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Role of the Gut Vascular Barrier in metastatic Colon Cancer

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*"Happiness can be found, even in the
Darkest of time, if one only
Remembers to turn on the light"*
A.S.

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LIST OF ABBREVIATIONS

ANGPT4	Angiopoietin Like-4
AOM	Azoxymethane
APC	Adenomatous Polyposis
AJs	Adherens Junctions
CC	Colon Cancer
CCL2	C-C motif ligand 2
CCSCs	Colon Cancer Stem Cells
CFUs	Colony Forming Units
CIN	Chromosomal Instability
CSCs	Cancer Stem Cells
C3aRKO	C3a Receptor Knock Out
DSS	Dextran Sulphate Sodium
ECs	Endothelial Cells
ECM	Extra Cellular Matrix
EGF	Epithelial Growth Factor
EGFR	Epithelial Growth Factor Receptor
EMT	Epithelial to mesenchymal transition
FAP	Familial Adenomatous Polyposis
FDS	Fenestrated Diaphragms
FFPE	Formalin Fixed Paraffin Embedded
FISH	Fluorescent In Situ Hybridization
FMT	Fecal Microbiota Transplantation
GF	Germ Free
GVB	Gut Vascular Barrier
GVU	Gut Vascular Unit
G-CSF	Colony-Stimulating Factor
IBD	Intestinal Bowel Disease

IEB	Intestinal Epithelial Barrier
LN	Lymph Nodes
mCC	metastatic Colon Cancer
IMC	Imaging Mass Cytometry
MET	Mesenchymal to Epithelial Transition
mLN	mesenteric Lymph Nodes
MSI	Micro Satellite Instability
MMP	Metalloproteinase
MMR	Miss Match Repair
NRP-1	Neurophilin-1
PLVAP	Plasmalemma Vesicle Associated Protein
PKs	PolyKetide Synthase
PMN	Pre-Metastatic Niche
SDS	Stomatal Diaphragms
SOX9	Sex-Determining region Y (SRY)-box9
TECs	Transendothelial Channels
TGF- β	Transforming Growth Factor- β
TNF- α	Tumor Necrosis Factor- α
TNM	Tumor-Node-Metastases
TJs	Tight Junctions
VEGFR	Vascular Endothelial Growth Factor
ZO-1	Zonula Occludens-1

ABSTRACT

Distant metastasis is the second leading cause of colon cancer-related mortality. After surgical resection of the primary tumor, 30% to 40% of colon cancer (CC) patients develop distant metastases in five years, meaning that these patients at the time of the diagnosis have already some disseminating cancer cells. Spreading of neoplastic cells from CC to regional lymph nodes (LN) is often associated with distant recurrence. However, a number of clinical trials have shown that lymphadenectomy is not increasing CC patient's survival. This phenomenon is probably due to a metastatic dissemination that occurs via the systemic blood circulation, rather than the lymphatic vessels. So far it has not been fully described how such dissemination is achieved.

In the present thesis the aim was to verify whether the disruption of the Gut Vascular Barrier (GVB) in colon tumors could be linked to cancer cell dissemination at distant sites. Therefore, we tested the expression of PV-1, a marker of deranged GVB, in primary tumor of CC patients that have developed or not distant metastases (i.e. liver or lungs) within five years. We demonstrated that PV-1 is highly expressed in patients that experience distant recurrence and this expression is independent from the presence of metastases in LN. Interestingly, these patients with dismantled GVB exhibit also bacterial presence inside distant metastatic lesions. Therefore, we additionally assessed whether cancer-related bacteria could take advantage of the GVB disruption and colonize distant organs triggering the formation of a premetastatic niche and fostering distant metastases formation. Using a model of tumor-bearing mice that develop spontaneous tumors in the colon, we studied bacteria dissemination and consequent premetastatic niche formation. We showed that bacteria presence could generate an immunosuppressive microenvironment characterized by the recruitment of myeloid cells (i.e. macrophages, neutrophils and inflammatory monocytes). Also, we demonstrated that this altered hepatic microenvironment is able to foster the recruitment of disseminating cancer cells when injected intrasplenically.

Taken together these results indicate that GVB impairment can shed light into the process of colon metastases and that PV-1 can be used as a new prognostic marker

for distant recurrence. The presence of a dismantled GVB can also allow the spreading of cancer related bacteria at distant sites where they can reside and form a premetastatic niche able to favour the seeding of disseminating cancer cells. Hence, we speculate that targeting the GVB and/or disseminating bacteria could be used as a therapeutic intervention to impair distant metastases formation.

1 INTRODUCTION

1.1 Colon cancer

Colon cancer (CC) is one of the leading causes of cancer-related mortality in industrialized country(Hagggar and Boushey 2009). It is a multifactorial disease driven by different factors such as genetic mutations, diet and inflammation. In the recent years it has become clear that colon cancer-related dysbiosis could drive colon cancer progression and influence the formation of distant metastases(Garrett 2015b; Ma et al. 2018; Sears and Garrett 2014) (see paragraph 1.3).

1.1.1 Colon cancer disease from a genetic point of view

Colorectal carcinogenesis is believed to be a multi-stage process that originates with a localized adenoma, which linearly progresses from an intra-mucosal carcinoma, to an invasive lesion, and metastatic cancer (Irraz??bal et al. 2014; Marian 2004) (see paragraph 1.3) (Figure 1-1).

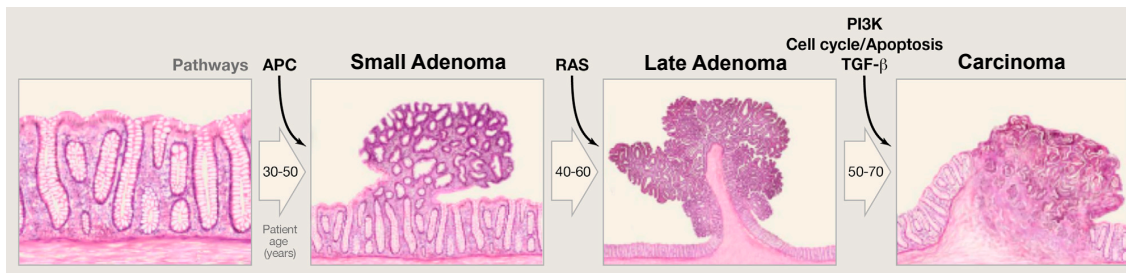


Figure 1-1 Colorectal cancer developement from benign adenomas to invasive carcinomas. The most common initiator events are mutations in APC and in mismatch repair genes (MMR). Subsequent accumulation of mutations leads to progression and development of invasive cancer (Figure adapted from Sears and Garrett 2014).

Among CC patients, 10% are genetically predisposed to develop hereditary CC. Familial adenomatous polyposis (FAP) arises from an autosomal dominant mutation in the adenomatous polyposis (apc) tumor suppressor gene(Irraz??bal et al. 2014;

Marian 2004). This disease is characterized by the early onset of hundreds to thousands of adenomatous polyps throughout the colon. If left untreated, all patients with this syndrome will develop CC by the age of 35-40 years.

The remaining 90% of CC arises sporadically due to microsatellite instability (MSI), aberrant CpG island methylation pathway, and chromosomal instability (CIN) in the hyperplastic epithelium (Figure 1-2) (Bogaert and Prenen 2014). MSI is caused by mutations in DNA mismatch repair (MMR) genes characterized by insertion, depletion or frame shift mutation in noncoding regions. CIN instead, is a process that generates gene deletion, duplication and chromosome rearrangement (Bogaert and Prenen 2014). Among the oncogenes and oncosuppressors that are mutated during CC progression, the Apc mutation is commonly considered as the initiating event in adenoma formation (Bogaert and Prenen 2014).

Apc protein is involved in the Wnt/ β -catenin signalling pathway, which is fundamental to control several processes in adult homeostasis, including cell proliferation and polarity (Figure 1-2) (Moon et al. 2004). Wnt ligands are a group of 19 secreted glycoproteins that below a certain concentration are sequestered by Frizzled related protein (Fzd) and Dickkopf (DKK), two secreted antagonists, in order to switch-off the signalling pathway. In the absence of Wnt ligands, β -catenin level is controlled by β -catenin degradation complex. This complex is composed of different proteins like APC that target β -catenin for proteasome degradation (Moon et al. 2004; Najdi, Holcombe, and Waterman 2011). When Wnt concentration increases or APC is mutated, β -catenin degradation process is impaired and it can translocate to the nucleus and interact with DNA-bound TCF and LEF family members to activate the transcription of target genes mainly related to cell proliferation such as *C-Myc* (Moon et al. 2004; Najdi, Holcombe, and Waterman 2011).

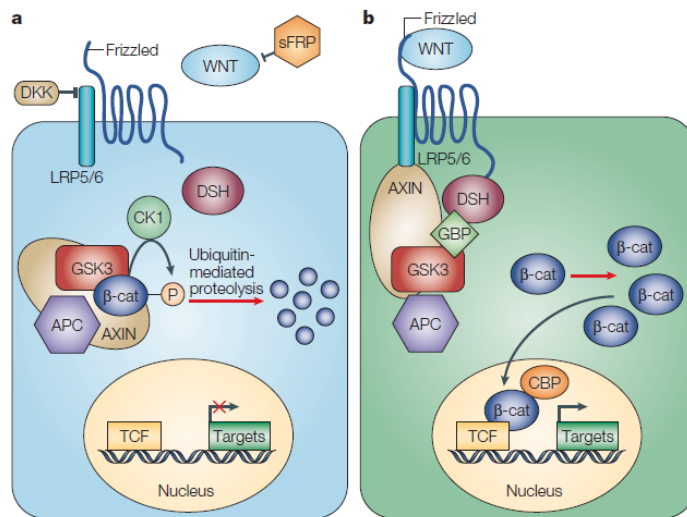


Figure 1-2 Wnt signalling pathway. (A) Under certain level wnt ligands are not able to bind to the specific receptor thus β -catenin degradation complex target β -catenin to degradation. (B) When wnt ligands bind to specific membrane receptor or APC protein is mutated, β -catenin is not targetted to degradation thus it can translocate into the nucleus and activate the trascription of proliferative genes such as *C-Myc* (Figure adapted from Moon et al. 2004).

APC mutation is then followed by the accumulation of multiple mutations in several genes (i.e. KRAS, SMAD4, BRAF, TP53), which are triggering aberrant cell proliferation, evasion of the immune system, and metastatic invasion leading to carcinoma formation. At this late stage of carcinogenesis, colon cancer cells start to acquire a more aggressive and invasive phenotype in order to metastasize to distant organs through the lymphatic and blood systemic circulation.

1.1.2 Colon cancer disease from a clinical point of view

Approximately, 20% of patients with CC also show distant visceral metastases at the time of diagnosis (synchronous metastases) and 50% of patients with localized disease ultimately develop distant metastases within five years after the removal of the primary tumor (metachronous metastases)(Risio 2012). Detecting in advance the possible relapsing patients is the main point of surveillance after the resection of the primary tumor. The pathological staging system is the most accurate prediction of

recurrence. In colon cancer, the Tumor-Node-Metastases (TNM) staging system considers tumor size and the degree of local tumor invasion (T); the size and location of metastatic regional lymph nodes (N), and the presence or absence of distant metastases (M).

The risk assessment of recurrence is important to decide the optimal treatment option for each patient (Labianca et al. 2013). CC patients could be treated with chemotherapy alone or in combination with biological therapies. Adjuvant therapy is a systemic treatment administered after primary tumor resection with the aim of reducing the risk of recurrence. The current chemotherapies used in the clinic are: 5-fluoracil with leucovorin (5-FU-LV), capecitabine, 5-FU-LV in combination with oxaliplatin (FOLFOX) and capecitabine in combination with oxaliplatin (CAPEOX) (Carrato 2008). Biological therapy or “molecular target therapy” consists of drugs targeting specific cell growth factors, receptors or molecular pathways. In particular, Cetuximab and Panitumumab block epithelial growth factor receptor (EGFR) and inhibit downstream RAS-ERK pathway activation, which normally drives tumor proliferation and invasion. Bevacizumab is considered a “tumor-starving” biologic agent (or anti-angiogenic), which is directly neutralizing vascular endothelial growth factor (VEGF) impairing the formation of new vessels and normalizing the existing vasculature in order to improve the delivery of chemotherapy (Wolpin and Mayer 2008).

Synchronous metastatic patients (M1) are treated with the most aggressive therapy, which combines biological therapies and chemotherapies (Labianca et al. 2013). Patient without distant metastases (M0) could be treated or not with adjuvant chemotherapies. As you can see from Figure 1-3, patients considered to be at high-risk of recurrence (with lymph node metastases) are always treated with adjuvant chemotherapy. Instead, patients at low risk of recurrence (with no lymph node involvement) rarely receive any treatment even if the tumor is massively invading the bowel wall (T3 or T4). This schedule is based on the idea that colon carcinoma confined to the colonic mucosa cannot metastasize at distant sites due to the paucity

of lymphatic vessels at this site. Whereas tumors that invade the submucosal layer can permeate the lymphatic system and disseminate systemically.

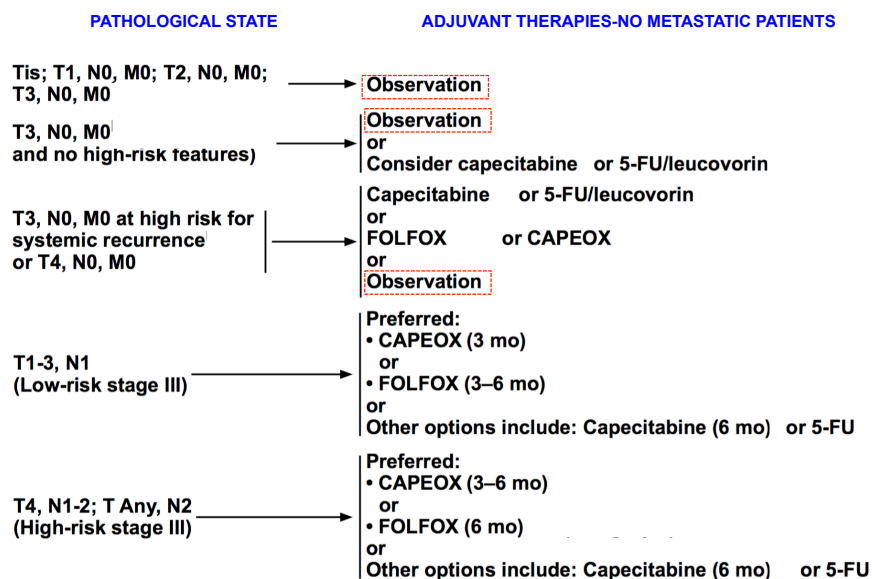


Figure 1-3 Possible treatment strategies for CC patients without distant metastases.

CC patients without distant metastases could be treated or not with adjuvant therapies up to the presence of regional lymph node involvement. The adjuvant therapies available in the clinic are: 5-FU-FL, capecitabine, FOLFOX and CAPEOX. Patients without lymph node involvement are preferentially not treated (observation) even if the pathological stage is considered at high risk (T3/T4) (Figure adapted from NCCN Clinical practice guidelines in oncology 2018).

Despite the surgery of the primary tumor and the following possible therapies, 50% of patients with colon cancer will relapse and most of them will die from metastases. This suggests that, besides the mere involvement of lymphatic vessels, additional factors should be considered during patient stratification. Specifically, the invasion of the blood circulation which could be also involved in the complex machinery of the metastatic process. However, up to now, there are no means to measure blood invasion by tumor cells.

1.1.2.1 Conundrum between lymphatic and blood vessels dissemination

Until now, the involvement of regional lymph nodes has been considered the only clinical feature that can predict CC recurrence. Despite the fact that lymph node involvement appears to be a central staging parameter for CC patients, still, 30% of patients with histopathology-negative lymph node metastases die from metachronous distant metastases (Van Wyk et al. 2014). Indeed, the role of lymphatic dissemination remains controversial and debated (Gervasoni, Sbayi, and Cady 2007). Some clinical trials have demonstrated that lymphadenectomy did not statistically improve the overall patient survival suggesting that lymph node metastases may not be the only responsible for distant lesions (Fioranelli et al. 2017; Gervasoni, Sbayi, and Cady 2007). As a matter of fact, tumor cells can disseminate to distant organs through two ways: the vascular and the lymphatic circulation (Risio 2012; Van Wyk et al. 2014). The blood vessels can transport tumor cells directly to distant organs (i.e. the liver and the lung), whereas, the intestinal lymphatic circulation may transfer tumor cells from regional lymph nodes to distant lymph nodes (Lanciault and Jacobson 1976; Pereira et al. 2018; Van Wyk et al. 2014) and from there through the thoracic duct to the blood circulation. Therefore, metastases to the liver and lung can occur independently of lymphatic spread via the blood circulation. Naxerova et al. have examined genetic distances between lymph node metastases, primary tumor and corresponding distant metastases (Naxerova et al. 2017). From the phylogenetic analyses, they have found that lymph node and liver distant metastases were more closely related to a primary tumor region than to each other, suggesting that different sub-clones in the primary tumor were able to metastasize either to the lymph node or to distant organs (Naxerova et al. 2017). These findings support the idea that lymph node metastases and distant metastases are independent. Since distant metastases can occur through blood vessel circulation, it would be necessary to introduce in the CC staging system a specific marker for blood vessels permeability.

1.2 The Gut Vascular Barrier (GVB)

The gastrointestinal mucosa is constantly exposed to a dense community of bacteria ($\geq 10^{12}/\text{cm}^3$ intestinal matter)(L. V. Hooper, Littman, and Macpherson 2012) called the “microbiota”, which increases in quantities along the gut surface from the duodenum to the colon. Microbiota and food antigens are confined to the intestinal lumen thanks to the Intestinal Epithelial Barrier (IEB) that acts as a physical and immunological barrier. In normal conditions, microbiota can cross the IEB and reach the mesenteric Lymph Nodes (mLNs) through the lymphatic vessels to induce local tolerance against them. During intestinal inflammation instead, bacteria can translocate into the liver, which acts as a “firewall” of our body(Balmer et al. 2014; Mowat 2003).

In our laboratory, it has been demonstrated that beyond the epithelium there is another barrier involving the intestinal endothelium, namely the Gut Vascular Barrier (GVB)(Spadoni et al. 2015). Under homeostatic conditions, the GVB acts in concert with the IEB to control the entrance of nutrients and the influx of ions and water into the human body(Daneman and Rescigno 2009; Spadoni et al. 2015; Spadoni, Fornasa, and Rescigno 2017). The GVB is composed by endothelial cells, which are tightened to each other through Tight Junctions (TJs) and Adherens Junctions (AJs) proteins. The TJs proteins expressed in the gut endothelium are occludin, zonula occludens 1 (ZO-1), JAM-A and claudin-5, which are mainly regulating paracellular permeability and maintaining cell polarity(Bazzoni 2004). Among the AJs proteins present in the gut endothelium, VE-cadherin is the most represented one, which acts as a regulator of cell-cell adhesion(Giannotta, Trani, and Dejana 2013; Spadoni et al. 2015).

Gut micro vessels are also in close contact with enteric glial cells (astrocytes-like GFAP positive cells) and pericytes (α -SMA and Desmin positive cells) to cooperate and create a Gut Vascular Unit (GVU) (Spadoni et al. 2015). Although the role of the GVU remains to be elucidated, it has been shown that GFAP positive enteric glial cells present in the gut mucosa, reach the epithelial cells and blood vessels with their

projections(Gulbransen and Sharkey 2012; Hanani and reichenbach 1994; Savidge, Sofroniew, and Neunlist 2007). These cells are able to release several factors, such as transforming growth factor β (TGF- β 1), which regulates gut barrier functions through the induction of ZO-1 and occludin expression(Flamant et al. 2011; Gulbransen and Sharkey 2012).

Under non-homeostatic condition, GVB perturbation results in increased blood vessels permeability triggering improper entrance of molecules into the systemic circulation, which are then able to reach secondary organs. Indeed, under *Salmonella typhimurium* infection, bacteria are able to reach the liver by inducing GVB damage. This damage results in impaired vascular permeability assessed by the extravasation of FITC dextran (70 Kda) from the blood stream into the lamina propria(Spadoni et al. 2015). Leaky vessels express high level of PV-1 protein encoded by Plasmalemma Vesicle Associated Protein gene (PLVAP), a downstream target of Wnt/ β -catenin signalling pathway. Under hazardous conditions, together with PV-1 over expression, Endothelial Cells (ECs) up-regulate other genes involved in angiogenesis and epithelial/endothelial to mesenchymal transition, which are crucial steps in the metastatic process(Spadoni et al. 2016) (see paragraph 1.3).

Mice gain of function for β -catenin in its active form in an endothelial cells-specific manner (Cdh5 (PAC)-CreERT2/ β -catenin^{lox(ex3)/lox(ex3)}(VEC-PAC)) under hazardous conditions do not show either PV-1 up-regulation or bacteria spreading to the liver suggesting that β -catenin forced activation can prevent GVB breakage and blood systemic dissemination(Spadoni et al. 2015).

In conclusion, the intestinal epithelial barrier and the GVB cooperate to shape the intestine as an “immune privileged” organ(Spadoni, Fornasa, and Rescigno 2017). Indeed, here the microbiota can be tolerated by the immune system and it is confined inside the lumen. Under hazardous conditions, harmful bacteria can cross the epithelial barrier and mediate GVB damage(Spadoni et al. 2015, 2016; Spadoni, Fornasa, and Rescigno 2017). GVB impairment, assessed by PV-1 up-regulation,

allows bacteria to enter the blood circulation and disseminate up to the liver (Spadoni et al. 2015, 2016; Spadoni, Fornasa, and Rescigno 2017).

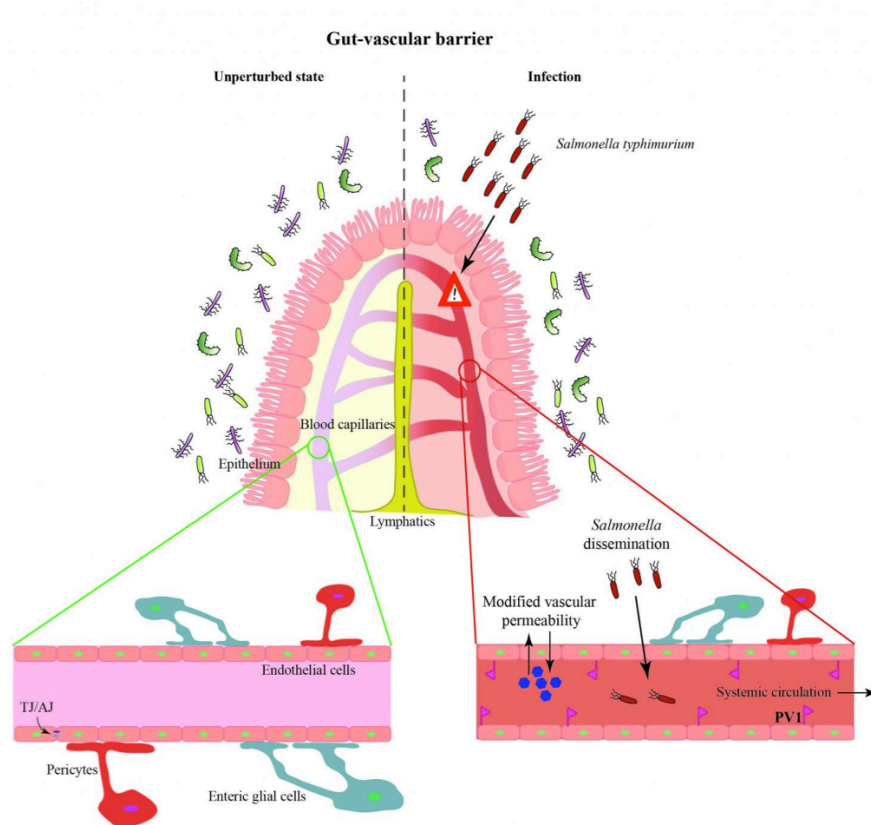


Figure 1-4 The Gut Vascular Barrier at steady state and upon *Salmonella* infection.

Gut endothelial cells are tightly connected each other by TJs and AJs proteins. Moreover, gut endothelial cells are in contact with pericytes and astrocytes forming a GVB (left part of the figure). Under adzardous conditions, like *Salmonella* infection, this barrier is wiped out and *Salmonella* is able to disseminate systemically reaching the liver. Exhausted GVB is witnessed by the expression of PV-1 (image adapted from Spadoni et al. 2015).

1.2.1 PV-1: a multifaceted protein

Endothelial cells have evolved several phenotypes due to the diverse biochemical and structural function of the organ they belong to (Tse and Stan 2010). Endothelial cells can be classified in three structural types: the continuous, fenestrated and discontinuous endothelium (Tse and Stan 2010). The mucosal vessels, concentrated at the centre of the villi, are fenestrated, allowing the homeostatic passage of water and soluble factors from the intestinal lamina propria to the blood circulation (Clementi and Palade 1969).

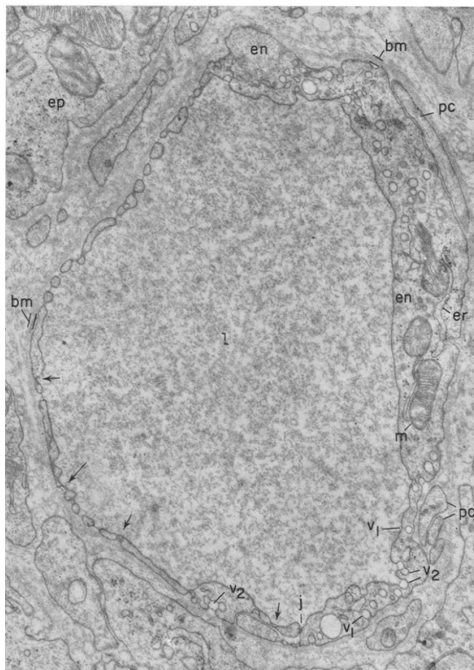


Figure 1-5 Cross section of endothelial cell in the gut.

The thin part of the endothelial cell (en) is facing the intestinal epithelium (ep). It is made up with numerous fenestrae composed by diaphragms (arrow) and vesicles (V). This part of the cell takes up to the 40% of total surface of the cell. The thicker part (perikaryon) of the endothelial cells is turned towards the centre of the villus. It is composed of a thick basement membrane (bm), which holds mitochondria (m) and ER structures. (Figure adapted from Clementi et al JCB 1969).

A Cross-section of gut capillary (Figure 1-5) reveals that the endothelial layer consists of two faces. The thicker face, made up of a continuous basement membrane pointing towards the centre of the villi (Clementi and Palade 1969); while the attenuated face, facing the epithelium, has a discontinuous endothelium interrupted by aperture fenestrae, caveolae and transendothelial channels (TECs) (Clementi and Palade 1969).

Caveolae and TECs are provided with stomatal diaphragms (SDs), whereas fenestrae are assembled by fenestral diaphragms (FDs). Both these diaphragms are composed by a cationic homodimeric endothelial membrane glycoprotein, namely PV-1 (R. V. Stan et al. 1999; R V Stan, Kubitza, and Palade 1999; Radu V. Stan, Tkachenko, and Niesman 2004; Tse and Stan 2010). Indeed, *in vivo* studies

conducted on PV-1 deficient mice have revealed loss of all diaphragms and perinatal mortality(Radu V. Stan, Tkachenko, and Niesman 2004).

PV-1 is a vascular endothelial cell specific protein(Spadoni et al. 2015; Radu V. Stan et al. 2012), constituted by type II integral membrane glycoprotein that dimerize *in vivo* and binds to heparin at physiological pH(Guo et al. 2016). Besides its transmembrane domain, PV-1 has a short intracellular tail and a long extracellular C-terminus domain with 4 N-glycosylation sites(Guo et al. 2016). Based on the literature, SDs and FDs are made of radial fibrils which interweave in the central knobs of the diaphragm(Tse and Stan 2010) (Figure 1-6 A). These fibrils are composed by PV-1 dimers, where PV-1 C-termini form the centre of the diaphragms. These structures are connected via the heavy glycosylation that involves PV-1 N-glycosylation sites nearby the membrane(Tse and Stan 2010) (Figure 1-6 B). Moreover, PV-1 C-termini interaction and other extracellular protein further stabilize the diaphragms architecture(Tse and Stan 2010).

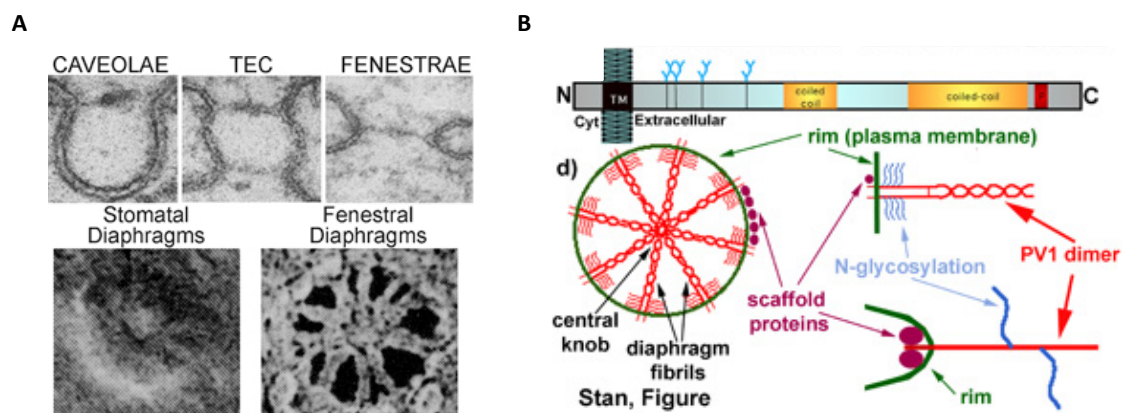


Figure 1-6 Cartwheel-like flat diaphragm structures.

(A) Perpendicular sections (upper images) of Caveolae, TEC and Fenestrae. En face view of stomatal and fenestral diaphragms (bottom images). Both images are shown by deep-etch rapid freeze techniques. (B) Schematic representation of PV-1 integration in endothelial diaphragms. (Images adapted from (R. V. Stan 2007)).

Besides its structural function on cell surface, PV-1 has also been defined as the “guardian” of pores (diaphragms-like structures) in liver sinusoidal lymphatic endothelial cells(Rantakari et al. 2015). The authors have assumed that PV-1 was

controlling the size-selective entry of lymphocytes into the lymph nodes parenchyma(Rantakari et al. 2015). Since single PV-1-guarded pore is too small to allow cell passage, it has been speculated that this protein is only indicating the easily accessible path to access the circulation(Rantakari et al. 2015). Additionally, in 2016 it has been demonstrated that PV-1 not only has a passive function as a diaphragms “guardian” but it actively regulates monocytes egress from the fetal liver(Rantakari et al. 2016). Interestingly, PV-1-heparin complexes in the diaphragms assist monocytes exit by giving an adhesive substrate or by immobilizing chemotactic molecules like VEGF (Rantakari et al. 2016). In line with these studies a blocking antibody against PV-1 (MECA-32), in a model of acute peritonitis, can reduce the leukocytes migration inside pathologic tissue and consequently decreasing disease status (Keuschnigg et al. 2009). Together with acute peritonitis, PV-1 expression is linked also to the development of other diseases characterized by leaky blood vessels such as cancer. A recent studies have revealed that PV-1 was up-regulated in highly vascularized human brain malignant tumors(Carson-Walter et al. 2005) and its expression could be enhanced by VEGF (Carson-Walter et al. 2005; Shue et al. 2008) providing evidence that PV-1 has a role in cancer angiogenesis. Moreover, there is increasing interest in the potential of PV1 function to be harnessed in devising new anti-cancer drugs(Deharvengt et al. 2012; Guo et al. 2016; Wang et al. 2014).

In addition to cancer, PV-1 up-regulation is associated to other diseases that develop along the Gut-liver axes, such as celiac disease(Spadoni et al. 2015) and ulcerative colitis (*Carlioni et al, unpublished*). Indeed, ulcerative colitis patients exhibit higher PV-1 expression in the colonic vasculature compared to healthy individuals. Precisely, this up-regulation correlates with increased bacteria dissemination to the liver. Then, by using a mouse model of ulcerative colitis, we have demonstrated that a bile acid analogue is able to rescue GVB leakage, reducing PV-1 expression, resolving disease development and bacteria liver dissemination. These data suggest that acting on GVB could be a possible therapeutic strategy to avoid extra-intestinal manifestations of ulcerative colitis (*Carlioni et al, unpublished*). Thus, controlling GVB status, reducing

blood vessels permeability, could impact on the progression of disease that develops along the Gut-Liver axis like the metastatic colon cancer.

1.3 Metastatic process

Colon cancer metastases are the major cause of colon cancer-related mortality(T. Brabletz 2012a). The liver is recognized as the preferential site of CC metastases because the majority of intestinal drainage enters the hepatic portal venous system(Zarour et al. 2017). The metastatization process can be summarized into two major phases: first dedifferentiated tumor cells disseminate from colon primary tumor to distant organs by shedding cells into the blood stream (intravasation); second, seeded colon cancer cells adapt to the foreign microenvironment in order to form a secondary tumor (colonization)(Chaffer and Weinberg 2011).

Colon tumor is usually composed by differentiated epithelial cells tightly connected to each other and to the underlying basement membrane through several junctions(T. Brabletz 2012a; Chaffer and Weinberg 2011; Chiang and Massagué 2008; Joyce and Pollard 2009). Cancer cells that circumvent this organization are undergoing epithelial to mesenchymal transition (EMT). The EMT is a very complex mechanisms during which epithelial cells acquire a mesenchymal phenotype losing their cell-cell contact in order to gain an aberrant cellular motility and trigger cancer cell dissemination and metastases(T. Brabletz 2012a; Chaffer and Weinberg 2011; Chiang and Massagué 2008; Joyce and Pollard 2009). It has been demonstrated that loss of E-cadherin, a tight junction protein which binds other E-cadherin molecules on the surface of adjacent cells, results in increased tumor growth and metastases(Derksen et al. 2006; Onder et al. 2008). Nevertheless, loss of E-cadherin is necessary but not sufficient for the EMT; indeed recent studies suggest that different transcription factors work in concert with specific microRNAs to control EMT(T. Brabletz 2012a). Among different transcription factors, ZEB1 is a major driver of metastases both in human and mouse(T. Brabletz 2012a). It has been reported that ZEB1 favour EMT by inhibiting the transcription of not only different epithelial genes but also of its repressor miR-200, which in turn controls ZEB1 transcription and EMT transition(T. Brabletz 2012a). However, it has been demonstrated that few mice with pancreatic cancer and

concomitant depletion of *Zeb1* have developed metastases meaning that other transcription factors not targeted by miR-200 could achieve tumor cells spreading (T. Brabletz 2012a; Krebs et al. 2017) such as the negative feedback loop through SNAIL1 and miR-34 (S. Brabletz, Brabletz, and Stemmler 2017). Recent evidence has suggested that EMT could induce cancer cells to become cancer stem cells (CSCs), which are defined as the “seed” that initiate and drive secondary lesion formation (Sampieri and Fodde 2012; Zeuner et al. 2014). Several evidences have revealed that in CC exists a heterogeneous population of CSCs responsible for different processes each of them expressing several cell surface markers (i.e. CD44, CD26). A fundamental aspect of cancer stem cells is their dynamic state defined as cell plasticity (Sampieri and Fodde 2012; Zeuner et al. 2014). This plasticity means that CSC have the ability to shift from a non CSC-to-CSC state and vice versa; this shift can be modulated by microenvironmental signals and it is impacting on distant metastases formation (Zeuner et al. 2014). Indeed, some CSCs in secondary adapted microenvironment can lapse back to epithelial state lacking stem cells properties. On the other hand, CSCs can also remain quiescent for years inside secondary organs forming micrometastases which can eventually turn into macrometastases only if CSCs switch from cell cycle arrest to proliferation (Chaffer and Weinberg 2011).

One of the major regulators of the shift from a non CSC-to-CSC state is the transcription factor Sex-determining region Y (SRY)-box9 (SOX9). SOX9 is a downstream target of Wnt/ β -catenin signalling pathway whose activation is relevant for colon cancer initiation and the maintenance of a CSC state. SOX-9 activation, essential for retaining metastatic CC-CSC properties at distant sites (Carrasco-Garcia et al. 2016), is triggered by different environmental factors like different bacterial pathogens such as *Shigella* and *Salmonella* (Fernandez et al. 2008; Rodriguez et al. 2012).

Once the cancer cells from the primary tumor become invasive acquiring a mesenchymal phenotype or becoming CSC-like, in order to reach secondary organs they need to invade tumor blood and/or lymphatic vessels, which provide a circuit for

their dissemination(Chaffer and Weinberg 2011; Hanahan and Weinberg 2011; Nishida et al. 2006).

Angiogenesis is essential for tumor growth and metastases because it provides tumor mass with nutrient and oxygen(Chaffer and Weinberg 2011; Hanahan and Weinberg 2011; Nishida et al. 2006). Hence tumor, in the absence of tumor angiogenesis becomes necrotic or even apoptotic(Nishida et al. 2006). The immature tumor vessels are characterized by functional and structural abnormalities. These vessels are leaky because endothelial cells are losing cell-cell contact and they have altered pericytes coverage, which are mural cells necessary to maintain vessels stability(Angiolini and Cavallaro 2017; Francavilla, Maddaluno, and Cavallaro 2009; Magrini et al. 2014). Vascular endothelial-cadherin (Ve-cadherin) promotes homotypic endothelial cell-to-cell adhesion, which is fundamental to control vascular barrier integrity(Giannotta, Trani, and Dejana 2013; Trani and Dejana 2015). Several mediators such as VEGF and thrombin can mediate and sustain aberrant vascular permeability and tumor angiogenesis(Giannotta, Trani, and Dejana 2013). Indeed, high level of VEGF is associated with poor prognosis in CC patients and it is linked to lymph node and distant metastases occurrence (Bendardaf et al. 2008). Nowadays, restoring tumor vessels integrity is considered a valuable therapy to decrease tumor invasion and metastases formation, however, we are still far from understanding the mechanisms and the mediators involved(Ehling and Mazzone 2015; Saif 2013; Trani and Dejana 2015).

Once circulating tumor cells reach the preferred target organ they have to extravasate from systemic circulation and transit back to an epithelial state through the mesenchymal to epithelial transition (MET) process in order to form distant metastases(T. Brabletz 2012a; Chaffer and Weinberg 2011; Joyce and Pollard 2009). The tropism of neoplastic cells for specific organs could be directly mediated by the expression of chemo attractants secreted by secondary organs which are able to attract chemokine receptor-expressing neoplastic cells(Joyce and Pollard 2009). It has been demonstrated that CXCR4 and CCR7 are highly expressed on breast

cancer cells whereas their ligands SDF1 and CCL21 showed high level of expression in the metastatic organs of breast cancer patients(Joyce and Pollard 2009). Furthermore, *in vivo* experiments showed that using neutralizing antibodies against both SDF1 and CXCR4 could elicit metastases formation suggesting that chemokines and their receptors can have a critical role in dictating the site of metastases formation(Joyce and Pollard 2009). Besides the role on chemotaxis, recent studies suggest that tropism to secondary organs could be directly mediated by the primary tumor through the release of exosomes and other tumor-derived factors able to trigger the formation of a pre-metastatic niche at distant sites(Hoshino et al. 2015; Peinado et al. 2012, 2017). Interestingly, it has been shown that exosomes are acting as a cargo of specific RNA and proteins (i.e. MET and MIF) essential for fostering the pre-metastatic niche formation(Hoshino et al. 2015; Peinado et al. 2012).

1.3.1 Premetastatic niche

The idea that the microenvironment of distant organs can recruit and support macrometastases formation was first hypothesized in 1889 by a pivotal discovery of Stephen Paget (Paget 1889). He conducted post-mortem analyses on 735 women with breast cancer and he reported that in this cohort of patients the metastases were not randomly distributed (Paget 1889). Paget suggested that the metastases only occur when cancer cells (“seed”) are implanted inside their suitable adapted microenvironment (“soil”) (Paget 1889). This preferential microenvironment is arranged before cancer cell seeding and it is called “premetastatic niche” (PMN) while the “metastatic niche” is started and shaped upon cancer cells arrival (Peinado et al. 2017) (Figure 1-7). The PMN formation is a conclusion of sequential and distinct phases: *Priming*, *Licensing*, *Initiation* and *Progression* phase (Figure 1-7). As previously mentioned, PMN is mainly triggered by primary tumor-derived factors which stimulate the recruitment of innate immune cells priming the future metastatic organ (Y. Liu and Cao 2016; Sleeman 2012). This *Priming* phase is crucial for the formation of an immature pre-metastatic niche which is not yet well prepared for the seeding or colonization of metastatic tumor cells. This first phase is followed by the *Licensing* phase, which is the link between the maturation of the PMN and initiation of tumor micrometastases (*Initiation* phase), followed by macrometastases onset (*Progression* phase). Here, the PMN is characterized by a pro-tumorigenic microenvironment and an inflammatory milieu whereby Cd11b⁺ myeloid cells such as macrophages, neutrophils and inflammatory monocytes, are constantly recruited inside secondary metastatic organs (Peinado et al. 2017) (Figure 1-7). These myeloid cells are specifically mobilized at distant sites through the action of specific tumor cell-derived chemokines and inflammatory mediators. An important tumor cell-derived chemokine is chemokine C-C motif ligand 2 (CCL2) (Peinado et al. 2017). CCL2 expression has been showed to be associated with tumor cell migration and metastases formation in different types of cancer (Loberg et al. 2007; Peinado et al. 2017), and high level of

CCL2 in the primary tumor correlates with colon cancer poor-prognosis(Itatani et al. 2016). It has been demonstrated that both inflammatory monocytes and macrophages can be recruited inside the PMN through CCL2-CCR2 signalling pathway both in mouse and human(Headley et al. 2016; Kitamura, Qian, and Pollard 2015; B. Z. Qian et al. 2011). More importantly, *in vivo* blockade of CCL2 with a neutralizing antibody has been shown to inhibit inflammatory monocytes recruitment, reduce metastases burden and prolong tumor-bearing mice survival (van Deventer et al. 2013; Loberg et al. 2007; B.-Z. Qian et al. 2011; B. Z. Qian et al. 2011). By contrast, neutrophils are mainly recruited inside secondary organs through granulocyte colony-stimulating factor (G-CSF)(Jablonska et al. 2017), and it has been shown that mice orthotopically inoculated with tumor cells in the fat pad had reduced metastatic foci if injected with anti-G-CSF antibody(Kowanetz et al. 2010; Peinado et al. 2017).

Once mobilized at distant sites, these recruited and suppressive immune cells are supporting PMN maturation before cancer cell seeding. Macrophages can support metastases formation through different mechanisms such as suppressing CD8⁺T cell infiltration and supporting subsequent cancer cell invasion(DeNardo et al. 2011; Headley et al. 2016; Kitamura, Qian, and Pollard 2015; Peinado et al. 2017). Moreover, macrophages together with neutrophils release high level of IL-10 known to be required for PMN maturation. Indeed, it has been shown that high expression of IL-10 results in rapid growth of liver metastases due to the down-regulation of local anti-tumor immunity and promotion of tumor cell proliferation(West, McCuaig, et al. 2015). Besides their role in modulating the PMN towards a more immune suppressive microenvironment, it has been reported that macrophages and neutrophils can actively facilitates circulating tumor cells (CTCs) entrance inside the PMN. Indeed, at this pro-metastatic stage neutrophils are secreting soluble factors such as TNF- α , IL-12 and other proteases that improve cancer cells vascular adhesion at distant sites. As a matter of fact, mice treated with an antibody against neutrophils (anti-GR1 antibody) 24 hours prior intrasplenic injection of tumor cells, displayed reduced formation of liver metastases compared to non-treated mice(Spicer et al. 2012). In

addition to that, *in vivo* intravital imaging has shown that disseminating neoplastic cells could invade secondary organs together with perivascular macrophages. First, lung perivascular macrophages are making clots enhancing the homing and the survival of tumor cells, then cancer cells secrete colony-stimulating factor-1 (CSF1) to promote macrophages production of epithelial growth factor (EGF), which in turn enhance tumor cells extravasation.

Macrophages and neutrophils can also passively regulate CTCs extravasation through the release of TNF- α which can trigger the up-regulation of different adhesion molecules, such as E-selectin and V-CAM1(Benedicto, Romayor, and Arteta 2017). E-selectin, V-CAM-1 and ICAM-1 are crucial for cancer cell adhesion and extravasation from vessels around the pre-metastatic niche(Benedicto, Romayor, and Arteta 2017; Peinado et al. 2017; Spicer et al. 2012). Indeed, it has been demonstrated that blocking the expression of ICAM-1 leads to the abrogation of organ metastases formation, indicating a fundamental role of adhesion molecules during the growth of distant metastases(Benedicto, Romayor, and Arteta 2017; Brodt 2016; Francavilla, Maddaluno, and Cavallaro 2009). Interestingly, silencing of β 2 integrin on tumor cells and ICAM-1 on endothelial cells results in decreased transmigration of CC neoplastic cells inside liver parenchyma (Benedicto, Romayor, and Arteta 2017). Furthermore, neutrophils express also high levels of metalloproteinases such as MMP9 that trigger extracellular matrix (ECM) remodelling(Peinado et al. 2017).

Indeed, ECM remodelling is another crucial step for PMN establishment (Figure 1-7) and it is mainly driven by increased deposition of fibronectin by stromal activated fibroblasts, which are called, in the liver, hepatic stellate cells (HSCs)(Brodt 2016; Eveno et al. 2015; Peinado et al. 2017). These activated cells acquire a myofibroblast-like phenotype (α -SMA⁺) and produce ECM rich in collagen increasing *in vivo* liver metastases(Brodt 2016; Eveno et al. 2015; Peinado et al. 2017). Moreover, *in vivo* co-injection of HSCs and tumor cells showed increase metastatic burden with an organized vascular network surrounded by laminin positive cells(Brodt 2016; Eveno et al. 2015; Peinado et al. 2017). Interestingly, a similar pattern is also seen in liver

metastases of colorectal cancer patients(Brodt 2016; Eveno et al. 2015; Peinado et al. 2017). In addition to fibronectin deposition of MMPs have long been recognized as major contributors of the proteolytic degradation of extracellular matrix during tumor invasion. MMP-9 deficient mice presented considerably fewer liver metastatic lesions when CC cells were injected in their spleen.

As last step of the *Licensing* phase the PMN have to increase angiogenesis and vasculature permeability to allow metastases formation(Y. Liu and Cao 2016; Peinado et al. 2017)(Figure 1-7). Before cancer cell seeding, the de-regulation of vasculature integrity at distant sites can be mediated by different cytokines like TGF- β and proteins belonging to MMP. Indeed, it has been demonstrated that high level of TGF- β primes breast tumor cells to seed the lungs by increasing the permeability of lung vasculature through the expression of angiopoietin like-4 (ANGPT4)(Padua et al. 2008).

At the end of the *Licensing* phase the PMN is mature and fertile for CTCs colonization (initiation phase) and macrometastases formation (progression phase).

Targeting the PMN-promoting factors to repress PMN formation could be a promising strategy for cancer therapeutics. As emerging evidence, colon cancer tumor-associated bacteria are one of the major factors affecting directly primary tumor progression and recent studies have suggested that cancer-related dysbiosis could indirectly influence metastases formation(Garrett 2015a; Ma et al. 2018) (see paragraph 1.4.1).

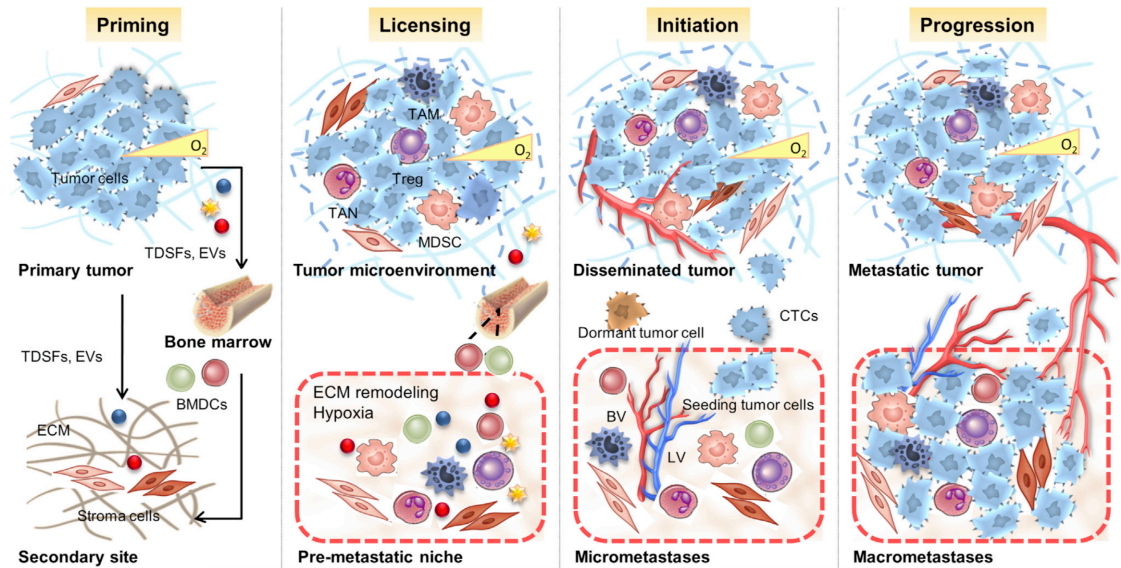


Figure 1-7 Premetastatic niche is triggering macrometastases formation.

Firstly primary tumor are sending, through the blood circulation, extracellular vesicle (EVs, like exosomes) and tumor-derived factors in secondary organs (TDSFs) in order to trigger the formation of a immature pre-metastatic niche. Then tumor associated neutrophils (TAN), tumor associated macrophages (TAM) and other myeloid derived suppressor cells are recruited inside secondary organs in response to TDSFs/EVs. Here they trigger the remodelling of the extracellular matrix and altering vessels permeability in order to allow the extravasation of circulating cancer cells (CTCs) inside secondary organ. The fertile premetastatic niche is able to support the proliferation of neoplastic cells resulting in micrometastases. In the advanced state of the metastatic process the premetastatic niche is substituted by the metastatic niche which leads to the expansion of tumor cells (macrometastases) (Figure adapted from Y. Liu and Cao 2016).

1.4 Healthy Human gut microbiome

The human intestinal microbial community consists of 500~1000 distinct bacterial species which belong to only a few of known bacterial phyla(Lora V. Hooper and MacPherson 2010). The human microbiota is defined as a metabolic active organ. It is very dynamic and changes along different individuals and during the life of a human being(Arumugam et al. 2011; Lora V. Hooper and MacPherson 2010).

Along the gastrointestinal tract, the commensal bacteria are differentially distributed. Bacterial density increases longitudinally from the stomach to the colon and transversely along the tissue-lumen axis from low colonization of the epithelium to high representation of bacteria into the lumen(Macpherson and McCoy 2013).

The host and the microbiota establish a symbiotic relationship between them. In fact, the host provides the microbiota with a favourable environment and constant supply of nutrients while intestinal harmless bacteria are involved in the digestive process and immune cell maturation(Eberl 2010). Due to this strict relationship between the gut flora and the host, new concepts of “superorganism”(Eberl 2010) and “metagenoma” have been developed(Lora V. Hooper and MacPherson 2010). The superorganism is the combination of the host cells with its symbiotic bacteria to create a mutualistic partnership between them(Eberl 2010). Instead, the metagenome refers to the genome of the microbiota and contains more than 5 million genes encoding for proteins able to expand the biochemical and metabolic capability of the host(Lora V. Hooper and MacPherson 2010). For this reason the intestine is defined as an immune privileged organ(Spadoni, Fornasa, and Rescigno 2017) due to the homeostatic equilibrium maintained between the gut microbes and the immune system.

The composition of the gut microbiota changes during life and it is affected by several factors. It has been demonstrated that human beings have acquired the pool of “pioneer” commensal microbes from the mother during delivery and lactation(Blaser 2014; Rescigno 2017). The “fit” microbiota is subsequently adjusted by diet and environmental factors leading to the formation of an ecosystem in which different

phyla occur in high or low abundance(Arumugam et al. 2011). In general, Bacteroidetes and Firmicutes are the phyla more represented inside the human gut(Arumugam et al. 2011) and among the different genera Bacteroides are more represented(Arumugam et al. 2011).

Microbes inhabiting the human healthy gut are clustered between three different Enterotypes: Enterotypes 1, 2 and 3; even if it is not known which environmental or genetic factors are selecting these clusters(Arumugam et al. 2011), each of them is pinpointed by the variation in level of specific genera and they can characterize an individual.

As shown in Figure 1-8, *Bacteroides* are mainly represented in Enterotype 1. In concomitant with the co-occurring genera, they are suitable to take energy from carbohydrates and protein through fermentation(Arumugam et al. 2011).

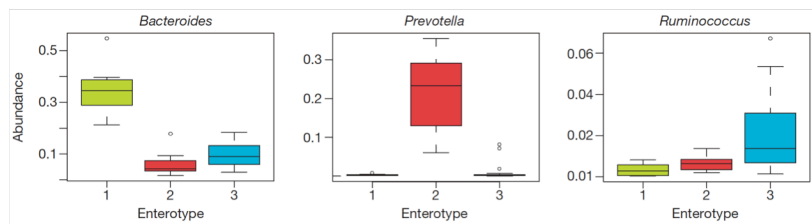
Mucins degradation and desulphation are the main functions allocated to microbes belonging to Enterotype 2, prevalently symbolized by *Prevotella* and other six genera. Finally, *Ruminococcus* and *Akkermansia* drive Enterotype 3, which is also associated to mucins degradation and sugar transportation(Arumugam et al. 2011).

Several factors including age, genetics and diet may influence microbiota composition. Of these, it has been shown that diet is particularly strongly associated with enterotype partitioning(G. D. Wu et al. 2011).

Enterotypes could be important to stratify patients that respond differentially to different diet or drugs. It has been shown that tumor-bearing mice housed in germ free (GF) conditions do not show sign of tumor regression after anti-CTLA4 treatment. Further, recolonization of GF tumor bearing mice with different species belonging to Bacteroides phylum (Enterotype 2) restore the anti-cancer effect of anti-CTLA4 blocking antibody (Vétizou et al. 2015).

Emerging evidence has shown that gut microbiota dysbiosis can lead to alteration of the host physiology fostering several disease which of colon tumorigenesis(Garrett 2015b).

A



B

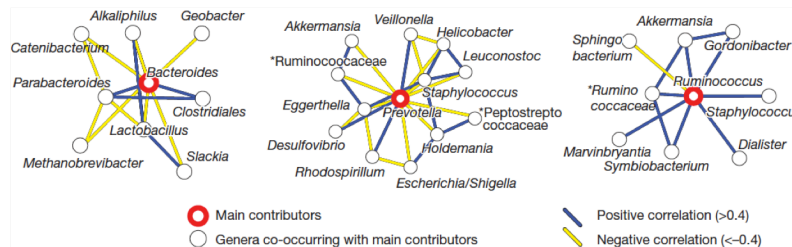


Figure 1-8 Enterotypes of Human healthy gut microbiome.

(A) Graphs are showing the main bacterial genus and (B) the co-occurrence network of bacteria characterizing each human enterotypes. Data are obtained from a Sanger metagenome (Figure adapted from (Arumugam et al. 2011)).

1.4.1 Microbiota and Cancer

Mutations in human genes are considered to be the main initiator of CC development, but other environmental factors can drive tumor progression (Garrett 2015a; Sears and Garrett 2014). Microorganism pervasively colonizes the gastrointestinal tract. The interaction between the tumor and the microbiota upon tumorigenesis has been widely investigated in the recent years. Indeed, microorganisms colonizing mucosal sites can be seen as a collection of gene networks affecting tumor growth, cancer genomic stability, metabolism and immune responsiveness (Garrett 2015b; Sears and Garrett 2014). In fact mice reared in germ free conditions or treated with antibiotics have showed reduced tumor penetrance, suggesting that intestinal bacteria trigger tumor initiation and progression (Sears and Garrett 2014). Accordingly, it has been proposed the bacteria “driver-passenger” model (S. Li et al. 2017; Tjalsma et al. 2012). Driver bacteria can induce both epithelial cells genotoxicity causing CC initiation and they can communicate with other commensal microbes through quorum sensing and secretion of hormones skewing the colonic microbiota towards a pro-tumorigenic

network of microbes(S. Li et al. 2017; Sears and Garrett 2014; Tjalsma et al. 2012; Vijay-Kumar et al. 2010). Alterations of the tumor microenvironment triggered by driver bacteria induce pro-inflammatory responses but can also have a genotoxic effect. Indeed it has been shown that *Apc*^{Min/+} mice chronically colonized with enterotoxigenic *Bacteroides fragilis* (ETBF) have increased tumorigenesis in a TH17-driven colonic inflammation (S. Li et al. 2017; Sears and Garrett 2014; Tjalsma et al. 2012; Zhu et al. 2013), and mediates the cleavage of E-cadherin resulting in cell proliferation and increased intestine permeability. *Escherichia coli*, member of the Enterobacteriaceae family, is a driver bacterium able to induce double-strand breaks through the polyketide synthase (*pks*). It has been demonstrated that AOM-treated IL-10^{-/-} mice, a murine model of colon carcinogenesis associated to genetic driven inflammation, mono-colonized with *Escherichia coli* develop invasive mucinous adenocarcinoma(Arthur et al. 2012; Garrett 2015b; Sears and Garrett 2014).

Driver bacteria are known to contribute to the initiation of premalignant lesions and the transition to carcinoma status(Tjalsma et al. 2012). At this tumor stage, the IEB is more permeable, there is a change in cellular metabolisms, which alter the tumor microenvironment causing a selective pressure on local microbiota(Tjalsma et al. 2012). Indeed, driver bacteria are outcompeted by passenger bacteria, which are more adept to live inside this new adapted tumor microenvironment(S. Li et al. 2017; Tjalsma et al. 2012). Among the bacteria described as mostly associated to late stage CC are members of the Streptococcaceae family. In particular, a recent meta-analysis has showed that 43% of patients with colon adenoma are infected with *Streptococcus gallolyticus*(Boleij et al. 2011). It has been shown that *Streptococcus gallolyticus* directly influence colon cancer neoplasia through the induction of cyclooxygenase 2 expression, which has been linked to CC worse prognosis(Blumberg and Powrie 2012). This bacterium has also been shown to be able to trigger the production of angiogenic factors in the colonic mucosa, such as IL-8, thus favoring CC progression (Abdulmir, Hafidh, and Bakar 2011; Blumberg and Powrie 2012). On the basis of recent microbiome studies *Fusobacterium spp* has

been described as a common passenger bacterium colonizing this opportunistic flora. Indeed, it has been reported to be the dominant phylotype colonizing tumor colonic mucosa of advanced CC patients (Bullman et al. 2017; Sears and Garrett 2014). *Apc*^{Min/+} mice colonized with *Fusobacterium nucleatum* were shown to develop more intestinal tumors both in the small intestine and in the colon with increased intestinal recruitment of myeloid cells, tumor associated macrophages, tumor associated neutrophils and dendritic cells (Kostic et al. 2013; Sears and Garrett 2014). Recently, it has been demonstrated that CC dysbiosis is also fostering extra intestinal modifications able to trigger the formation of distant metastases. Ma et al. have demonstrated that gut colonization with *Clostridium scindens* increases the production and subsequent liver translocation of secondary bile acids. Here, secondary bile acids lock the recruitment of anti-tumorigenic NKT cells fostering liver metastases (Ma et al. 2018). Interestingly, treating *Clostridium scindens* colonized mice with antibiotics reduces metastases formation restoring NKT cells recruitment. This study has proved that gut dysbiosis and its altered metabolites have an effect at distant sites promoting liver metastases (Ma et al. 2018).

Furthermore, clinical observations have highlighted a connection between CC and septicemia from several bacterial species (Tjalsma et al. 2012). *Streptococcus bovis* has been isolated from blood cultures of asymptomatic CC patients, meaning that it can evade the immune system and survive in the blood stream. The authors have postulated that presence of bacteria inside the blood circulation was due to a leaky colonic mucosa inside the neoplastic tissue (Kwong et al. 2018). Further, CC associated bacteria could be detected not only inside blood circulation but also in distant metastatic lesions (Bullman et al. 2017). Bullman et al. have showed that primary colon cancer and paired liver metastases were colonized by identical bacteria such as *Fusobacterium nucleatum*, *Bacteroides fragilis* and *Prevotella*. Among all of them *Fusobacterium* was the most represented in this cohort of human mCC patients. Interestingly, performing a mouse xenotransplantation of *F. nucleatum* culture positive colon cancer cells (COCA32) they have demonstrated that upon antibiotic treatment

this mice developed less tumors compared to untreated mice suggesting that *F. nucleatum* is triggering tumor growth. It is still unknown whether bacteria are colonizing distant organs before, after or concomitantly to cancer cell seeding and which is their role inside secondary organs.

2 AIM OF THE STUDY

Spreading of neoplastic cells from colon cancer to regional lymph nodes (LN) is often associated with distant recurrence. Nevertheless, metastases can occur independently from lymphatic spread and reach the systemic circulation. How such dissemination is achieved is unknown.

We have shown that beside the intestinal epithelial barrier (IEB), another physiological barrier exists in the human and murine intestine, the Gut Vascular Barrier (GVB). The GVB is fundamental to manage the homeostasis between the intestinal lumen and the organism, and works in concert with the epithelial barrier to keep the bacteria outside the systemic circulation. It has been shown that upon *Salmonella typhimurium* infection the GVB is disrupted, as revealed by the up-regulation of plasmalemma vesicle associated protein 1 (PV-1). GVB damage favors bacterial spreading to systemic organs such as the liver. Hence, we hypothesized that GVB deregulation might control the translocation of neoplastic cells through the portal circulation from colon cancer tumor to distant organs. Using both murine and human systems, we addressed the following questions:

- Assess whether PV-1 expression correlates with the development of distant metastases within five years after the resection of the primary tumor;
- Evaluate whether PV-1 is a new marker of distant recurrence independent from the involvement of metastatic regional lymph nodes;
- Assess whether PV-1 expression in human colon cancer correlates also with the dissemination of bacteria inside distant metastatic organs;
- Investigate whether liver bacteria translocation precedes distant metastasis onset and correlates with the formation of pre-metastatic niches;
- Address whether bacteria depletion can abrogate pre-metastatic niche formation;

- Evaluate whether metastases formation is mediated by the presence of a premetastatic niche.

3 MATERIALS AND METHODS

3.1 Mice

In vivo experiments were done using: C57Bl/6 male mice purchased from Charles River Laboratories, Wild-Type (WT), $Apc^{Min/+}$ (C57BL/6J-Apcmin), $Apc^{Min/+}$ C3arKO (C57BL/6J-APCmin/+C3arKO) and C3arKO (C57BL/6J-C3arKO) mice that were bred and maintained at the animal facility located at IFOM-IEO campus.

C57BL/6J- Apc^{Min}/J (referred to as $Apc^{Min/+}$) mice were obtained as a result of genetic mutation induced by treatment with N-ethyl N-nitrosurea. T to A transversion at position 2549 of the *apc* gene causes the generation of a premature stop codon. These mice develop multiple intestinal neoplasia (min) characterized by adenomas in the small intestine and colon (Moser, Pitot, and Dove 1990). $Apc^{Min/+}$ mice colony was maintained as heterozygous in our animal facility by breeding $Apc^{Min/+}$ males with WT female $Apc^{+/+}$ littermates. At weaning mice were screened for the presence of the min allele by multiplexed PCR on genomic DNA using primers listed in Table 3-1.

Primer	Sequence (5'→3')	Allele recognized
oIMR0033	GCCATCCCTTCACGTTAG	Wild type
oIMR0034	TTCCACTTTGGCATAAGGC	Common
oIMR0758	TTCTGAGAAAGACAGAAAGTTA	Mutated

Table 3-1 Primer sequences for $Apc^{Min/+}$ genotyping assay

The cycling protocol used is described in Table 3-2.

Temperature (°C)	Time	N. of cycles
94	3 minutes	1
94	30 seconds	x 35 cycles
55	1 minute	
72	1 minute	
72	2 minutes	1

Table 3-2 Thermal cycler protocol used for $Apc^{Min/+}$ genotyping assay

The assay amplifies a 600 bp band for the wild type allele and a 340 bp band for the min allele as shown in Figure 3-1.



Figure 3-1 Apc^{Min/+} genotyping assay. The gel shows bands for min apc (marked with red arrow) and WT allele (marked with red asterisk).

C3aR^{-/-} mice were generated in the laboratory of Craig Gerard (Childrens' Hospital Boston, Harvard MS). C3aR is the gene encoding the receptor for the component C3a of the complement and is located on the chromosome 6. In the knockout mice, a targeting vector replaces 736 bp of the N-terminal region of the C3aR gene with a mouse phosphoglycerate kinase promoter driven neomycin resistance gene. This construct was then electroporated in embryonic stem cells subsequently injected in C57BL/6 blastocysts. C3aR^{-/-} mice are fertile and display no developmental or morphological defects under SPF conditions. The animals showed decreased airway responsiveness as demonstrated in an ovalbumin- induced model of allergic asthma. C57BL/6J-Apc^{Min}/JC3arKO mice were generated crossing male APC^{Min/+} and female C3arKO mice littermates (Guglietta et al. 2016a). Colony was maintained crossing male APC^{Min/+} C3arKO with female APC^{+/+} C3arKO (C3arKO) littermates.

The C57BL/6J-Apc^{Min}/JC3arKO mice, in combination with knock out for the complement anaphylatoxin-3 receptor, have also a heterozygous germline mutation in the tumor suppressor adenomatosis polyposis coli (APC) gene.

This result in tumor development mainly in the colon compared to the APC^{Min/+}, resembling better the human colorectal cancer situation (*Guglietta et al, under submission*).

At weaning mice were screened for the presence of the min allele (Figure 3-1) and C3arKo band by independent multiplexed PCR on genomic DNA. In order to check the presence of knock out band in the C3ar gene, we are using primers listed in Table 3-3.

<i>Primer</i>	<i>Sequence (5'→3')</i>	<i>Allele recognized</i>
oIMR4180	AGC CAT TCT AGG GGC GTA TT	Common
oIMR4181	CAT GGT TTG GGG TTA TTT CG	WT Reverse
oIMR8162	TGG ATG TGG AAT GTG TGC GAG	Mutant Reverse

Table 3-3 Primer sequences for the C3arKO genotyping assay

The cycling protocol used is described in Table 3-4.

<i>Temperature (°C)</i>	<i>Time</i>	<i>N. of cycles</i>
94	3 minutes	1
94	30 seconds	x 35 cycles
55	30 seconds	
72	30 seconds	
72	2 minutes	1

Table 3-4 Thermal cyclor protocol used for Apc^{Min/+} C3arKO genotyping assay

The assay amplifies a 250 bp band for the wild type allele, 400 and 250 bp bands for the heterozygous allele and 400 bp band for the ko allele as shown in Figure 3-2.

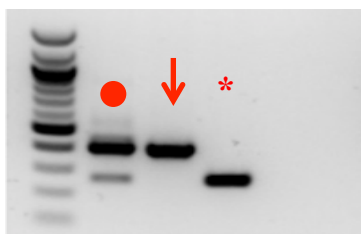


Figure 3-2 C3arKO genotyping assay. The gel shows bands for the heterozygous allele (marked with red circle), the knock out allele (marked with red arrow) and WT allele (marked with red asterisk).

Experiments were performed using both male and female. Wild type mice used for experiments are littermates from our Apc^{Min/+} mice colony or C57BL/6J mice purchased from Harlan Laboratories.

All mice were maintained in micro isolator cages in a specific pathogen-free animal facility and experiments were performed in accordance with the guidelines established

in the Principles of Laboratory Animal Care (directive 86/609/EEC) and approved by the Italian Ministry of Health.

3.2 Human samples

Formalin fixed paraffin-embedded (FFPE) samples of colon cancer and healthy colon were retrieved from IEO Biobank. Each patient was enrolled into a clinical trial IEO/0799 entitled "*Ruolo della barriera vascolare intestinale nell'ambito dei tumori del colon*". A total of 57 patients were included in the study:

- 34 CC patients did not develop distant metastases within 5 years;
- 23 CC patients have developed metastases within 5 years.

As ancillary study we have analysed 10 colon samples belonging to healthy patients.

Eligible patients have had an histologically proven colon adenocarcinoma, they have undergone surgery with curative intent between 2000-2012 (in order to have 5 years of follow up) at the European Institute for Oncology (IEO) in Milan, male and female were equally included in the study and they were aged 18-75 years at the time of diagnosis (Table 3-5). Patients were excluded if: they have had previous or concomitant malignancies, they have had any serious co-morbidity which could impair 5 years of prognosis, Inflammatory Bowel Disease (IBD) patients, they have had a rectal tumor and they have undergone radiotherapy/chemotherapy before surgery. This retrospective study has been designed as a case-control study.

A case patient was defined as an eligible patient who has developed distant metastases within 5 years from the date of radical surgery. All cases have been included in the analysis. Case patient will be defined, in other thesis paragraph, as "metachronous metastatic patients".

A control patient was defined as an eligible patient who did not develop distant metastases within 5 years. It has been randomly selected from the IEO Biobank in order to obtain a representative sample. Patients belonging to this group will be defined, in other thesis paragraph, as "not metastatic patients".

Variable	Value
Total Patient (n)	57
Mean Age (yr)	63±10
Gender (n)	
Male	34
Female	23
Primary Tumor	
Adenocarcinoma	57
TNM, Colon Cancer Staging System	
Depth of infiltration (T)	
T1 (n)	2
T2 (n)	4
T3 (n)	35
T4 (n)	16
Regional lymph-node involvement (N)	
Yes (n)	36
No (n)	21
Metastases at the time of diagnosis (M)	
M1 (n)	0
Recurrence within 5 years	
Metachronous metastatic patients	23
Not metastatic patients	34

Table 3-5 Human patients table

3.3 Flow cytometry on liver isolated cells

Flow cytometry analyses were performed on cells isolated from livers of $Apc^{Min/+}$, $Apc^{Min/+}$ C3arKO, C3arKO, AOM/DSS treated, DSS treated and WT mice at 20 and 25 weeks of age.

Mice were perfused with sterile PBS and livers were harvested in complete medium (RPMI, 5% fetal bovine serum North American, Penicillin Streptomycin 1% and Glutamine 1%). Livers were enzymatically digested for 30 minutes at 37 °C with 1 mg/mL Collagenase D. Then samples were filtered through a 70 µm cell strainer using a syringe plunger. The harvested cells were submitted to a discontinuous Percoll gradient (80/40) to separate mononuclear cells from hepatocytes and stromal cells through centrifugation for 20 minutes at 2000 rpm. Erythrocytes were lysed with red blood cell lysis buffer (6 mM NH_4Cl , 140 mM $NaHCO_3$, 16 mM EDTA, pH 7,4). Then, the immune cells were incubated with anti-FcR antibody and stained the following antibodies: CD45.2 Pacific Blue (BD clone 104), CD3 Percp 5.5 (BD clone 145-2c11),

CD11b FITC (BD clone M1/70), F4/80 PECy7 (Invitrogen clone BM8) , Ly6C APC (BD clone AL-21), Ly6G PE (BD clone 1A8), MHCII APC Cy7 (BD clone M5/114.15.2) and Fixable Viability Dye eFluor® 450 (eBioscience) to exclude dead cells. After 1 hour of antibody mix incubation and three washing steps, cells were fixed for 10 minutes at 4° with PFA 1% and maintained in FACS Buffer.

Samples were acquired at FACSCanto II (BD Biosciences) flow cytometer and analyzed with flow jo.

Inflammatory monocytes were identified as CD11b⁺ Ly6C^{high} cells in the CD45⁺ CD3⁻ gate; while neutrophils were identified as CD11b⁺ Ly6G⁺ cells in the CD45⁺ CD3⁻ gate and macrophages were identified as Cd11b⁺ F4/80⁺ cells in the CD45⁺ CD3⁻ gate. Every gate where done on live cells.

3.4 RT-qPCR assay

Liver samples from Apc^{Min/+} C3arKO, C3arKO and WT mice were collected at 25 weeks of age and stored in Trizol (Invitrogen) at -80. Tissues were homogenised using Tissue Lyser (QIAGEN). RNA was extracted adding 100 µl of chloroform, precipitating the aqueous phase with 300 µl of 70% ethanol and purifying RNA with Quick-RNA MiniPrep (ZYMO RESEARCH) according to the manufacturer's guidelines. Genomic DNA contamination was removed with DNase-Free DNase set (ZYMO RESEARCH). Total RNA extracted from liver tissues was stored at -80°. Then, 1ug RNA was retro-transcribed with ImProm-II Reverse Transcriptase kit (Promega) following manufacturer's instruction and random primers (0,5 ug/ul). qPCR assay was performed with Fast Sybr Green Master Mix (Life Technologies) on 10 ng cDNA template/reaction using exon-spanning primers (final concentration 1 µM).

The relative expression levels were calculated by the $\Delta\Delta C_T$ method after normalization to the average of endogenous control 60S ribosomal protein gene for RPL32.

For the detection of *Il10*, *Mm9*, *Tnf- α* , *Tgf- β 1* QuantiTect Primer Assays (Qiagen) QT00106169, QT00108815, QT00104006, QT00145250, respectively, were used. Primers used are listed in Table 3-6.

<i>Gene</i>	<i>Forward primer (5'→3')</i>	<i>Reverse primer (5'→3')</i>
Rpl32 (SIGMA)	TAACCGATGTTGGGCATCAG	AAGCGAACTGGCGGAAC
ICAM1 (PRIMM)	TTCACACTGAATGCCAGCTC	GTCTGCTGCTGAGACCCCTCTTG
CCL2 (PRIMM)	CAGGTCCTGTCATGCTTCT	GTCAGCACAGACCTCTCTCT
IFN- γ (PRIMM)	CTGGACCTGTGGGTGTTG	GCGTCATTGAATCACACCTG
Mmp7 (SIGMA)	AGGAAGCTGGAGATGTGAGC	CAAGGCCAAAGAACTTCTGCA

Table 3-6 qPCR primers

3.5 Bacterial CFUs from liver tissue

Livers from AOM/DSS treated, DSS treated, Apc^{Min/+}, Apc^{Min/+}C3arKO, C3arKO, WT (Apc^{Min/+} littermates) and C57BL/6 mice at either 20 or 25 weeks of age were harvested and incubated with gentamycin (50 μ g/ml) to eliminate extracellular contamination. Then the organs were digested with collagenase D (1 mg/ml, Roche) and reduced to single-cell suspension through a 70 μ m cell strainer using a syringe plunger. Erythrocytes were lysed with red blood cell lysis buffer (6 mM NH₄Cl, 140 mM NaHCO₃, 16 mM EDTA, pH 7.4). Total cells were counted and cell pellet was plated onto Columbia agar plates (5% sheep blood) together with a same volume of sodium deoxycholate 1% (DOC 1%) to lyse cells and so quantify intracellular bacteria. Columbia plates were incubated at 37 °C for 3-5 days under aerobic and anaerobic conditions.

3.6 ALT detection

Alanine transaminases were detected in sera of Apc^{Min/+}C3arKO, C3arKO and WT control mice with ALT Microwell Autom kit (Sentinel Diagnostics) following the manufacturer's instructions. Samples were diluted 1:10 in PBS. Transaminases are involved in protein metabolism and are present in different organs, but their detection in the serum is associated with liver cell damage. The kit is based on an enzymatic reaction cascade resulting in the development of a colorimetric signal detected at 550 nm.

3.7 Antibiotic treatment protocol *in vivo*

Age matched mice were administered with Ampicillin (1 g/L), Vancomycin (500 mg/L), Neomycin sulfate (1 g/L) *ad libitum* in drinking water for one week (Rakoff-Nahoum et al., 2004). Since it was known that mice generally keep off drinking the antibiotic cocktail for the bad taste of metronidazole, we gavaged mice, every other day, with metronidazole (2 mg/mouse in 200 µl).

After the treatment we confirmed the depletion of intestinal microbiota plating the feces and livers on Columbia agar plates.

3.8 Bacteria DNA extraction from fecal samples

DNA from fecal pellets was extracted with G'NOME DNA isolation kit (MP) following the literature (Furet et al. 2009). Briefly, fecal pellets, stored at -80°C, were homogenised in 550 µl Cell Suspension Solution (G NOME DNA Kit). After addition of 50 µl RNase Mix (G NOME DNA Kit) and 100 µl Cell Lysis/Denaturing Solution (G NOME DNA Kit) samples were incubated at 55°C under shaking for 30 minutes. After adding 25 µl Protease Mix (G NOME DNA Kit) samples were incubated for further 2 hours at 55°C under shaking. Samples then underwent mechanical disruption of bacterial cells with 0,1 mm glass beads (BioSpec Products) in FastPrep®-24 homogenizer (MP Biomedicals). Lysates were retrieved. Glass beads were washed three times with 400 µl of TENP buffer (50 mM Tris pH 8, 20 mM EDTA pH 8, 100 mM NaCl, 1% PVPP). Supernatants were pooled with the original lysate and precipitated with isopropanol. DNA pellet was resuspended in 400 µl water and incubated with 100 µl of Salt.Out Mixture (G NOME DNA Kit) to remove impurities. Samples were then precipitated in 100% ethanol and DNA pellet washed with 70% ethanol. DNA pellets were dried and resuspended in water.

3.9 Bacteria DNA extraction from organ samples

Murine colons and livers were harvested in 2 mL tubes and stored at -80 °C until further usage. After tissue homogenization we used DNA Microbiome Kit (QIAamp®, Cat. No. 51704, QIAGEN®, U.S.A.) to deplete the host DNA (eukaryotic DNA) from the sample. After mechanical homogenization and series of washing step, we proceeded with two steps of incubation with benzonase and proteinase K in order to degrade only the host exposed nucleic acids. Once the eukaryotic DNA was lysed, in order to ensure efficient lysis of both Gram-negative and Gram-positive bacteria we used an optimized cellular mechanical disruption method: Bead beater. Starting from this step, we followed the G NOME DNA isolation kit (MP).

Lysates were retrieved. Glass beads are washed three times with 400 µl of TENP buffer (50 mM Tris pH 8, 20 mM EDTA pH 8, 100 mM NaCl, 1% PVPP). Supernatants were pooled with the original lysate and precipitated with isopropanol. DNA pellet was resuspended in 400 µl water and incubated with 100 µl of Salt.Out Mixture (G NOME DNA Kit) to remove impurities. Samples were then precipitated in 100% ethanol and DNA pellet washed with 70% ethanol. DNA pellets were dried and resuspended in water.

3.10 16s rRNA analysis

Fecal material and organs were sampled from Apc^{Min/+}C3arKO, C3arKO and WT mice at 25 weeks of age and DNA purified as described in paragraph 1.08 and 1.09.

The subsequent steps involving sample preparation for sequencing and bioinformatics analysis were conducted in the laboratory of Prof. Marco Ventura (GenProbio s.r.l., Parco Area delle Scienze n.11/A 43124 University of Parma, Italy) and will be here summarized.

V3 hypervariable regions of bacterial 16S rRNA were amplified using primer pair Probio_Uni and /Probio_Rev (Milani et al. 2013). 16S rRNA gene amplification and amplicon checks were carried out as previously described (Milani et al. 2013).16S

rRNA gene sequencing was performed using a MiSeq (Illumina) at the DNA sequencing facility of GenProbio srl (according to the protocol previously reported (Milani et al. 2013)). Following sequencing, the obtained individual sequence reads were filtered by the Illumina software to remove low quality and polyclonal sequences. All Illumina quality-approved, trimmed and filtered data were exported as “.fastq” files and processed using a custom script based on the QIIME software suite (Caporaso et al. 2010). Paired-end reads pairs were assembled to reconstruct the complete Probio_Uni / Probio_Rev amplicons. Quality control retained sequences with a length between 140 and 400 bp and mean sequence quality score >20 while sequences with homopolymers >7 bp and mismatched primers were omitted. In order to calculate downstream alpha diversity measure, 16S rRNA Operational Taxonomic Units (OTUs) were defined at $\geq 99\%$ sequence homology using uclust (Edgar 2010) and OTUs with less than 10 sequences were filtered. All reads were classified to the lowest possible taxonomic rank using QIIME (Caporaso et al. 2010) and a reference dataset from the SILVA database (Quast et al. 2013). Biodiversity of the samples (alpha-diversity) were calculated with Chao1 and Shannon indexes.

3.11 Immunofluorescence

Murine colon tissues were fixed over night (o/n) in PLP (paraformaldehyde (PFA), L-Lysine pH 7.4 and NaIO₄) fixative buffer) and then washed, dehydrated with 20% sucrose for at least 4 hours and included in OCT. 10 μ m cryosections were rehydrated, blocked with 0.1M Tris- HCl pH 7.4, 2% fetal bovine serum (FBS), 0.3% Triton X-100 before staining them with PV-1 (clone MECA-32, 1:100 BD Bioscience) and CD34- A488 (clone RAM34, 1:100 eBioscience) antibodies. Primary antibodies were incubated o/n at 4°C. Slices were then incubated with the appropriate fluorophore-conjugated secondary antibody. Before imaging, nuclei were counterstained with 4',6-diamidin-2-fenilindolo (DAPI). Confocal microscopy was performed on a Leica TCS SP5/SP8 laser confocal scanner mounted on a Leica DMI 6000B inverted microscope equipped with motorized stage. Violet (405nm laser diode), blue (488nm argon laser)

and yellow (561nm laser diode) laser lines that have been used for excitation. All images were acquired with a HCX PL APO 40X (NA 1.25) oil immersion objective. Software used for all acquisitions was Leica LAS AF and ImageJ for images analysis.

3.12 Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized incubating the glass slides in Histolemon (Bio-Clear, Bio-optica) three times for 10 minutes. Tissue sections were re-hydrated via a graded alcohol series (100% twice, 95%, 70% and H₂O) for 5 minutes each. Afterwards epitopes were retrieved in different buffer (for PV-1 immunohistochemistry 1 mM EDTA (pH 8), for ZO-1/CD31 immunofluorescence 10mM Tris- 1mM EDTA Tween 0.05% pH 9) at 95 °C for 50 minutes. The slides were cooled down at room temperature (RT) for 20 minutes and washed with water. The activity of endogenous peroxidases was inhibited with 3% H₂O₂ for 5 minutes at RT after which slides can be handled for immunohistochemistry and immunofluorescence.

- Immunohistochemistry staining: after the deparaffinization and antigen retrieval steps, the slides were washed in Tris-buffered saline (TBS) 1X and blocked with antibody (Ab) mix (TBS 1X, bovine serum albumin 2%, fetal bovine serum North American 2%, Tween20 0.02%) for 20 minutes at RT. Then, specimens were incubated: first with the primary Ab for 2 hours and second with secondary Ab (ready-to-use DAKO Envision system HRP) for 30 minutes at RT. The primary antibodies used are: anti-PV1 (Rabbit anti-Human, 1:200 SIGMA ALDRICH) and CD31 (# M0823 1:30 DAKO). Afterwards, incubation in peroxidase substrate solution (DAB DAKO) was performed during 2 to 10 minutes at RT, which reaction was blocked using water. The slides were counterstained with haematoxylin for 5 minutes, rinsed with tap water, and de-hydrated using graded alcohol series (70%, 90%, and

100% twice) and Histolemon at RT. Lastly, the specimens were mounted using Eukitt (Bio-Optica) and covered with glass slides.

- Immunofluorescent staining: after the deparaffinization and antigen retrieval steps, the slides were washed in Tris-buffered saline (0.1M pH7.4) for 10 minutes. Then, we blocked aspecific sites with 30 minutes incubation with Blocking Buffer (2% SA serum, 0.3% Triton X-100 and Tris 0.1M). We incubated for O/N at 4° primary antibody anti-CD31 (Ab 28364 Rabbit anti Human 1:50 ABCAM). Then, after two steps of washing, the slides were incubated with secondary antibody (Donkey anti-Rabbit Alexa 488 1:300) and anti ZO-1 conjugated antibody (Mouse anti-Human Alexa 594 1:400 Invitrogen). Before imaging, nuclei were counterstained with 4',6-diamidin-2-fenilindolo (DAPI). Confocal microscopy was performed on a Leica TCS SP8 laser confocal scanner mounted on a Leica DMI 6000B inverted microscope equipped with motorized stage. Violet (405nm laser diode), blue (488nm argon laser) and yellow (561nm laser diode) laser lines that have been used for excitation. All images were acquired with a HCX PL APO 63X (NA 1.25) oil immersion objective. Software used for all acquisitions was Leica LAS AF and ImageJ for images analysis. ZO-1 staining intensity over the CD31⁺ cells was calculated using Fiji Plugin called “mask versus signal Bio Formats Batch Analysis”.

3.13 Fluorescent In Situ Hybridization (FISH)

Formalin Fixed paraffin embedded tissues were sectioned 5 μ m thickness. Deparaffinization and re-hydration is performed as described above in paragraph 3.12. Samples were washed in 0.1 M Tris (pH 7.4) for 15 minutes, after which gram-positive bacterial cell walls were hydrolyzed using lysozyme (10 mg/mL) for 30 minutes at 37 °C. The three used eubacteria probes (EUB 1, 2 and 3, see table 1-6 below) were designed to specifically target different regions of the bacterial 16S ribosomal RNA (16S rRNA). The scramble probe (Table 3-7) is the ideal FISH control; it represents a “non-sense probe”. All the probes were manufactured by SIGMA and labelled with Alexa 488. Probes were applied to slides at a concentration of 5 ng/ μ l in prewarmed hybridization buffer (0.9 M NaCl, 20 mM Tris pH 7.4, 0.01% SDS). Slides were incubated at 55°C in a humid chamber for O/N, and washed two times at 55°C in pre-warmed washing buffer (0.9 M NaCl, 20 mM Tris pH 7.4). The slides were counterstained with DAPI (1:5000) and mounted in the VECTA SHIELD. Confocal images were acquired using SP8 confocal microscope through HCX PL APO 40X(NA 1.25) oil immersion objective.

<i>Oligo Name</i>	<i>Oligo #</i>	<i>Sequence (5'-3')</i>
Eubacteria 1 (EUB1)	8810663008-000030	[A488]GCTGCCTCCCGTAGGA
Eubacteria 2 (EUB2)	8810663008-000010	[A488]GCAGCCACCCGTAGGTGT
Eubacteria 3 (EUB3)	8810663008-000020	[A488]GCTGCCACCCGTAGGTGT
Scramble probe	8021349462-000010	[Cyanine5]CGACGGAGGGCATCCTCA

Table 3-7 FISH probes.

3.14 Imaging Mass Cytometry (IMC)

3.14.1 Tissue Preparation and Staining

Paraffin-embedded tissue section of human metastatic liver, taken from IEO Biobank and belonging to a patient enrolled in the clinical trial (see above section), was dewaxed in xylene followed by alcohol series rehydration steps. Heat-induced epitope retrieval was conducted in antigen retrieval reagent pH 9 1X (Catalog number

S236784-2 Agilent) for 30 minutes at 96°C. After immediate cooling and washing in Maxpar Water, the slide was blocked in 3% BSA in Maxpar (blocking buffer) water for 45 minutes at RT.

Sample was incubated in a wet chamber over-night at 4°C with a mixture of 12 metal-conjugated antibodies (see Table 3-8 for antibodies and dilution details), labelled by the use of Max-Par labelling kit (Fluidigm, South San Francisco CA, USA) and diluted in blocking buffer. Samples were then washed in 0.2% Triton X in Maxpar PBS for 8 minutes and exposed to Cell-ID™-intercalator-Ir 1:400 (Catalog number 201192AFluidigm) for 30 minutes at RT for nuclei staining. Slides were washed in Maxpar water twice for three minutes. Air-dry the section at least for 20 minutes at room temperature before IMC analysis.

Target	Clone	Tag	Product #	Dilution
α-SMA	1A4	141Pr	3141017D	1:200
CD11b	EPR1344	149Sm	3149028D	1:100
CD3	Polyclonal, C-Terminal	170E	3170019D	1:100
CD31	EPR3094	151Eu	3151025D	1:100
CD4	EPR6855	156Gd	3156033D	1:100
CD44	IM7	153Eu	3153029D	1:100
CD45	CD45-2B11	152Sm	3152016D	1:100
CD8a	C8/1448	162Dy	3162034D	1:100
Collagen type I	Polyclonal	169Tm	3169023D	1:400
Cytokeratin 7	RCK105	164Dy	3164028D	1:100
Ki-67	B56	168Er	3168022D	1:50
SOX-9	EPR14335	147Sm	3147022D	1:50

Table 3-8 Antibodies used for Imaging Mass Cytometry

3.15 Imaging Mass Cytometry Acquisition

Images were acquired with a Fluidigm Hyperion Laser Scanning Module coupled to a Helios Mass Cytometer. Acquired data, generated by Fluidigm's CyTOF software were available in .mdc and .txt files.

Slide was inserted into a laser ablation chamber (Giesen et al. 2014), with time-of-flight detection, where the sample was scanned by a pulsed UV laser focused to different regions of interest (ROIs) of 1µm² area each. ROIs were totally vaporized

and the plumes were delivered into the inductively coupled plasma ion source for simultaneous analysis by CyTOF Helios instrument. The metal isotopes associated to each spot were quantified and categorized against the location of each spot. The tissue was scanned spot-by-spot along a scan line and at the end an intensity map of target proteins was yielded.

For image processing we used Fiji and Histo CAT(Schapiro et al. 2017). HistoCat is open source software develop to analyse highly multiplex mass spectrometry images.

3.16 AOM/DSS

C57BL/6J male mice at 8 weeks of age were treated with azoxymethane (AOM) 10 mg/Kg of body weight by intra-peritoneal injection. After 7 days AOM injected mice received Dextran Sulphate Sodium (DSS) 1% w/v in drinking water. Other control groups of mice received respectively nothing and Dextran Sulphate Sodium (DSS) 1% w/v in drinking water (groups DSS and UNTREATED). Two cohorts received DSS 1% w/v in drinking water for 7 days (AOM/DSS and DSS). Both groups were allowed for recovery for 14 days. This schedule was repeated for 3 cycles. Body weight and survival were assessed periodically. After one week of recovery from the last cycle of DSS, mice were intrasplenically injected at day 56 of treatment.

3.17 Intra-splenic injection

A colon cancer cell line (MC38) stably transfected with the Luc gene was generated. To establish a model of liver metastasis AOM/DSS, DSS and untreated C57BL/6J male mice were anesthetized with avertin. Then the spleen was exposed to allow direct intrasplenic injections of 2×10^5 Luc-labelled MC38 (MC38pLuc) cells in 50 μ L PBS 1mM EDTA on day 86 after the AOM/DSS protocol (3.16). Ten minutes after the injection of tumor cells, spleen was removed using a cauterizer. Mice were sacrificed 26 days after injection of the tumor cells, and liver metastases were analysed immediately. For *Ex vivo* imaging, mice were administered with d-luciferin (150 mg/kg;

Promega) by intra-peritoneal injections. Five minutes later, the liver were collected and acquired using counted using the *ex vivo* IVIS Lumina III system (Perkin Elmer). Collected images were analysed using Living Image 4.3.1 software (Perkin Elmer): ROIs were designed in order to appropriately select each organs and radiant efficiency calculated. Approval for these studies was obtained from the Committee on Animal Research of Minister of Health.

3.18 Statistical analysis

Results are represented as mean $\pm$ SEM. Statistical significance was evaluated with Mann-Whitney/Student's t-test (for comparison between two groups) or Kruskal-Wallis (for comparison between more then two groups without Gaussian distribution) or One-Way ANOVA (for comparison between more then two groups with Gaussian distribution) test were evaluated using GraphPad Prism software. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Outliers were calculated using GraphPad Prism software. For Human part, linear regression and contingency analyses were done using GraphPad Prism. Fisher's exact test was done to calculate statistical significant related to contingency analysis.

4 RESULTS

4.1 Role of the Gut Vascular Barrier in Human Metachronous Colon Cancer Patients

4.1.1 PV-1 is highly expressed in colon cancer vessels compared to healthy colon vessels

It has been demonstrated that PV-1 is a marker of damaged Gut Vascular Barrier (GVB) and of increased blood vessel permeability. Since GVB breakage favours bacteria spreading to systemic organs from the gut to the liver (Spadoni et al. 2015), we wondered whether this event could be implicated in diseases developing along the Gut-Liver axis as metastatic Colon Cancer (mCC).

Therefore, we investigated at first the state of the GVB in colon vessels from CC patients compared to healthy controls. We carried out a retrospective analysis on resected human colon cancer (n=57) and healthy colons (n=10), and performed an immunohistochemistry analysis to detect PV-1 expression in resected formalin fixed paraffin embedded (FFPE) colon samples taken from the European Institute of Oncology (IEO) BioBank. The pathologist has calculated the amount of PV-1⁺ cells among CD31⁺ endothelial cells (L. Liu and Shi 2012), and from our analysis, we have observed that more PV1⁺ CD31⁺ endothelial cells were present in CC patients compared to healthy control samples (Figure 4-1 B). Then, among the PV-1⁺ CD31⁺ cells we tested PV-1 intensity by using the IxP index. IxP was calculated by multiplying the highest score of PV-1 intensity by the percentage of vessels with that score. In PV-1⁺ CD31⁺ endothelial cells we have observed that the staining for PV-1 was higher in colon tumor compared to healthy control samples (Figure 4-1 B).

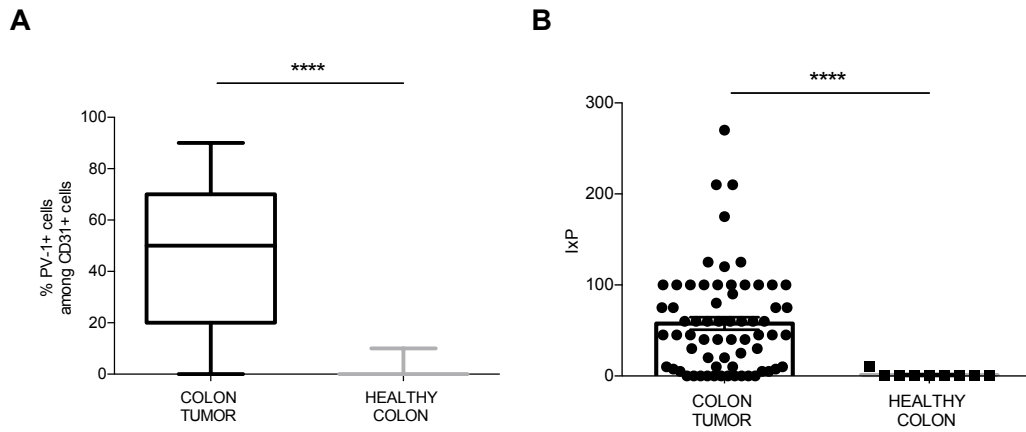


Figure 4-1 PV-1 expression in human colon tumors compared to healthy control samples. (A) Percentage of PV-1⁺ cells among CD31⁺ cells and (B) the corresponding IxP, index of PV-1 expression, in human CRC tumors and healthy controls. We have analyzed 10 healthy colons and 58 colon tumors. Each data point represents one sample. Error bars represent SEM. Statistical significance was evaluated by Mann Whitney unpaired test. *P < 0.05, **P < 0.01, **** P < 0.0001.

4.1.2 Metachronous CC patients have more PV-1⁺ endothelial cells inside the primary tumor compared to not metastatic patients.

Half of colon cancer patients develop distant recurrence within five years from the resection of the primary tumor; they are defined as metachronous metastatic patients.

Here, we wanted to verify if the higher PV-1 expression in CC patients correlated with the development of metachronous distant metastases in a follow up of 5 years.

Hence, we analysed PV-1 expression in resected FFPE colon samples from patients with metachronous distant metastases (n=23) and patients without distant metastases (n=34). As you can see from Figure 4-2, metachronous CC patients, at the time of the diagnosis of the primary tumor, exhibited more PV-1⁺ CD31⁺ positive cells compared to patients who did not develop distant metastases in the analysed 5-year period (Figure 4-2 A). Indeed, as shown by the quantification in Figure 4-2 B the percentage of PV-1⁺ CD31⁺ endothelial cells was statistically higher in primary tumor vessels of recurrent compared to the not recurrent patients.

Although PV-1 intensity is not altered between the groups (Figure 4-2 B), plotting the IxP values for both groups of patients we observed that the values of patients without metastases were forming two clusters: one with high (upper rectangle) and one with

low PV-1 intensity (lower rectangle) (Figure 4-2 C). We thus have hypothesized that other component of the GVB, like ZO-1, is balancing PV-1 expression, maintaining cell-cell contact, blocking the passage of cancer cells and impairing metastatization.

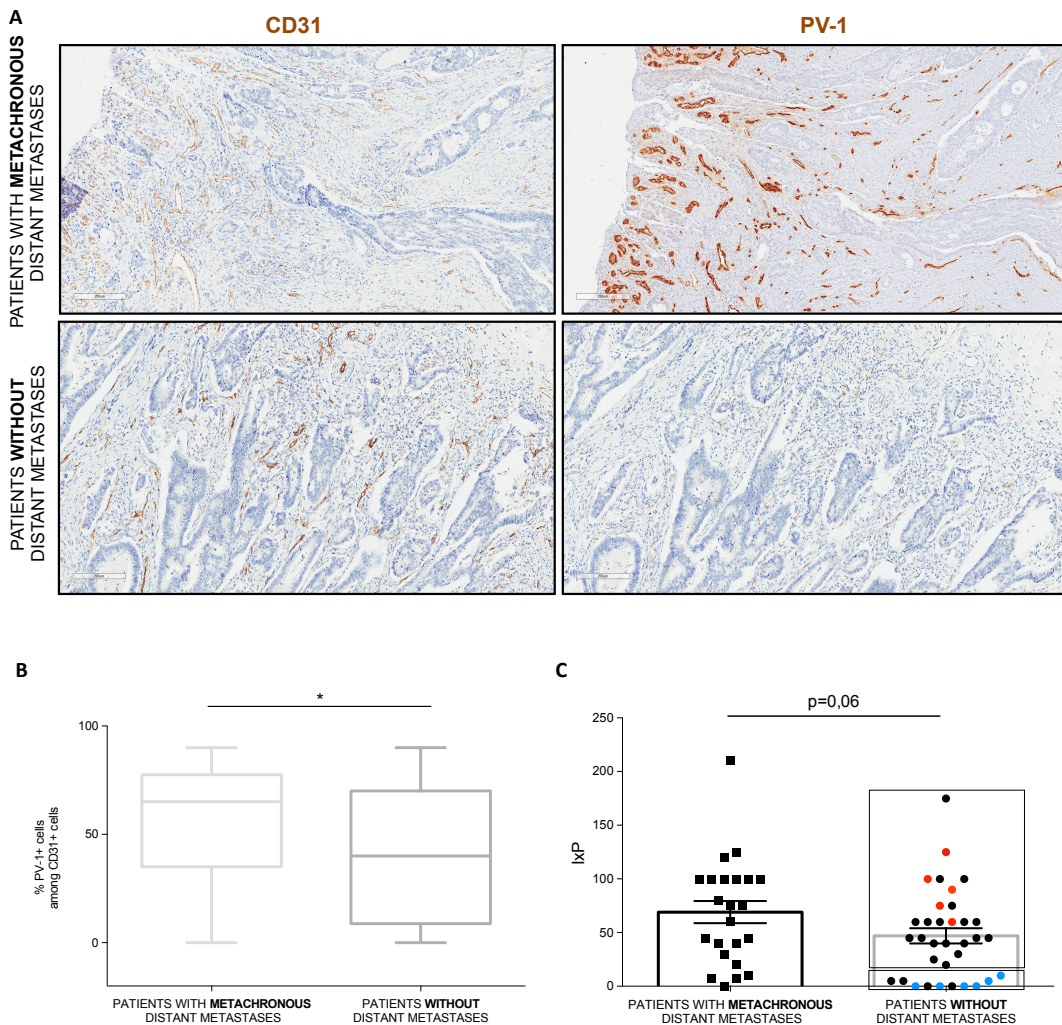


Figure 4-2 GVB impairment in metachronous colon cancer patients. (A) Immunohistochemical staining to determine the distribution of CD31⁺ cells (left panels) and PV-1⁺ vessels (right panels) in paraffin-embedded specimens from colorectal cancer patients with (upper panels) or without (lower panels) distant metastases (magnification, 10x). Scale bar: 200 μ m. (B) Quantification of PV-1⁺ cells shown as percentage among CD31⁺ cells in human CRC patients with metachronous metastases or without distant metastases and (C) the corresponding IxP. Upper and low rectangles are highlighting two clusters of patients with high and low PV-1 intensity, respectively. Red and blue circles represent patients analysed for ZO-1 expression. Each data point represents one sample. Error bars represent SEM. Statistical significance was evaluated by Mann Whitney unpaired test. *P < 0.05, **P < 0.01, **** P < 0.0001.

As detailed in the introduction another characteristic feature of the endothelial cells is the presence of elaborated junctional complexes that includes TJ and AJ

proteins(Spadoni et al. 2015). It has been shown that ZO-1 expression was decreased in primary colon cancer tumor of patients with liver metastases(Kaihara et al. 2003), and ZO-1 expression was considered a good prognostic factor able to hinder metastasis onset(Kaihara et al. 2003; Ni et al. 2013). Hence, we have analysed by immunofluorescence the expression of ZO-1 among the CD31⁺ cells in the primary colon tumor of 10 patients, all-arising from our cohort of not metastatic patients: 5 from the upper cluster (red circle) and 5 from the bottom cluster (light blue circle). Interestingly, we have found that in patients with high PV-1 intensity, ZO-1 was more expressed in CD31⁺ blood vessels compared to patients with low PV-1 intensity (Figure 4-3 A and B).

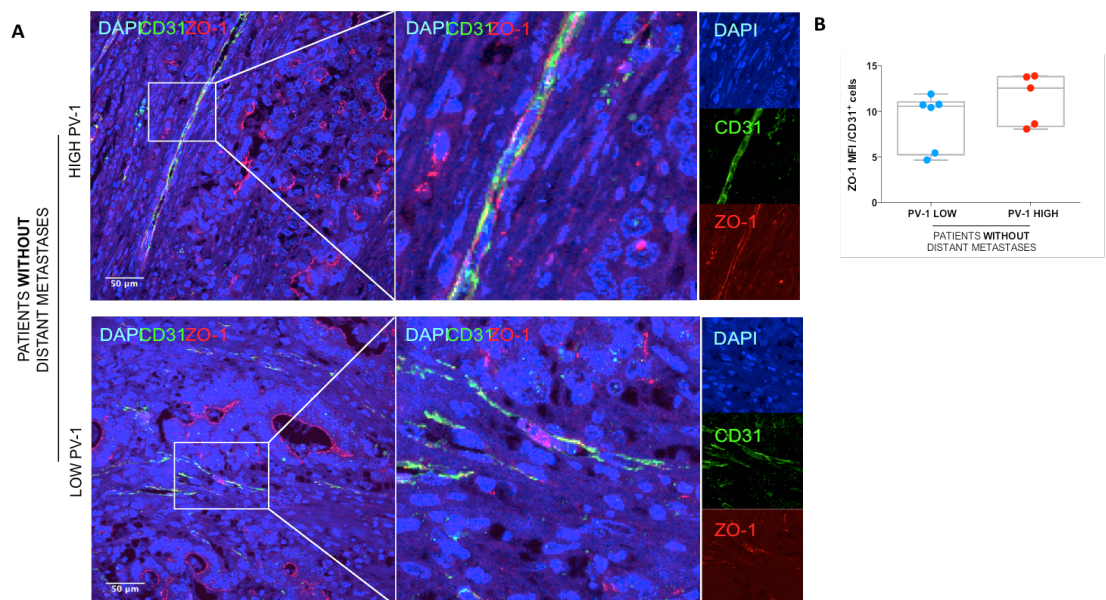


Figure 4-3 ZO-1⁺ CD31⁺ endothelial cells in colon tumor of patients who do not develop distant metastases. (A) Confocal images show ZO-1 expression (in red) on CD31 blood vessels (in green) in FFPE human colon tumors with high (upper figure) or low (bottom figure) PV-1 intensity. All images are counterstained with DAPI, in order to mark cell nuclei. Scal bar: 50 µm. (B) Mean Fluorescent Intensity (MFI) of ZO-1 positive cells among the CD31 blood endothelial cells.

This could suggest that ZO-1 and PV-1 have a compensatory mechanism in controlling metastatization. Meaning that, despite PV-1 expression and the consequent GVB leakage, endothelial cells of these patients are maintaining cell-to-cell contact impairing neoplastic cells vascular systemic dissemination. It would be

interesting to evaluate whether in metastatic patients where PV1 is higher, instead the level of ZO-1 is reduced.

Taken together these data reveal that CC tumor vessels exhibit an impaired GVB compared to healthy colon samples. Moreover, this impairment is much higher in CC patients that will develop metachronous distant metastases.

4.2 PV-1 as a new predictive marker of human CC distant recurrence

Until now, the involvement of regional lymph nodes is considered the only clinical feature that can predict the occurrence of distant metastases within five years (Ong and Schofield 2016). As a matter of fact, tumor cells can disseminate to distant organs through two ways: the vascular and the lymphatic circulation (T. Brabletz 2012b; Cheung and Ewald 2016; Enquist et al. 2014). Blood vessels can transport tumor cells directly to distant organs (i.e. the liver and the lung), whereas, the intestinal lymphatic circulation may transfer tumor cells from regional lymph nodes to distant lymph nodes (Lanciault and Jacobson 1976). Therefore, metastases to the liver and lung can occur independently of lymphatic spread but via the blood circulation. Our data demonstrated that metachronous colon cancer patients had more PV-1⁺ blood endothelial cells already at the time of the diagnosis of the primary tumor (Figure 4-2 B). Thus, we have performed a correlation analysis to highlight if there was a correlation between the amount of PV-1⁺ endothelial cells and the number of metastatic regional lymph node. Interestingly, we have found that, in our cohort of metachronous cancer patients, the presence of PV-1⁺ cells did not correlate with the number of metastatic regional lymph nodes at the time of the diagnosis (Figure 4-4). This suggests that the number of metastatic regional lymph nodes and the amount of PV-1⁺ endothelial cells are two independent factors, depicting two different routes of cancer cells dissemination.

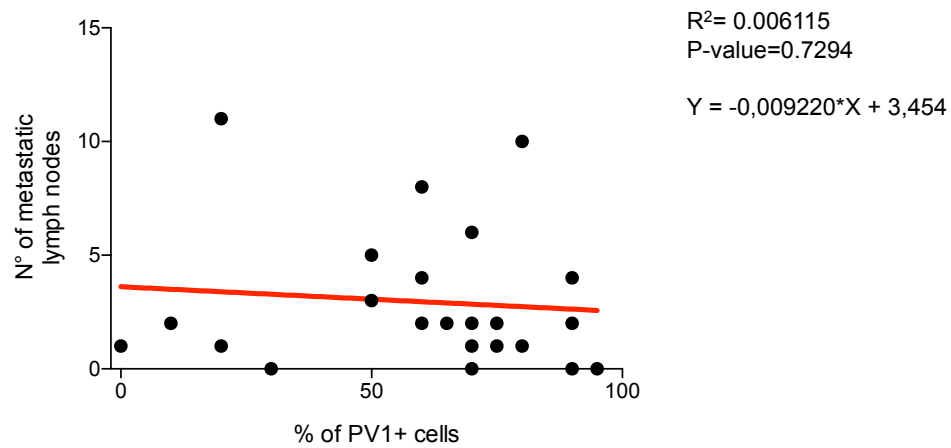


Figure 4-4 Correlation between PV-1 positive blood endothelial cells and number of metastatic regional lymph nodes. Linear regression analysis shows no correlation between number of metastatic lymph nodes and percentage of PV-1 positive blood endothelial cells. Each data point represent one patient (n=23). Red line represents the ideal match between the data. R^2 and p value are shown.

Therefore the metachronous CC patients, that at the time of diagnosis had high PV-1 expression but no lymph node involvement, has been wrongly considered as not metastatic and did not receive the correct treatment.

Moreover, performing contingency analyses, we were able to predict the risk of developing distant recurrence in relation to the % of PV-1⁺ cells. We have found that patients with more than 50% of PV-1⁺ cells inside the primary tumor are significantly associated with distant metastases recurrence ($p\text{-value} = 0,002$) and they have 2,43 times higher probability to develop distant metastases compared to patients with less than 50% of PV-1⁺ cells (Figure 4-5).

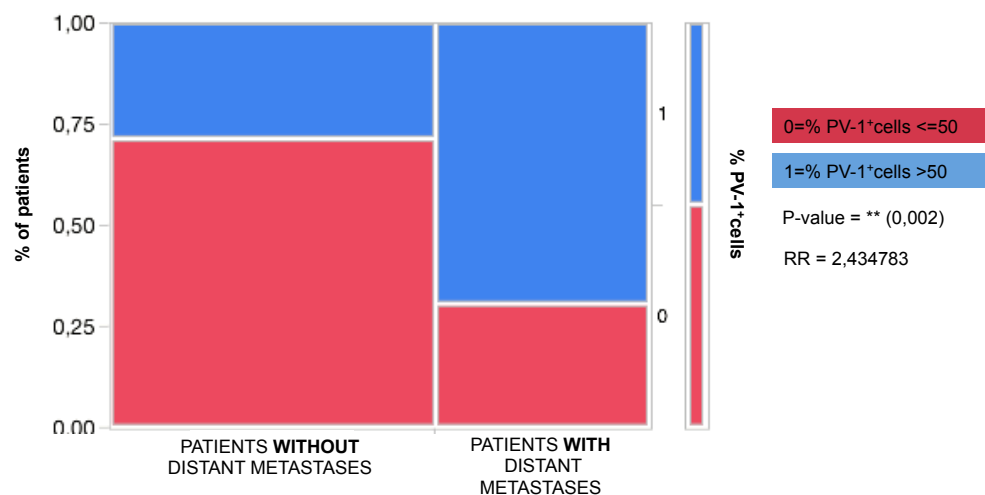


Figure 4-5 Contingency analysis of the probability to develop metachronous distant metastases in relation to the amount of PV-1 positive tumoral blood endothelial cells. Contingency analysis shows the relative risk (RR=2,434) of developing distant metastases when patients have more than 50% PV-1 positive blood endothelial cells. Statistical significance was evaluated with Fisher's exact test. ** P< 0,01.

Moreover in this cohort of patients we conducted the same contingency analyses to predict the risk of developing recurrence in relation to the presence or not of lymph-node metastases. Interestingly, we have found that the presence of lymph-node metastases is not statistically linked with the development of distant metastases (p-value=0,1862) and metachronous CC patients have 1,3 times the probability to develop distant metastases (Figure 4-6).

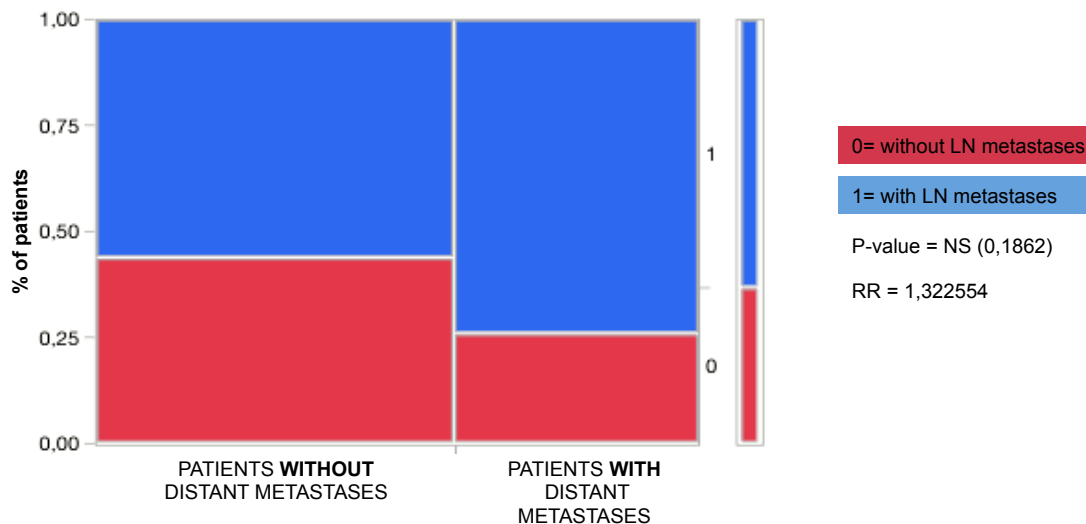


Figure 4-6 Contingency analysis of the probability to develop metachronous distant metastases in relation to the number of metastatic regional lymph-node. Contingency analysis shows the relative risk (RR=1,32254) of developing distant metastases when patients have metastatic regional lymph-node. Statistical significance was evaluated with Fisher's exact test. ** P< 0,01.

Taken together these data reveal that GVB is modified in metastatic CC patients independently of LN metastases. From a therapeutic standpoint, PV-1 is a novel marker able to predict distant recurrence, likely to be important for preventing metastases in patients who have been diagnosed for early cancer lesions.

4.3 Human GVB impairment correlates with bacterial presence inside the corresponding distant metastases.

4.3.1 Metachronous cancer patients have bacteria inside secondary metastatic organs.

We have previously shown that damaged GVB results in bacteria systemic dissemination and liver colonization (Spadoni et al. 2015). We have showed that the GVB is impaired inside the primary tumor and this is correlated with metachronous metastases in colon cancer patients (Figure 4-2). Since, it has been shown that *Fusobacterium nucleatum* colonizes primary tumors, is found in the corresponding distant metastases, and it is essential for tumor growth and proliferation (Bullman et al. 2017), thus we have hypothesized that cancer related bacteria could take advantage of GVB breakage in primary tumor to reach metastatic secondary organs.

Thus we have analysed the presence of bacteria in 6 metastatic organs (4 livers, 1 ovary and 1 lung) paired with primary colon tumor belonging to our cohort of metachronous cancer patients. To determine the spatial distribution of bacteria, we took advantage of Fluorescent In Situ Hybridization (FISH) technique to detect bacteria inside different secondary organs. We used a combination of three universal probes for eubacteria that allow us to detect all bacterial species and the equivalent scrambled probes to exclude unspecific staining.

Interestingly, bacteria were observed in all metastatic liver samples. They were all localized near cells whose histomorphology was consistent with colon cancer cells (Figure 4-7), while occasionally stromal associated bacteria could be seen as well. In all the samples we did not observe any biofilm aggregates. Unfortunately, the same analysis could not be performed on livers coming from healthy individuals due to obvious ethical issues.

We have observed bacteria also in other analysed metastatic organs such as the ovary and the lung (data not shown). In accordance with the literature (Dickson, Erb-Downward, and Huffnagle 2013; O'Dwyer, Dickson, and Moore 2016), we have detected bacteria also in the healthy part of the lung. Indeed, it was documented the

existence of a lung microbiome which was altered under pathological conditions such as changes in oxygen tension, ventilation and inflammation (Dickson, Erb-Downward, and Huffnagle 2013; O'Dwyer, Dickson, and Moore 2016).

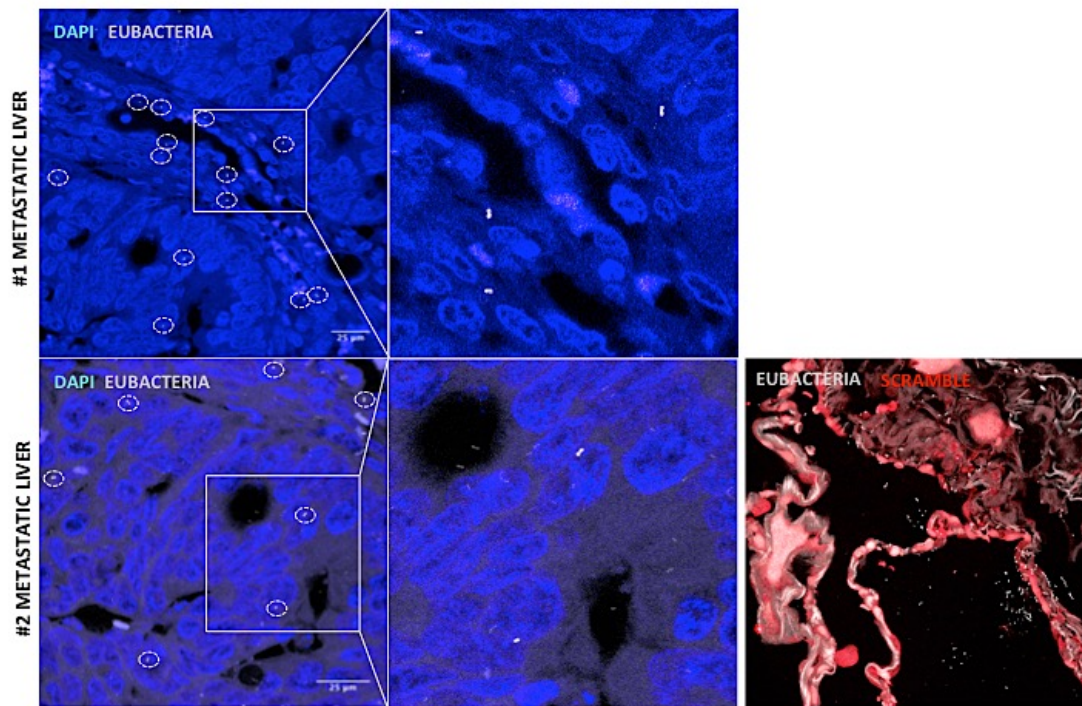


Figure 4-7 Bacteria in liver metastatic organs of metachronous colon cancer patients. Confocal images show fluorescence in situ hybridization analysis of 2 representative human metastatic liver. Eubacteria (green) were stained using three general bacterial probes (See Materials and Methods). All the sections were stained with DAPI (blue), to stain the nuclei. Bottom right figure shows co-stain with Eubacteria probe (in green) and scramble probe (in gray). Indeed, eu-probe specificity was verified by the use of scramble probe, which has been designed with the same nucleotides of the eu-probe but scrambled. Scale bar: 25 μ m.

Our data demonstrates that metachronous metastatic CC patients have permeable vessels inside the tumor due to GVB damage (Figure 4-2), therefore we can suggest that altered blood vessels permeability allows both cancer cells and cancer-associated bacteria to disseminate systemically and colonize distant organs (Figure 4-7). We cannot exclude that bacteria are migrating inside cancer cells.

4.3.2 Bacteria are located around SOX9⁺Ki67⁺ highly expressing cells.

Next, we wanted to better characterize the tumor metastatic microenvironment at distant sites. Precisely, we wanted to specifically define the localization of the bacteria within the human metastatic lesions. To achieve this purpose, we took advantage of an innovative technique called Imaging Mass Cytometry (IMC, Hyperion), which has allowed us to stain simultaneously the sample with 12 metal-target antibodies directed against 12 different antigens (See materials and methods 3.14).

We used antibodies targeting different proteins known to be expressed by different type of cells involved in the metastatic process. Highly proliferative cells (Ki67⁺ cells) and SOX9⁺ cancer stem cells were surrounded by remodelled extracellular matrix marked with α -Sma and collagen type I positive cells. Additionally, liver lesions were enclosed by a vascular network of CD31⁺ blood vessels which are likely supplying oxygen and nutrient to tumor tissue(Trani and Dejana 2015). Specific regions of this altered microenvironment were infiltrated by cells belonging to the adaptive (CD45⁺CD3⁺CD4⁺/CD8⁺Tcells) and innate (CD45⁺CD3⁻CD11b⁺cells) immune system. Notably other regions whose histomorphology was consistent with colon cancer cells were deprived of immune cells infiltrate (Figure 4-8).

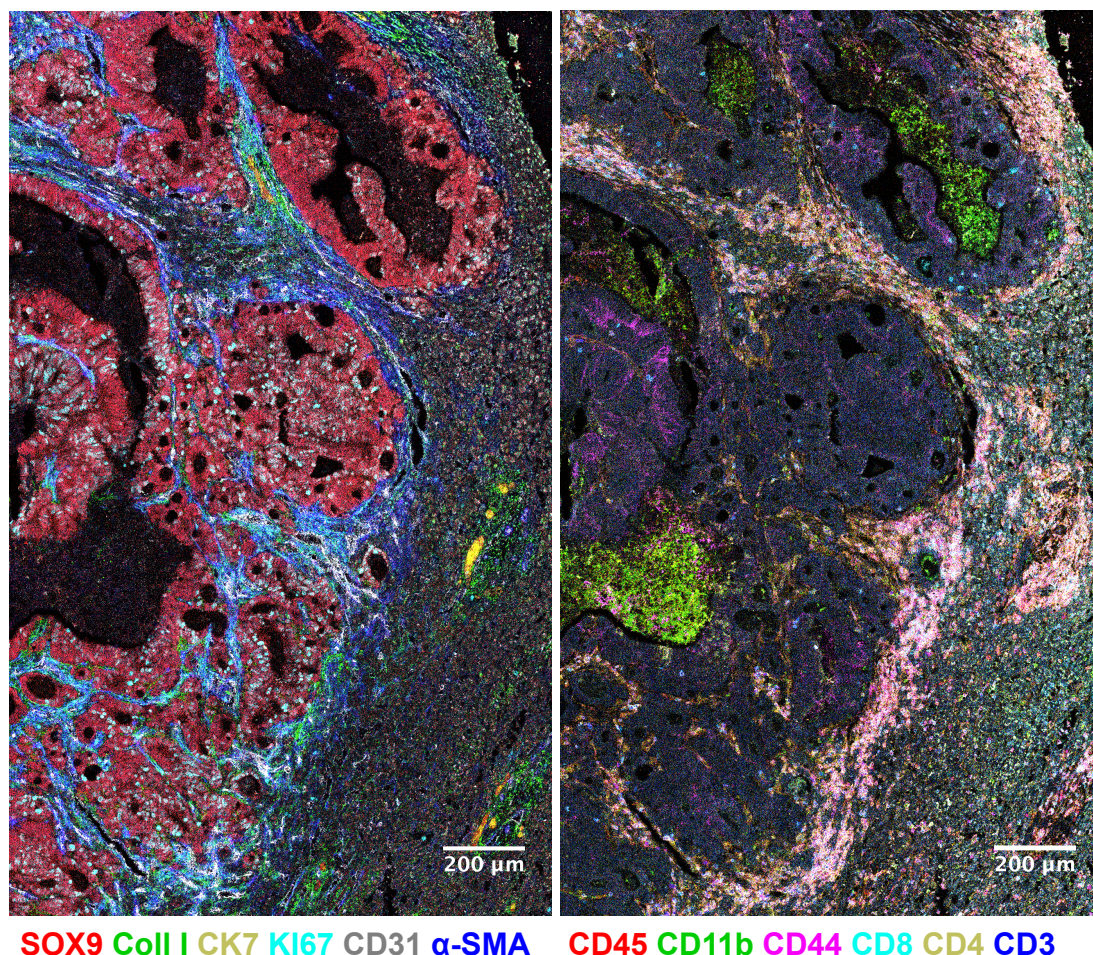


Figure 4-8 Liver metastatic lesion microenvironment. Liver section was stained simultaneously with 12 different metal-target antibodies targetting 12 differetn proteins well known to be involved in the metaastatic process. Mass cytometry left image shows the distribution of SOX9, COLL III, ,CK7, Ki67, CD31, and α-SMA positive cells. Righ image is showing the expression of CD45, CD11b, CD44, CD8, CD4 and CD3 positive cells. Scale bar 200 μm.

In order to assess the exact bacteria localization in this already described metastatic microenvironment (Figure 4-8), we analysed serial sections of the same specimen by FISH (Figure 4-9 B). In this way we were able to identify the region (Region 1) of interest (ROI) containing bacteria as shown in Figure 4-7. Then we have defined 3 additional ROIs without bacteria (Region 2, 3, 4 Figure 4-9 A) and used them as a control.

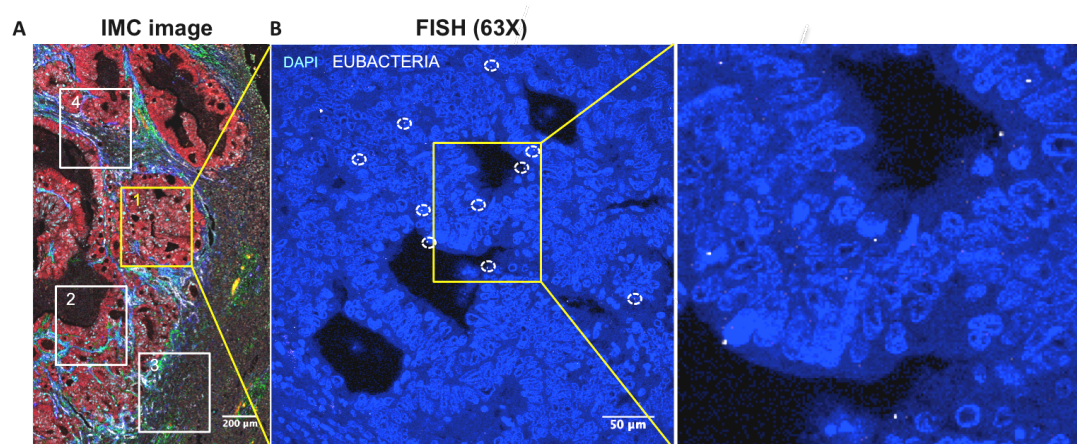


Figure 4-9 Region of interest defined by the presence of bacteria (yellow line) inside metastatic liver lesion. (A) The IMC image showing liver metastases surrounded by the metastatic niche previously characterized. Region 2, 3 and 4 are the one without bacteria whereas region 1 is containing bacteria as it is witnessed by the FISH (B). Bacteria are encircled by white dashed circle. Scale bar: 200 and 50 μ m.

We have quantified the expression of all the 11 markers previously used in all the four regions and we observed that Region 1, the one containing bacteria, exhibited higher expression of SOX9 and Ki67 positive cells compared to the other three regions (Figure 4-10). Interestingly Region 4 shows a mild expression of SOX9 despite deprived of bacteria, suggesting that other bacteria could be found in this area if serial sections of the same specimen would be analysed by FISH.

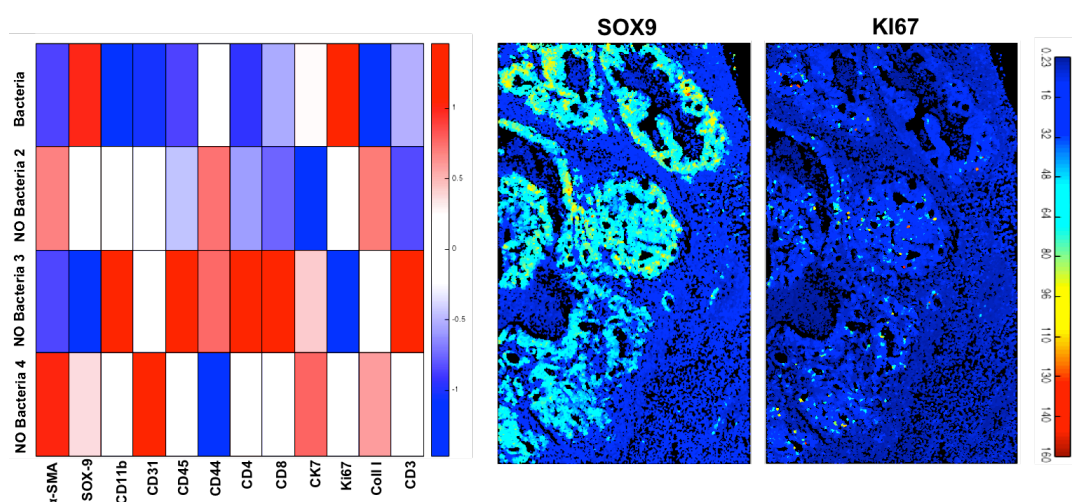


Figure 4-10 Expression profile of different protein present in human metastatic liver associated to region with bacteria or region without bacteria (2,3,4).

From these data we confirmed previous studies demonstrating that bacteria are colonizing distant metastatic organs(Bullman et al. 2017). Interestingly, translocating bacteria are localized in proximity to highly expressing Ki67 and SOX9 colon cancer cells. Additionally we showed that immune cells infiltrated only few regions of this adapted microenvironment.

4.4 Bacteria reach the liver before metastases formation and concomitant with the onset of a premetastatic niche

4.4.1 Bacteria are colonizing the liver before cancer cell seeding.

It has been reported that cancer related bacteria drive host cell transformation by causing genomic instability, resistance to cell death, and proliferative signalling (Garrett 2015b). Hence, we have decided to use an animal model to demonstrate that bacteria from the gut can actively trigger the metastatization process. Specifically, we have used the $Apc^{Min/+}$ C3arKO mice, a mouse model of spontaneous tumorigenesis in the colon developed in our laboratory (Guglietta et al. 2016a). $Apc^{Min/+}$ C3arKO mice combine the $Apc^{Min/+}$ mutation with deficiency in the C3a complement anaphylatoxin receptor (C3ar). These mice develop fewer tumors in the small intestine compared to $Apc^{Min/+}$ single mutant mice (Guglietta et al. 2016b) but increased tumor burden in the colon resembling human CC (Guglietta et al, unpublished data). As controls we have WT and C3arKO mice that do not develop tumors and have no other pathological conditions.

First, we have tested PV-1 expression among CD34⁺ endothelial cells in colon samples coming from $Apc^{Min/+}$ C3arKO, C3arKO and WT mice at 25 weeks of age. From the $Apc^{Min/+}$ C3arKO we have collected both colon tumors and healthy-associated colon tissue. Our results showed that 25 aged $Apc^{Min/+}$ C3arKO mice had higher expression of PV-1 as compared to healthy colon of WT or C3arKO, meaning that these mice had an altered GVB (Figure 4-11 A and Figure 4-11 B). Notably, we did not find differences in terms of PV-1 expressing cells comparing colon tumor and healthy tissue coming from the same $Apc^{Min/+}$ C3arKO mouse (Figure 4-11 B).

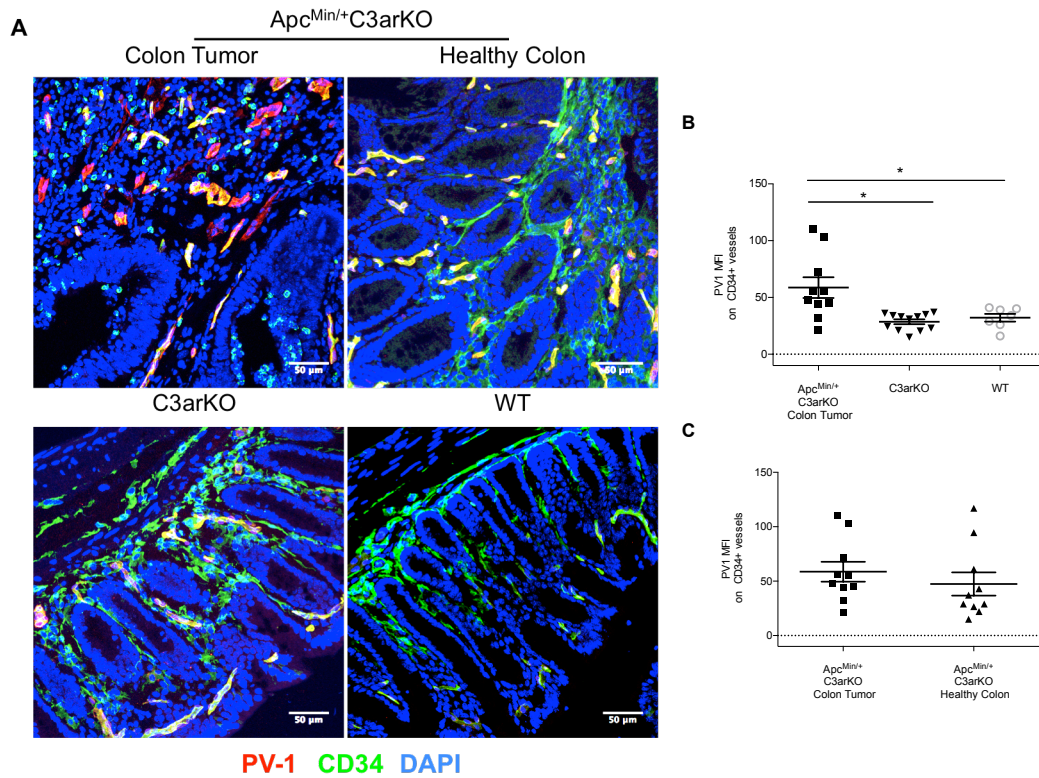


Figure 4-11 Expression of PV-1 as a marker of GVB injury in $Apc^{Min/+}C3arKO$ at 25 weeks of age. (A) PV1 (red) expression was evaluated in colon blood vessels stained with CD34 (green). Cell nuclei were stained with DAPI (blue). Scale bars, 50 μm . (B) Mean fluorescence intensity of PV1 was measured to quantify the up-regulation of the protein in the intestinal vessels of $Apc^{Min/+}C3arKO$, C3arKO and WT mice at 25 weeks of age. Statistical comparisons were based on one-way analysis of variance (ANOVA). * $P < 0.05$, ** $P < 0.01$. (B, bottom panel) (C) Mean fluorescence intensity of PV1 in $Apc^{Min/+}C3arKO$ colon tumors was compared with its healthy counterpart and statistical comparison was based on Wilcoxon paired test * $P < 0.01$. Each data point represents an individual mouse (n =7 to 12). Error bars represent SEM.

Together with GVB damage, we observed that in the $Apc^{Min/+}C3arKO$ mice more intracellular aerobic and anaerobic bacteria could be detected inside the liver parenchyma compared to C3arKO and WT mice (Figure 4-12).

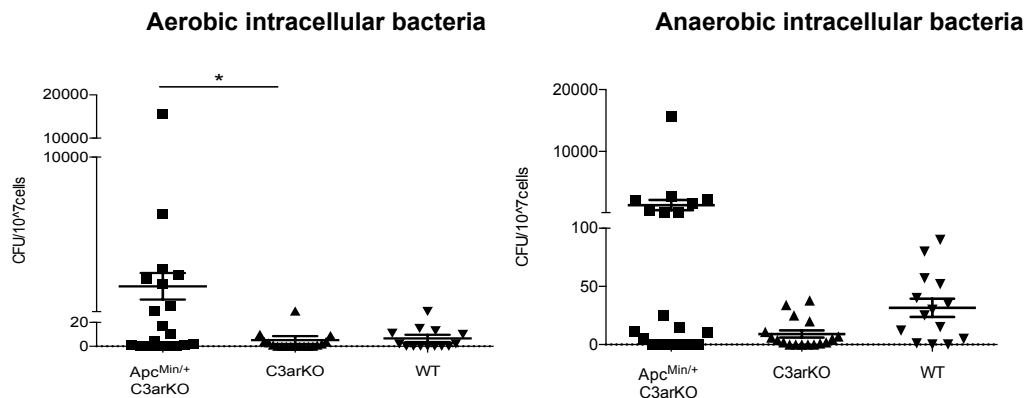


Figure 4-12 Increased aerobic and anaerobic liver bacteria in $Apc^{Min/+}$ C3arKO mice at 25 weeks of age. Intracellular aerobic and anaerobic bacteria inside the liver parenchyma of $Apc^{Min/+}$ C3arKO, C3arKO and WT mice at 25 weeks of age. Statistical comparisons were based on Kruskal-Wallis unpaired test.

In order to include the $Apc^{Min/+}$ mice in the analysis, due to their shorter lifespan, we checked the GVB status and liver bacteria dissemination at earlier time points (20 weeks of age) in $Apc^{Min/+}$, $Apc^{Min/+}$ C3arKO, C3arKO and WT mice.

As it is shown in Figure 4-13, neither colon PV-1 expression nor the number of liver bacteria were different between the groups. Even though we could detect a mild increase of aerobic bacteria in the liver of $Apc^{Min/+}$ C3arKO mice compared to the other control groups. This difference is 80 times less compared to the liver intracellular aerobic bacteria present at later time points (Figure 4-12), suggesting that these might be resident bacteria.

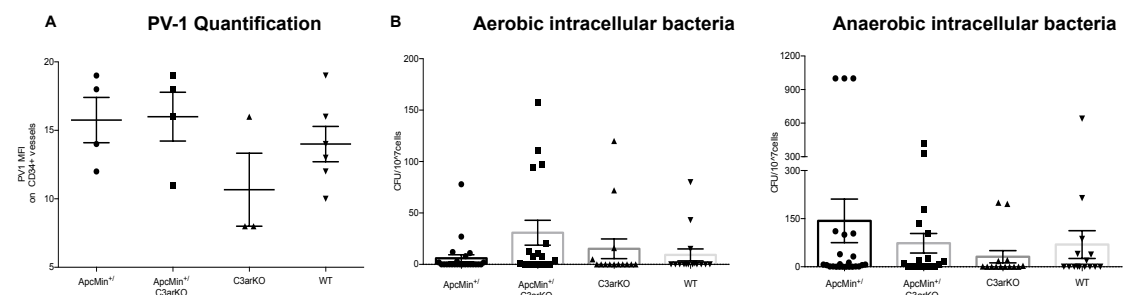


Figure 4-13 PV-1 expression and liver bacteria colonization in $Apc^{Min/+}$ C3arKO, $Apc^{Min/+}$ C3arKO and WT mice at 20 weeks of age. (A) Mean fluorescence intensity of PV1⁺CD34⁺ colon vessels in $Apc^{Min/+}$ C3arKO, $Apc^{Min/+}$ C3arKO and WT mice at 20 weeks of age. Each data point represents one mouse (n =3 to 6). Error bars represent SEM. (B) Intracellular aerobic and anaerobic intracellular bacteria presence inside the liver parenchyma of different group of mice. Each data point represents one mouse (n =14 to 24). Error bars represent SEM.

These data together suggest that the effect of colon GVB impairment in $Apc^{Min/+}$ C3arKO tumor bearing mice can mediate the dissemination of bacteria, recapitulating the human counterpart. Since these mice did not show signs of distant liver metastases, we believe that these changes precede metastases onset. In our condition, bacteria are not changing ALT levels in the serum (

Figure 4-14).

These results are in line with human data, showing that only 30% of colon cancer liver metastatic patients have increased ALT activity(X. Z. Wu, Ma, and Wang 2010).

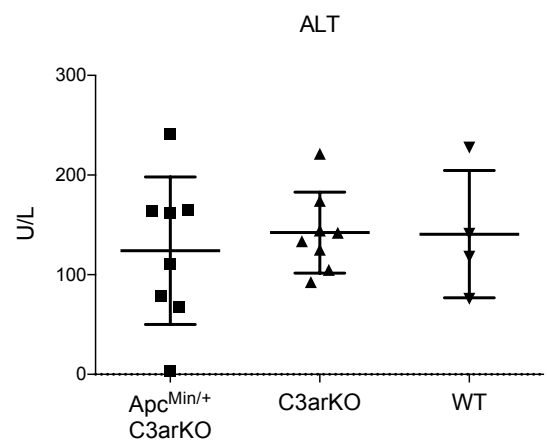


Figure 4-14 ALT serum level in Apc^{Min/+} C3arKO, C3arKO and WT mice at 25 weeks of age.

4.4.2 Characterization of a liver premetastatic niche

It has been demonstrated that tumors might metastasize to preferred organ sites (Hoshino et al. 2015; Kenific, Nogués, and Lyden 2016; Peinado et al. 2017), and that the immune system plays an important role in regulating tumor growth and metastasis (Hanna et al. 2015). In particular, it has been shown in different mouse models that innate immune cells accumulate in the lung/liver prior to the arrival of metastatic cells (Hanna et al. 2015).

Since we could detect bacteria within the liver of $Apc^{Min/+}$ C3arKO mice (Figure 4-12), we wondered whether their presence was correlated with other cellular/molecular changes required to facilitate tumor cell growth. First, we have analyzed by flow cytometry the innate immune cells infiltrating inside the liver of $Apc^{Min/+}$ C3arKO, C3arKO and WT at 25 weeks of age. We found that both monocytic (inflammatory monocytes, $Cd11b^+Ly6C^+$) and granulocytic (neutrophils, $Cd11b^+Ly6G^+$ and macrophages like $Cd11b^+F4/80^+$) myeloid cells were recruited preferentially in liver parenchyma of $Apc^{Min/+}$ C3arKO mice (Figure 4-15).

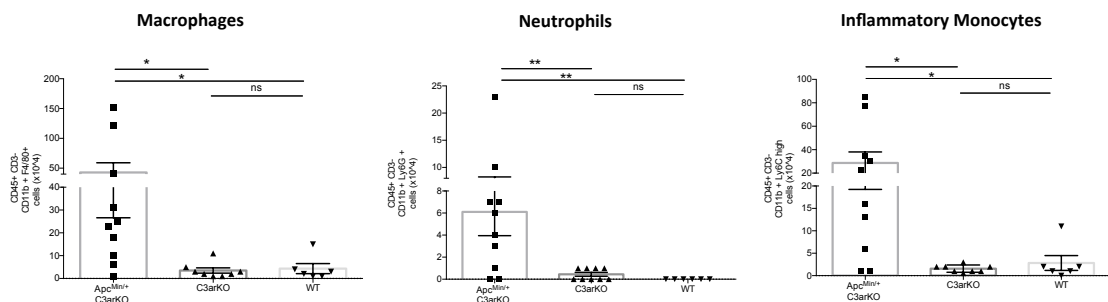


Figure 4-15 Innate immune cells recruitment in the liver of $Apc^{Min/+}$ C3arKO at 25 weeks of age. FACS analysis for $Cd11b^+ F4/80^+$ (macrophages like), $Cd11b^+ Ly6G^+$ (neutrophils) and $Cd11b^+ Ly6C^{high}$ (Inflammatory Monocytes) cells in the liver of $Apc^{Min/+}$ C3arKO, C3arKO and WT mice at 25 weeks of age. Each data point represents an individual mouse (n=6 to 10). Error bars represent SEM. Statistical significance has been evaluated by Kruskal Wallis unpaired test. *P < 0.05, **P < 0.01.

Furthermore, we tested the presence of innate immune cells in the liver of $Apc^{Min/+}$, $Apc^{Min/+}$ C3arKO, C3arKO and WT mice at 20 weeks of age. Interestingly, we could not detect any difference between the groups (data not shown). Since we did not find any difference in liver bacteria translocation, immune cell recruitment, and colon PV-1 expression at this time point between groups, we have decided to proceed only with mice at 25 weeks of age.

It has been demonstrated that tumors not only modulate the recruitment of immune cells into secondary organs but also drive the expansion of distant immunosuppressive microenvironment (Y. Liu and Cao 2015). Using quantitative PCR, we observed that concomitant with the recruitment of innate immune cells; the livers of $Apc^{Min/+}$ C3arKO expressed more TGF- β , TNF- α , IL-10 and CCL2 compared to C3arKO and WT mice (Figure 4-16). No changes among groups were detected at the gene expression level for IFN- γ (Figure 4-16). In addition, it has been widely reported that different factors such as matrix metalloproteinases (MMP) and adhesion molecules (i.e. ICAM-1) could accelerate tumor cell invasion into the parenchyma as well as promote tumor cell proliferation and angiogenesis, thereby enhancing liver metastases (Deryugina and Quigley 2006) (Duarte et al. 2015) (Zeng et al. 2002). All the livers isolated from $Apc^{Min/+}$ C3arKO mice displayed increased gene transcription levels of MMP-7, MMP-9 and ICAM-1 compared to C3arKO and WT mice (Figure 4-16). Additionally, these results showed that C3arKO mice had moderate decrease of TNF- α , IL-10, CCL2, IFN- γ and MMP-7 compared to WT mice, suggesting that C3arKO mice had less inflamed hepatic microenvironment compared to WT mice.

In conclusion, the hepatic microenvironment of the $Apc^{Min/+}$ C3arKO double mutant mice were altered compared to age matched C3arKO and WT mice. The livers of these double mutant mice showed recruitment of innate immune cells similarly to the ones already seen in the human liver. We observed also increased expression of metalloproteinases typically involved in tissue ECM modification, a step that precedes cancer cell seeding (Deryugina and Quigley 2006) (See paragraph 1.3.1).

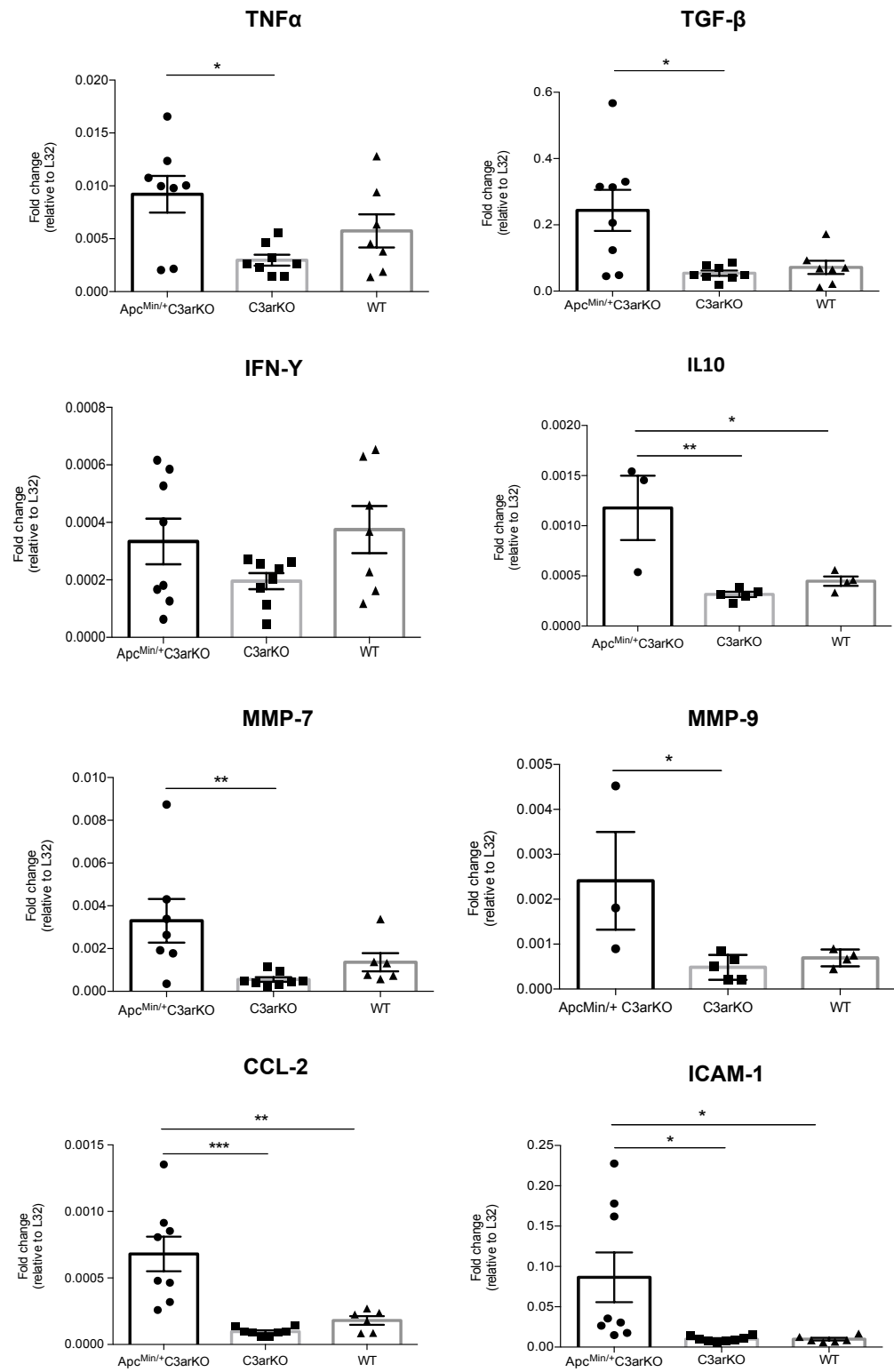


Figure 4-16 q-PCR analysis of frozen liver harvested from Apc^{Min/+}C3arKO, C3arKO and WT mice at 25 weeks of age. The expression of TGF- β , TNF- α , IL-10, IFN γ , CCL2, MMP9, MMP7 and ICAM1 were assessed by qPCR. Each data point represents an individual mouse (n=3 to 8). mRNA expression was normalized to L32 gene expression. Error bars represent SEM. Statistical significance was evaluated using one-way ANOVA. *P < 0.05, **P < 0.01, **** P < 0.0001.

4.4.3 Identification of bacteria related to GVB impairment and liver colonization

In the recent years it has been shown that cancer-related bacteria could have a role in altering tumor growth, guiding immune system function, impairing epithelial barrier integrity and influencing metabolism of host-produced factors and pharmaceuticals(Blumberg and Powrie 2012).

Since it has been reported that fecal microbiota might reflect a “part of the whole” alterations associated with colon cancer dysbiosis and the subsequent liver metastases, we have decided to take into account also bacteria associated to colonic mucosa and the corresponding liver microbiome.

$Apc^{Min/+}$ C3arKO liver microbiota

To assess liver microbiome composition, we harvested livers from $Apc^{Min/+}$ C3arKO, C3arKO littermates and WT at 25 weeks of age and sequenced the V3 hypervariable regions of bacterial 16S rDNA. We calculated Shannon and Chao1 indexes. Shannon index is an alpha-diversity index that considers both richness and evenness; it is used to measure the diversity within the community. Chao1 index instead is an estimator of diversity and it is used to measure diversity between different communities. Our data showed that neither richness nor diversity were different comparing $Apc^{Min/+}$ C3arKO, C3arKO and WT liver microbiome (Figure 4-17). This underlined that the $Apc^{Min/+}$ C3arKO liver microbiome was comparable to control groups in terms of richness and diversity at 25 weeks of age. Even if Shannon index is showing a mild decrease of diversity in WT mice compared to the other groups.

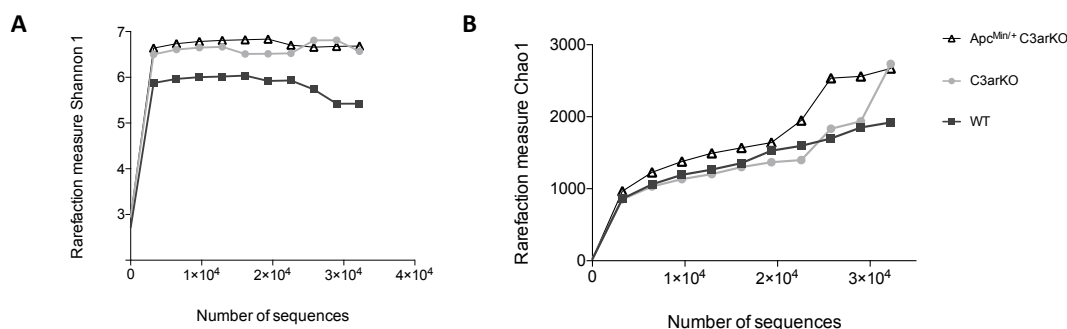


Figure 4-17 Liver Shannon and Chao indexes of $Apc^{Min/+}$ C3arKO, C3aKO and WT mice at 25 weeks of age. Metagenomic sequencing was performed on fecal samples from $Apc^{Min/+}$ C3arKO, C3arKO and WT mice at 25 weeks of age. Shannon (A) and Chao (B) diversity indexes relative are shown. $n=8$. Graphs show relative measures.

Analysis of liver bacterial composition at phylum level revealed that the $Apc^{Min/+}$ C3arKO liver microbiome was not giving any difference compared to the C3arKO and WT mice (Figure 4-18).

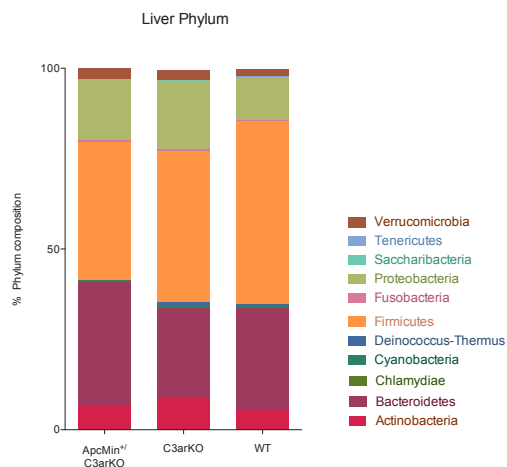


Figure 4-18 Phylum liver abundance in $Apc^{Min/+}$ C3arKO, C3arKO and WT mice. Metagenomic sequencing was performed on liver samples from $Apc^{Min/+}$ C3arKO, C3arKO and WT mice at 25 weeks of age. Phylum abundance are shown as bar plots, $n=8$.

$Apc^{Min/+}$ C3arKO feces and colon microbiota

To assess fecal microbial composition, we have collected feces from $Apc^{Min/+}$ C3arKO, C3arKO littermates and WT at 25 weeks of age and sequenced the V3 hypervariable regions of bacterial 16S rDNA.

Our data showed that both Chao and Shannon index were not highlighting any differences statistically significant comparing $Apc^{Min/+}$ C3arKO, C3arKO and WT fecal microbiome (Figure 4-19 A). Even if Chao index showed a mild increase of richness in $Apc^{Min/+}$ C3arKO, C3arKO fecal microbiome compared to WT mice (Figure 4-19 B).

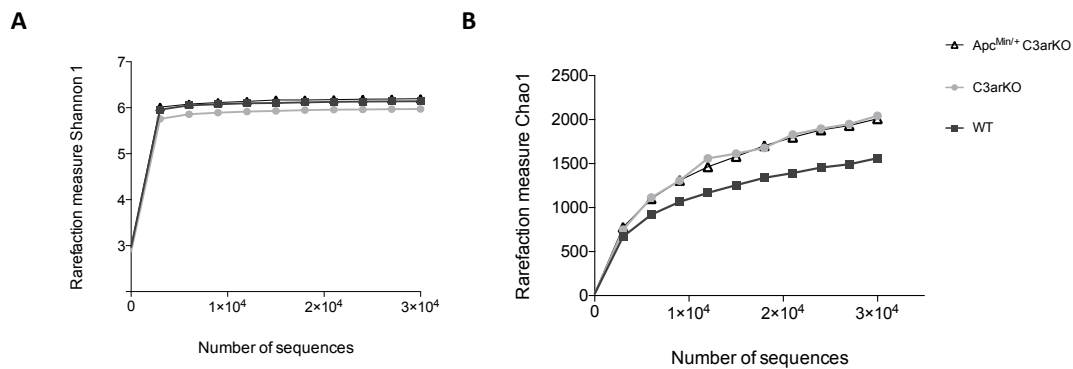


Figure 4-19 Fecal Shannon and Chao indexes of Apc^{Min/+} C3arKO, C3arKO and WT mice. Metagenomic sequencing was performed on fecal samples from Apc^{Min/+} C3arKO, C3arKO and WT mice at 25 weeks of age. Shannon (A) and Chao (B) diversity indexes relative are shown. n=8. Graphs show relative measures.

Although fecal bacterial composition at phylum levels did not show differences, family level was much more informative (Figure 4-20). In fact, we have observed increased abundance of taxa belonging to Bacteroidaceae and reduced abundance of Bacteroidales S24-7 group in tumor bearing mice compared to C3arKO and WT mice (Figure 4-20).

Interestingly, Lactobacillaceae and Erysipelotrichaceae were more abundant in WT mice and reduced in both Apc^{Min/+} C3arKO and C3arKO fecal sample. On the other hand, bacteria belonging to Lachnospiraceae family were decreased in WT mice compared to Apc^{Min/+} C3arKO and C3arKO mice.

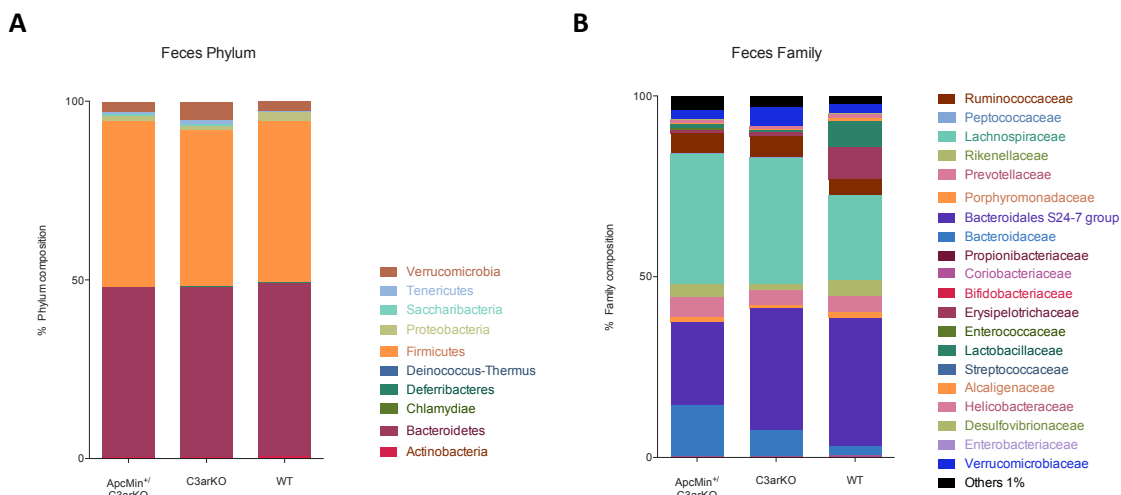


Figure 4-20 Fecal phylum abundance in $Apc^{Min/+}$ C3arKO, C3arKO and WT mice. Metagenomic sequencing was performed on fecal samples from $Apc^{Min/+}$ C3arKO, C3arKO and WT mice at 25 weeks of age. Phylum (A) and Family (B) abundance are shown as bar plots, n=8.

We then have analysed phylum and family abundance variations at the level of colon-mucosa associated microbiome over the different groups of mice. In line with data obtained from the fecal microbiome, $Apc^{Min/+}$ C3arKO colonic mucosa-associated bacteria composition revealed a lower abundance of Erysipelotrichaceae compared to WT and C3arKO mice (Figure 4-21).

Together with changes in Erysipelotrichaceae family, relative abundance of Bifidobacteriaceae, Streptococcaceae, Enterococcaceae, Enterobacteriaceae and Lactobacillaceae family were increased in $Apc^{Min/+}$ C3arKO colonic mucosa compared to C3arKO and WT mice. Furthermore, Lachnospiraceae relative abundance was higher in C3arKO colonic mucosa compared to $Apc^{Min/+}$ C3arKO and WT mice (Figure 4-21).

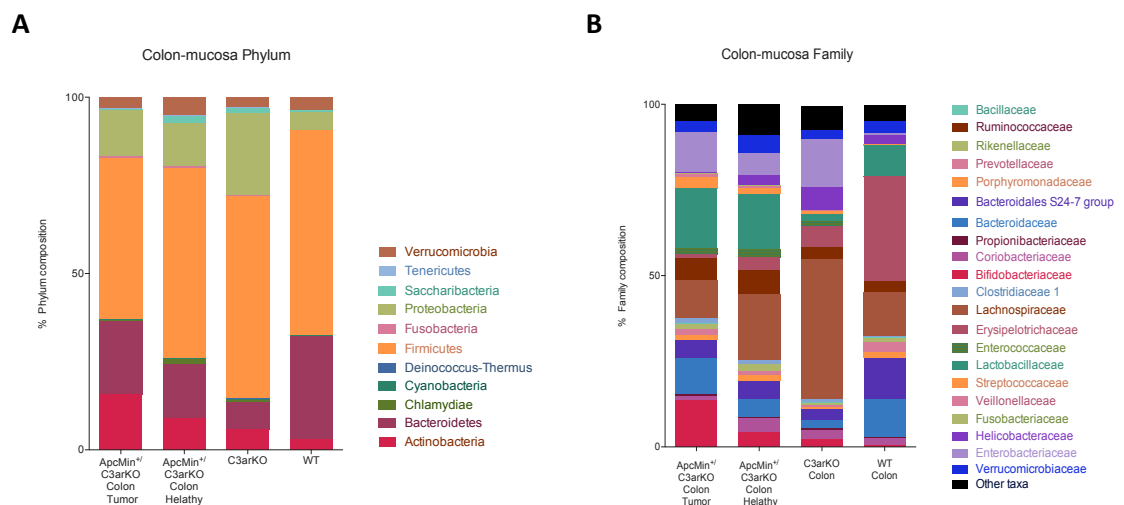


Figure 4-21 Colonic mucosa Phylum and Family abundance in $Apc^{Min/+}$ C3arKO, c3arKO and WT mice. Metagenomic sequencing was performed on colonic mucosa samples from $Apc^{Min/+}$ C3arKO, C3arKO and WT mice at 25 weeks of age. Phylum (A) and Family (B) abundance are shown as bar plots, n=8.

Interestingly, we could not detect big differences comparing $Apc^{Min/+}$ C3arKO tumor and healthy mucosa either at phylum or at family level (Figure 4-21). Indeed, as it is

shown in Figure 4-22 panel A, they were statistically more diverse compared to C3arKO and WT colonic mucosa-associated bacteria. No major differences were detected, among groups, in terms of richness (Figure 4-22 panel B).

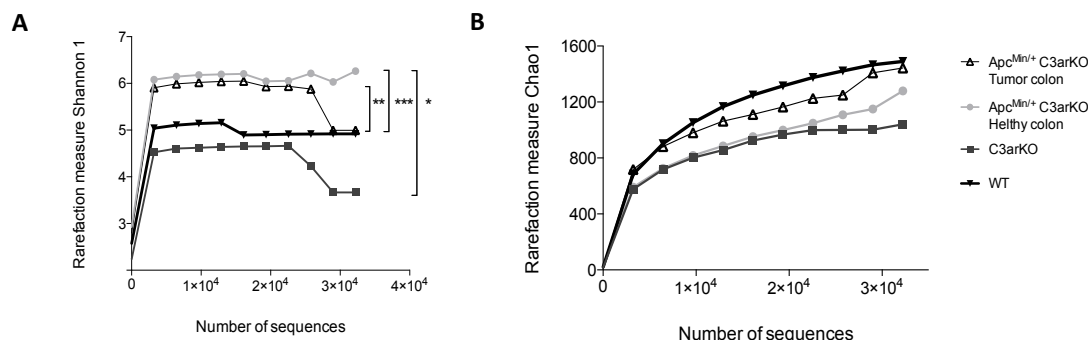


Figure 4-22 Colonic mucosal Shannon and Chao indexes of Apc^{Min/+} C3arKO, C3arKO and WT mice. Metagenomic sequencing was performed on colonic mucosa samples from Apc^{Min/+}C3arKO, C3arKO and WT mice at 25 weeks of age. Shannon and Chao diversity indexes relative are shown. n=8. Graphs show relative measures.

These data show that there is not a clear separation of microbial communities between tumor and near healthy areas of these tumor-bearing mice. Consistently, we could not detect any differences in terms of PV-1 expression comparing tumor and paired healthy mucosa of Apc^{Min/+} C3arKO mice (Figure 4-11 B bottom panel).

Therefore, we analysed genus abundance variations over the groups, considering Apc^{Min/+} C3arKO tumor and paired healthy mucosa as part of the same group. As it is shown in Figure 4-23, the bar plot showed a statically negative abundance of Erysipelotrichaceae family, Meiothermus, env.OPS 17 family and Eisenbergella in the Apc^{Min/+} C3arKO mice compared to C3arKO and WT mice. Whereas other bacteria were statistically more abundant in colon mucosa of Apc^{Min/+} C3arKO mice compared to C3arKO and WT mice, which include: Rikenella, Streptococcus, Lactococcus, Enterococcus, Bifidobacterium, Succinivibrio and Klebsiella. In accordance with literature, species belonging to Streptococcus Klebsiella and Enterococcus, like *Fusobacterium*, have been associated to late stage of carcinogenesis(Blumberg and Powrie 2012; S. Li et al. 2017).

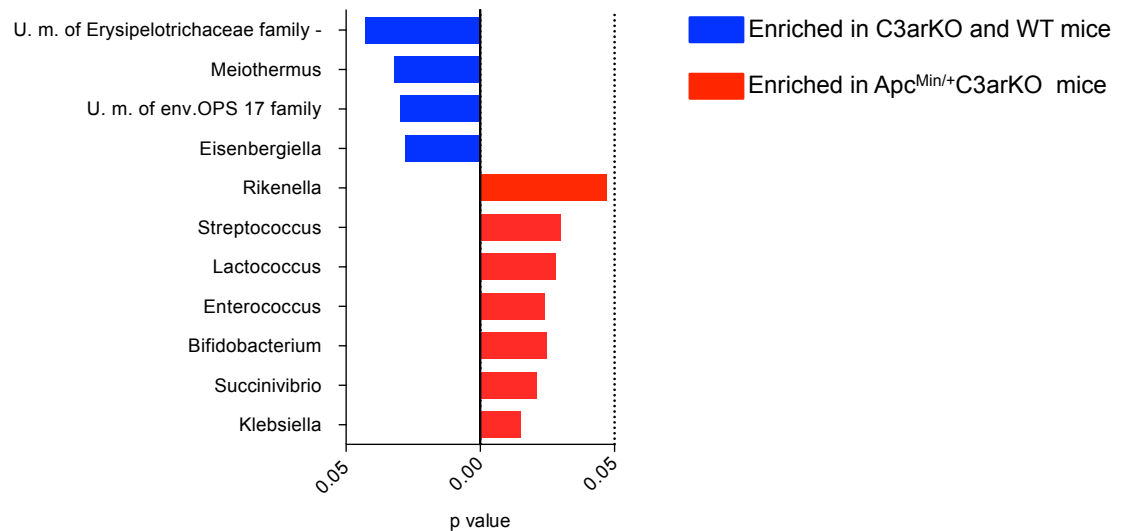


Figure 4-23 Genus colonic mucosa abundance in Apc^{Min/+}C3arKO, C3arKO and WT mice. P-value comparison between genus relative abundance of different taxa belongin to colonic mucosa of Apc^{Min/+}C3arKO, C3arKO and WT mice at 25 weeks of age (n=8). The bar plot shows bacteria gene that are enriched in correlltion (red bars) or inverse correlation (blue bars) with Apc^{Min/+}C3arKO mice. Statistical significant differences were calculated usign One-Way Anova test.

Surprisingly, Bifidobacterium is statistically (p-value=0,025) more associated with Apc^{Min/+} C3arKO colon tissue. This difference was already present at phylum level where Actinobacteria, phylum which includes Bifidobacterium genus, were more abundant in Apc^{Min/+} C3arKO mice compared to C3arKO and WT mice (Figure 4-21 A). Of note, three species were over represented in association with Apc^{Min/+} C3arKO colon area: Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium longum (Figure 4-24).

Bifidobacterium is commonly considered associated with healthy colon and able to boost anti-tumor responses(Zitvogel et al. 2018). An *in vivo* study shows that a specific subtype of *B.longum* (BI 372) cannot resolve DSS induced colitis inflammation and mucosal barrier loss(Srutkova et al. 2015). In line with these data, our data might suggest that either Bifidobacterium is effective only in the right microenvironmental context or tumor selects strains of the Bifidobacterium that are less beneficial than others, despite belonging to the same species.

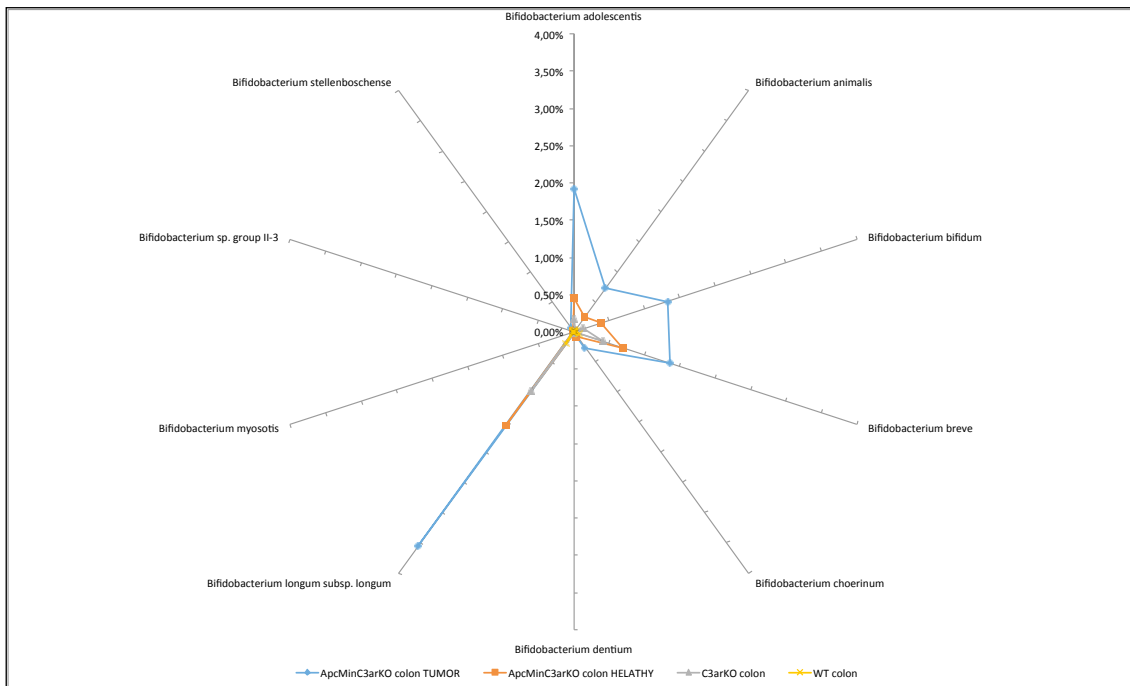


Figure 4-24 Bifidobacterium Species colonic mucosa abundance in $Apc^{Min/+}$ C3arKO mice. Bifidobacterium species abundance in colonic mucosa of $Apc^{Min/+}$ C3arKO, C3arKO and WT mice at 25 weeks of age, n=8.

Taken together our results reveal that colonic mucosa of $Apc^{Min/+}$ C3arKO mice were colonized by bacteria normally associated with late stage of colon cancer. Moreover, these bacteria were reported to alter mucosal barrier permeability.

4.5 Bacteria depletion abrogates the formation of liver premetastatic niche

It has been demonstrated that pathobionts contribute to CC development and progression (Garrett 2015a; Sears and Garrett 2014). Different *in vivo* studies have suggested that bacteria manipulation, through antibiotics treatment, could have a fundamental role in preventing colorectal cancer growth (Bullman et al. 2017) and liver metastases progression (Kronenberg and Hartmann 2018; Ma et al. 2018; Yoshimoto et al. 2013), although in the clinic the efficiency of antibiotic treatment in cancer therapy was debated (Zitvogel et al. 2018). Thus, we wondered whether decreasing the overall number of bacteria in the gut, we could reduce liver bacterial presence and subsequent formation of liver premetastatic niche.

To assess this hypothesis we treated $Apc^{Min/+}$ C3arKO, C3arKO and WT mice at 25 weeks of age with broad-spectrum antibiotic mix (ampicillin, vancomycin, neomycin sulphate e metronidazole) for short-term (one week) treatment (Figure 4-25).

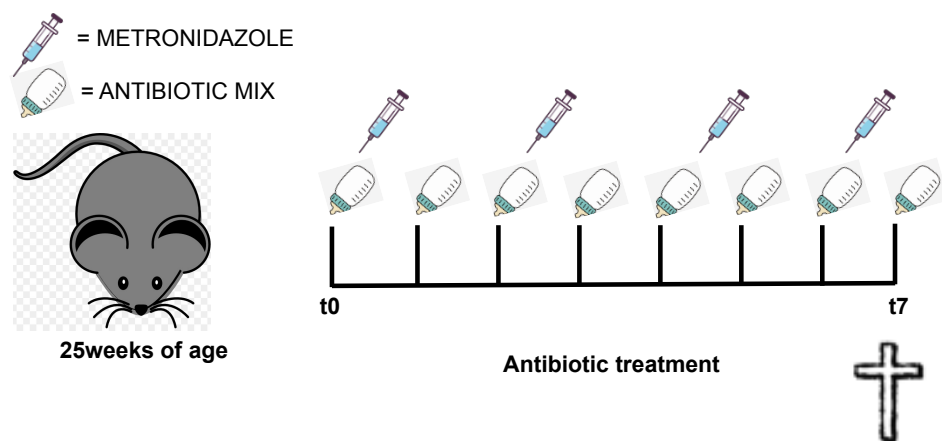


Figure 4-25 Scheme of the short term antibiotics treatment. To minimize the microbiome, $Apc^{Min/+}$ C3arKO, C3arKO and WT mice at 25 weeks of age received, for one week, a cocktail of three antibiotics (ampicillin, vancomycin, neomycin sulphate) *ab libitum* in the drinking water. Then, every other day all mice receive were gavaged with metronidazole which is depleting the strictly anaerobic bacteria.

To ensure the efficacy of the antibiotic treatment, we plated feces from treated and not treated mice. As expected, we could not detect culturable bacteria in most of treated mice compared to the untreated ones (Figure 4-26 A and B).

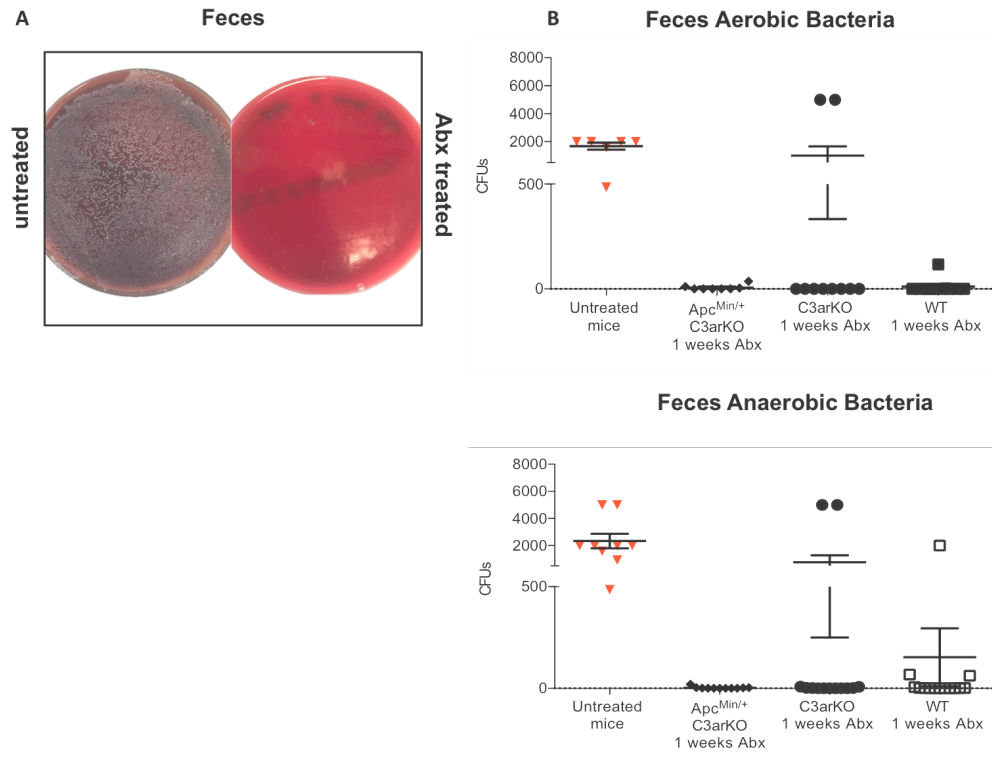


Figure 4-26 Fecal microbiota depletion after one week of antibiotics treatment.

(A) Pictures are showing the efficacy of short-term antibiotic treatment. (B) Colony Forming Units of fecal culturable bacteria of untreated and $Apc^{Min/+}$ C3arKO, C3arKO and WT treated mice. Each data point represent one individual mouse (n= 9-10).

One week of antibiotic treatment was able to decrease not only fecal microbiome but also liver bacteria. Indeed, as it is shown in panel A and B Figure 4-27, in both anaerobic and aerobic conditions, we could assess a statically significant reduction of liver bacteria CFUs comparing treated mice with $Apc^{Min/+}$ C3arKO untreated mice.

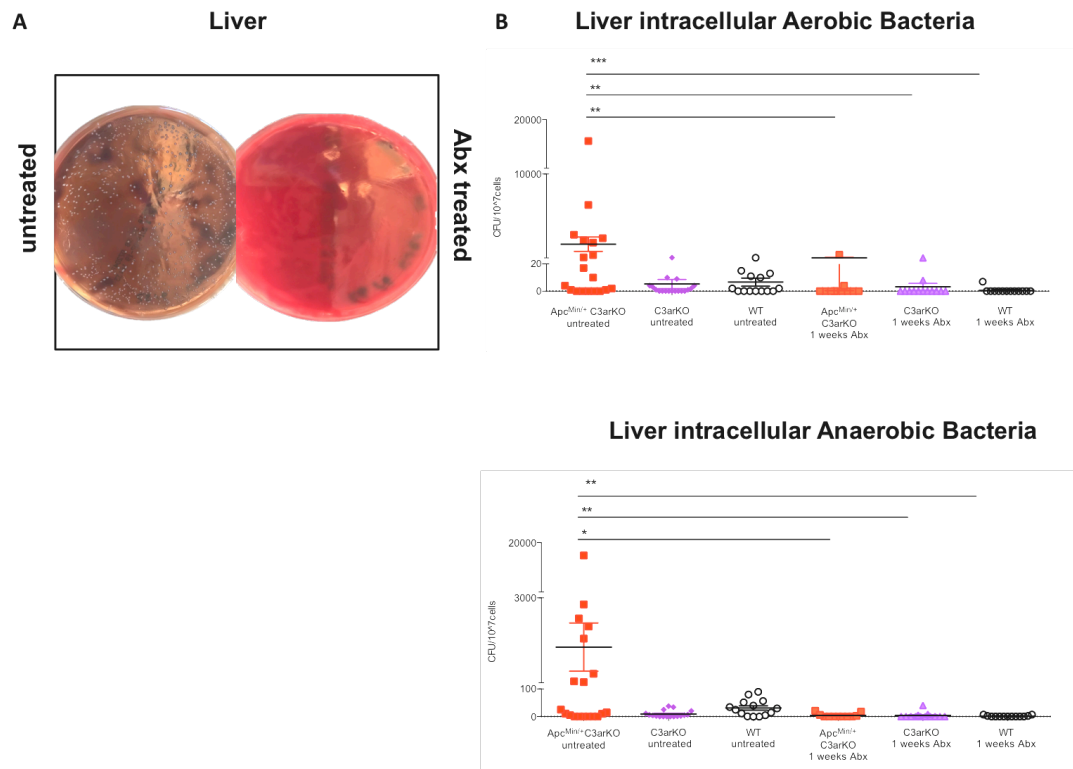


Figure 4-27 Liver microbiota depletion after one week of antibiotics treatment.

(A) Pictures are showing the efficacy of short-term antibiotic treatment. (B) Colony Forming Units of fecal culturable bacteria of untreated and Apc^{Min/+} C3arKO, C3arKO and WT treated mice. Each data point represent one individual mouse (n= 9-10).

The short-term antibiotic treatment did not affect tumor growth (Figure 4-28), whereas it has been shown in the literature in other model of tumor-bearing mice, that long-term (four weeks) treatment prevents tumor growth (Zackular et al. 2016). In accordance with the literature, we confirmed that long term treatment is reducing colon tumor number in Apc^{Min/+} C3arKO long-term antibiotic treated mice compared to

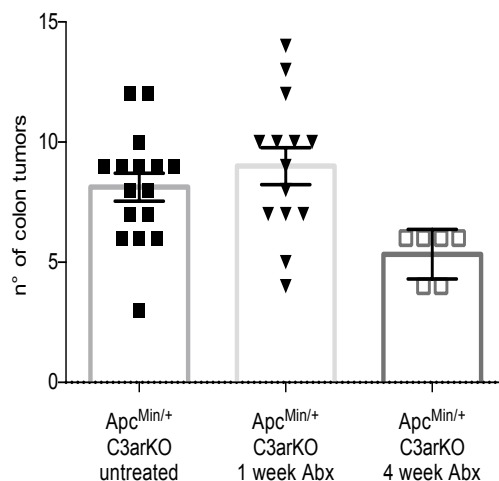


Figure 4-28 Number of colon tumor between untreated, short term and long term antibiotic treated Apc^{Min/+} C3arKO mice. The graph shows number of colon tumor between untreated short-term (one week) antibiotic treated and long-term (four week) antibiotic treated Apc^{Min/+} C3arKO mice. Each point represent a mouse (n=6-16).

no treated mice (Figure 4-28).

Since short-term antibiotic treatment minimized liver microbiota without affecting primary tumor growth, we wondered whether bacteria depletion could also directly impair innate immune cell recruitment inside the liver of $Apc^{Min/+}$ C3arKO mice. Then, we have analysed by flow cytometry the innate immune cells infiltrating inside the liver of antibiotic treated and untreated mice at 25 weeks of age. Although the livers from antibiotic treated double mutant mice showed still higher numbers of infiltrating neutrophils and macrophage-like cells in comparison to C3arKO and WT antibiotic treated mice (Figure 4-29 A), infiltrating immune cells were drastically reduced when compared to those of un-treated $Apc^{Min/+}$ C3arKO mice. Thus it is clear that antibiotics drastically reduce the homing of innate immune cells in the liver (Figure 4-29 B), presumably via the elimination of liver bacteria, which induce the recruitment of immune cells. This effect was even stronger after four weeks of treatment (data not shown).

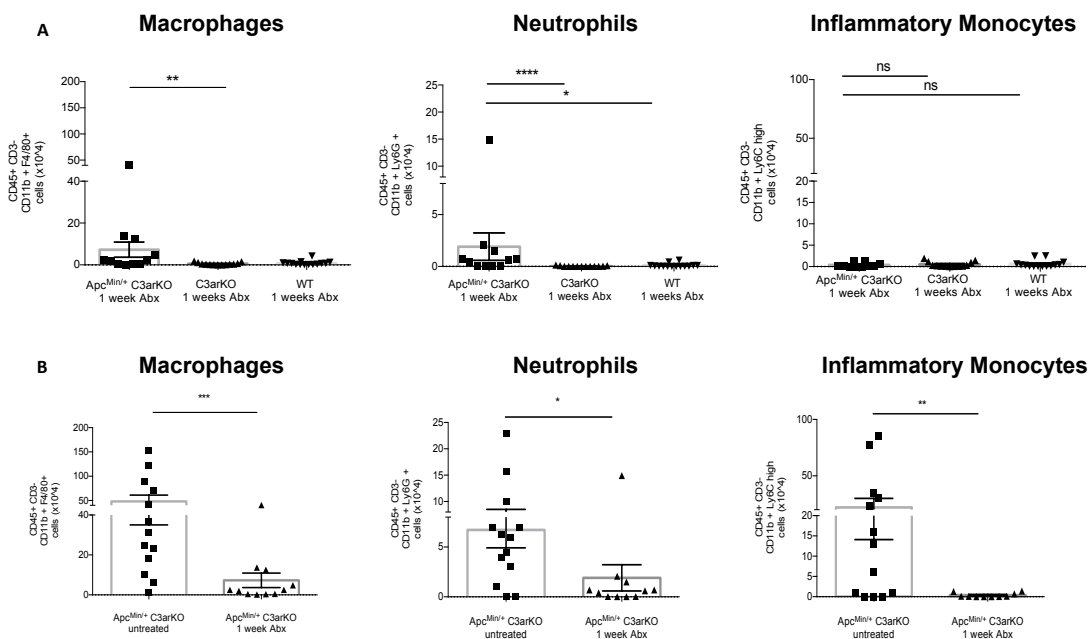
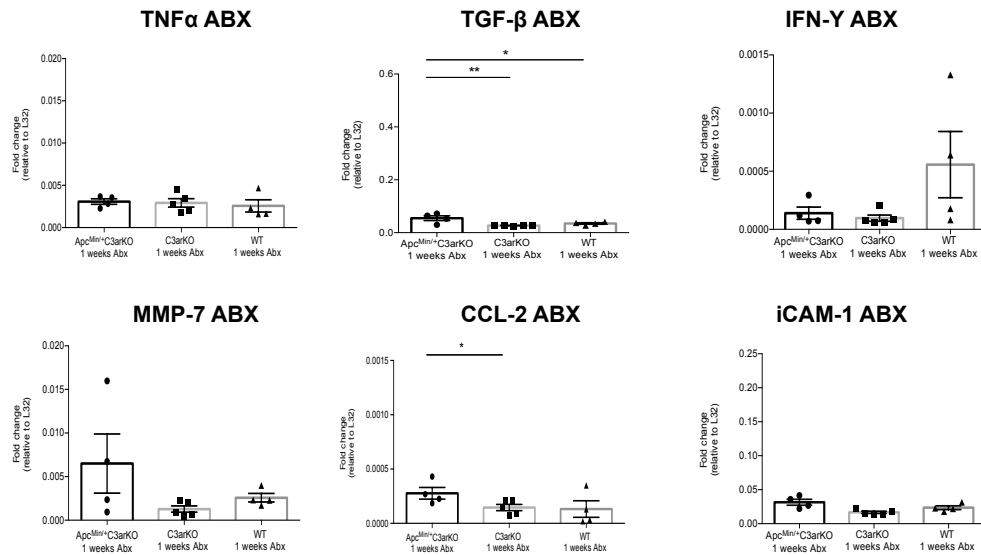


Figure 4-29 Loss of immune cells recruitment after antibiotics treatment.

(A) FACS analysis for Cd11b⁺⁺F4/80⁺ (macrophages-like), Cd11b⁺Ly6G⁺ (neutrophils) and Cd11bLy6C^{high} (Inflammatory Monocytes) cells in the liver of Apc^{Min/+}C3arKO, C3arKO and WT treated mice at 25 weeks of age. Each data point represents an individual mouse (n= 11). (B) FACS analysis show immune cells different recruitment between treated and untreated Apc^{Min/+}C3arKO mice at 25 weeks of age. Each data point represents an individual mouse (n=11 to 13). Error bars represent SEM. Statistical significance has been evaluated by (A) Kruskal Wallis unpaired test. *P < 0.05, **P < 0.01 and (B) Mann Whitney unpaired test *P < 0.05, **P < 0.01.

We have also tested the status of inflammation at the transcriptional level. After antibiotic treatment the significance in gene up-regulation of TNF- α , MMP7 and ICAM1 mRNA level was lost (Figure 4-30 A), but not that of CCL2 and TGF- β when comparing ApcMin/+C3arKO to C3arKO and WT treated mice (Figure 4-30 A). However, these differences were modest when we compared the antibiotic treated versus the untreated ApcMin/+C3arKO mice. Indeed, as shown in Figure 4-30 panel B, depletion of gut and liver bacteria resulted in a reduction of TNF- α , TGF- β , IFN- γ , CCL2 and ICAM1 gene transcriptions. We have shown that, without affecting primary tumor growth, liver inflammation was alleviated administering antibiotics. These findings support our hypothesis that bacterial presence directly promotes the recruitment of innate immune cells and the formation of an inflammatory environment. This confirms that gut microbiota may promote primary tumor growth and the formation of a premetastatic niche.

A



B

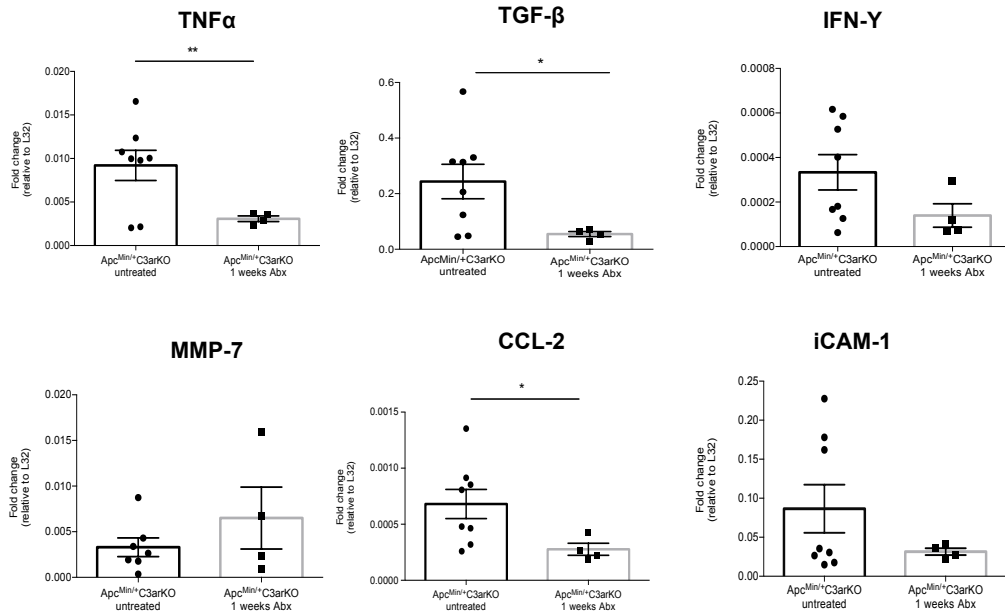


Figure 4-30 Loss of Premetastatic niche phenotype after one week of antibiotic treatment. The expression of TNF- α , TGF- β , IFN γ , MMP7, CCL2, and iCAM1 are assessed byqPCR. (A) mRNA expressions, normalized to L32 gene trascription, show differences between *Apc^{Min/+}C3arKO*, *C3arKO* and WT antibiotic treated mice at 25 weeks of age. Each data point represents an individual mouse (n=4 to 5). (B) mRNA expressions, normalized to L32 gene trascription, show differences between treated versus untreated *Apc^{Min/+}C3arKO* mice at 25 weeks of age. Each data point represents an individual mouse (n=3 to 8). Error bars represent SEM. Statistical significance was evaluated using (A) one-way ANOVA and (B) Student's t-test. *P < 0.05, **P < 0.01, **** P< 0.0001.

4.6 Evidence of metastases formation

4.6.1 Spontaneous liver lesions in old $Apc^{Min/+}$ C3arKO mouse

We showed that tumor bearing mice had more permeable colon vessels due to GVB impairment, which might have allowed bacteria to reach the blood circulation and colonize the liver altering the hepatic microenvironment. Since the liver of these double mutant mice has displayed a more inflammatory microenvironment, we wanted to test whether elder $Apc^{Min/+}$ C3arKO mice could develop spontaneous liver lesions.

To corroborate this hypothesis, we searched for the presence of spontaneous liver lesions in aged $Apc^{Min/+}$ C3arKO mice (more than 25 weeks of age). As shown in Figure 4-31 panel A, we found white macroscopic liver lesions located in all liver lobes. The haematoxylin and eosin (H&E) sections of these macroscopic lesions showed unusual necrosis inside the parenchyma (nuclear debris in a patchy distribution) and aberrant structures surrounded by fibrotic tissue (Figure 4-31 B). The liver parenchyma showed signs of cholestasis, due to bile deposits.

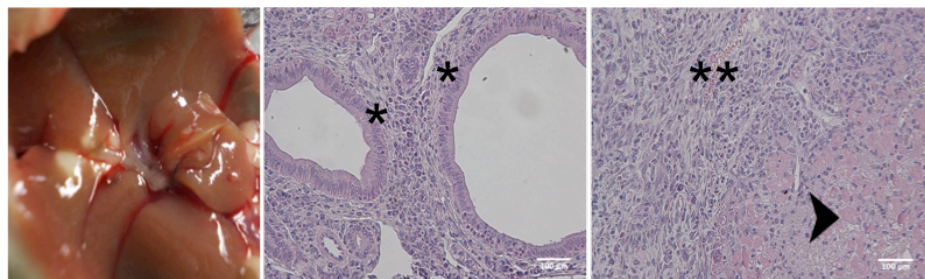


Figure 4-31 Aberrant liver lesions in aged $Apc^{Min/+}$ C3arKO mouse. Pictures show macroscopic hepatic lesions and the corresponding hematoxylin and eosin (H&E) stainings. Asterisk marks fibrosis (**), arrowhead necrotic area and double asterisk marks (*) aberrant structures. Scale bar: 100 μ m.

Cytokeratin 20 (CK20), a marker of intestinal epithelial cells was used to detect for aberrant structures (Figure 4-32). This marker is currently used in the clinic to detect CRC human liver metastases (Tot 2002). As shown in Figure 4-32, we could detect a significant infiltration of $KI67^{+}$ proliferative cells confirming our human data (Figure 4-8).

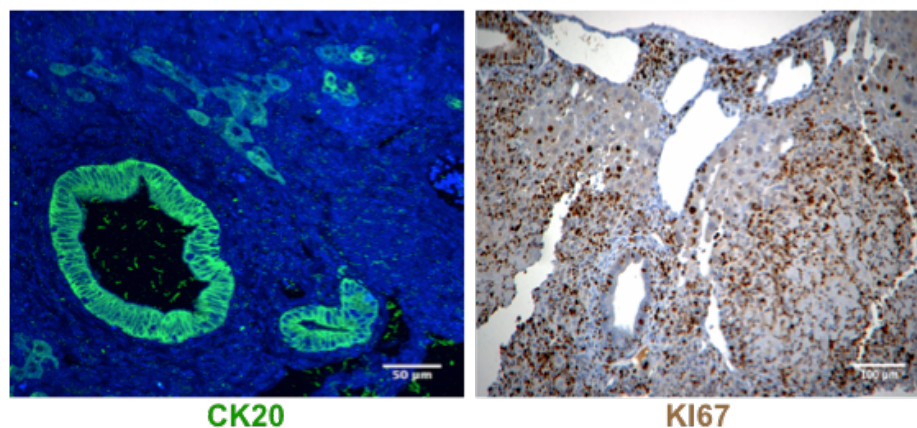


Figure 4-32 Characterization of liver lesions in aged $Apc^{Min/+}$ C3arKO mouse. Confocal images show aberrant liver structures positive for Cytokeratin 20 (green), marker of intestinal epithelial cells. Nuclei were stained in blue (DAPI). Scale bar: 50 μ m.

Furthermore, colon vessels of this $Apc^{Min/+}$ C3arKO mouse expressed higher levels of PV-1 compared to age-matched $Apc^{Min/+}$ C3arKO and WT mice without liver lesions (Figure 4-33).

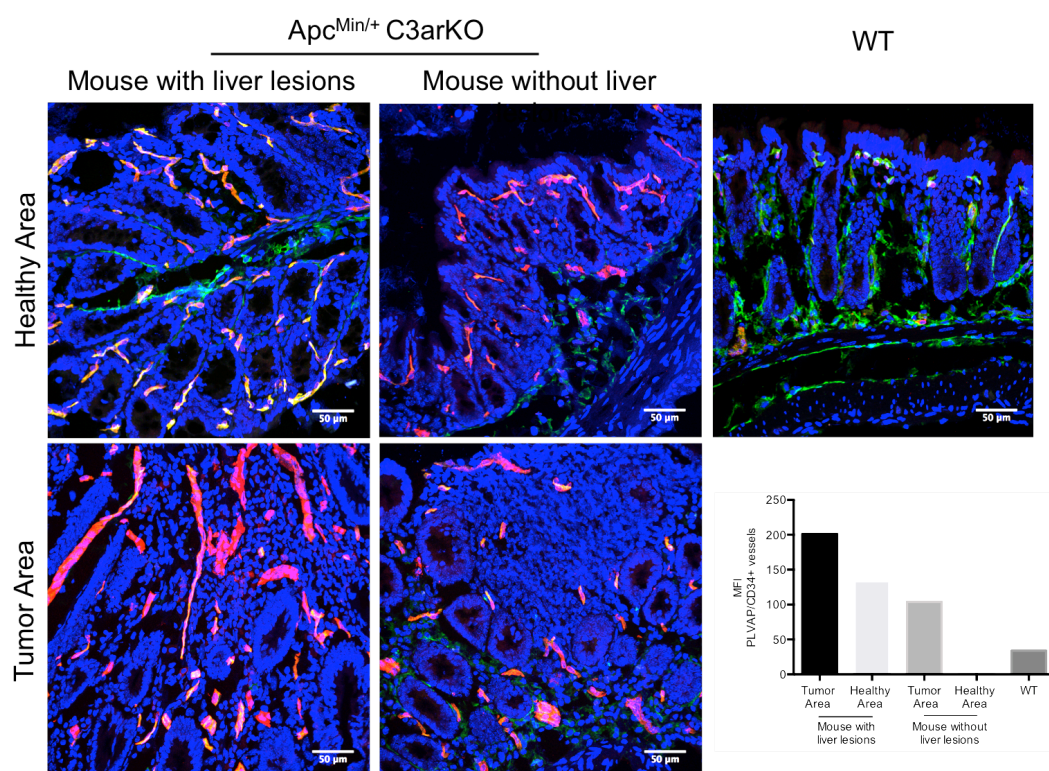


Figure 4-33 GVB impairment in aged $Apc^{Min/+}$ C3arKO mouse with aberrant spontaneous liver lesions. Confocal images show intestinal CD34⁺ blood vessels (green), PV-1 (red) and nuclei (blue, DAPI) in cry sections from healthy and colon tumor. Scale Bars: 100 μ m. Mean Fluorescent intensity of PV-1 in CD34⁺ vessels, related to the images. Scale Bars: 50 μ m.

As shown in $Apc^{Min/+}$ C3arKO mice at 25 weeks of age, we could detect bacteria within the liver, confirming that also inside these pathologic livers with aberrant lesions we could detect bacteria infiltrating hepatic parenchyma (Figure 4-34).

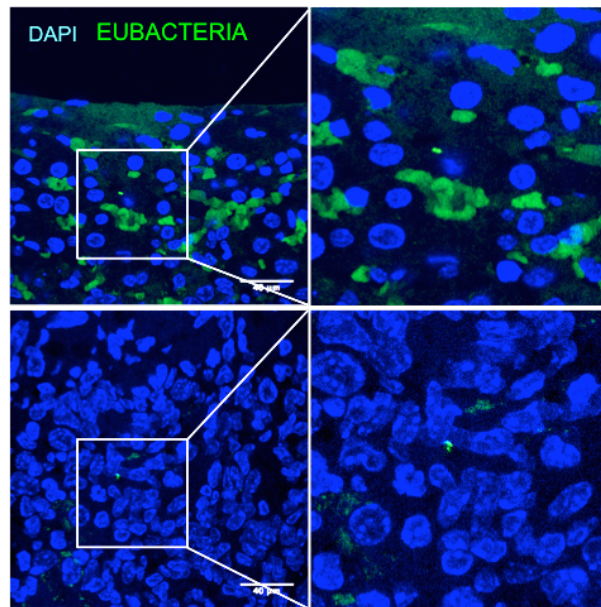


Figure 4-34 Eubacteria in aberrant liver lesions in aged $Apc^{Min/+}$ C3arKO mouse. Confocal images show fluorescence in situ hybridization analysis of liver. Eubacteria (green, white circle) were stained using three general bacterial probes (See Material and Methods). All the sections were stained with DAPI (blue), to stain the nuclei. Scale bar: 40 μ m.

The appearance of these CK20⁺Ki67⁺ liver lesions in aged in $Apc^{Min/+}$ C3arKO mice suggests that bacteria translocation to the liver, throughout GVB disruption, might be possible and could create a preferential soil for liver lesion formation.

4.6.2 Bacteria mediate liver metastases in another murine model of colon cancer.

Our *in vivo* model is probably the only spontaneous model of CC metastatization in mice as we showed the formation of liver lesions in old $Apc^{Min/+}C3arKO$ mouse. However, this is a rare event because it is occurring in anaemic and sick old tumor bearing mice at the end of their lifespan. Thus, we decided to verify the frequency of metastatic lesions and the characterization of the premetastatic niche using another currently accepted model of CRC metastatization which is not spontaneous, but is based on chemically induced CRC model, the AOM/DSS model (Neufert, Becker, and Neurath 2007) followed by intrasplenic injection of murine colon cancer cells.

Mice were subjected to azoxymethane (AOM 10mg/kg) as a mutagen and treated with 1 % dextran sodium sulphate (DSS) to induce intestine inflammation. In Figure 4-35 panel A was reported the treatment scheme that we have followed and the weight curve proving treatment efficacy.

Only mice treated with both AOM and DSS develop colon tumors. Whereas DSS treated mice are losing weight upon treatment without developing tumors.

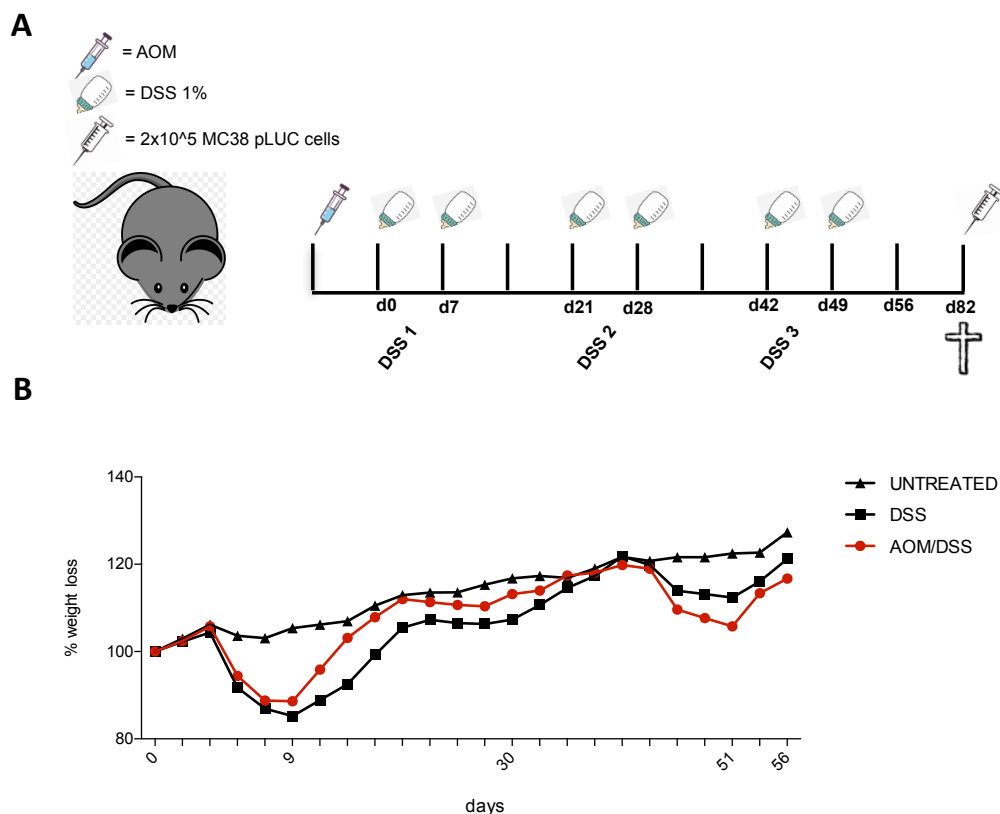


Figure 4-35 Scheme of treatments and weight curve of AOM/DSS and Intrasplenic injected mice.

(A) C57BL/6 males (n=3) aged 8 weeks were treated with azoxymethane (AOM). Two cohorts received dextran sodium sulphate (DSS) 1% in drinking water for 7 days (AOM/DSS and DSS). Both groups were allowed for recovery for 14 days but while group AOM/DSS received water upon DSS stop. This schedule was repeated for 3 cycles. (B) Weight was monitored throughout the experiment and represented as % of the initial body weight. Graph shows mean $\pm$ SEM. n=3.

From our previous result, we know that AOM/DSS treated mice exhibit more aerobic and anaerobic bacteria inside liver parenchyma compared to untreated mice (Figure 4-36).

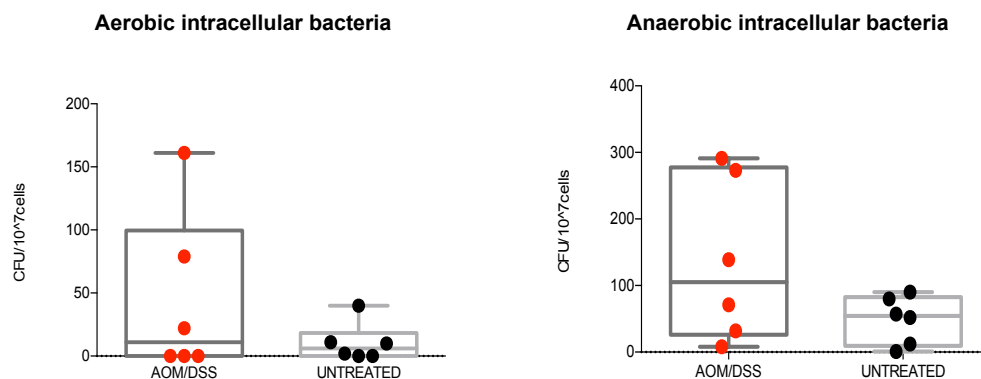


Figure 4-36 Liver intracellular aerobic and anaerobic bacteria in AOM/DSS and untreated C57BL/6 mice. Blots are showing intracellular aerobic and anaerobic bacteria inside the liver parenchyma of C57BL/6 AOM/DSS treated and untreated mice. Each data point represents one mouse (n =6). Error bars represent SEM.

At day 56, after the third cycle of DSS, AOM/DSS tumor-bearing mice showed colon tumors and translocation of bacteria inside the liver. At this time point we performed, an intra-splenic injection of 2×10^5 (in 50 μ l) MC38 pLUC cells in all groups of mice (Figure 4-35 panel A). Following intrasplenic injection, tumor cells entered the portal circulation and reached the liver, mimicking the metastatization process (Soares et al. 2014). To follow tumor spreading and the progressive formation of liver metastases *in vivo* we have generated MC38-luc cell line that was derived from MC38 invasive murine adenocarcinoma cell line through the transfection with a luciferase expression plasmid.

We have monitored the detection of light emitted by live MC38 pLUC cells, using the IVIS Bioluminescence imaging. After 26 days from intrasplenic injection, mice were sacrificed and livers were harvested for *ex-vivo* quantification of metastases accumulation.

Bioluminescent *ex vivo* analysis showed more metastatic foci in livers of CC tumor-bearing mice compared to control mice (Figure 4-37). As it is shown in Figure 4-37, in the same mice we have also observed macroscopically larger liver metastases compared to control mice. In these control mice, metastatic foci were stochastically distributed whereas in CC tumor bearing mice metastatic foci were more aggregated in one lobe. Interestingly, this difference was not simply related to an increase in intestinal inflammation as DSS only treated mice displayed a number of liver lesions comparable to those of untreated (only intrasplenically injected mice).

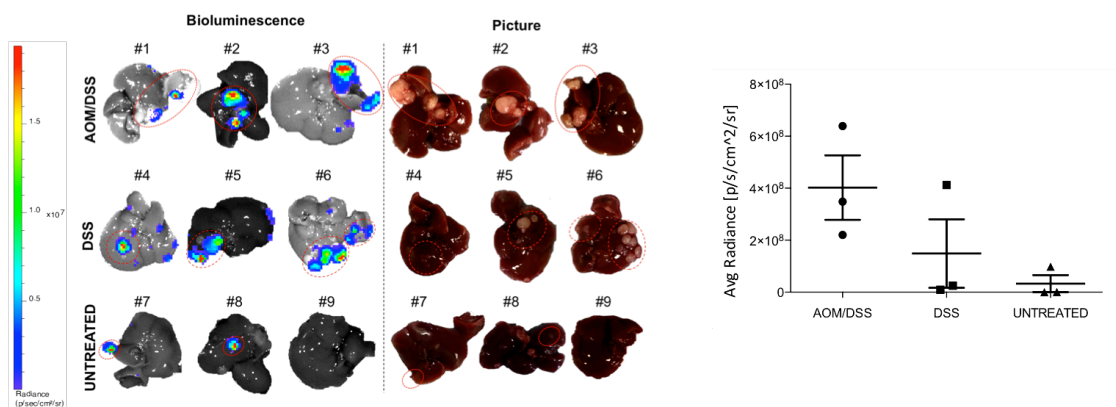


Figure 4-37 Ex-vivo imaging detection of MC38pLUC⁺ liver metastatic foci.

Ex vivo analysis of the liver of C57BL/6 AOM/DSS, DSS and untreated intra-splenically injected mice. The colorbar shows the Radiant Efficiency. Dashed red circles indicate the accumulation of MC38pLUC⁺ cells and the corresponding area in the picture. Quantification of the *ex vivo* analysis of MC38pLUC⁺ cells accumulation in the liver of C57BL/6 AOM/DSS, DSS and untreated intra-splenically injected mice. Data are presented as min and max whiskers plot.

Since we hypothesized that liver cancer cell homing was mediated by bacteria translocation and a more receptive liver microenvironment, we checked for the presence of culturable liver bacteria in this model. Thus, we lysed and plated liver cells collected from AOM/DSS, DSS and untreated mice. We found a mild difference in liver aerobic bacteria CFUs; bacteria concentration was higher in AOM/DSS treated

compared to DSS and untreated mice, while anaerobic bacteria were equally colonizing the livers of all mice.

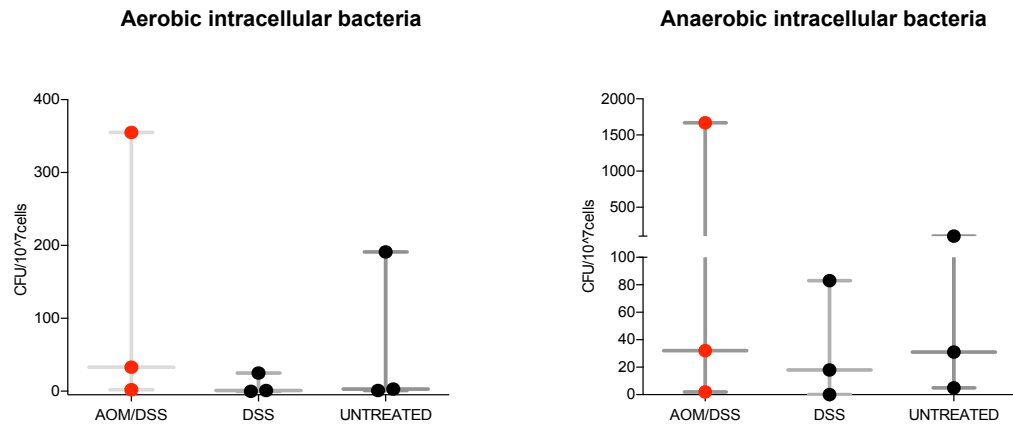


Figure 4-38 Intracellular aerobic and anaerobic liver bacteria in AOM/DSS, DSS and Untreated intrsplenically injected mice. Graphs show intracellular aerobic and anaerobic bacteria inside the liver parenchyma of C57BL/6 AOM/DSS, DSS and untreated intrsplenically injected mice. Each data point represents one mouse (n =3). Error bars represent SEM.

Among the intrasplenically injected untreated mice, the only mouse that showed liver metastases was also the one that had higher CFUs in the liver compared to the other untreated mice (Figure 4-39). Additionally, the liver of untreated and AOM/DSS treated mice could be colonized by different bacteria which are derived from different commensals.

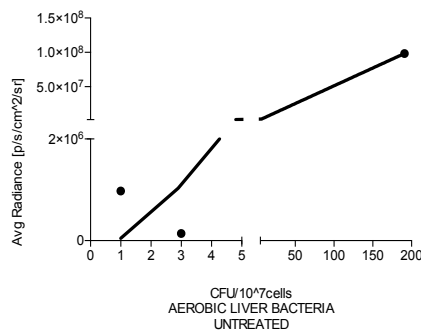


Figure 4-39 Positive correlation between aerobic bacteria and metastatic foci in untreated mice. Linear regression analysis between liver CFUs of Aerobic untreated mice and Avr Radiance [p/s/cm²/sr]. Black line represents the ideal match between the data. R^2 and p value are shown.

In line with this data, concomitant with the development of liver metastases, the livers of AOM/DSS tumor-bearing mice attracted more macrophages, neutrophils and inflammatory monocytes in comparison to DSS treated and untreated mice (Figure 4-40).

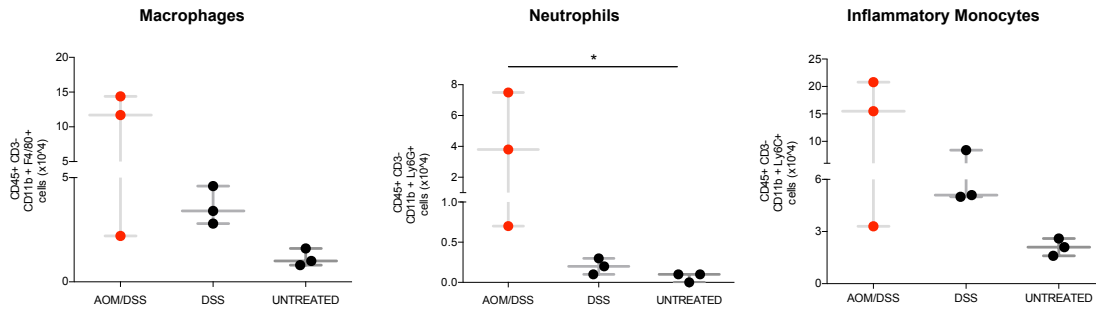


Figure 4-40 Innate immune cells recruitment in AOM/DSS intrasplenically injected mice. FACS analysis for Cd11b F4/80 (macrophages-like), Cd11b Ly6G (neutrophils) and Cd11b Ly6c⁺ (Inflammatory monocytes) cells in the liver of AOM/DSS, DSS and untreated intrasplenically injected mice. Each data point represents an individual mouse (n=3). Error bars represent SEM. Statistical significance has been evaluated by Kruskal Wallis unpaired test. * P < 0.05.

5 DISCUSSION

In this work we provide evidence that PV-1, an indicator of Gut Vascular Barrier (GVB) impairment, is a new prognostic biomarker of distant recurrence for colon cancer (CC) patients. Indeed, we proved that CC patients with high presence of PV-1⁺ vascular endothelial cells (>50%) have two times greater risk to develop distant metastases from the primary tumor resection. We also showed that in tumor bearing mice with GVB damage, bacteria reach the liver and trigger the formation of a premetastatic niche, fostering cancer cell recruitment and macrometastases formation.

Globally, 20-25% of CC patients have clinically detectable distant metastases at the time of initial diagnosis. A further of 40-50% of CC patients develop distant metastases after resection of the primary tumor (metachronous CC patients)(Chuang et al. 2011).

Despite numerous studies, the metastatic process remains highly unknown. It is still clinically difficult to predict which patient will most likely develop distant non-lymph node recurrence after CC resection. So far, the presence of lymph node metastases is the most important factor predicting survival in CC patients and it is also considered an indication of chemotherapy treatment(Laubert et al. 2012). Nevertheless, recent clinical trials suggested that curative surgery of the primary tumor followed by lymphadenectomy did not increase patient survival, suggesting that cancer cells may spread through another route to colonize distant organs. Venous blood from the intestine reaches the liver directly through the portal vein, therefore it is possible that liver metastases are preferentially seeded hematogenously(Naxerova et al. 2017). Indeed, using an *in vivo* intravital approach Wyckoff et al. discovered that metastatic cells cluster around blood vessels and intravasate into the systemic circulation using an amoeboid-like movement(Wong and Hynes 2006; Wyckoff et al. 2000). So far, no clinical prognostic marker is available to stratify CC patients based on the tumor blood vessels status. We have previously demonstrated the existence of a Gut Vascular

Barrier (GVB)(Spadoni et al. 2015). Under hazardous conditions the GVB is impaired and bacteria reach the liver(Spadoni et al. 2015).

In this thesis, we proved that GVB disruption, assessed by increased PV-1 detection, correlates with the occurrence of distant metastases in metachronous CC patients. Performing a contingency analysis, we provided evidence that CC patients with more than 50% of PV-1⁺ endothelial cells had two times greater probability to develop distant metastases after the diagnosis. These data further support that PV-1 is a marker to highlight CC blood vessels permeability and it is able to predict distant metastasis recurrence through the blood systemic circulation. We also saw that the percentage of PV-1⁺ endothelial cells was independent from the number of metastatic lymph nodes. This was also true in the absence of lymph node metastases, suggesting that cancer cell dissemination through the blood stream is independent from the lymphatic circulation. This assumption is further supported by a recent study indicating that different cancer cell clones from the primary tumor colonize either the lymph node or distant sites proving that seeding of lymph nodes and distant metastases are independent(Naxerova et al. 2017). Based on current CC patient's stratification, metachronous CC patients with high PV-1⁺ endothelial cells, but no lymph node involvement, were wrongly considered as not metastatic and did not receive the correct treatment. Recently, in order to evaluate a decrease in metastases occurrence, CC patients with positive lymph node involvement were enrolled in different clinical trials and treated with a more aggressive biological therapy(Labianca et al. 2013). However, all these trials (i.e. AVANT, NCCTG NO147 and PETACC-8) reported negative results, probably due to different biological characteristics between early and advanced CC(Labianca et al. 2013) or because the enrolled patients could not be stratified with a correct marker of distance recurrence. Indeed, our data showed that the involvement of regional lymph nodes did not statistically correlate with distant recurrence. Thus, all these data suggest that PV-1 can be used as a marker of vessels permeability in primary tumors ensuring a better stratification of CC patients.

At the moment it is still unknown whether PV-1 could have a direct role in cancer intravasation. PV-1 is a transmembrane glycoprotein originally discovered as a structural component of endothelial fenestrated and stomatal diaphragms(R V Stan, Kubitza, and Palade 1999; Radu V. Stan, Tkachenko, and Niesman 2004; Tkachenko et al. 2012). Recently, it has been discovered that PV-1, expressed on liver sinusoids, can actively control monocytes egress from fetal liver(Rantakari et al. 2016). Interestingly, Rantakari and colleagues showed that PV-1 forms complexes with neuropilin-1 (NRP-1), cell surface protein of circulating monocytes, allowing their extravasation from the hepatic parenchyma(Rantakari et al. 2016). Interestingly, recent studies revealed that NRP-1 was over-expressed on colon cancer cells and its inhibition could suppress metastases formation(L. Li et al. 2016; Parikh et al. 2004). Thus, we can hypothesize that PV-1 may actively mobilize colon cancer metastatic cells through the physical interaction with NRP-1. To prove this hypothesis, we should assess if PV-1 and NRP-1 colocalize in our cohort of human CC patients suggesting a possible active role of PV-1 in colon cancer metastatization. This evidence might prove that not only PV-1 can be used as a new prognostic marker of distant recurrence, but that it could also be harnessed as a target for blocking cancer cell dissemination.

Still, we do not know whether in tumor PV-1 is up regulated or it is just detectable because of a change in vessels conformation that makes PV-1 more accessible to the MECA-32 antibody. Indeed, the mechanisms that control PV-1 detection in tumor endothelial cells remain elusive. Recently, it has become increasingly clear that colon cancer-related dysbiosis can further fuel tumor progression at different levels impairing intestinal barrier properties(Gagliani et al. 2014; Garrett 2015a; Sears and Garrett 2014). Thus, to determine which colon cancer-related bacteria are interconnected with PV-1 accessibility (GVB disruption), we took advantage of a murine model of spontaneous colon tumorigenesis, the $Apc^{Min/+}C3arKO$ mice. These mice combine the $Apc^{Min/+}$ mutation with the C3a complement anaphylatoxin receptor (C3ar) deficiency(Guglietta et al. 2016b). They develop fewer tumors in the small

intestine compared to $Apc^{Min/+}$ single mutant mice, but they are characterized by an increased tumor burden in the colon, resembling human CC (*Guglietta et al, unpublished data*). As controls in the experimental setting, we included WT and C3arKO mice that do not develop tumors and have no other pathological conditions. We noticed many similarities between the $Apc^{Min/+}$ C3arKO murine model and the human CC situation. We observed a dismantling of the GVB in tumor-bearing mice. Moreover, when we analysed microbiota composition, we found a statistically significant increase in *Streptococcus*, *Enterococcus* and *Succinivibrio* known to be associated to advanced human colon tumors (Garrett 2015b; S. Li et al. 2017; Sears and Garrett 2014). In particular, a recent meta-analysis showed that 43% of patients with colon cancer were infected with *Streptococcus gallolyticus* and that this bacterium was able to trigger the production of angiogenic factors, such as IL-8, fostering CC progression (Blumberg and Powrie 2012; Sears and Garrett 2014). Contrary to our expectations, in this mouse model we saw a statistically significant enrichment of *Bifidobacterium* compared to healthy control groups. *Bifidobacterium* is usually associated with a healthy gut (Zitvogel et al. 2018), and it has been shown to be enriched in responding patients treated with anti-cancer therapies such as PD-1 blockade immunotherapy (Sivan et al. 2015; Zitvogel et al. 2018). However, an *in vivo* study has shown that a specific subtype of *Bifidobacterium longum* (BI 372) cannot resolve DSS induced colitis inflammation and mucosal barrier loss (Srutkova et al. 2015). Furthermore, in a mouse model of autoimmune arthritis, *in vivo* administration of *Bifidobacterium adolescentis* elicited an increase in Th-17 cells and exacerbated arthritis disease symptoms (Tan et al. 2016). In line with these data, our results might suggest that either *Bifidobacterium* is effective only in the right microenvironmental context, or that some tumor selects strains of *Bifidobacterium* that are less beneficial than others, or that there is an expansion of beneficial bacteria to counteract the presence of a tumor. All these hypotheses need to be evaluated.

We have previously shown that *Salmonella typhimurium* was able to directly mediate GVB damage (Spadoni et al. 2015), hence we hypothesized that cancer-related

bacteria may trigger GVB impairment. To address this point, we have already isolated several strains that are overrepresented in the tumor area. We now would like to evaluate whether these strains have the ability to dismantle the GVB. To do so, we will stimulate *ex-vivo* healthy human colon with different cancer-associated bacteria. Accumulating evidence has supported the idea that specific harmless bacteria had a positive role in fighting cancer(Zitvogel et al. 2018). The Erysipelotricachaceae was one of the bacteria families that we saw statistically significant lower in the Apc^{Min/+}C3arKO mice are. Other data produced in our laboratory showed that Apc^{Min/+} mice had a contraction in Erysipelotricachaceae both in the feces and colon mucosa associated bacteria compared to WT littermates. In this study, Zagato and colleagues have demonstrated that administration of an Erysipelotricachaceae family bacterium to Apc^{Min/+} mice was anti-tumorigenic. Furthermore, *ex-vivo* stimulation of colon cancer specimens with this bacterium showed reduced tumor proliferation. Taken this evidence, we could not exclude that harmless bacteria could have also a beneficial role in restoring GVB damage. To address this point, we will-stimulate *ex-vivo* human colon cancer specimen with healthy commensal bacteria.

Although the functions of cancer-associated intestinal dysbiosis were known to be exerted mainly in the gut, recent evidence suggests that altered microbiota could impact on extra-intestinal organs fostering colon cancer metastases formation(Gagliani et al. 2014; Ma et al. 2018). Indeed, it was shown that *Clostridium scindens* increased the production of secondary bile acids in the gut and their subsequent translocation to the liver. Here, secondary bile acids blocked the recruitment of anti-tumorigenic NKT cells fostering liver metastases(Ma et al. 2018). Furthermore, studies conducted on human CC patients showed an association between CC and septicaemia(Kwong et al. 2018; Lin et al. 2010), suggesting that colon cancer-associated bacteria are not only confined within the gut lumen but they could also invade the systemic circulation. In addition, Bullman et al. showed that primary colon tumor and paired liver metastases were colonized by co-occurring bacteria (such as *Fusobacterium nucleatum* and *Bacteroides fragilis*)(Bullman et al.

2017). Thus we hypothesized that GVB impairment could allow systemic dissemination not only of colon cancer cells, but also of cancer-associated bacteria. Accordingly, we detected bacterial DNA inside distant metastatic lesions of CC patients. In line with this, several clinical reports showed that often CC patients experience occurrence of liver abscesses close to liver metastatic lesions surrounded by inflammatory margins(Alvarez et al. 2004; Huang et al. 2012; Lin et al. 2010). Similarly, we showed that inflammatory cells, mainly belonging to the innate immune system, were surrounding metastatic foci. In cases where myeloid cells had infiltrated the lesions, they were forming aggregates deprived of bacteria, suggesting that they were involved in bacterial clearing.

We detected bacteria near colon cancer metastatic cells expressing high level of SOX9, which is a downstream transcription factor of WNT- β catenin signalling pathway. We can speculate that bacteria are coming from the primary tumor with/within SOX9 positive colon cancer cells, which are then hiding bacteria from the immune system. Thus, to assess this idea we will verify whether in the primary tumor bacteria are near or inside SOX9 highly expressing colon cancer cells.

Our human data, in line with other studies(Bullman et al. 2017), proved that bacteria can colonize distant metastases. Nonetheless we still do not know whether they arrive simultaneously or before circulating cancer cells. To investigate whether bacteria are preceding cancer macrometastases formation, we tested the presence of liver bacteria in the $Apc^{Min/+}C3arKO$ mice at 25 weeks of age in which mice have numerous colon tumors but no liver metastases. These mice showed a dismantled GVB concomitant to high amount of bacteria within the liver parenchyma. By contrast, younger double mutant mice exhibited neither GVB disruption nor increased liver bacteria translocation compared to control mice. Thus, our data showed that GVB modification in the primary tumor favours the entrance of cancer-related bacteria inside the bloodstream and subsequent colonization of the hepatic parenchyma. Moreover, since these mice did not show any sign of distant metastases we think that bacteria translocation precedes metastases dissemination. To functionally

demonstrate that these liver bacteria are coming from the colon due to GVB impairment, we will take advantage of a mouse model in which the GVB is constitutively closed: the *VEC-PAC* mice. The *VEC-PAC* (*Cdh5* (*PAC*)-*CreERT2*/ β -*catenin*^{*lox(ex3)/lox(ex3)*}*VEC-PAC*) mice are gain of function for β -catenin in its active form in an endothelial cells-specific manner. Under hazardous conditions they do not show either PV-1 up-regulation or bacteria spreading to the liver suggesting that β -catenin forced activation can prevent GVB breakage and bacteria systemic dissemination(Spadoni et al. 2015). *VEC-PAC* mice and control WT mice will be chemically injected with a carcinogen in order to trigger the development of colon tumor. Then, both groups will be injected with FITC dextran to mark blood vessels, and inoculated with a fecal microbiota transplantation (FMT) of fluorescent labelled-bacteria to track bacteria movement. By using an *in vivo* Cellvizio-imaging technique(Mielke et al. 2015) we will verify whether bacteria are able to enter the blood circulation due to GVB impairment and reach the liver. This dissemination should be blocked in *VEC-PAC* mice, which have the GVB constitutively closed.

We did not address whether bacteria were translocating freely or inside specific cells, but one possibility is that they were disseminating inside phagocytic cells such as macrophages and neutrophils. Of note, intracellular survival of *Klebsiella pneumoniae* inside neutrophils was shown to foster liver abscesses in CC patients(Lin et al. 2010). Another possibility could be that bacteria were released through specialized vesicles such as exosomes. Exosomes are known to allow packaging of complete pathogens or pathogen-derived factors allowing their escape from the immune system favouring their dissemination(Zhang et al. 2018). It has been demonstrated that exosomes, loaded with tumor-derived factors, carry different types of integrins which are responsible for exosome specific distant organ uptake(Hoshino et al. 2015). In this way, primary tumor-derived factors are “educating” distant target organs leading to the formation of a fertile soil for subsequent cancer cell seeding which is called the pre-metastatic niche (PMN)(Peinado et al. 2012; Shao et al. 2018). Hence in our model

we hypothesized that disseminating bacteria may be the trigger for the formation of a liver PMN.

The PMN consists of sequential phases which form a complex microenvironment characterized by inflammatory immune cells, stromal cells and remodelled extracellular matrix (ECM)(Gagliani et al. 2014). By analysing the liver of $Apc^{Min/+}C3arKO$ we demonstrated that the presence of bacteria was concomitant with the recruitment of innate immune cells such as macrophages, neutrophils and inflammatory monocytes. Together with the recruitment of innate immune cells we demonstrated that the liver of $Apc^{Min/+}C3arKO$ mice showed high gene expression of CCL-2, IL10, TNF- α , TGF- β compared to controls. CCL-2 may be directly involved in potentiating the recruitment of inflammatory cells. Indeed, CCL-2 expression is known to be associated to tumor cells migration and metastases formation in different type of cancer(Loberg et al. 2007; Peinado et al. 2017).

The recruitment of innate immune cells at distant sites has been demonstrated to foster PMN maturation before micrometastases formation(Peinado et al. 2017). Interestingly, it has been shown that macrophages could work in concert with neutrophils to elicit a switch to a more mature PMN through the release of TGF- β and IL-10. Indeed, high level of IL-10 resulted in rapid growth of liver metastases due to the down-regulation of local anti-tumor immunity(West, Mccuaig, et al. 2015). Further, it has been reported that innate immune cells could form clots on vasculature favoring the anchor to circulating tumor cells to actively facilitating their egress inside the PMN. Precisely, *in vivo* intravital imaging demonstrated that perivascular macrophages physically interact with neoplastic cells during intravasation process.

It has been also shown that E-selectin, V-CAM-1 and ICAM-1 are crucial for cancer cell adhesion(Francavilla, Maddaluno, and Cavallaro 2009) and extravasation from vessels around the pre-metastatic niche (Brodt 2016). Of note, it has been reported that blocking the expression of ICAM-1 leads to the abrogation of organ metastases formation (Kang et al. 2015), indicating a fundamental role of adhesion molecules during distant metastases growth.

If adhesion molecules are fundamental for tumor cell extravasation, metalloproteinases are essential for tumor cell engraftment. They are recognized as major contributors of extracellular matrix remodeling before tumor invasion. In an experimental metastasis assay, MMP-9-deficient mice present considerably fewer liver metastatic lesions when CC cells are intrasplenically injected (Gorden et al. 2007). This highlights the importance of host-derived MMPs in the metastatic process. Consistently, we found enhanced gene transcription level of ICAM-1 and different metalloproteinases such as MMP9 and MMP7 in $Apc^{Min/+}C3arKO$ livers compared to control mice.

In order to prove that bacteria are the key factors able to boost the formation of a premetastatic niche, we treated $Apc^{Min/+}C3arKO$ mice and control mice with a broad-spectrum antibiotic for one week (short-term treatment). After short-term antibiotic treatment without impairing primary tumor growth, the infiltrating immune cells were drastically reduced when compared to the un-treated $Apc^{Min/+}C3arKO$ mice. In line with these results, also different genes (i.e. TGF- β , CCL2 and MMPs) that we have previously shown to be upregulated in liver premetastatic niche were down regulated after bacteria depletion. Thus, to our knowledge we provided evidence for the first time that bacteria directly promoted the recruitment of innate immune cells and the formation of a liver premetastatic niche. Phenomenon that was abrogated after antibiotic-mediated bacteria depletion. Thus, we believe that depleting cancer-related bacteria could be a promising therapeutic strategy to prevent distant metastases formation.

Finally, we checked for spontaneous pathological liver lesions in old $Apc^{Min/+}C3arKO$ mice to corroborate the existence of a premetastatic niche able to initiate and boost metastases. We found an old $Apc^{Min/+}C3arKO$ mouse with dismantled GVB at primary tumor site and a compromised liver status. Indeed, this mouse displayed necrosis inside the parenchyma (nuclear debris in a patchy distribution) surrounded by fibrotic tissue. Inside these altered hepatic microenvironment we could detect CK20⁺ aberrant structures surrounded by several Ki67⁺ proliferative cells. To our knowledge, this *in*

vivo model is probably the only spontaneous model of CC metastatization. However, metastases formation was a rare event because it was occurring in anaemic and sick old tumor bearing mice at the end of their lifespan. Thus, we decided to verify the frequency of metastatic lesions and the characterization of the premetastatic niche using another currently accepted model of CC metastatization, the AOM/DSS model. This is a chemically induced CC model, followed by intrasplenic injection of murine colon cancer cells (Robertis et al. 2011). The obtained results showed that the liver of CC tumor-bearing mice, together with an increased liver bacteria concentration and recruitment of innate immune cells, had more metastatic foci compared to control mice. Interestingly, these differences were not only related to an increase in intestinal inflammation since only DSS-treated mice displayed a number of liver lesions comparable to the untreated group. Altogether these findings confirmed that the PMN was able to boost the formation of distant metastases and that, as discussed earlier, bacteria presence was essential for its formation.

To functionally demonstrate that bacteria are not only necessary for PMN formation but also sufficient to trigger metastatic foci formation, we will repeat the same experiment treating mice with antibiotics.

With this work we provided strong evidence that a strict relationship exists between GVB disruption, bacteria dissemination and distant metastases formation. We observed that metachronous CC patients with high presence of PV-1⁺ cells, marker of GVB damage, had high risk to develop distant recurrence. Moreover, the percentage of PV-1⁺ endothelial cells was independent from the number of metastatic regional lymph nodes, which in clinic is currently considered the most important parameter predicting CC distant recurrence. Thus, we proved that PV-1 could be considered as the marker to highlight vasculature permeability suggesting that high presence of PV-1⁺ endothelial cells could be the alert for already vascular dissemination of neoplastic cells. Furthermore, we demonstrated that GVB impairment correlates with bacteria dissemination inside the liver both in metastatic CC patients and tumor bearing mice. Precisely, in mice we proved that bacteria were colonizing the liver before cancer cells

seeding and here they fostered the formation and maturation of a preferential soil defined as premetastatic niche. In conclusion, we functionally proved that this preferential soil was able to foster cancer cells recruitment and macrometastases formation.

In the attempt to reconcile the observations obtained in the different experimental conditions, here we propose a model describing the events occurring during CC progression and involving GVB, gut/liver microbiota, liver premetastatic niche formation followed by macrometastases growth.

According to this model PV-1 detection, thus GVB modification, in colon tumor is the first event which results in altered blood vessels permeability. Then bacteria (2nd Event) invade the blood stream and reach the liver. They could translocate freely or engulfed by immune cells or inside specialized exosomes. Here, bacteria can foster the recruitment of macrophages, neutrophils and inflammatory monocytes, which are supporting the maturation of the premetastatic niche. Upon antibiotic treatment, instead, reduction of bacteria load prevents the formation of the premetastatic niche (3rd Event), giving the strong evidence that they are necessary for hepatic microenvironment modifications. At this stage, mature PMN exert a tissue tropism mechanism on cancer cells, which in turn can disseminate systemically due to GVB breakage (4rd Event). We cannot exclude that at this point other bacteria are disseminating with/inside neoplastic circulating cells as well. We proposed that either GVB disruption could passively provide “the path” for neoplastic cell dissemination or PV-1 could actively interact with NRP-1, expressed on colon cancer cells, allowing cancer cells blood vessels intravasation.

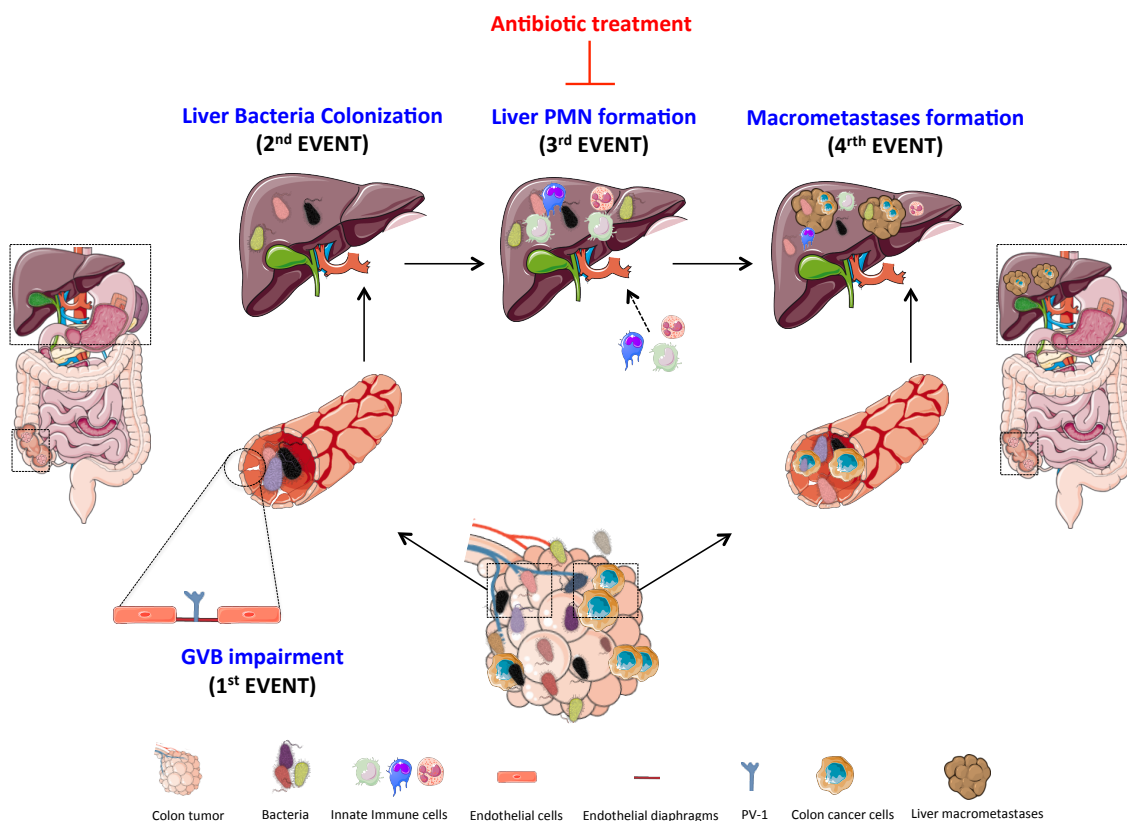


Figure 5-1 Proposed model for distant metastases formation caused by Gut Vascular Barrier impairment.

As previously discussed, other experiments are needed to functionally prove this model. However, we believe that acting both on GVB impairment and cancer-related dysbiosis would be a promising strategy for future CC patient's treatment.

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