Discussion

Approximately 90% of all cancer related deaths arise from the metastatic spread of tumors, which turns metastatic disease formation as the most clinical, but less understood, aspect of cancer progression [315]. In CRC, escape of malignant cells from the primary tumor to reach the circulation, constitutes one of the main routes for cancer dissemination, with liver as the principal target site for metastasis formation [306]. During the last years, the scientific community has greatly focused in the study of CTCs as a, although not definitively proved, highly probable responsible for cancer spreading towards distant sites through the bloodstream. Indeed, mCRC patients with 3 or more CTCs per 7,5mL of blood displayed a significantly reduced survival, when compared with those having 2 or less CTCs, which indicates the prognostic role of CTC measurement [76,80]. Based on clinical data, molecular analysis of CTCs has increasingly gained attention. From the very first CTC gene expression profile published in 2005 [211], several groups have focused on the genomic, transcriptomic and functional characterization of CTCs, in order to obtain a more comprehensive picture of the metastatic process from a minimally invasive liquid biopsy [171,172,179].

CTC extravasation constitutes the first step of the process of distant organ colonization from a cell being in the bloodstream. Although widely studied in leucocytes, as the normal process for cell exit from the circulation at inflammation sites, much less in known about extravasation in cancer metastasis [150,312]. In this regard, CTC characterization could provide more detailed information about specific molecules involved in the regulation of this process. We have recently published a set of mCRC CTC-specific genes [213], that mainly comprised genes related to adhesion pathways, reinforcing the role of adhesion-related molecules for CTC biology. From these results, we selected Talin1 as a potential extravasation-driving gene, and thus, as a metastasis-related marker and a putative therapeutic target against cancer dissemination. In the present study, we analyzed the role of Talin1 on cancer cell adhesion, invasion and migration, specially focusing on cellular features related to CTC extravasation.

The first barrier that a potentially metastatic CTC finds in the process of escape from the circulation is the luminal endothelial layer of blood capillaries. During extravasation, cancer cells must dynamically interact with endothelial cells to finally transmigrate through them into the underlying matrix and stroma [312]. It has been hypothesized, based on in vitro observations, that CTCs may roll over the endothelial layer, in an early process termed “docking”, (mediated by cancer cell glycoproteins and corresponding endothelial selectins), and later establish firmer adhesions with endothelial cells (“locking”) (usually mediated by CAMs and integrins) before final transendothelial migration [312,313,316]. Here we show that Talin1 depletion significantly reduces

tumor cell adhesion to HUVE cells, used as a model of endothelial blood vessel layer, both under static and more physiological flow conditions. Adhesion times used in these experiments were relatively short, indicating a possible role for Talin1 in the early "docking" step, probably through activation of integrins in the plasma membrane. We also found that, mimicking later steps of cancer cell extravasation, Talin1 failed to form membrane protrusions that could allow cancer cells to squeeze in between endothelial cells, with a posterior consequence in endothelial cell retraction, and finally in transendothelial migration. As observed in vitro, reduction in FA turnover time after Talin1 depletion could explain the reduced ability of those cells to establish membrane protrusions over the endothelial layer. Moreover, β1 integrins (commonly activated through Talin1 and expressed in HCT116 and CT26 cells) have been related to the process of endothelial cell retraction by the induction of VE-cadherin phosphorylation, which dissociates β-catenin from the VE-cadherin complex, finally disrupting endothelial adherens junctions in breast cancer [317]. Although not proven, we speculate that a similar mechanism could be occurring in our CRC model.

As mentioned, attachment of cancer cells to the endothelium prior to extravasation relies on the interaction between endothelial selectins and CAMs with their cancer cells counter-receptors (SLeX glycoproteins and β1 and β3 integrins, among others) [120,150,312,313]. Here, we showed an additional attachment mechanism, mediated by the expression of fibronectin fibers on the surface of endothelial cells, which colocalized with cancer cells FAs, accounting approximately for 20% of cancer cell-endothelial adhesiveness, as demonstrated by antibody blocking of endothelial fibronectin. To our knowledge, this is the first work reporting these types of contacts, which could represent a novel mechanism for CTC firm adhesion to the endothelium, prior to TEM. The involvement of Talin1 in this particular mechanism relies on the reduced ability of cancer cells to form FAs after Talin1 depletion, and thus, the establishment of endothelial-cancer cells contacts through FA-fibronectin interactions. Interestingly, data in lung cancer suggested that plasma fibronectin could confer a pro-metastatic ability to cancer cells by activating αVβ3 integrins, further promoting tumor cell invasion [318]. To this regard, production of fibronectin at endothelial cells surface and/or deposition of circulating plasma fibronectin could also be mediating metastasis formation by promoting CTC extravasation.

Cell adhesion strength and migration speed have been related in a biphasic manner [319,320]. Migration velocity increases from low to medium adhesion strengths, while an excessive increment in cell adhesion causes a dramatic reduction in cell movement. Talin1 depletion directly causes an increase in cell speed in 2D fibronectin coated substrates, probably due to a reduction in adhesion strength, likely caused both by a reduction in FA number and turnover. In contrast, Talin1 depletion decreased the ability of cells to migrate in a Transwell assay. This can be explained because the formation of mature and stable FAs (absent in Talin1 knockdown) is a necessary event for the interaction with the cellular acto-myosin cytoskeleton, which can transmit cell-substrate traction throughout the cell [321], further allowing migration through the pores of the Transwell insert. Because a gradient of FBS was established to perform this kind of assays, one could speculate that the reduced expression of Talin1 in cancer cells could also be influencing directional migration of cells towards a chemical gradient. However, no differences in directional migration were found for CT26 cells in Dunns chemotaxis assays (data not shown), discarding a role for Talin1 in chemotaxis.

After TEM, CTCs must deal with the presence of the endothelial basement membrane, invading through it to effectively colonize the final metastatic site. To address the role of Talin1 in this later step, we used a native BM invasion assay, which represents the closest model to the in vivo situation, as a native mouse BM is used. Reduced expression levels of Talin1 led to a significantly reduced invasion ability of the highly invasive CT26 CRC cell line, suggesting an important role for Talin1 in this process. Cancer cells used F-actin-based structures called invadosomes to establish close contact with the ECM and invade through it, due to their ability to degrade ECM components [322].

As Talin1 is involved in invadosome formation, we hypothesize that reduced ability of CT26 to invade BM structures could be, at least in part, due to a reduction in invadosomes number and/or activity. Matrix degradation assays, as well as Matrix Metalloproteinases (MMP) expression analysis would be of great interest to elucidate the mechanism by which Talin1 could be modulating cell invasiveness. These results, together with the reduced ability of Talin1 depleted cells to adhere, induce retraction and TEM of endothelial cell monolayers, indicate the relevance of Talin1 in the whole process of CTC extravasation from the circulation.

Based on our in vitro observations, we analyzed the role of Talin1 in a mouse model of metastatic dissemination. Metastasis formation was dramatically decreased for human HCT116 cancer cells, a better model than mouse CT26 cells to test CRC dissemination, after Talin1 depletion, with a reduced number of finally detected CTCs on injected animals. Clinically, we describe an association between Talin1 and the sequential acquisition of an aggressive phenotype, based on Talin1 expression levels in primary and metastatic lesions, in agreement with some works that have already reported the role of Talin1 in cancer progression [140,311]. Moreover, it has been shown to be a target for mir-9, a microRNA with tumor suppressor activity in ovarian carcinoma [323]. These results, together with the observation that patients with high Talin1 expression in CTCs displayed much less survival times (PFS and OS) than those with low-Talin1, reinforced the role of Talin1 as a CTC and metastasis marker and as a potential therapeutic target against cancer dissemination.

Importantly, we also found that Talin1 expression on CTCs had a predictive value, due to the fact that, variations in Talin1 CTC expression during treatment, predicted therapy response in terms of patient survival. Talin1 loss of function was previously associated to Docetaxel chemosensitivity in triple negative breast cancer [324] and elevated Talin1 expression in metastatic cancer cells has also been linked to anoikis (detachment-induced cell death) resistance [140], and to TRAIL-induced apoptosis [325]. Talin1 function could represent a mechanism by which CTCs acquire resistance to chemotherapy, explaining our patient survival data. It is also reasonable to hypothesize that, in our metastasis mouse model, Talin1 depleted cells could undergo anoikis after failure to attach the blood vessels endothelium.

Currently, several experimental therapies are being tested in clinical trials, to assess the validity of targeting integrin activation, in which Talin1 is involved. αVβ3 binding inhibition to ECM was effectively blocked by small peptides (Cilengitide, EMD121974, Merck) or by monoclonal antibodies (DI17E6), showing promising results in clinical trials for different tumor types, including mCRC (DI17E6 in combination with Cetuximab, NTC01008475) [326]. Based on our results, here we propose Talin1 as a direct target for pharmacological inhibition, especially for the treatment of metastatic disease.

In summary, the evidence presented supports a particular role for Talin1 in circulating tumor cell extravasation, through the specific interaction with blood vessels endothelial cells, mediating adhesion, transendothelial migration and further basement membrane invasion, which finally results in metastasis formation. Our clinical data suggest Talin1 as a valuable marker both to predict patient prognosis based on Talin1 levels in CTCs, but also as a tool to monitor treatment effectiveness in mCRC. Further studies will pursue the evaluation of specific Talin1 inhibitors, what will place this molecule in a relevant condition to be considered as a new therapeutic target to tackle metastatic spreading.

Acknowledgements

We gratefully thank all the patients for their willingness to participate in the study and the staff of the medical oncology department for their collaboration in sample collection and clinical data analysis. The authors also want to thank NIKON Imaging Facility at Institut Curie, Paris. Jorge Barbazán is recipient of a FPU fellowship from the Spanish Ministry of Education, Culture and Sports. Lorena Alonso-Alconada was supported by a fellowship from the Vasque government.

Supporting Information

Supporting Figures

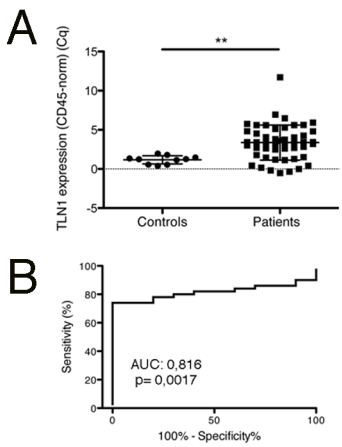


Figure S1. Talin1 is expressed in CTCs from mCRC patients. A) Talin1 genes expression levels in CTCs immunoisolated from mCRC patient's blood and healthy controls. **B)** ROC curve of mCRC patients Talin1 expression in CTCs versus healthy controls. (**): p<0,01

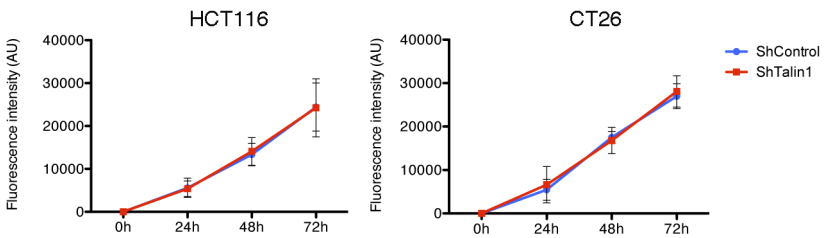


Figure S2. Talin1 knockdown does nor affect tumor cell proliferation. Proliferation of HCT116 and CT26 (ShControl and ShTalin1) cells after 24, 48 and 72h.

Supporting Movies

These files can be downloaded here:

Movie S1

https://dl.dropboxusercontent.com/u/42562496/Videos%20Tesis/Movie%20S1.avi

Movie S2

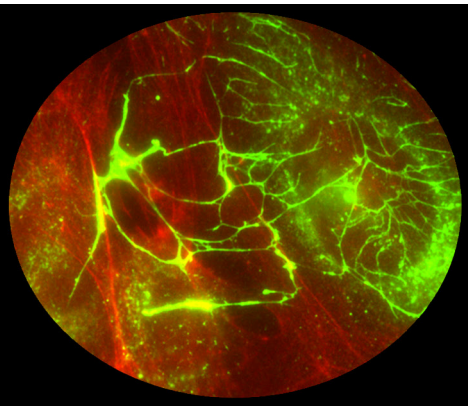
https://dl.dropboxusercontent.com/u/42562496/Videos%20Tesis/Movie%20S2.avi

Movie S3

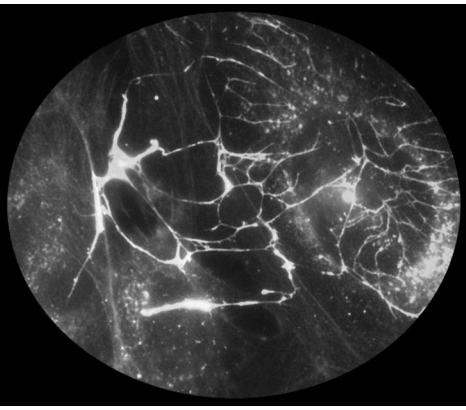
https://dl.dropboxusercontent.com/u/42562496/Videos%20Tesis/Movie%20S3.avi

Chapter VI

General Discussion and Conclusions



General Discussion



Colorectal cancer (CRC) represents the third most diagnosed cancer type worldwide, accounting for more than 600.000 deaths in 2012, which approximately represents 8,5% of all cancer-related deaths [1]. Taking into account that incidence rates are still continuously increasing in developing countries [3,4], research in CRC screening, diagnosis and treatment is underscored.

During the last years, significant advances have been made in the early detection and treatment of localized CRC, which has importantly contributed to the reduced mortality rates and allowing for five-year survival rates over 90% [7]. However, the appearance of disseminated disease is still a milestone that invariably determines patient's prognosis, with reduced 5-year survival rates that reach 10% in the worst cases [7]. This makes the occurrence of metastasis the main actual bottleneck in the management of CRC patients.

In this regard and despite patients with advanced CRC stages cannot routinely undergo surgery, progress in the discovery of new therapeutic agents have substantially improved survival rates and general patient welfare. However, the development of more specific and active cancer drugs, must go hand by hand with the establishment of new and more effective prognostic and predictive factors, to reliably estimate patient's life expectancy, allow the selection of adequate therapies, and evaluate their response to anti-tumor agents.

Notwithstanding considerable achievements have been made to this regard, prognostic, and especially predictive markers often fail to show results close to the expected. This could be probably due to their analysis in primary tumor tissue samples, and not in metastatic lesions, which are those that truly determine the prognosis of this type of patients [328]. Moreover, given that cancer is a continually evolving disease at the molecular level, it is controversial whether a single primary tumor biopsy at a specific time point represents the patient's disease [329]. Sequential analysis of primary tissue during the course of a given therapy, or the evaluation of molecular alterations in metastatic lesions would thus probably be a better indicator of tumor characteristics, leading to an optimized therapy response prediction. However, subjecting patients to serial invasive biopsies (from primary or metastatic tissues) is often impractical and confer a considerable risk to the patient [171]. In addition, single site biopsies are unlikely to capture the complexity of the genomic landscape of a patient's tumor, normally due to the presence of high intratumor heterogeneity [330]. Thus, having a more comprehensive picture of the overall tumor content is required for a better patient management. The analysis of Circulating Tumor Cells (CTCs) provide innovative and promising solutions to these problems, as they can be quantified and characterized repetitively in a non-invasive way, through the obtaining of a simple blood sample, thus serving as a surrogate patient's "liquid biopsy".

CTCs correspond to tumor cells that have been shed into the bloodstream by primary tumors and, at least a subpopulation of these CTCs, has the ability for the generation of overt metastatic lesions [172]. Moreover, CTCs found in the circulation can also come from distant metastases or bone marrow [117,169,170], proposing CTCs as metastatic intermediates and making their analysis of a great relevance for the better understanding of dynamic cancer complexity. CTC counts have been shown to predict CRC patient's prognosis, among other

several cancer types [76,77,177], and can be also used as an early marker of therapy response [108,109,112]. Moreover, and considering the biological metastasis-related characteristics that CTCs potentially hold, their molecular characterization is expected to open a completely new avenue in the understanding of the life-threatening metastatic cascade. However, CTC detection, quantification and characterization methodologies, although promising, are still in their initial phases, and important steps forward are needed to fulfill the contribution of CTCs in oncology. In this thesis, we have described a number of advances in the field of CTCs, specifically in metastatic CRC, in terms of detection and quantification, global molecular characterization, identification of predictive CTC-related biomarkers and finally, in the elucidation of molecular determinants linked to the inherent CTC metastatic ability.

CTC detection and quantification

Although several methodologies have been reported in the last years for CTC isolation from blood and their quantification, the CellSearch® system remains the gold standard method, as the only approved by the American FDA for CTC quantification in metastatic prostate, breast and colon cancers [76,199,200]. As mentioned, the scarcity of CTCs, even in patients with overt metastases, together with the overwhelming number of peripheral blood mononuclear cells amongst which they have to be identified, evidences the need of more specific and sensitive CTC detection techniques.

In the first part of this thesis we have developed an alternative method for CTC isolation and quantification based on the use of anti-EpCAM coated magnetic beads coupled to indirect CTC detection using qPCR-based selected markers (chapter II, page 65).

Contrary to what happens in other tumor types, the frequency of EpCAM-negative colorectal tumors is low for all tumor stages, grades and histologies, which supports the use of EpCAM as a target molecule for CTC enrichment [331]. However, cellular plasticity processes linked to EMT could be downregulating EpCAM before intravasation of CTCs, suggesting that at least a subpopulation of EpCAM-negative (EMT positive) CTCs could be lost using this methodology [305]. While this may be so, EpCAM+ CTCs represent an essential CTC subpopulation with demonstrated metastasis formation abilities as reported by Baccelli et al [172]. This, together with the widely evidenced prognostic value of the presence of EpCAM+ CTCs, as repeatedly reported in CellSearch®-based studies [77,80,177], clearly upholds EpCAM-based CTC isolation in CRC, and reinforces the validity of our strategy.

Even after CTC enrichment, a large number of unspecifically isolated cells are present in the sample, which hinders a reliable and specific CTC detection and quantification. In fact, technologies like CellSearch depend on the use of positive markers (in this case, cytokeratins) for the identification of CTCs, but also of lymphoid markers (e.g. CD45) to get rid of the noise produced by the presence of leucocytes in the sample [77,175-177]. Taking this into account, we screened for genes which expression was previously reported as high in intestinal epithelial tissues, including *VIL1*, *TBX20*, *GPA33*, *FAM132A*, *PREX2*, *EPCAM*, *CK20*, *TM4SF3*, *CDX2* and *CDH17*. Moreover, and to maximize CTC detection sensitivity, we included in

this method a preamplification step by which genetic material coming from EpCAM-enriched CTCs is moderately amplified before final qPCR. By applying this methodology, only *VIL1*, a gene encoding for the Villin protein, specifically expressed at the intestinal villus [332] and colorectal carcinomas [333], and thus detecting epithelial cells from intestinal origin, was found to be significantly expressed in CTCs to be detected above background. Interestingly, and even after CTC enrichment using anti-EpCAM magnetic beads, we could not find significant EpCAM expression in patient's samples. However, expression of EpCAM protein at cell surface need not necessarily correlates with its mRNA expression. This, together with differential rates of RNA degradation between transcripts, might explain the lack of detectable EpCAM expression. We also included a probe for *GAPDH*, a housekeeping gene ubiquitously expressed, as an indicator of the global sample cellularity, and *CD45*, to evaluate the levels of leucocyte contamination. Importantly, expression levels of *CD45* did not differ between patient and control samples, demonstrating similar levels of unspecificity between samples, and finally allowing its usage as a normalizer transcript to, at least partially, eliminate background noise present in the other two genes analyzed.

By combining the expression of *GAPDH* and *VIL1* (both normalized to *CD45*), we finally generated a CTC detection model that effectively separate mCRC patients from healthy volunteers, classifying 73% of mCRC patients as CTC positive. CTC positivity rates for the CellSearch® system in the same type of patients do not normally exceed 50% [334]. This, together with the prognostic value found for these CTC-markers, clearly highlights the validity of our method for an improved CTC detection and quantification.

Moreover, the versatility of this technology, where different or additional probes can be incorporated for CTC detection in other malignancies, together with its reduced cost compared with other CTC-detection strategies, and its relative simplicity, are attributes that make it an interesting alternative for the evaluation of CTC burden in cancer patients.

Advances in CTC molecular characterization

Going beyond CTC counting and employing their features as prognostic factors, the exploration of CTC molecular characteristics represents one of the most attractive, and at the same time challenging, actual aspects of CTC research. The evaluation of CTC-specific gene expression profiles would potentially shed light into the still largely unknown process of tumor dissemination, since they are considered as one of the main metastatic intermediates [117,169,170]. Moreover, this type of analyses are of special interest as the obtaining of new CTC-derived biomarkers will provide with valuable tools for their detection and quantification, prognostic and predictive markers and, interestingly, potential therapeutic targets to specifically tackle CTCs and thus, metastatic spreading.

Based on the CTC enrichment method previously described, and with the aim of having a wider picture of CTC transcriptional profile, we performed a global molecular characterization of CTCs from mCRC patients using gene expression microarrays, which interrogate a very large list of mRNA transcripts (chapter III, page 75).

Despite the large information volumes that this kind of methodologies can yield, they still depend on the use of relatively high amounts of starting genetic material, which cannot normally be obtained from a CTC sample. To overcome these shortcomings, we performed a whole transcriptome amplification (WTA) method, to linearly amplify the total CTC RNA, in order to obtain enough material for further analysis. The optimization of this methodology, demonstrated the feasibility of the global molecular expression of limited-cellularity samples, and reinforces the compatibility of the previously described CTC enrichment/quantification method with subsequent molecular analyses. This allows the quantification of CTC burden at the same time that enables CTC profiling, in a single sample, which gives it an advantage over other relatively closed systems like CellSearch®.

By comparing patient samples containing CTCs with normal blood from healthy volunteer samples, we finally obtained a set of 410 genes specifically expressed in the CTC population. Interestingly, the whole gene set was found to be positively expressed in patient's samples, which fits the idea of having an extra cellular population (CTCs) in this sample type, absent in healthy donors, validating this strategy for CTC profiling. Of note, bioinformatic interpretation of the whole CTC-derived dataset, suggested a CTC phenotype based on cell adhesion, migration and invasion abilities, including at the same time transcripts previously associated with chemorresistance (*ABCC3*, *ABCC4* or *ABCC5* [335]), which, as a whole, indicated a potential CTC aggressive behavior.

Array results were validated in a larger sample (20 patients and 10 controls) by qPCR, for 11 different transcripts (*TGFβ1*, *APP*, *CD9*, *CLU*, *ITGB5*, *LIMS1*, *TIMP1*, *VCL* and *BMP6*), selected based both on their relative expression levels (microarray signals) between control and patient samples, and on the cellular roles played by their encoded proteins. As we had previously hypothesized, all validated CTC-transcripts showed a significant prognostic value, and patients with higher marker expression levels displayed reduced progression free survival times, which confirms the potential clinical utility of CTC profiling. Of particular interest is the fact that most of those CTC-specific validated transcripts, were found to be sequentially upregulated during the process of metastatic dissemination (sequence invasive primary tumor-metastasis). As CTCs are supposed to be an intermediate step between primary tumors and the formation of overt metastases, the progressive upregulation of these genes in particular, but probably of more of those included in the whole array set, would probably be linked to the ability of these cells to escape the primary tumor and reach a target organ, through all the intermediate processes included in the metastatic cascade.

However, and despite these results are promising, they must be carefully interpreted as we cannot ensure if gene expression differences between patients come from their intrinsic expression in CTCs, or merely depend on the number of CTCs that a single patient has. Future work must be done in this field to specifically assess the relative gene-expression levels of this gene-set in individual CTCs, which may then be directly compared with primary or metastatic tumor tissue.

Altogether, the results presented in the second part of this thesis highlight the importance of CTC profiling as a powerful strategy to identify markers linked to the metastatic process,

which, at se same time that provide clues of its molecular framework, allow the identification of prognostic factors that, once clinically validated, could help in the better patients management and stratification.

CTC-markers for therapy response evaluation

Bearing in mind the relationship that has been extensively found between CTC counts and patient's prognosis, one would expect that its quantification, together with their molecular characterization, might provide valuable insights for the individual treatment of cancer patients and its subsequent monitoring.

As treatment has become more effective for colorectal cancer, decision-making has also become more complicated. The actual clinical standard considers a treatment change after several weeks or months if there's evidence of progression. However, after initiation of systemic treatment, current methodologies, usually imaging, do not often allow for an accurate and early assessment of clinical benefit. Thus, patients may be either treated for prolonged periods with an inactive therapy or a potentially active therapy might be discontinued prematurely. CTCs have been proposed as a novel therapy response evaluation tool as they can be analyzed repeatedly during the course of a treatment from a minimally invasive blood sample. Changes in the number of CTCs along treatment have shown to identify patients showing a response to therapy from those who do not [108,109,111,112]. Moreover, molecular profiling of CTCs for the detection of specific gene mutations or proteins that are relevant for the efficacy of certain cancer drugs, confers CTCs a substantial advantage compared to the methods routinely used for therapy response evaluation [207-210].

To further validate the role of CTCs as indicators of treatment benefit, we have reported the usefulness of a panel of selected CTC-markers, detected by qPCR after CTC-enrichment, for the early identification of patients at high risk of treatment failure (chapter IV, page 93).

The combination of CTC-related transcripts previously used for patient prognosis (*GAPDH*, *VIL1* and *CD45*), with CTC-specific genes coming from our global CTC expression profile (*CLU* and *TIMP1*), together with *LOLX3* and *ZEB2* as EMT-related transcripts, due to the importance that this process potentially has in metastatic dissemination. Their analysis at various time points along treatment, allowed us to effectively discriminate between patients responding or not to a given therapy. Of note, from the global array of EMT transcription factors analyzed in this study (*SNAIL1*, *SNAIL2*, *TWIST1*, *LOXL2*, *LOXL3*, *ZEB1*, *ZEB2* and *E47*), only *LOXL3* and *ZEB2* were found to be CTC-specific. This highlights the need to assess non-classical EMT markers in CTC samples, for their better quantification and understanding, and again reinforces the importance of the EMT process in the field of CTC research.

Importantly, using this method, we were able to assess treatment effectiveness, for most of the interrogated patients, only after 4 weeks, which represent a significant advantage compared to radiological imaging. Comparison of the results obtained using this methodology with the therapy response evaluation performed by computed tomography (CT) in this patient

cohort, showed that a significant percentage of patients (≈25%) might be being incorrectly classified by CT as responders, when indeed they are not. Those patients, identified as non-responders by CTC-markers, displayed a significant reduction in survival times when compared to patients classified as responders by both methods, strongly suggesting the evaluation of CTCs as an effective alternative for therapy response prediction.

Even the insights shed by this methodology should be carefully interpreted due to limitations in sample size, its potential implementation in the routine clinical practice is currently under evaluation. In fact, these results have been patented (application number: 1438107.2), and PrediCTC, a kit for early therapy response evaluation in mCRC patients is under development. Ideally, after its validation in a large patient cohort, PrediCTC would be useful to select patients non-responding to therapy, candidates for a treatment change based on clinical standards.

However, a parallel evolution in the field of cancer-drugs research is urgently needed to guarantee the existence of more effective alternative treatments to be applied to those patients identified as non-responders.

Insights into cancer dissemination: lessons from CTCs

Evaluating patient prognosis and therapy response by means of the analysis of CTCs might provide beneficial information for the clinical management of patients with the final goal of personalized cancer treatment. However, the CTC-derived knowledge goes far beyond. The identification of CTC-specific molecules involved in some of the key processes enabling metastasis formation from CTCs, is critical for the better understanding of cancer dissemination, and at the same time holds the possibility of the discovery of CTC-based therapeutic targets.

To this regard, from the list of CTC-related genes described in chapter III, we have identified Talin1 as a molecule potentially implicated in the promotion of CTC extravasation from the circulation, which is a prerequisite for effective target organ colonization and subsequent metastasis formation (Chapter V, page 109).

Talin1 is a protein that belongs to the integrin signaling pathway, and plays a key role in the connection of the cytoplasmic tails of β-integrins and the F-actin, thus providing a physical link between the extracellular matrix (recognized by integrins in the membrane) and the inner cellular cytoskeleton [234]. Although the relationship of the integrin pathway with some of the steps of cancer invasion and metastasis has been widely investigated [218,310,336], little is known about the role of the Talin1 protein per se to this regard. Some previous reports have attributed a role for Talin1 in the process of cell death resistance under non-attachment conditions, also known as anoikis, or in the acquisition of an aggressive cellular phenotype leading to increased cancer cell invasion and metastasis formation [140,311].

Here, we found Talin1 as a CTC-specific marker, as its expression levels were significantly higher in CTC-enriched samples from mCRC patients compared with healthy controls. As the focus of the last part of this thesis, we investigated how Talin1 expression in CTCs could be involved in the process of CTC extravasation. We found that Talin1 depleted cells failed

to adhere to a monolayer of endothelial cells, the first barrier that CTCs find in their way out of the circulation. This could be, at least partially, because correct focal adhesion formation, and their interaction with fibronectin fibers superficially synthesized by endothelial cells, seemed to be, at least partially, relevant for this early cancer cell-endothelium interaction. Interestingly, we found that after Talin1 depletion, cancer cells also failed to both disrupt the endothelial layer and migrate through it to reach the underlying basement membrane, which also confers an obstacle to Talin1-depleted cells as proved in native basement membrane invasion assays.

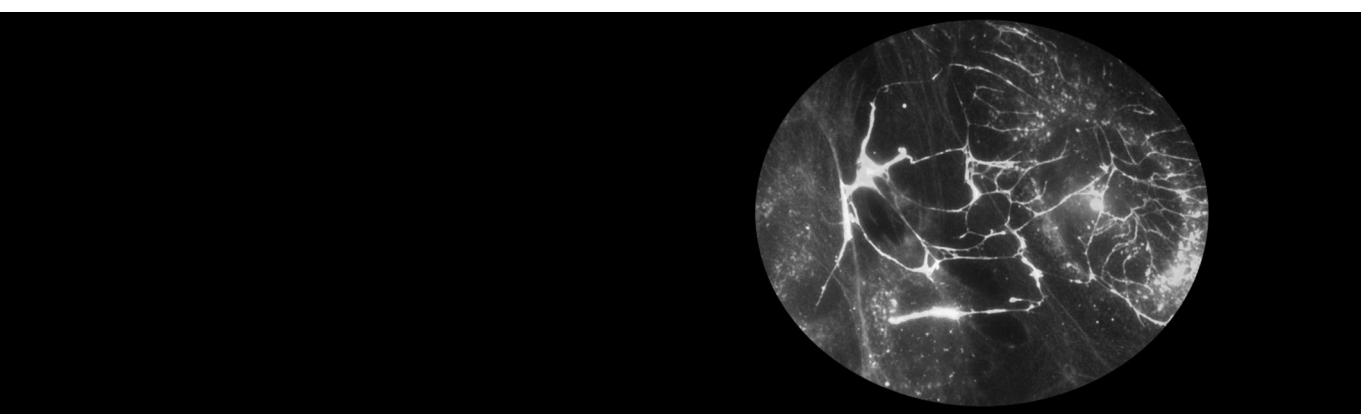
Altogether, these in vitro results can explain the marked metastasis formation ability of Talin1-depleted cells when tested into a cancer dissemination animal model. This, in conjunction with the fact that Talin1 expression in CTCs showed both prognostic and predictive attributes, confer an interesting value to this molecule, both as a CTC-related biomarker, but also as a potential therapeutic target that could potentially deprive CTCs from their metastatic ability. A next step in this project is based on the high-throughput screening for active and specific anti-Talin1 compounds, which may finally translate this molecule into the clinics.

It is important to mention that the role of Talin1 seems to be cell or tumor type dependent. Very recently, it has been shown that in hepatocellular carcinoma (HCC), Talin1 may be repressing invasion and metastasis, and high Talin1 expression levels in primary HCC could confer good patient prognosis [337]. Therefore, the need of future work is clearly evidenced to finally elucidate the role of this molecule in cancer progression.

The results presented in this last part, together with the multimarker panel for therapy response prediction in mCRC, widely validate the strategy of developing an improved method for CTC detection and its molecular characterization as a source of CTC-related information. At the same time, highlights the importance of basic research in this field that opens a room for improvement of current tools available at the clinical setting.

In summary, the potential clinical value of CTCs is clear: early detection and treatment of metastatic disease are key for patient outcome and overall, there is increasing evidence that CTCs reflect cancer progression in real time, providing information that might cover the main fields related to cancer research: prevention, detection, treatment guidance and target discovery. However, there's still a long way to go. Despite CellSearch® CTC counting has opened a promising way to the clinics, results obtained are still far from the global potential that CTCs might provide. Deeper understanding of CTC biology in terms of the elucidation of the differences between CTC subpopulation as well as the isolation and molecular characterization of potential tumor initiating cells, would open completely new avenues in the field of oncology. Although technically challenging, the application of the constantly improving single cell "omics", or the optimization of CTC culture protocols for the real-time and patient-specific testing of drugs tackling metastasis, would ultimately determine the real clinical potential of CTCs.

Conclusions



1. Anti-EpCAM magnetic immunoisolation of CTCs combined with their quantification by the assessment of *VIL1*, *GAPDH* and *CD45* gene expression, generated an improved method for the analysis of circulating tumor burden in patients with metastatic colorectal cancer, in terms detection sensitivity and specificity.

2. The logistic model built by combining *VIL1*, *GAPDH* and *CD45* has shown a prognostic value, separating mCRC patients based on their clinical outcome, and identifying patients at high risk of disease progression.

3. We have developed an effective methodology for the evaluation of the global gene expression profile of samples containing very low amounts of starting genetic material. This technique, based on the combination of EpCAM-based CTC immunoisolation, whole transcriptome amplification and microarray hybridization, allowed the molecular characterization of CTCs from mCRC patients.

4. A specific set of 410 genes was found to characterize the population of CTCs isolated from mCRC patients. At the molecular level, this gene expression signature was related to a potential CTC phenotype mainly characterized by its adhesive and migratory abilities.

5. The specific expression of *APP*, *CD9*, *CLU*, *ITGB5*, *LIMS1*, *RSU1*, *TIMP1*, *TLN1*, *VCL* and *BMP6* genes in CTC-enriched samples from mCRC patients, analyzed by qPCR, significantly validated our microarray analysis data. Moreover, the prognosis value of these CTC-markers, as well as their elevated expression levels in metastatic tissues compared to primary tumors, evidenced the usefulness of our methodology for the discovery of new and improved metastasis-related clinical biomarkers.

6. We have developed a panel of CTC-related markers, composed by *GAPDH*, *VIL1*, *CLU*, *TIMP1*, *LOXL3*, *ZEB2* and *CD45*, for the evaluation of patient prognosis but more importantly, for the assessment of therapy response prediction in mCRC patients undergoing first-line treatment.

7. We have shown that the sequential analysis of CTC-related transcripts along treatment constitutes an improved method for therapy response evaluation, when compared with standard computerized tomography. The implementation of this methodology at the clinical setting, in combination with routine imaging techniques, would improve current therapy effectiveness evaluation protocols. At this time, PrediCTC, a therapy response evaluation kit based on this methodology, is being designed and clinically validated on a multicenter trial.

8. We have demonstrated that the depletion of Talin1, a CTC-related molecule, in HCT116 and CT26 colorectal cancer cell lines, reduced their ability to attach and migrate through a monolayer of endothelial cells, key features in the process of CTC extravasation from blood.

9. We have identified a potentially new cancer cell-endothelium interaction mechanism, driven by the formation of focal adhesions by CT26 cancer cells at fibronectin-rich sites in the surface of endothelial cells. Talin1 represents a key component for the correct formation of these contact types.

10. We have proved that Talin1 represents a crucial cellular component for the correct establishment and turnover of focal adhesions in HCT116 and CT26 cell lines and that, functionally, Talin1 regulates cell-ECM adhesiveness, migration and basement membrane invasion.

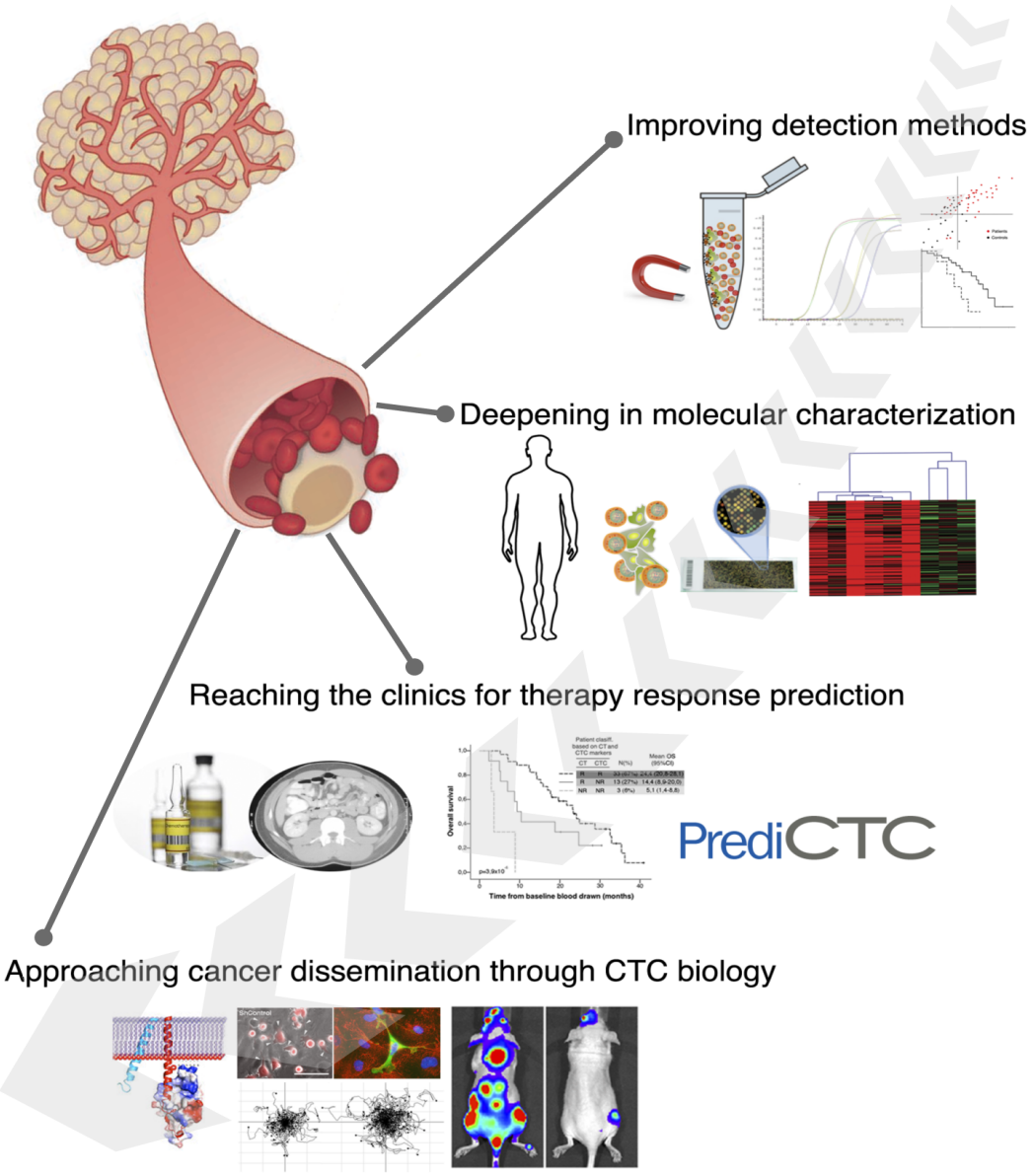
11. We have shown up the role of Talin1 in cancer dissemination mediated by CTCs in an *in vivo* model, highlighting its biological importance in metastasis formation, reinforced by its increased expression, in human tissue samples, as tumor progression advances. Moreover, we have validated the gene expression analysis of Talin1 in CTC-enriched samples as a prognostic and predictive factor in mCRC patients.

12. In closing, this thesis encompasses a global approach that, focusing on the study of circulating tumor cells in the process of cancer metastasis, aims to improve the current clinical tools available for a better patient management. As a whole, the establishment of new prognosis and predictive biomarkers, as well as potential therapeutic targets such as Talin1, widely evidence the clear translational commitment of this thesis. It represents an effort to transfer basic CTC-derived knowledge into a clinical application, with the ultimate intention of improving patient's welfare.

Circulating tumor cells in metastatic colorectal cancer

from basic understandig to clinical practice

Conclusions graphic overview



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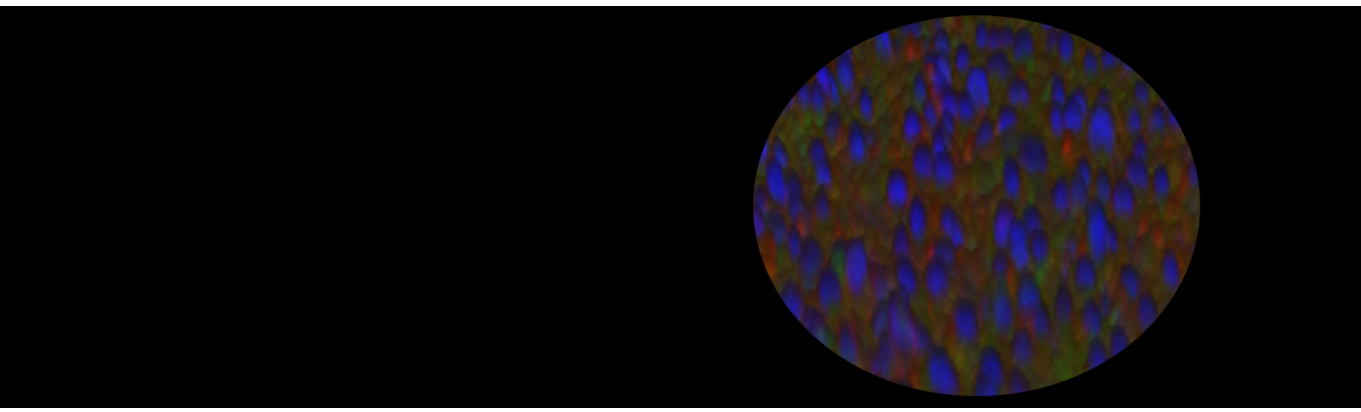
List of abbreviations

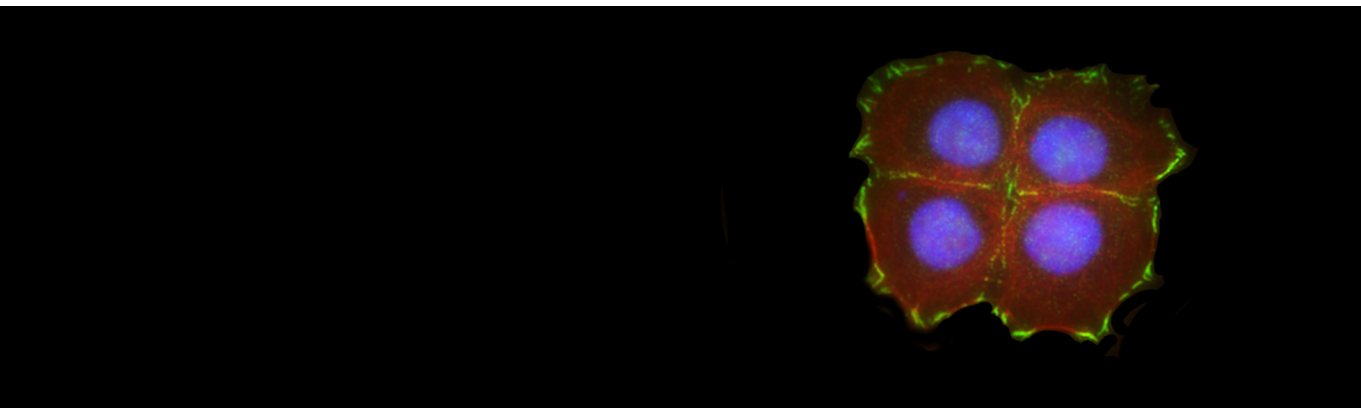


2D	Two dimensional
3D	Three dimensional
5-FU	5-Fluorouracil
AJCC	American Joint Committee on Cancer
AOM	Azoxymethane
ATCC	American Type Culture Collection
AUROC/AUC	Area Under ROC curves
BCA	Bicinchoninic Acid assay
BM	Basement Membrane
CAF	Cancer Associated Fibroblast
CAM	Aell Adhesion Molecule
cDNA	Comlementary DNA
CEA	Carcinoembrionic Antigen
CGH	Comparative Genome Hybridization
CI	Confidence Interval
CIMP	CpG Island Methylator Pathway
CIN	Chromosomal Instability
CK	Cytokeratin
CR	Complete Response
CRC	Colorectal Cancer
CT	Computed tomography
CTC	Circulating Tumor Cell
ctDNA	Circulating deoxyribonucleic acid
DAPI	4',6-diamino-2-phenylindole
DCBE	Double-Contrast Barium Enema
DFF	Dean Flow Fractionation
DMBA	3,2'-dimethyl-4-aminobiphenyl
DMH	Dimethyhydrazine
DNA	Deoxyribonucleic Acid
DPD	Dihydropyrimidine-dehydrogenase
DSS	Dextran Sulfate Sodium
EC	Endothelial Cell
ECM	Extracellular Matrix
ECOG	Eastern Cooperative Oncology Group
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
FA	Focal Adhesion
FAK	Focal Adhesion Kinase
FAP	Familial Adenomatous Polyposis
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FDG-PET	18-fluoro-2deoxy-D-glucose-Positron Emission Tomography
FISH	Fluorescence In Situ Hybridization
FIT	Fecal Immunochemical Test
FOBT	Fecal Occult Blood Test
GEMM	Genetically Engineered Mouse Model

GFP	Green Fluorescent Protein
GTP	Guanosine Triphosphate
HCC	Hepatocellular Carcinoma
HDMB	Hexadimethrine Bromide
HER2	Human Epidermal Growth Factor Receptor 2
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
HP	Hyperplastic Polyps
HR	Hazard Ratio
HUVEC	Human Umbilical Vein Endothelial Cell
ICC	Immunocytochemistry
KM	Kaplan-Meier
LA	Leukapheresis
LOH	Loss of Heterozygosity
LV	Leucovorin
mBC	Metastatic Breast Cancer
mCRC	Metastatic Colorectal Cancer
MMP	Matrix Metalloproteinase
MMR	Mismatch Repair
MNC	Mononuclear Cell
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MoAb	Monoclonal Antibody
MSI	Microsatellite Instability
MSS	Microsatellite Stable
NR	Non-Responder
NSCLC	non small-cell lung cancer
OS	Overall Survival
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Progression Disease
PET	Positron Emission Tomography
PFS	Progression Free Survival
PIP2	Phosphatidylinositol 4,5 Biphosphate
PKC	Protein Kinase C
PR	Partial Response
PS	Performance Status
PSA	Prostate Specific Antigen
qPCR	Quantitative Polymerase Chain Reaction
R	Responder
RNA	Ribonucleic acid
ROC	Receiver Operating Characteristic
RT	Room Temperature
SD	Stable Disease
SD	Standard Deviation
ShRNA	Short Hairpin RNA
SSA	Sessile Serrated Adenoma
TEM	Transendothelial Migration
TF	Transcription Factor
TGCA	The Cancer Genome Atlas

TIC	Tumor Initiating Cell
TKI	Tyrosine Kinase Inhibitor
TS	Thymidylate synthase
TSA	Traditional Serrated Adenoma
U.S.	United States
UGT1A1	UDP-Glucuronosyl Transferase 1A1
USC	Universidade de Santiago de Compostela
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cell
WTA	Whole Transcriptome Amplification

Resumo



O Cancro Colorrectal

O cancro colorrectal (CCR) representa o terceiro tipo de cancro en canto a incidencia a nivel mundial, e o cuarto no referente a taxas de mortalidade, con aproximadamente 690.000 mortes relacionadas con CCR no ano 2012, sendo lixeiramente máis habitual entre a poboación masculina [1], e cunha tendencia ao incremento especialmente en países en vías de desenvolvemento [3,4]. A pesares disto, as taxas de mortalidade en países coma España rexistran unha redución paulatina dende o ano 2001 para ambos sexos [6], á que hai que sumar tamén un incremento progresivo das taxas de supervivencia dos pacientes con CCR [8,9], todo iso debido ás melloras en prevención, diagnóstico e tratamento deste tipo de tumores nos últimos anos. As taxas de supervivencia a 5 anos de pacientes afectados de CCR varían enormemente en función do estadio no que este sexa diagnosticado, cun 90,1% para CCR en estadios localizados, que se reduce a un 69,2% trala extensión tumoral cara a órganos adxacentes ou nódulos linfáticos, ou a un 11,7% xa nos casos máis avanzados nos que existe unha diseminación a órganos máis distantes [7].

Aproximadamente un 5-10% dos casos de CCR son considerados de índole hereditaria, sendo o Cancro Colorrectal Hereditario Non Polipósico ou a Polipose Adenomatosa Familiar (HNPCC ou FAP, polas súas respectivas siglas en inglés), as dúas formas principais de CCR [10,11]. A maioría dos casos de CCR teñen unha etioloxía esporádica, e factores como a idade, a presenza previa de pólipos no colon, ou certos factores ambientais teñen sido relacionados coa etioloxía deste tipo de cancro [10].

A nivel molecular, xa no ano 1990, Fearon e Vogelstein describiron un modelo de carcinoxénese colorrectal no que se propoñía a súa formación a partires da progresión paulatina de adenomas preexistentes [16], a través de mutacións secuenciais que resultan na alteración de polo menos 4-5 xenes (activación de oncoxenes e inactivación de xenes supresores de tumores). Aínda que se seguen a conservar as premisas formuladas neste modelo inicial, nas últimas dúas décadas describíronse outras rutas que contribúen ao proceso de carcinoxénese colorrectal. Estas inclúen a denominada inestabilidade de Microsatélites (ou MSI) [27,29], a ruta de metilación de illas CpG (CIMP) [31,32], a de inestabilidade cromosomal (CIN) [18], ou a máis recentemente descrita “Serrated pathway” (denominación inglesa pola morfoloxía en forma de dentes de serra das lesións nas que se observa) [34-36]. En conxunto, todas estas características tumorais proporcionan información a nivel molecular que permite unha clasificación dos tumores dunha forma máis exacta, o que finalmente repercute nun mellor manexo e tratamento deste tipo de cancros.

Se temos en conta a dramática redución das taxas de supervivencia nos estadios máis avanzados desta enfermidade, xunto coa lenta evolución da mesma (dende a presenza de adenomas ata o desenvolvemento de CCR) ponse claramente de manifesto a importancia da detección temperá neste tipo de tumores. Diversas metodoloxías que permiten a detección de sangue ou de determinadas proteínas asociadas ao CCR nas feces (FOBT e FIT) [57-59], ou de marcadores de CCR no soro sanguíneo (SEPT9, MMP7, PTGS2 ou CEA) [65-67], foron descritos como ferramentas útiles para a detección precoz de CCR. Neste senso, dende

o ano 2013, está implantado na comunidade galega un programa de detección precoz deste tipo de cancro, baseado no uso da técnica FOBT. Non obstante, o uso de técnicas de imaxe, que permiten a visualización e extirpación de lesións nun mesmo procedemento, constitúen o tipo de técnicas mais amplamente utilizado, polas consecuencias positivas que supoñen para o doente [57]. Independentemente da técnica utilizada, a diagnose final do CCR faise en base ao resultado dunha colonoscopia, conxuntamente cunha biopsia do propio tumor e coa aplicación doutras técnicas de imaxe coma a tomografía de emisión de positróns (PET) para a detección de diseminación tumoral a distancia. Finalmente, a cada tumor asígnaselle un estadio en función dos resultados das probas anteriores, segundo a clasificación TNM que ten en conta tanto o tumor primario, coma a presenza de afectación nodular ou de metástases a distancia.

A cirurxía constitúe o único tratamento curativo para este tipo de tumores, sempre e cando se encontren en estadios localizados [84]. Aínda nestes casos, e para eliminar riscos de recorrencias pola presenza de células tumorais remanentes trala extirpación do tumor, é habitual a administración de terapias adxuvantes (complementarias á cirurxía) [85,338,339]. Tamén, aínda que é máis frecuente no caso de cancro rectal, se poden aplicar terapias neo-adxuvantes en determinadas situacións, para diminuír o tamaño do tumor antes da intervención cirúrxica [87]. No caso da presenza de enfermidade metastática a distancia, a quimioterapia constitúe practicamente a única alternativa, e é aplicada normalmente cun fin paliativo, para mellorar a calidade de vida do paciente. A mesma, está baseada principalmente no uso de 5-fluoracilo normalmente en combinación con outros axentes (irinotecan ou oxaliplatino), e con terapias biolóxicas dirixidas (Bevacizumab ou Cetuximab) [10,11].

A existencia de factores prognóstico (asociados coa evolución do doente en ausencia de terapia), ou predictivos (que permiten a identificación de pacientes con máis probabilidade de responder a unha terapia determinada), considérase hoxe en día fundamental para un mellor manexo dos pacientes con CCR. Un exemplo de factor prognóstico son os niveis do Antíxeno Carcinoembriónico (CEA) en soro, cuxos niveis elevados están asociados cunha peor evolución de enfermidade [72]. Por outra banda, a presenza de mutacións activadoras do xene *KRAS*, constitúe un dos factores predictivos máis estudados en CCR, estando asociado coa resistencia a terapias anti-EGFR, ruta na que está implicada o propio *KRAS* [103].

O proceso de metástase e as Células Tumorais Circulantes

Aínda que causante de máis do 90% da mortalidade relacionada co cancro [115,116], o proceso de metástase dista moito de ser completamente descifrado debido á súa elevada complexidade. O mecanismo polo cal as células epiteliais nos tumores primarios son capaces de xerar finalmente unha masa metastática a distancia, produce unha serie de eventos en serie, denominados en inglés “metastatic cascade [117]”. Nun primeiro lugar, ditas células tumorais han de invadir a nivel local, para acabar atravesando a membrana basal, que separa o

compartimento tumoral do estroma veciño [118,119], para finalmente alcanzar os vasos, tanto sanguíneos como linfáticos, situados nas zonas adxacentes ao tumor primario, e entrar nos mesmos nun proceso denominado intravasación [115]. Dito proceso de invasión caracterízase pola adquisición dun fenotipo máis mesenquimal por parte das células tumorais epiteliais, a través dun fenómeno denominado "Transición Epitelio-Mesénquima" ou EMT polas súas siglas en inglés. Grazas a isto, as células tumorais adquiren unha maior capacidade migratoria e invasiva, que lles permite avanzar cara a vasculatura e penetrar finalmente na mesma [134,161,294]. Aínda que potencialmente ditas células tumorais teñen acceso tanto á circulación sanguínea como á linfática, no caso do CCR, tense descrito que a ruta principal de diseminación, e polo tanto de formación de metástases, é a hematóxena [115].

Unha vez no compartimento circulatorio, estas Células Tumorais Circulantes ou CTCs teñen virtualmente acceso a tódolos órganos do corpo. Con todo, na maioría dos tumores obsérvase un claro patrón de metástase, con órganos diana preferenciais para cada tipo de tumor primario. No caso do CCR, o fígado constitúe o órgano principal en canto a formación de metástases, cos pulmóns en segundo lugar [141]. A propia disposición anatómica pode explicar parcialmente este patrón, debido á existencia dunha circulación mesentérica que conecta directamente fígado e intestino [133]. Emporiso, outros fenómenos como a liberación de factores solubles [148] ou microvesículas [147] por parte do tumor primario, poden colaborar na preparación do tecido diana antes da propia chegada das células tumorais que darán lugar á formación das metástases, xerando o que se denomina un "nicho premetastático" [145,146].

Unha vez na súa localización final, as CTCs han de abandonar a circulación para a posterior colonización no tecido diana, nun proceso denominado "extravasación", no cal, estas se adhiren primeiramente ás células endoteliais que conforman a cara luminal dos vasos sanguíneos, e migran a través das mesmas (migración transendotelial ou TEM) [150,340]. Finalmente, e tras alcanzar o estroma do tecido diana, unha subpoboación destas CTCs, as denominadas Células Iniciadoras de Tumores (ou TICs) [160] dará lugar á formación de micrometástases que serán as que finalmente orixinarán metástases macroscópicas [116].

A pesar do impacto que a aparición de metástases ten na supervivencia dos doentes [116], estudos en modelos animais suxiren que o proceso de formación das mesmas é altamente ineficiente, e calcúlase que unicamente un 0,02% do número total de CTCs liberadas á circulación a nivel do tumor primario darán lugar finalmente á formación de masas metastáticas macroscópicas [159]. Aínda así, a formación das mesmas segue a representar a principal causa de mortalidade relacionada con cancro.

O feito de que as CTCs representen un dos principais intermediarios no proceso de diseminación tumoral, con capacidade para, a lo menos a través dalgunha das súas subpoboacións, dar lugar á formación de metástases [172], fixo que nos últimos anos numerosos grupos de investigación se dedicasen ao seu estudo en profundidade. A propia bioloxía das mesmas, convérteas en candidatos moi atractivos para a análise do proceso de diseminación tumoral dunha forma dinámica e pouco invasiva, cos beneficios que iso pode reportar para a calidade de vida do doente. É importante ter en conta que, a día de hoxe, a maior parte das análises realizadas para determinar o prognóstico dun doente con cancro,

fanse a nivel do tumor primario [328]. Non obstante, nun contexto de enfermidade metastática, a evolución do doente vén determinada pola presenza das propias metástases, tanto polo seu número e localización, como pola súa bioloxía intrínseca. Se, adicionalmente, temos en conta que o cancro constitúe unha enfermidade cun alto compoñente de variación temporal, a análise dunha única biopsia do tumor primario probablemente non estea reflectindo as características reais da enfermidade global do doente. Porén, a obtención de repetidas mostras de tecido (tanto do tumor primario como das propias metástases), representa un risco considerable para o doente. Se temos en conta que as CTCs poden provir tanto dos tumores primarios, coma das metástases a distancia xa formadas [117,169,170], xunto coa relativa accesibilidade ás mesmas a través da obtención de mostras de sangue, que poden ser repetidas ó longo do tempo, converte este tipo celular nun candidato realmente atractivo para a avaliación das características globais da enfermidade, na forma dunha "biopsia líquida".

Unha das limitacións máis importantes para a análise das CTCs vén dada pola súa escasa frecuencia de aparición, estimada nunha CTC por cada $\approx 10^7$ células brancas por mililitro de sangue [173,174]. Isto esixe o desenvolvemento de metodoloxías altamente específicas e sensibles para o illamento e cuantificación de CTCs. Nos últimos anos, un número elevado de tecnoloxías foron descritas con dita intención, baseadas no emprego de determinadas propiedades diferenciais entre as CTCs e o resto de células presentes de forma normal no sangue. A expresión de proteínas de membrana ou xenes específicos, así como o seu maior tamaño comparado coas células brancas, ou algunhas propiedades funcionais (por exemplo, invasividade), son algunhas das características mais empregadas para a captura, cuantificación e posterior caracterización molecular das CTCs (resumidas na táboa 9 da sección "introduction").

Na actualidade a única metodoloxía aprobada pola "Food and Drug Administration, FDA" americana, para a cuantificación de CTCs en cancros de mama, colorrectal e próstata metastáticos, é a plataforma CellSearch (Janssen Diagnostics, Raritan, NJ, USA). Esta basease no illamento de CTCs mediante o uso de partículas magnéticas acopladas a anticorpos anti-EpCAM (molécula de adhesión altamente expresada en células epiteliais), e na súa posterior detección por marcaxe positiva para as citoqueratinas 8, 18 e 19 (epiteliais), e negativa para CD45 (marcador de células brancas) [175-177]. Aplicando esta metodoloxía en doentes con CCR metastático (CCRm), diversos grupos de investigación demostraron que a presenza de 3 ou máis CTCs (en 7,5mL de sangue) antes do inicio do tratamento, estaba claramente relacionada cun prognóstico desfavorable para o doente [76,77,177]. Resultados similares en cancros de mama [175,199,201], próstata [200,202], ou pulmón [203], poñen de manifesto o valor prognóstico da cuantificación de CTCs neste tipo de doentes.

De forma complementaria, a cuantificación de CTCs ao longo do tratamento mostrou ter un valor predictivo de resposta á terapia, pois reducións no número das mesmas despois da aplicación dun tratamento determinado, foron asociadas cun beneficio do doente en termos de supervivencia, tanto en mCRC [108,109,111,112], como en cancro de mama metastático [206]. Ademais, a análise das CTCs permite a avaliación de xenes concretos,

cuxo estado mutacional pode ser relacionado coa maior probabilidade de responder a unha terapia dirixida. Neste sentido, a determinación de mutacións en *KRAS* ou *BRAF* [207] en CTCs procedentes de doentes con mCRC, así como no *EGFR* en doentes con cancro de pulmón de células grandes (NSCLC, polas súas siglas en inglés) [208], preséntase como unha alternativa que, aínda pendente dun proceso de optimización metodolóxica, pode abrir novas vías no eido da predición de resposta a tratamento.

As análises relacionadas coas CTCs non quedan exclusivamente restrinxidas á súa contaxe ou á avaliación de mutacións puntuais en xenes de interese, senón que a determinación do seu perfil molecular estase a converter nun dos retos máis importantes a día de hoxe neste campo de estudo. Tanto a análise do perfil de expresión xénica destes tipos celulares [211,212], como a avaliación da presenza de alteracións xenéticas a nivel global [216], constitúen ferramentas que abren a posibilidade de atopar novos marcadores prognósticos e predictivos baseados en CTCs, ao mesmo tempo que proporcionan una información moi valiosa acerca da propia bioloxía de ditos dipos celulares. Iso abre unha nova vía para a mellor comprensión do proceso de metástase, e a identificación de potenciais dianas terapéuticas especificamente deseñadas para atacar de forma dirixida ao proceso de diseminación tumoral. No entanto, feitos como o reducido número de CTCs, ou os elevados niveis de contaminación por células sanguíneas presentes nas mostras, incluso despois do enriquecemento en CTCs, representan factores que limitan a obtención de información con este tipo de metodoloxías. Deste xeito, a mellora dos sistemas de illamento e cuantificación de CTCs, a implementación de novas metodoloxías que permitan o procesamento de volumes maiores de sangue para a obtención de maiores cantidades de material tumoral, ou o perfeccionamento das tecnoloxías de análise de célula única, son fundamentais para o avance deste campo de estudo que, aínda con moito camiño por percorrer, se considera prometedor.

Deste xeito, e tendo en conta tanto as características do cancro colorrectal, do proceso de formación de metástases, e a importancia que o estudo das CTCs achega para o mesmo, nesta tese formúlanse os seguintes obxectivos principais:

1. O desenvolvemento dunha metodoloxía mellorada para o illamento e cuantificación de CTCs en doentes con cancro colorrectal metastático.

2. A caracterización molecular de CTCs en doentes con cancro colorrectal metastático, en base ao seu perfil de expresión xénica.

3. A avaliación dun panel de marcadores de CTCs como unha ferramenta prognóstica e predictiva para a análise de resposta a terapia en doentes con cancro colorrectal metastático.

4. A caracterización do papel de Talina1 no proceso de diseminación metastática mediado por CTCs, e a análise de Talin1 como unha nova potencial diana terapéutica en cancro colorrectal metastático.

Detección e cuantificación de CTCs

Na primeira parte desta tese descríbese a optimización dun método alternativo para o illamento e cuantificación de CTCs en doentes con CCRm, baseado no emprego de micropartículas magnéticas acopladas a anticorpos anti-EpCAM para a purificación de CTCs, combinado coa súa posterior detección mediante técnicas de PCR a tempo real. Ademais, o emprego dun método de extracción de RNA optimizado para mostras con escasa carga celular, xunto coa realización dun proceso de preamplificación previo á PCR, proporcionan niveis de sensibilidade adecuados para a efectiva detección de CTCs.

A presenza de células illadas de forma inespecífica na mostra final (principalmente células brancas), fai que sexa necesario o uso dunha metodoloxía con elevada especificidade para a detección de CTCs. Finalmente, a combinación de *VIL1* (xene expresado de forma específica no tecido intestinal), *GAPDH* (xene "housekeeping", expresado de forma ubicua, e empregado como indicador da celularidade global presente na mostra), e *CD45* (marcador específico de células de estirpe linfocitaria, empregado como avaliador dos niveis de illamento inespecífico), deron lugar a un modelo para a efectiva detección e cuantificación de CTCs en doentes con CCRm. Valores de área baixo a curva ROC próximos a 0,86 evidencian as altas taxas de sensibilidade e especificidade alcanzadas por este método de detección, con valores superiores os doutras metodoloxías previamente descritas. Ademais, encontrouse unha relación significativa entre os niveis destes marcadores e a evolución clínica dos doentes, sendo peor naqueles con marcadores elevados, evidenciando o valor prognóstico desta metodoloxía. Aínda que a nivel de proba de concepto, estes resultados poñen de manifesto a utilidade deste sistema de detección para a súa incorporación en ensaios clínicos para a súa validación.

Avances na caracterización molecular das CTC

Trala optimización dun método de illamento e detección de CTCs, xorde a necesidade de avanzar un paso máis cara a obtención dun perfil molecular completo deste tipo celular, o cal representa un dos aspectos máis atractivos, á par que desafiantes, da investigación no campo das CTCs

Neste contexto, e baseándonos no método descrito anteriormente, na segunda parte desta tese levouse a cabo un estudo do perfil de expresión xénica xeral de CTCs illadas de doentes con CCRm. Trala amplificación lineal do seu transcriptoma completo, que permite a obtención de cantidades maiores de material xenético, levouse a cabo a hibridación en "arrays" de expresión xénica. Iso, combinado co emprego de mostras de doentes e controis sans, permitiu a identificación dun panel de 410 xenes específicos de CTCs neste tipo de doentes, principalmente implicados en funcións celulares como adhesión, migración, proliferación ou sinalización celular. Ditos resultados foron validados nun grupo de 20 doentes e 10 controis sans, mediante qPCR para 11 xenes provenientes da lista inicial (410), obtendo diferenzas significativas entre ambos grupos para todos eles. É destacable

o feito de que, tras unha análise de supervivencia, tódolos xenes validados mostraron un valor prognóstico significativo, separando ós doentes en función da súa probabilidade de supervivencia, ademais dunha maior expresión (a diferentes niveis, dependendo do xene) en tecido metastático comparado co tumor primario.

En conxunto, os resultados presentados nesta parte reforzan a importancia da identificación do perfil de expresión xénica das CTCs, como unha ferramenta para a identificación de posibles marcadores, tanto diagnósticos como prognósticos, ao mesmo tempo que proporciona información acerca da propia bioloxía de ditas CTCs, e polo tanto do proceso de metástase en si, moi relevante para o deseño de futuras terapias dirixidas.

CTCs e predición de resposta a tratamento

A selección de terapias adecuadas para o tratamento do CCR metastático resulta crítica para un manexo efectivo deste tipo de doentes, cun impacto importante na supervivencia dos mesmos. Actualmente, a avaliación de resposta a tratamento efectúase principalmente mediante técnicas de imaxe, en base ao resultado das cales se decide o mantemento ou cambio dunha terapia determinada. Porén, este tipo de metodoloxías non proporcionan información tan rápido nin efectivamente como moitas veces é necesario na práctica clínica diaria. Deste xeito, moitos doentes poden estar sendo tratados durante longos períodos de tempo cunha terapia inefectiva, coas respectivas consecuencias para os mesmos, normalmente relacionadas coa propia toxicidade dos tratamentos.

Nesta tese propoñemos a utilización dun panel de marcadores de detección de CTCs en doentes con CCRm, para a avaliación de resposta a tratamento dunha forma eficaz e temperá. A análise de CTCs con este fin presenta certas vantaxes fronte ás metodoloxías convencionais, xa que poden ser analizadas de forma repetida ao longo do tempo de tratamento, dunha forma minimamente invasiva e inocua para o doente, á vez que poden proporcionar información dunha forma máis rápida e menos custosa que as técnicas de imaxe.

A combinación de marcadores específicos para a detección CTC, obtidos nos traballos correspondentes ás primeiras partes desta tese (*GAPDH*, *VIL1*, *CLU* e *TIMP1*), xunto con outros relacionados co proceso de EMT (*ZEB2* e *LOXL3*), todos eles normalizados pola expresión de *CD45*, xerou un panel de marcadores efectivo tanto para a avaliación do prognóstico dos doentes, como para a predición de resposta a tratamento nos mesmos. A avaliación dos niveis de expresión de ditos xenes levouse a cabo en 50 doentes con CCRm antes e despois do inicio do tratamento. Empregando esta metodoloxía, fomos capaces de avaliar a resposta a tratamento na maioría dos doentes tras tan só catro semanas despois do inicio do mesmo, moito antes que as técnicas de imaxe convencionais (normalmente tres meses). Aqueles doentes que presentaron unha redución dos niveis de marcadores de CTCs, mostraron unha maior supervivencia, comparados nos que se encontrou un aumento dos mesmos. É de destacar a existencia dunha porcentaxe de doentes (≈25%) clasificados como respondedores por técnicas de imaxe, e que esta metodoloxía identificou como non

respondedores. Estes doentes presentan un tempo de supervivencia significativamente máis curto que aqueles clasificados como respondedores por ambos métodos, o que pon de manifesto a clara superioridade do emprego destes marcadores de CTCs para a avaliación temperá da resposta a terapia.

Os resultados desta parte da tese deron lugar á solicitude dunha patente europea, e actualmente estase a desenvolver un kit de predición de resposta a terapia, que será avaliado nun número máis amplo de doentes con CCRm nun ensaio clínico multicéntrico. Todo iso, co fin de poder implantar esta metodoloxía na práctica clínica diaria, co evidente beneficio que pode supoñer para a calidade de vida dos doentes.

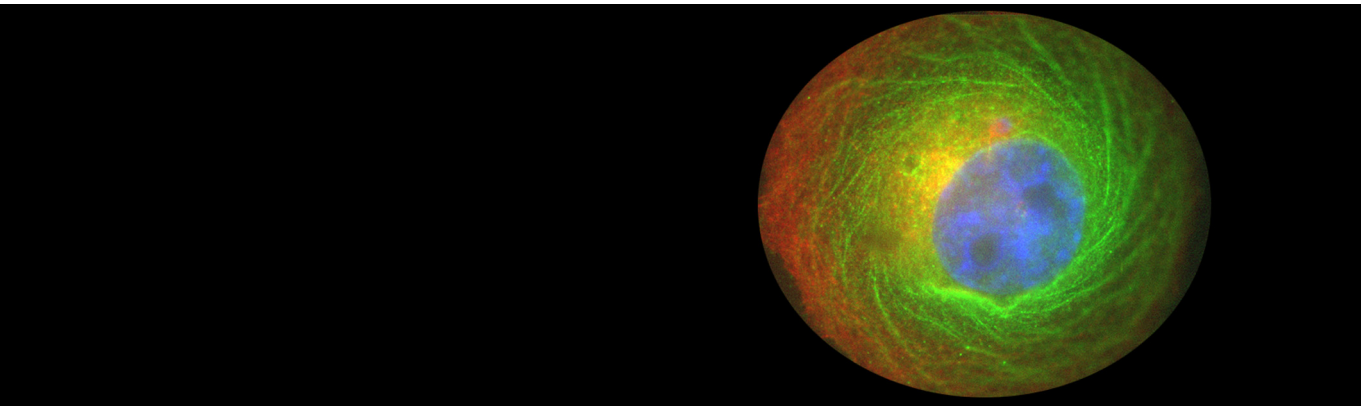
CTCs e a bioloxía do proceso de metástase

A información que as CTCs poden chegar a proporcionar vai mais alá do seu valor prognóstico ou predictivo. Como xa se comentou, a identificación de moléculas CTC-específicas, involucradas en procesos chave na diseminación tumoral, resulta crítica para unha mellor comprensión da mesma, e ao mesmo tempo abre unha nova vía para o establecemento de novas dianas terapéuticas máis efectivas, dirixidas de forma específica contra o proceso de metástase mediado por CTCs.

Neste sentido, na derradeira parte desta tese identificouse Talina1, molécula pertencente á ruta das integrinas e involucrada na adhesión das células á matriz extracelular que as rodea, como un marcador específico de CTCs en doentes con CCRm. O bloqueo in vitro da expresión de Talina1 en liñas celulares de CCRm deu lugar a unha redución no número de adhesións focais, así como á diminución das capacidades tanto adhesivas como migratorias destes tipos celulares. Ademais, encontramos que Talina1 xoga un papel fundamental no proceso de adhesión das células tumorais ás células endoteliais, primeira barreira que encontra unha CTC durante o proceso de extravasación, no seu camiño cara a colonización dos tecidos diana para a posterior formación de metástases. Describimos tamén que dita adhesión se leva a cabo, a lo menos en parte, grazas á interacción que se establece entre as adhesións focais das células tumorais e fibras de fibronectina que as células endoteliais producen na súa superficie. A maiores, despois do bloqueo da expresión de Talina1, as células tumorais mostraron unha habilidade diminuída para migrar a través dunha monocapa de células endoteliais e da membrana basal situada inmediatamente debaixo das mesmas. Todos estes resultados in vitro explican a escasa capacidade das células para, en ausencia de Talina1, dar lugar á formación de metástases trala inxección das mesmas nun modelo animal. Se a iso lle sumamos a capacidade tanto prognóstica como predictiva que presentaron os niveis de expresión de Talina1 en CTCs de doentes con CCRm, queda claramente reflectida a importancia clínica desta molécula a tódolos niveis.

En resumo, o traballo recollido nesta tese engloba o establecemento dunha nova metodoloxía para o illamento e cuantificación de CTCs provenientes de doentes con CCRm, dunha forma sensible e específica, que posibilitou a posterior avaliación do seu perfil de expresión xénica, clave para o mellor entendemento da súa propia bioloxía. Ademais, e partindo da información obtida nos dous primeiros traballos, levouse a cabo o deseño dun panel de marcadores para a detección de resposta a terapia dunha forma sinxela, rápida e efectiva. Por último, a validación de Talina1 como unha molécula relevante durante o proceso de extravasación das CTCs da circulación e o posterior establecemento de metástases, e polo tanto como unha potencial diana terapéutica para o control do mesmo, pon de manifesto o valor global do estudo das CTCs en tódalas facetas relacionadas co manexo clínico dos doentes con CCRm.

Agradecementos



Cinco anos… dinse pronto…

Xa non é a primeira vez que escoito aquilo de que as teses en Bioloxía levan a palma en canto aos agradecementos. E é soamente cando chegado este momento, un bota a vista atrás e dáse conta de que quizais este sexa un caso máis que axude a alimentar tales lerias.

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Non cambiaría nin un só intre de todos cantos me tocou vivir nesta época.

Grazas, a todos.

...one thing is certain, that if they came from an existing cancer structure,
they must have passed through the greater part of the circulatory system to have arrived
at the internal saphena vein of the sound leg...

...cells identical with those of the cancer itself being seen in the blood may tend to
throw some light upon the mode of origin of multiple tumours existing in the same person...

Thomas Ashworth, 1869