A possible IDO1- TSP1 role in breast cancer dormancy

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Doctor of Philosophy

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Summary

Disseminated breast cancer cells have been found in the perivascular niche of lung, brain and bone marrow. Research has suggested that thrombospondin 1 (TSP1), an antiangiogenic protein secreted by endothelial cells, is involved in cancer dormancy. My research aims to clarify the role of TSP1 in cancer dormancy. On the other hand, breast tumours overexpress indoleamine 2,3-dioxygenase (IDO1) which degrades intracellular tryptophan, a key amino acid of TSP1. I aim to investigate whether cancer cells induce endothelial IDO1 expression and therefore limit TSP1 synthesis. I hypothesize that the decrease of TSP1 might enable cancer cell proliferation and angiogenesis.

To evaluate whether endothelial cells can induce cancer dormancy, MDA-MB-231 cells were cultured on the top of an endothelial monolayer or treated with endothelial conditioned medium. Ki67, p21 and cell cycle analysis showed that endothelial cells induce cell cycle arrest in MDA-MB-231 cells but not senescence. ki67 was also decreased when MDA-MB-231 cells were cultured with TSP1. MDA-MB-231 revealed to be more resistant to docetaxel, a breast cancer drug, when pre-cultured with TSP1.

Conditioned medium experiments showed that MDA-MB-231 cells are capable of inducing endothelial IDO1 expression and it also increased tryptophan degradation, which was prevented by siRNA IDO1 knockdown. Interestingly, endothelial TSP1 secretion was revealed to be decreased under low tryptophan concentration. Immunohistochemistry of breast cancer tissue showed that there was a negative correlation between vascular IDO1 and stromal TSP1. IFNγ, a potent inducer of IDO1, showed to be able to induce endothelial IDO1 and a decrease in endothelial TSP1.

Taken together, the data presented here suggests that endothelial cells induce breast cancer dormancy and drug resistance via TSP1. My research also suggests that an IFNγ/IDO1 pathway might decrease TSP1 synthesis leading to cancer cell proliferation and angiogenesis.

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Publications

- **Lopes-Bastos, B**. M., Jiang, W. G. and Cai, J. 2016. Tumour-endothelial cell communications: important and indispensable mediators of tumour angiogenesis. *Anticancer Research* 36(3), pp. 1119-1126.

- He Misi, Wang D, Zou D, Wang C, **Lopes-Bastos B**, Jiang W, Chester J, Cai j. 2016. Re-purposing of curcumin as an anti-metastatic agent for the treatment of epithelial ovarian cancer: in vitro model using cancer stem cell enriched ovarian cancer spheroids. *Oncotarget*

- **Lopes-Bastos B**, Jin L, Ruge F, Owen S, Sanders A, Jiang W, Cai J. A possible non-canonical role of interferon-γ (IFNγ) /indoleamine 2,3-dioxygenase 1 (IDO1)/thrombospondin 1 (TSP1) axis in microvascular niche-dominated growth arrest of breast invasive ductal carcinoma cells (*To be submitted to Oncotarget*).

Abstracts and conference presentations

- **Lopes-Bastos, B. M.**, Jiang, W. G. and Cai, J. Regulatory role of IDO1-TSP1 link in breast cancer dormancy [abstract]. In: China- United Kingdom Cancer (CUKC) conference; 2015 Jul 17-18; Cardiff, Wales, UK: Anticancer research, 2015. Abstract nr 4329.

- **Lopes-Bastos, B. M.**, Jiang, W. G. and Cai, J. Possible role of IDO1/TSP1 in breast cancer metastasis [abstract]. In: European Cancer Congress; 2015 Sep 25-29; Vienna, Austria: European Journal of Cancer; 2015. Abstract nr 1978.

Abbreviations

1-MT: 1-methyl-tryptophan

3-HANA: 3-hydroxyanthranilic acid

3-HAO: 3-hydroxyanthranilic acid 3,4-dioxygenase

3-HK: 3-hydroxykynurenine

5-HT: 5-hydroxytryptamine

Aa: Amino acids

APCs: Antigen-presenting cells

ATF-1: Activating Transcription Factor-1

BAI1: Brain-specific angiogenesis inhibitor

bFGF: Basic fibroblast growth factor

C: Carbon atom

CEACAM: Antigen-related cell adhesion molecule-1

CM: Conditioned medium

CNT: Control

-COOH: Carboxyl group

CTL: Cytotoxic T lymphocyte

CXCR4: C-X-C chemokine receptor type 4

DCIS: Ductal carcinoma in situ

DCs: Dendritic cells

DTCs: Disseminated tumour cells

EAA: Essential amino acids

ECM: Extracellular matrix

ECS: Elogin/cullin/SOCS

EDG2: Lysophosphatidic acid receptor

EGFR: Epidermal growth factor receptor

FBS: Fetal Bovine Serum

FMN: Flavin mononucleotide

GASs: IFNγ-activated sites

GCN2: General control nonderepressible 2

H: Hydrogen

HDAC: Histone deacetylase

HECV: Human endothelial vascular

HMVECad: Human Microvascular Endothelial Cells, adult dermis

IDO1: Indoleamine 2,3-dioxygenase 1

IDO2: Indoleamine 2,3-dioxygenase 2

IFNγ: Interferon-gamma

IGF1: Insulin-like growth factor 1

IL-1: Interleukin 1

IRF1: IFN regulatory factor 1

ISREs: IFNγ-stimulated response elements

ITAMs: Immunoreceptor tyrosine-based activation motifs

JAK: Janus kinase

KATs: kynurenine aminotransferases

Kcat: Catalytic rate

KMO: kynurenine 3-monooxygenase

KMO: kynurenine 3-monooxygenase

KYNA: kynurenic acid

LAP: Latency-associated peptide

LCIN: Lobular carcinoma in situ

LGM: Low glucose medium

LRP1: Lipoprotein receptor-related protein

NEAA: Nonessential amino acids

NF- κB: Nuclear factor κB

-NH2: Amino group

NM: Normal medium

NO: Nitric oxide

NR4A2: Orphan receptor 4A2

PGE2: Prostaglandin E2

PI3K: Phosphoinositide 3-kinase

PKCδ: Protein kinase Cδ

proto-IDO: Proto-indoleamine 2,3-dioxygenase

QUIN: Quinolinic acid

R: Side-chain specific to each amino acid

RFK: Arginine-phenylalanine-lysine

SDF1/CXCL12: Stromal cell-derived factor 1

SNP: Single nucleotide polymorphism

SOCS3: Cytokine signalling 3

TDO: 2,3-dioxygenase

Th: T helper cells

TLR: Toll-like receptor

TNFα: Tumour necrosis factor α

TPH: Tryptophan hydroxylase

Treg: Regulatory T-cells

TRP: Tryptophan

TSP1: Thrombospondin 1

TSPs: Thrombospondins

TSR: Thrombospondin type 1 repeat

TSRs: Structural homology repeats

TTS: Tryptophanyl-tRNA synthetase

WT1: Wilms' tumour suppressor gene

Chapter I: Introduction

1.1 Breast cancer

Breast cancer is the most frequent female cancer and the second cause of cancer related death for women worldwide (Druesne-Pecollo et al., 2012; Ferlay et al., 2015). In the US alone, the cost of cancer care in 2010 was 124.57 billion US dollars, among of which 16.50 billion US dollars was spent on female breast cancer care (Mariotto et al., 2011). Yet, breast cancer is a highly heterogeneous disease, at both pathology and biological level, and the outcome is greatly influenced by the stage of the disease at diagnosis (Ferlay et al., 2015; Verma et al., 2012).

1.1.1 Epidemiology, risk factors and prevention

Breast cancer accounted for 1.67 million new cases worldwide in 2012, about 25% of all cancer cases. The incidence rate varies worldwide. For instance, in the Middle Africa and Eastern Asia there were 27 cases diagnosed as breast cancer per 100,000 women, while it raises to 96 in Western Europe. There was also less prevalence of breast cancer cases in women from less developed countries, as shown in Figure 1 (Ferlay et al., 2015).

Breast cancer was responsible for 522.000 deaths in 2012 ranking as the fifth deadliest cancer overall. In undeveloped countries breast cancer is the leading cause of cancer related deaths in women (about 14.3% of total), while it was the second cause of death in more developed countries (15.4%) after lung cancer (Ferlay et al., 2015).

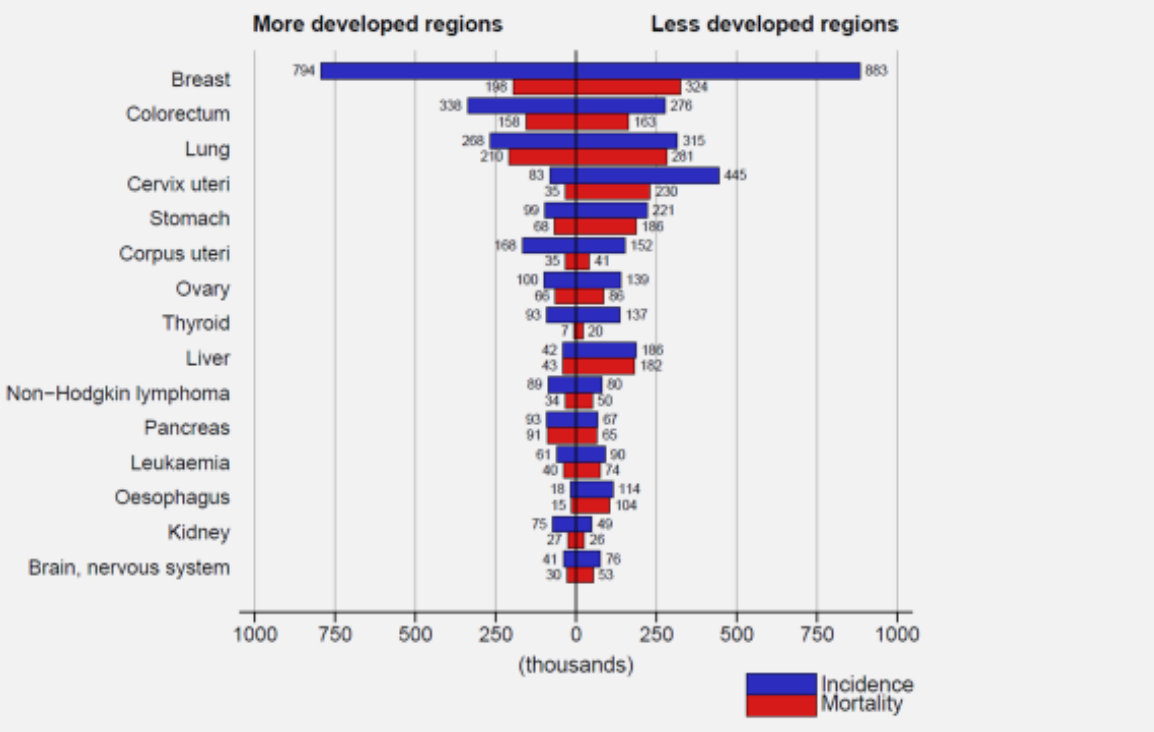


Figure 1: Estimated numbers of new cancer cases and deaths in more developed and less developed countries. Numbers are represented in thousands (Modified from (Ferlay et al., 2015).

Breast cancer is a major public health burden and due to the fact that incidence is increasing in most countries, the number of new cases will keep rising for the next 20 years (Arnold et al., 2015; Colditz and Bohlke, 2014; Eccles et al., 2013; Rahib et al., 2014). In most countries, the number of females with breast cancer risk factors has been on the rise. The risk factors include the lower age of the first menstrual period, first pregnancy at a late age, fewer pregnancies, shorter or no breastfeeding periods, and late age of menopause. Many life styles, such as alcohol consumption, obesity, hormone replacement therapy and inactivity are correlated with the incidence of breast cancer (Colditz and Bohlke, 2014). Genetic inherent-related risk factors for breast cancer has also increased in the past years. For instance, the breast cancer 2 (BRCA2) mutation has been estimated to have increased fourfold in Iceland over the last century (Tryggvadottir et al., 2006). Interestingly, the same study estimated that the incidence of

sporadic breast cancer in Iceland also increased fourfold in women under 70 years old (Tryggvadottir et al., 2006), suggesting that both familial and non-familial breast cancer risk factors have increased.

In 2002, a study estimated that the incidence of breast cancer would drop from 6.3 to 2.7 per 100 women under the age of 70 in developed countries, if women had more children and breastfed for longer periods (Collaborative Group on Hormonal Factors in Breast Cancer, 2002). Based on the effects of the current population growth, social, economic and health condition, current views suggest that women are not encouraged to increase the number of pregnancies or have earlier pregnancie However, breastfeeding may have preventative effects on breast cancer. Oestrogen is responsible for most reproductive risk factors for breast cancer, which can be reduced by an early oophorectomy, or by the inhibitors of oestrogen receptors, such as tamoxifen or raloxifene (Cuzick et al., 2013), or the inhibition of oestrogen synthesis [e.g., exemestane (Goss et al., 2011) and anastrozole (Brown, 2014; Cuzick et al., 2014)] Tamoxifen and raloxifene, which are cheap, safe and effective in antagonising oestrogen, are currently used in the NHS as preventive drugs for females with high risk of breast cancer. Prophylactic mastectomy is another preventive measure, although it is mostly intended for women from high risk families, for example families with inherited BRCA1 mutation (Domchek et al., 2010).

The life style and environment are other important variables, and it has been estimated that 26.8% of new breast cancer cases in the UK were caused by these two variables (Parkin et al., 2011). So, a healthy and balanced diet, exercise, maintenance of a healthy weight, quitting smoking and drinking alcohol are considered preventive measures.

1.1.2 Histological classification and biomarkers

Breast cancer can be catalogued into two major groups, carcinoma *in situ* and invasive carcinoma. The *in situ* carcinoma is subdivided into ductal carcinoma *in situ* (DCIS) when there are abnormal cells in the lining of the breast ducts or lobular carcinoma *in situ* (LCIS) when there are abnormal cells in the breast lobules. DCIS is the most common *in situ* breast carcinoma and has five subgroups of tumours (comedo, cribiform, micropapillary and solid). The five subgroups are very heterogeneous between them (Malhotra et al., 2010). The invasive carcinomas are also a heterogeneous group which is subdivided into infiltrating ductal, invasive lobular, ductal/lobular, mucinous, tubular, medullary and papillary carcinomas. Infiltrating ductal carcinoma accounts for 70 to 80% of the invasive carcinoma (Li et al., 2005). The differentiation level of infiltrating ductal carcinoma is graded according to the glandular formation, mitotic index and nuclear pleomorphism (Lester et al., 2009). There are three ranks of differentiation grade including grade 1 (well-differentiated), grade 2 (moderately differentiated) and grade 3 (poorly differentiated).

The classification described above has been used for decades but merely based on the histology of the tumour. The advance in breast cancer therapy and in particular the recent surge of personalised medicine has increased the necessity to identify new biomarkers including molecular markers to stratify the patients with breast cancer by improving the knowledge of the tumour according to its biological behaviour, route and course to progression, recurrence risk, sensitivity intervention and therapy response.

Breast cancer is subdivided according to its ER, PR and HER2/neu profile. These markers are well accepted for the characterization of infiltrating ductal carcinoma (Harris et al., 2007; Malhotra et al., 2010). As these markers can predict which patients are likely to respond to specific therapies, it is a helpful tool for clinical decision (Payne et al., 2008; Rakha et al., 2010). Ki67 and p53 are other two markers that have been extensively studied as prognostic and predictive tools in breast cancer (Nishimura et al., 2010; Ohara

et al., 2016). However, techniques and scoring methods have to be standardised to be used in everyday practice (Urruticoechea et al., 2005).

Breast cancer is also graded according to the TNM staging system, which evaluates the tumour on the basis of primary tumour (T), lymph-node (N) and metastasis (M). It was first established by Pierre Denoix in the 1940s, and it was revised by the American Joint Committee on Cancer and International Union against Cancer in 2002 (Table 1). The TNM classification of a tumour is based on a series of clinical examinations, including radiological imaging, biopsies and biochemical analysis (Greene and Sobin, 2002).

1.1.3 Current therapy

An appropriate evaluation of the tumour is crucial for the clinicians to choose the best treatment options. There are standard treatments that are currently available which includes surgery, radiation therapy, chemotherapy and hormone therapy. However, there are clinical trials that aim to test new drugs that target both conventional and new markers or innovative strategies, such as new devices and immunotherapies.

There are different types of surgery that can be performed in breast cancer patients, including lumpectomy, the removal of the lump, mastectomy, the removal of the breast or radical mastectomy, the removal of the breast and some axillary lymph nodes. The type of surgery executed depends on the stage of the tumour, and each one of the approaches carries different risks (Sharma et al., 2010).

Radiation therapy uses high energy X-rays or gamma rays to eliminate cancer cells. This method can be applied to the tumour before or after surgery. It is usually performed after the surgery and in particular for those with nodal involvement in order to kill any remaining cancer cells and it can last for up to seven weeks (Sharma et al., 2010).

The use of chemotherapy agents to eliminate cancer cells is known as chemotherapy. Chemo agent selection is highly specific to the tumour and stage of the

tumour and biomarkers. Age, medical history and overall health are also taking into consideration (Sharma et al., 2010). Chemotherapy is usually done in cycles. The patient can be treated for a period of time, then a recovery period, followed by another treatment. Chemotherapy administrated after surgery is called adjuvant therapy and studies have shown that delaying adjuvant chemotherapy by over 12 weeks increases the risk of recurrence and a decrease in survival (Lohrisch et al., 2006). There are several drugs available to treat early stage breast cancer patients, such as doxorubicin, cyclophosphamide, fluorouracil, paclitaxel and methotrexate. While the advanced disease is treated with a combination of gemcitabine and taxanes (Ferguson et al., 2007; Gudena et al., 2008). For the past two decades, chemotherapy prior to surgery, one type of neoadjuvant therapy, has become a choice for a majority of the patients. This has been shown to reduce the risk of recurrence, improve the survival of the patients and reduce the scale of surgical intervention (Kaufmann et al., 2010; Mamounas et al., 2012; Mieog et al., 2007).

Another type of cancer therapy involves removing or blocking the effect of hormones. Therefore, this kind of therapy is only used in a patient with hormone-receptor positive (ER or PR). Oestrogen is mainly produced in the ovaries and it stimulates the growth of some breast tumours. One strategy to reduce oestrogen production is to remove the ovaries by surgery (Coombes et al., 2007; Rao and Cobleigh, 2012). Oestrogen production can also be inhibited by aromatase inhibitors, such as anastrozole, exemestane or letrozole. These compounds inhibit the aromatase enzyme, which is responsible for the conversion of androgens to oestrogen (Miller and Larionov, 2012). Oestrogen receptors can also be blocked by tamoxifen (Coombes et al., 2007; Rao and Cobleigh, 2012), which directly binds to it and blocks the transcriptional activity of this receptor (Ali et al., 2016). Tamoxifen and aromatase inhibitors are effective therapies and they are less harmful when compared to chemotherapy. Although these therapies

improve the quality of life of ER-positive breast cancer patients, a large number of patients develop drug resistance (Jan 2012).

Table 1: TNM grading system for breast cancer. A- Definition of the TNM categories. B- Stage grouping according to the categories. (Information from (American_Joint_Committee_on_Cancer, 2002).

Definition of categories	
Primary tumour (T)	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: Intraductal carcinoma, lobular carcinoma in situ, or Paget's disease of the nipple with no tumour
T1	Tumour 2 cm or less in greatest dimension
T1a	0.5 cm or less in greatest dimension
T1b	More than 0.5 cm but not more than 1 cm in greatest dimension
T1c	More than 1 cm but not more than 2 cm in greatest dimension
T2	Tumour more than 2 cm but not more than 5 cm in greatest dimension
T3	Tumour more than 5 cm in greatest dimension
T4	Tumour of any size with direct extension to chest wall or skin
T4a	Extension to chest wall
T4b	Edema (including peau d'orange) or ulceration of the skin of breast or satellite skin nodules confined to same breast
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
Lymph node (N)	
Nx	Regional lymph nodes cannot be assessed (e.g. previously removed)
N0	No regional lymph node metastasis
N1	Metastasis to movable ipsilateral axillary lymph node(s)
N2	Metastasis to ipsilateral axillary lymph node(s) fixed to one another or to other structures
N3	Metastasis to ipsilateral internal mammary lymph node(s)
Distant Metastasis (M)	
Mx	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis (includes metastasis to ipsilateral supraclavicular lymph node(s))

Stage grouping			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T0	N1	M0
	T1	N1*	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	Any N	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M1

*Note: The prognosis of patient with N1a is similar to that patient with pN0

1.2 Metastasis

The formation of metastasis is a complex multi-step cascade where cancer cells migrate from the primary lesion to a distant organ. To metastasize, cancer cells have to go through a specific sequence of events: local invasion (including the basement and extracellular matrix), trans-endothelial migration into the bloodstream (intravasation), survival in the circulation, trans-endothelial migration into a new site (extravasation) and colonization of a distant tissue (Cristofanilli et al., 2004; Nguyen et al., 2009; Yang and Weinberg, 2008). They currently known that the primary tumour releases cancer cells into the circulation at early stages (Hüsemann et al., 2008) indicating that metastatic process may well start very early during cancer development and progression. Each one of these steps has natural barriers that cancer cells have to overcome, the metastatic cancer cells also have to fight against the established organisation and homeostasis of the target tissue (Gupta and Massagué, 2006). Thus, the metastatic cascade is highly inefficient. It has been estimated that 99.98% of disseminated tumour cells (DTCs) die before it forms a metastasis (Gupta and Massagué, 2006). Nevertheless, experimental studies have shown that even normal epithelial cells might be able to invade new tissues (Podsypanina et al., 2008; Rhim et al., 2012), less than 3% of cancer cells can survive in the new site (Kienast et al., 2010; Luzzi et al., 1998). Therefore, it is recognised that colonisation is the rate-limiting step in the metastatic process (Vanharanta et al., 2013). Cancer cells may face several fates once they reach the target organ: 1- cancer cells can die through spontaneous apoptosis or by the immune system; 2- DTCs can remain dormant; or 3- cancer cells can proliferate and develop early relapse.

1.2.1 Breast cancer metastasis pattern

Clinical observations have shown that different types of tumours have distinct patterns of metastatic spread, one example being their different preferential metastatic sites also known as organ tropism (Chiang and Massagué, 2008). This shows the importance of the microenvironment in developing a new metastasis. Breast cancer often

metastasizes to the bone, lung, liver and brain, which eventually leads to impaired function of those organs. Interestingly, the pattern of metastatic spread depends also on the subtype of the breast cancer (Kennecke et al., 2010). For instance, ER positive tumours exhibit initially the lowest incidence rate of metastasis, but the rate increases up to 40% after five years of follow-up. The preferable metastatic site of this subtype is the bone and the least is the brain. However, the triple-negative subtype has the worst prognosis, having a high metastatic incidence rate within the first and second year after diagnosis (Dent et al., 2009; Kennecke et al., 2010). Brain and lung are the organs more often affected by triple-negative tumours. Another aggressive breast cancer subtype is the HER2 positive tumours. Anti-HER2 therapy has improved the prognosis and survival of patients with HER-2 breast tumours (Kennecke et al., 2010). However, the blood-brain barrier restricts the penetration of transtuzumab (anti-HER2 antibody) to the brain, therefore limiting its efficacy in the brain (Pestalozzi and Brignoli, 2000; Stemmler et al., 2007) and trastuzumab-treated patient registered an increase in the incidence of brain metastasis (American_Cancer_Society_Cancer_Facts&Figures_2013", 2013; Tabouret et al., 2012; Wen and Loeffler, 1999).

The mechanism underlying the pattern of metastatic spread of each tumour type or subtype remains unclear (Valastyan and Weinberg, 2011). In 1889, Sir Stephen Paget suggested the “seed-and-soil” theory, which proposed that disseminated tumour cells (seeds) lead to the formation of metastasis once they reach a tissue (soil) that allow them to survive and proliferate(Paget, 1889). Since then, many genes have been identified as beneficial to metastasis formation increasing metastasis in specific sites (Bos et al., 2009; Hart and Fidler, 1980; Kang et al., 2003; Minn et al., 2005; Nguyen et al., 2009; Pencheva et al., 2012; Raz et al., 1980; Vanharanta et al., 2013), supporting the Paget hypothesis. The blood-metastasis can only explain this phenomenon in some tumour types, such as colon cancer. Colon cancer metastasis is more often observed in the liver and then in the lung via circulation (Gupta and Massagué, 2006). Breast cancer

metastasis are more common in bone (70.6%), followed by liver (54.5%) and lung (31.4%) (Savci-Heijink et al., 2015), which seems not fit into the previous blood stream theory.

It has been shown a link between chemokines and metastasis sites (Klein et al., 2002). For example, the stromal cell-derived factor 1 (SDF1/CXCL12), a cytokine secreted by bone, lungs, liver, lymph nodes and stromal fibroblasts in tumours, induced invasion and migration towards that SDF1 producing tissue by breast cancer cells that expressed the SDF1/XXL12 receptor, C-X-C chemokine receptor type 4 (CXCR4) (Müller et al., 2001). Interestingly, another study showed that high levels of CXCL12 and Insulin-like growth factor 1 (IGF1) in the bone promote survival of triple-negative breast cancer cells that highly colonises to the bone, compared to the lung and liver (Zhang et al., 2013). A better understanding of the site-specific signals responsible for attracting breast cancer cells could lead to more target therapies.

1.2.2 Cancer dormancy

As mentioned before, the treatment of metastasis is the main challenge of cancer treatment (Uhr and Pantel, 2011; Willis, 1952) due to the early dissemination of cancer cells from the primary tumour (Klein, 2011). The adjuvant therapy, which aims to prevent the relapse of the primary tumour and the development of metastasis, is often not efficient (Goss and Chambers, 2010) due to the ineffectiveness against disseminated tumour cells (DTC) (Klein, 2013). The biology of cancer cells in the primary tumour and DTCs is very different. DTCs can go through a period of dormancy and be resistant to cancer drugs (Aguirre-Ghiso, 2007; Goss and Chambers, 2010; Klein, 2011, 2013). For instance, most chemotherapeutic drugs target metabolic pathways that are needed for DNA replication, and only effective to proliferating cells, failing to eliminate DTCs (Mitchison, 2012).

Clinical dormancy is common in several solid tumours, such as renal, breast, thyroid, prostate and melanoma (Uhr and Pantel, 2011). It is defined as the disease-free period of time that goes from the treatment of the primary tumour to the relapse of metastasis (Demicheli et al., 2005; Klein, 2011; Uhr and Pantel, 2011).

Breast cancer is easier to treat when detected at an early stage, whilst the metastasised cancers are associated with a poor prognosis (Cardoso et al., 2009). However, a study showed that around 10% of patients who were metastasis-free at the diagnosis stage developed secondary tumours after breast cancer treatment (Noh et al., 2011). Thus, uncovering the molecular mechanism behind the switch from clinical dormancy to the development of metastasis is currently one of the main focuses of breast cancer research.

In the 1940s, Willis first time proposed cancer dormancy. In the 1950s Hadfield described it a transitory growth arrest (Willis, 1952) and mitotic arrest (HADFIELD, 1954). Current cancer dormancy is divided into three groups. Cellular dormancy refers to single or small groups of DTCs that are in the quiescent state, While angiogenic dormancy, is the situation the total volume of the micrometastasis is kept constant by a balance between dividing and apoptotic cells due to a lack of a good vascularisation system. In the immune-mediated dormancy, DTCs are proliferating but the total volume of the micrometastasis is kept constant due to the action of the immune system (Aguirre-Ghiso, 2007). Different types of cancer dormancy may occur at the same time and some molecular mechanisms of the different cancer dormancy might be overlapped.

It is estimated that only 3% of single dormant cells are able to grow into a micrometastasis, while only about 0.02% of dormant cells grow into clinically detectable metastasis (Luzzi et al., 1998). Cancer cells can come out of dormancy after years or even decades of the diagnosis of the primary tumour leading to a late relapse of the tumour. The cause of the regrowth of dormant cancer cells is not clear, but many factors

that control cancer dormancy, such as genetic changes, angiogenesis switch, the immune system and microenvironment, might be responsible for it.

Genetic changes

Genomic analysis showed that tumour cells from metastatic sites are homogeneous, while dormant DTCs from patients with no detectable metastasis were very heterogeneous (Klein et al., 2002; Klein et al., 1999; Schardt et al., 2005). Tumour genomic aberrations include mutations, loss of heterozygosity and polymorphisms. These findings suggest that DTCs might be originated from early stages of tumour progression and DTCs accumulate genetic changes over time that are required to metastasise. Although some studies have identified genes and molecular pathways that might be involved in tumour dormancy and the escape of DTCs from dormancy, further research is needed to identify a genetic signature that explains the outgrowth of dormant micrometastasis.

Almog and colleagues developed a tumour dormancy *in vivo* model for breast carcinoma, liposarcoma, glioblastoma and osteosarcoma (Almog et al., 2009). The authors compared the gene expression pattern of dormant tumours and switched fast-growing tumours by genome-wide transcriptional analysis. Their results showed that, in all tumour types studied, switch fast-growing tumours exhibited a downregulation of the expression of thrombospondin, an angiogenic inhibitor, and a decrease of the sensitivity to angiostatin, another natural angiogenic inhibitor (Almog et al., 2009). Their results suggested that tumour vascular niche plays a key role in the escape of dormancy.

Other molecular pathways, such as tissue inhibitor of metalloproteinase-3, insulin-like growth factor receptor, KISS1 metastasis-suppressor, epidermal growth factor receptor (EGFR), have also been identified in the cellular processes of cancer dormancy (Steeg et al., 2003).

MicroRNAs have also been implicated in cancer dormancy. For instance, 16 microRNAs were found to be overexpressed in dormant tumours and their downregulation was correlated with the switch of dormant status to growing malignancies (Almog et al., 2012).

Epigenetic alterations are another type of gene regulation in both dormant and proliferate cancer cells is. For instance, the expression of tissue inhibitor of metalloproteinases-3 (TIMP3) and E-cadherin (CDH1) was increased in dormant ovarian cancer cells and decreased in ovarian cancer cells transitioning to active growth. Both genes can be through promoter methylation (Lyu et al., 2013). Both TIMP3 and CDH1 also function as anti-angiogenic molecules (Qi et al., 2003; Yi Kim et al., 2007) which suggest again that angiogenesis may be a central cellular mechanism in the escape of cancer dormancy.

Angiogenesis

Tumour growth is dependent on angiogenesis, which relies on a balance between pro- and anti- angiogenic factors. Rapidly growing cancer cells rely on their easy access to nutrients and oxygen, which is provided by angiogenesis (Folkman, 1990). Dormant micrometastases, however, have been shown to be avascular (Hanahan and Folkman, 1996). Thus, micrometastases have to increase the secretion of pro-angiogenic factors and/or downregulate anti-angiogenic factors in order to grow and regrow.

Glioma cells, angiogenic-dependent cancer cells, are much earlier to form tumours in mice than angiogenic-independent cancer cells, osteosarcoma cells. Intriguingly, there is no difference of proliferation rates between these two types of cancer cells, indicating that the tumour formations are related to the angiogenesis-induction capacity of cancer cells rather than the quiescence mechanisms (Naumov et al., 2006b). However, cancer cells can evolve to an angiogenic phenotype, this process is known as

angiogenic switch, which is characterised by an increased in the expression of pro-angiogenic factors and a decrease in anti-angiogenic factors (Naumov et al., 2006a).

In a series of *in vivo* and *in vitro* assays, Ghajar and collaborators showed that the vascular niche also might be involved in maintaining single cell dormancy (Ghajar et al., 2013). They demonstrated that a single breast cancer cell did not proliferate when in close association with a stable microtubule, while it divided when in the microenvironment of a sprouting vessel (Ghajar et al., 2013). They attributed this phenomenon, at least in part, to the presence of a high concentration of thrombospondin 1 (TSP1) in the microenvironment of the stable vessel.

Immune system

The hypothesis of cancer immune-editing mechanism states that tumour development has to go through 3 stages: elimination, equilibrium and escape (Dunn et al., 2002). The innate and adaptive immune system can detect and eradicate cancer cells. If this mechanism fails, cancer cells might reach an equilibrium for a long period of time. Some of the cancer cells can even escape the immune system (Dunn et al., 2002).

The mechanism of how the immune system induces cancer dormancy is not established. Nevertheless, the following studies give some clues.

Patients with breast cancer, who had DTCs in the bone marrow, had a higher number of CD4 T cells and CD56+CD8+ cells, when compared to healthy subjects (Feuerer et al., 2001). Also, depletion of CD4+ and CD8+ T cells in mouse models led to the escape of cancer cells from dormancy state (Teng et al., 2008). Interestingly, a study showed that cancer cells could overcome the effect of the immune system by overexpressing B7 homolog 1 (B7-H1), inhibiting the activation of T cells and the cytotoxic T lymphocyte (CTL) response (Ge et al., 2009).

Also, many immune cells within the tumour microenvironment, including T cells and macrophages, secrete many mitogens and pro-angiogenic factors, cytokines and MMPs (Ribatti and Crivellato, 2009). These molecules might help cancer cells to escape dormancy and induce angiogenesis. Despite these interesting observation, the interplay between immune cells, bone marrow, microenvironment and angiogenesis is far more complicated. For example, Bonapace and collaborators have shown that another targeting chemokine, CCL2, whilst can suppress the occurrence of metastasis, the withdraw of anti-CCL2 agents led to a dramatic increase in metastasis and accelerated death (Bonapace et al., 2014). The effects of the interruption of anti-CCL2 therapy were attributed to the release of monocytes from the bone marrow and cancer cells from the primary tumour.

Microenvironment

Once cancer cells reach a new tissue and survive in that microenvironment, they have to go through a dormancy state before a possible proliferative state (Heyn et al., 2006; Murrell et al., 2014; Naumov et al., 2003). Interestingly, the higher numbers of DTCs are not necessarily correlated to the metastasis incidence in some organs than the others (Aguirre-Ghiso, 2007). For instance, gastric cancer patients have a high number of DTCs in the bone marrow. These DTCs have been shown to be non-proliferative, and bone marrow metastasis are rare in this patients (Aguirre-Ghiso, 2007; Hüsemann et al., 2008; Klein, 2011; Pantel et al., 2009; Schardt et al., 2005). These findings suggest a crucial role of the microenvironment in the growth of DTCs. For this reason, the different microenvironments have been classified as dormancy-permissive or dormancy-restrictive (Aguirre-Ghiso et al., 2013; Bragado et al., 2013; Bragado et al., 2012).

Normal stromal cells in the bone marrow secrete BMP7, which induced ERK inhibition, p38 activation, leading to and the dormancy of prostate cancer cells that were injected into the bone (Kobayashi et al., 2011). Knockout of BMPR2, the BMP7 receptor,

caused the injected prostate cancer escaping from dormancy (Kobayashi et al., 2011). Consistently, bone metastasis in prostate patients was inversely correlated with BMPR2 expression (Kobayashi et al., 2011), suggesting that the loss of BMPR2 might be a mechanism adapted by prostate cancer cells to escape dormancy in the bone marrow. The inhibition of BMP4, another BMPR2 ligand, secreted by lung, had a similar escaping effect on dormant breast DTCs in the lungs, indicating a similar role of BMPR2 in breast cancer cells. Other receptors in breast cancer cells have been linked to dormancy regulation, such as the lysophosphatidic acid receptor (EDG2) (Marshall et al., 2012).

Other studies have shown that fibroblasts and endothelial tip secrete periostin, which, promote the cancer cells escaping of dormancy (Ghajar et al., 2013; Malanchi et al., 2011).

Although the mechanisms of how cancer cells escape the dormancy still not clear, the above studies provide insight into the possible molecular mechanisms.

1.3 Tumour stroma

1.3.1 Cell-to-cell communication and angiogenesis

Cell-to-cell communication is important for proper cell coordination and plays a central role in the development and function of animal tissues and organs. The failure of cell-to-cell communication has been demonstrated to lead to the development of diseases, for instance, immune disorders (Allen et al., 1993) and heart failure (De Mello, 1996). Cells have different means of communication, cell communication can be through soluble factors, adhesion contacts, cell junctions or through vesicles, which can activate their own receptors (autocrine), reach a neighbouring cell (paracrine) or target distant cells (endocrine) (Camussi et al., 2010).

Tumours are formed by a collection of different cell types, such as cancer cells, fibroblasts and macrophages. Stromal cells, the non-cancer cells residing within the tumour stroma, create a perfect microenvironment so cancer cells can grow to allow

tumour progression. Cell-to-cell communication is fundamental for cancer cells to attract and also to transform stromal cells. The inhibition of the cell communication within tumour microenvironment has been shown to reduce growth and aggressiveness of the tumour (Lin et al., 2013; Matsuda et al., 2014).

Another type of stromal cells present in the tumour microenvironment are the endothelial cells. Tumour growth is dependent on a proper network of vessels; it has been shown that tumours do not grow beyond 2-3 mm in diameter without the proper vasculature support. Tumour Induced-angiogenesis – the formation of new blood vessels from an existing one, provides nutrients and oxygen to cancer and the other stromal cells allowing at the same time the removal of toxic metabolites. Tumour-endothelial cell communication is crucial to angiogenesis and the inhibition of those signals has shown to reduce tumour vasculature and tumour size (Vasudev and Reynolds, 2014).

Tumour cells can indirectly communicate with endothelial cells via soluble factors, adhesion receptors, gap junctions or vesicles, in order to induce angiogenesis, Figure 2 (reviewed in (Lopes-Bastos et al., 2016). Tumour cells can also attract and transform stromal cells, which in turn induce angiogenesis via secreting proteases leading to remodelling the extracellular matrix, or by other ways, such as changing the pH, temperature, or nutrient and oxygen availability, which have been shown to modify endothelial phenotype, Figure 2 (reviewed in (Lopes-Bastos et al., 2016)).

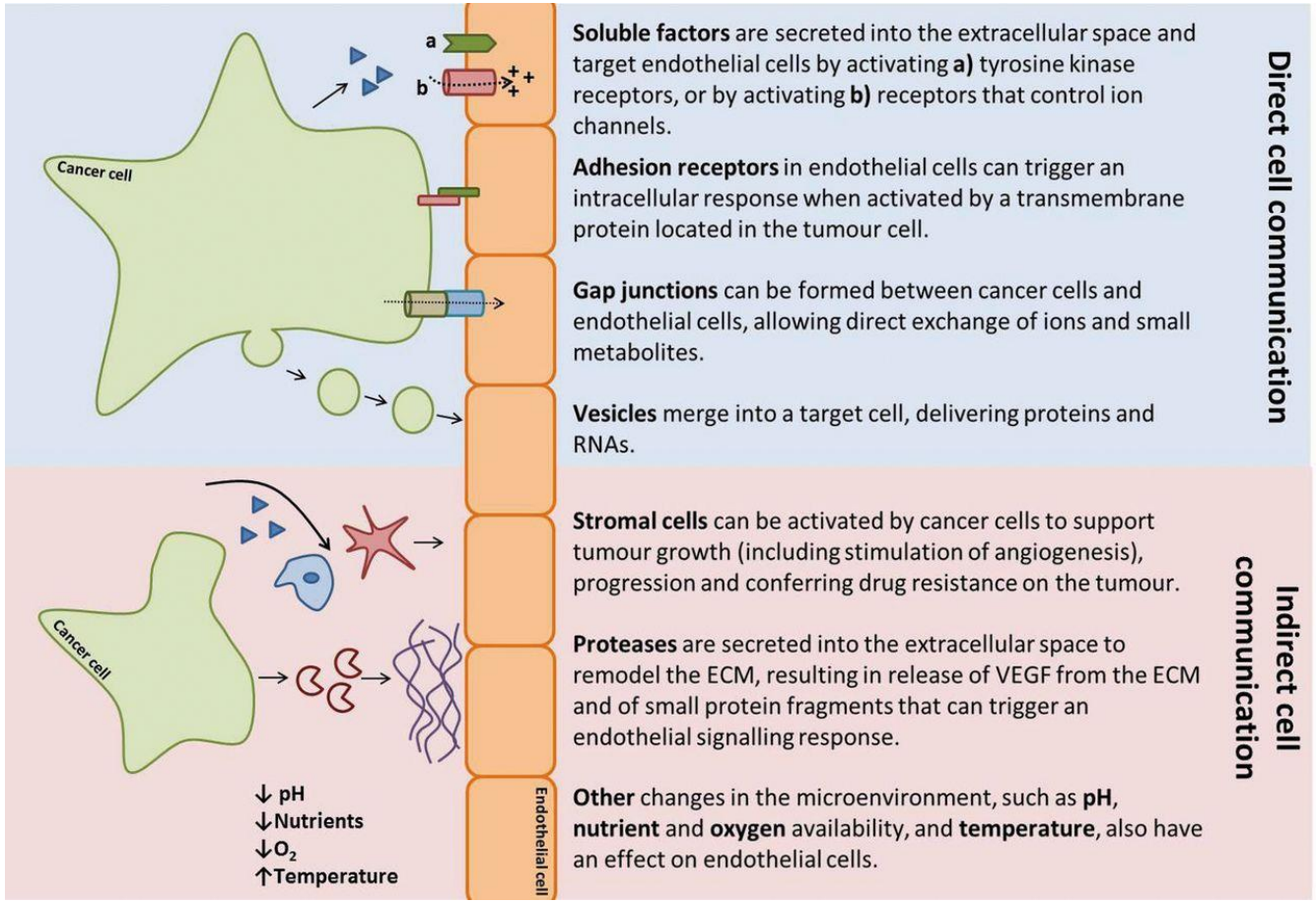


Figure 2: Schematic representation of the different types of cell-to-cell communication in order to cancer cells induce angiogenesis (Lopes-Bastos et al., 2016).

1.3.2 Extracellular matrix (ECM)

The microenvironment of a tissue or organ is defined by their specific cell types and vesicles, proteins and other molecules secreted by those cells into the extracellular space. The denominated extracellular component is a tissue-specific extracellular matrix (ECM). ECM contains proteins such as fibronectin, laminins, collagens, glycoproteins and proteoglycans. These proteins give support to the cells and maintain the integrity and sustainability of the tissue. ECM is extremely important in embryonic development, but it is also essential in the homoeostasis of adult tissue. Although ECM provides a structure to tissues, its role goes beyond that. For instance, ECM proteins bind and activate surface receptors, which can promote essential cellular signalling for key cell behaviour, including cell growth/ survival and mobility (Chong et al., 2012). ECM can also bind soluble growth factors and other molecules, and modulate distribution and activation of these molecules, and their availability to cells (Hynes, 2009).

The tumour ECM has been described as abnormal in composition and architecture. Cancer cells, but also infiltrating T-cells, macrophages and fibroblasts contribute to its composition, by matrix deposition and matrix modification. It is recognised that the tumour ECM is a crucial factor in cancer progression. For instance, tumours are composed of different subpopulations of cancer cells that are genetically heterogeneous. A study using a breast cancer model, demonstrated that a minor cancer cell subpopulation was responsible for the sustained growth of the tumour (Marusyk et al., 2014). The minor subpopulation of cells modulated the tumour microenvironment, including the organisation of the collagen (Marusyk et al., 2014).

1.3.2.1 Thrombospondin family

Thrombospondins (TSPs) are secreted proteins and are part of the ECM. However, TSPs do not have a role as a structural protein like most ECM proteins do. TSPs are denominated matricellular proteins with a wide range of functions. TSPs are

able to interact with several cell surface receptors but also with other ECM proteins, such as growth factors, proteases and collagens (Cockburn and Barnes, 1991; Greenaway et al., 2007; Gupta et al., 1999).

The TSP family is composed of 5 members divided into two groups based on their domain structure. The TSP group A includes TSP-1 and TSP-2, while TSP-3, TSP-4 and TSP-5 belong to group B (Adams, 2001; Adams and Lawler, 2004, 2011). TSP proteins in lower organism can be composed of only one protein or by multiple depending on the organism, but these proteins are very similar to human TSPs from the group B. Interestingly, the development of the vascular system coincides with the appearance of TSPs from group A, and these proteins evolve even more with the development of the cardiovascular and immune system (Bentley and Adams, 2010; Tucker et al., 2013) suggesting an important role of TSP group A in these processes.

TSPs have a wide range of roles involved in many physiologic and pathological cellular processes, such as regulation of inflammation (Frolova et al., 2010; Lopez-Dee et al., 2011; Mustonen et al., 2012; Stenina-Adognravi, 2013; Vanhoutte et al., 2013), maintenance of the integrity and function of the myocardium (Chatila et al., 2007; Cingolani et al., 2011; Frolova et al., 2012; Lynch et al., 2012; Roberts et al., 2012; Schroen et al., 2004; Swinnen et al., 2009; van Almen et al., 2011), fibrosis regulation (Sweetwyne and Murphy-Ullrich, 2012), synaptogenesis (Risher and Eroglu, 2012), immune response modulation (Martin-Manso et al., 2012; Miller et al., 2013), angiogenesis and cancer progression (Lawler and Lawler, 2012).

1.2.2.1.1 Thrombospondin 1 and angiogenesis

Judah Folkman proposed in 1971 the existence of endogenous angiogenic inhibitors in order to counterbalance known pro-angiogenic molecules (Folkman, 1971). In 1990, thrombospondin 1 (TSP1) became the first known endogenous inhibitor of angiogenesis (Good et al., 1990). TSP1 is a 142,000 KDa glycoprotein that was first

isolated from human platelets (Lawler et al., 1978). Several cell types secrete TSP1, such as endothelial cells, adipocytes, smooth muscle cells, fibroblasts, monocytes, macrophages and also malignant cells (Naganuma et al., 2004; Wight et al., 1985). A fragment of TSP1, identified in hamster kidney cells (BHK21/cl12), was demonstrated to inhibit cell migration of endothelial cells and angiogenesis in the rat cornea. In the same year, several independent groups reported that TSP1 inhibits endothelial cell proliferation (Bagavandoss and Wilks, 1990) and also antagonizes the effects of basic fibroblast growth factor (bFGF) on endothelial cells (Taraboletti et al., 1990). Since then, many other proteins have been identified that contain an amino acid sequence of TSP1 and TSP2 proteins called the thrombospondin type 1 repeat (TSR) sequence, such as the brain-specific angiogenesis inhibitor (BAI1) (Kaur et al., 2003). The identification of several endogenous inhibitors of angiogenesis led to the notion that angiogenesis in both physiological and pathological conditions is regulated by a controlled balance between pro- and anti- angiogenic proteins. “Angiogenic switch” (Hanahan and Folkman, 1996) indicates that the endothelial phenotype changes from quiescent to sprouting and also to changes in the endothelial microenvironment that triggers angiogenesis. It has been shown that the low level of TSP1 and TSP2 is a key factor in angiogenesis initiation, and the local TSP1 level has proven to be an efficient determinant of tumour growth (Zaslavsky et al., 2010). The discovery of angiogenic molecules initiated new therapeutic strategies of inhibition of angiogenesis, such as recombinant proteins and other cell-based strategies to increase TSP1, especially in cancer (Zhang and Lawler, 2007).

TSP1 domains

TSP1 contains specific domains that allow it to bind to membrane proteins, such as integrins, and other proteins present in the matrix which are expressed by the cells in the microenvironment. The ability of TSP1 to bind to the proteins of the extracellular matrix allows it to be retained in the matrix, where TSP1 can fold and acquired its 3D

conformation (Bornstein, 1995; Tan and Lawler, 2009). This mechanism permits the cells to control the retention of TSP1 in their microenvironment (Sottile and Hocking, 2002).

TSP1 is a large protein that contains 3 repeat domains (type I, II and III), a procollagen homology domain, and an amino and a carboxyl terminal which are globular (Lopez-Dee et al., 2011), as represented in Figure 3.

TSP1 is involved in several molecular pathways, specially linked to anti-angiogenic effects as summarised in Figure 4 and described along this subchapter.

The NH2-terminal of TSP1 can interact with low-density lipoprotein receptor-related protein (LRP1) releasing any metalloproteases bound to TSP1 modulating its enzymatic activity (Chen et al., 1996). TSP1 also binds to integrins and heparin sulphate proteoglycans via its NH2-termainal to regulate cell motility, chemotaxis adhesion and angiogenesis (Calzada et al., 2003). The thrombospondin structural homology repeats (TSRs), also called repeat domain I is only present in the TSP1 and TSP2 structure, while repeat domains II and III are present in all TSP members. The repeat domain type I is involved in the most well-known TSP1 pathway as an angiogenic inhibitor molecule, which interacts with the CD36 receptor in endothelial cells causing its dimerization which leads to endothelial apoptosis (Daviet et al., 1997; Jiménez et al., 2000). Other TSP1 domains can modulate this process by interacting with their receptors.

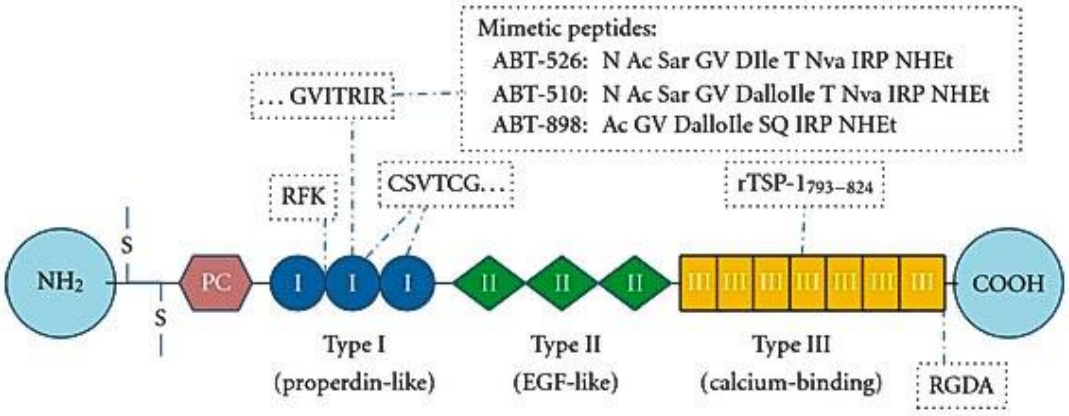


Figure 3: Representation of the TSP1 structure (Lopez-Dee et al., 2011).

TSP1 is the only TSP member that can activate TGFβ1. This cytokine is involved in key processes such as, cell proliferation, immune response, and wound healing and extracellular matrix formation. Mature TGFβ1 is found in the extracellular matrix in its latent form where is associated with latency-associated peptide (LAP). TSP1 binds to the latent TGFβ1-LAP complex which disrupts the complex turning TGFβ1 active (Young and Murphy-Ullrich, 2004). The amino acid sequence arginine-phenylalanine-lysine (RFK) is located in the TSR and it is responsible for the interaction between TSP1 and TGFβ1-LAP complex (Crawford et al., 1998; Schultz-Cherry et al., 1995; Young and Murphy-Ullrich, 2004).

TSP1 can activate neutrophils by interacting with neutrophil elastase through TSP1 type III repeat domain (Hogg et al., 1993; Majluf-Cruz et al., 2000). The same domain is able to reduce angiogenesis by blocking the binding of fibroblast growth factor to endothelial cells (Margosio et al., 2008).

The TSP1 repeat domain II contain EGF-like repeats. Although these repeats domains are recognised by integrins, no biological function has been attributed to such interaction (Calzada et al., 2004).

The TSP1 COOH-terminal also affects angiogenesis, immune response and cell proliferation by its ability to bind to CD47 (Kosfeld and Frazier, 1993), β1 and β2 integrins and proteoglycans (Calzada et al., 2003). The TSP1-CD47 interaction regulates nitric oxide (NO) modulating vasodilation and chemotaxis (Isenberg et al., 2006). Through a series of experiments using TSP1 and CD47 knockout mice or inhibiting the interaction between these two proteins, a research team has shown that TSP1-CD47 interaction is crucial in controlling angiogenesis (Isenberg et al., 2006). This study revealed much complex molecular mechanism has revealed to be much more complex, which indicating that CD47 can interact with VEGFR2 (Kaur et al., 2010), but the interaction between TSP1 and CD47 inhibits VEGFR2 phosphorylation and therefore angiogenesis (Kaur et al., 2014).

Although, the majority of studies suggests an anti-angiogenic role of TSP1, it has been shown that TSP1 can induce angiogenesis. The pro-angiogenic activity of TSP1 has been mainly described to be restricted to the N-terminal (BenEzra et al., 1993; Calzada et al., 2004; Chandrasekaran et al., 2000; Iruela-Arispe et al., 1999; Nicosia and Tuszynski, 1994), which should be taken into consideration when TSP1 peptide based therapy is being developed.

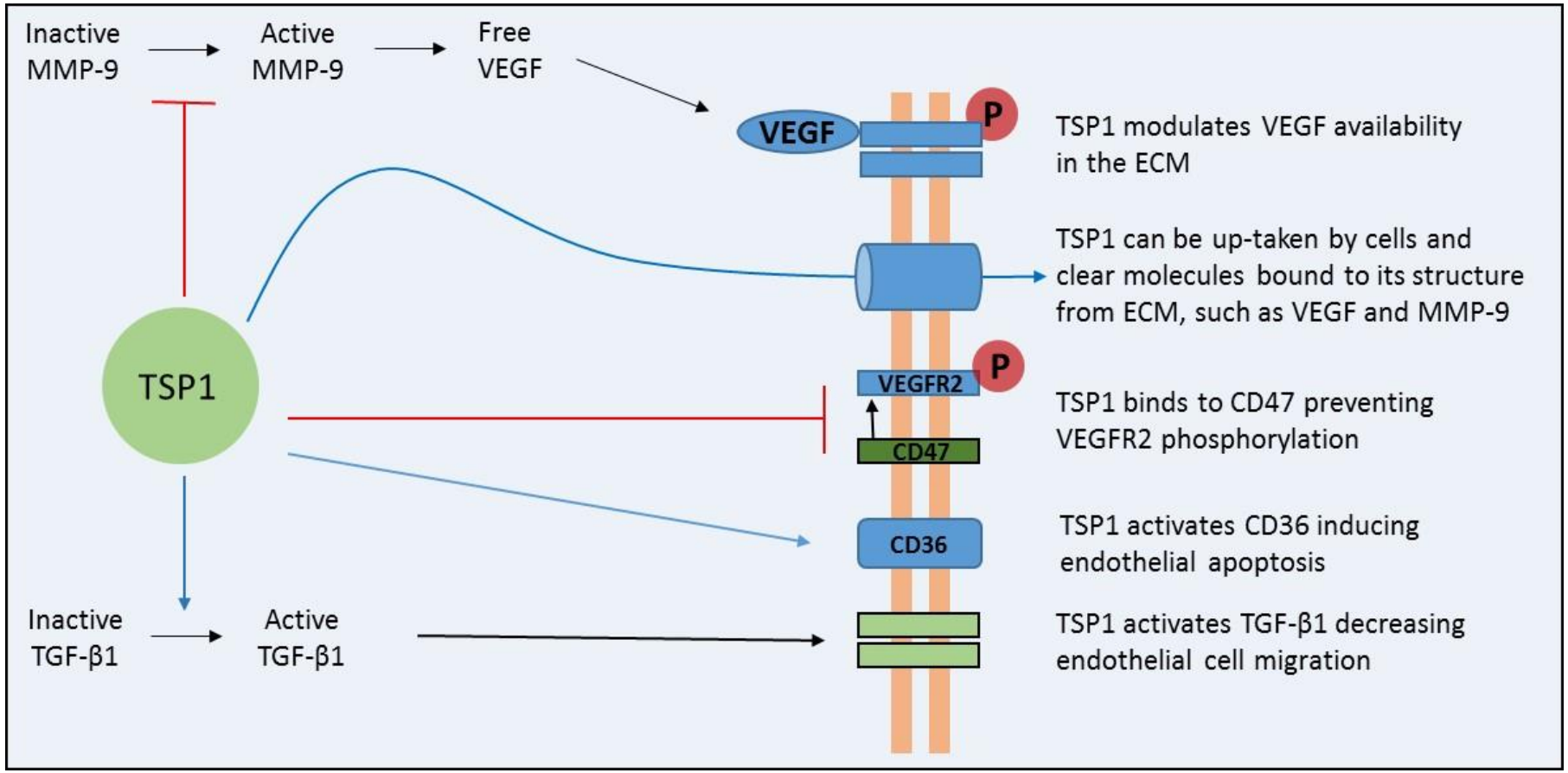


Figure 4: Schematic representation of the main TSP1 anti-angiogenic pathways that have been identified.

Regulation of TSP1 level in the ECM

As mentioned before, several cell types secrete TSP1 (Naganuma et al., 2004; Wight et al., 1985). Several studies have described a differential regulation of the TSP1 gene promoter in different cell types, suggesting tissue specific signals to modulate its expression. The activation of a promoter is not only dependent on the amino acid sequence, but also dependent on the availability of the target binding sites, transcription factors, cell signalling pathways to activate those transcription factors and protein co-factors. One of the best studied TSP1 stimuli is glucose. Most cell types of the vascular wall, such as fibroblasts, smooth muscle cells and endothelial cells, up-regulate TSP1 mRNA in response to high glucose (Stenina et al., 2003). Interestingly, the TSP1 up-regulation occurs at different times, it happens much earlier in endothelial cells than smooth muscle cells. Studies revealed that endothelial cells and smooth muscle cells contains different transcriptional regulation mechanisms of the TSP1 gene in response to high glucose. In endothelial cells, TSP1 transcription was regulated by a proximal fragment of the promoter, while those in smooth muscle cells were required a proximal and a distal fragment of the promoter (Dabir et al., 2008; Raman et al., 2011).

TSP1 is a significant protein in several metabolic disorders (Li et al., 2011; Moura et al., 2008), which leading to research on TSP1 regulation by leptin, a key hormone in diabetes and obesity. Leptin up-regulated TSP1 mRNA in a JAK/ERK/JNK dependent manner (Chavez et al., 2012).

Erg-1, a transcription factor involved in inflammation mechanisms, induced TSP1 transcription in combination with MYC, upon thrombin stimuli (McLaughlin et al., 2005). In inflammatory joint disease, a decrease in the orphan receptor 4A2 (NR4A2) led to a decrease in TSP1 expression suggesting a role of TSP1 in the resolution of the inflammation (McMorrow et al., 2013).

A series of experiments demonstrated that shorter TSP1 promoter deletion constructs have an inhibitory element (Dabir et al., 2008; Kang et al., 2004; Kang et al., 2003). A well-known human carcinogen, Nickel, decreased TSP1 promoter activity and downregulated its expression in hamster embryo cells. Nickel induced the expression of Activating Transcription Factor-1 (ATF-1) which was identified as the protein binding to the TSP1 promoter and repressing the promoter activity (Salnikow et al., 1994). The TSP1 promoter activity can be decreased by other proteins, such as c-Jun that interacts with YY-1 weakening the interaction of YY-1 to the TSP1 promoter (Kang et al., 2004).

The inhibition of TSP1 expression is an effective and common control mechanism employed by cells once TSP1 protein and mRNA are both unstable resulting in rapid effects (Chavez et al., 2012; El Btaouri et al., 2011; McGray et al., 2011; Raman et al., 2011; Raman et al., 2007).

In melanoma, methylation of the TSP1 promoter is a powerful inhibitor of TSP1 expression (Lindner et al. 2013), and reduced TSP1 expression has been associated with methylation of the TSP1 promoter in gastric carcinoma (Oue et al., 2003).

The TSP1 level in the extracellular matrix can also be regulated by secretion. TSP1 can be stored inside the cells and its secretion is regulated by Ca2+ (Veliceasa et al., 2007). Platelet is a major source of extracellular matrix TSP1 and pre-synthesised TSP1 is only released after platelets activation (Dawes et al., 1983; Lawler and Slayter, 1981). Cellular density can determine the secretion of TSP1. For instance, low density of endothelial cells, fibroblasts and smooth muscle cells secrete higher levels of TSP1 without changing TSP1 synthesis (Mumby et al., 1984).

TSP1 in cancer

TSP1 is a key protein in angiogenesis. TSP1 has been extensively studied in cancer relating to tumour angiogenesis. Several studies have found a correlation between changes in the expression of TSP1 and tumour angiogenesis. A decrease in

TSP1 expression correlates with higher vascular density in colon cancer (Kawakami et al., 2001), glioma cells (Harada et al., 2003) and invasive cervical cancer (Cinatl et al., 1999). An *In vivo* breast cancer study has shown that TSP1 inhibits angiogenesis constraining the growth of the primary tumour (Yee et al., 2009). TSP1 has been related to a decrease in angiogenesis and growth of the primary tumour, and metastasis (Incardona et al., 1995; Yee et al., 2009). Accordingly, many studies have presented low levels of TSP1 as a marker of poor prognosis in glioma (Perez-Janices et al., 2015), melanoma (Borsotti et al., 2015), ovarian and pancreatic carcinoma (Lyu et al., 2013; Nie et al., 2014; Pinessi et al., 2015).

Studies showed that the suppression of TSP1 in cancer led to an increase in angiogenesis and tumour growth. Wilms' tumour suppressor gene (WT1) has been shown to bind to the TSP1 promoter and inhibit TSP1 expression (Dejong et al., 1999). On the other hand, p53 induces the activity of the TSP1 promoter (Su et al., 2010). The transcription factor Id1 has also been identified as one of the regulators of TSP1 expression (Volpert et al., 2002). Hypermethylation has also been identified as a mechanism employed by several tumours to prevent the activation of the TSP1 promoter (Kanai et al., 2001; Lindner et al., 2013; Liu et al., 2005; Yang et al., 2003).

As an extracellular matrix protein, TSP1 interacting with many other proteins makes TSP1 difficulty to be studied. An *in vivo* study showed that the level of TSP1 was inversely correlated with tumour size, angiogenesis and the level of activated MMP-9 (Rodriguez-Manzaneque et al., 2001). MMPs are enzymes that degrade a wide range of extracellular proteins and TSP1 can bind MMPs via its TSR domain (Bein and Simons, 2000). The same study confirmed that in the presence of TSP1 there was a decrease in VEGF (Rodriguez-Manzaneque et al., 2001). This study concluded that TSP1 suppressed angiogenesis by VEGF retention in the extracellular matrix which is possible by suppressing MMP activity.

TSP1 can also bind directly VEGF contributing to retention of VEGF in the matrix (Greenaway et al., 2007; Gupta et al., 1999). Interestingly, it has been shown that TSP1 can be removed from the extracellular space by being transported into the cytosol. This cellular mechanism allows a fast decrease of extracellular TSP1, but also the reduction of proteins associated with TSP1, such as VEGF and MMPs. This process is regulated by low-density lipoprotein receptor-related protein *(*LRP) (Wang et al., 2004).

The repeat domain type III of TSP1 also inhibits VEGF signal by blocking the VEGFR2 phosphorylation at the tyrosine-1175 (Zhang et al., 2009). CD36 is a receptor of TSP1 and an interaction between CD36 and VEGFR2 was observed in the same study (Zhang et al., 2009). Interestingly, the inhibition of VEGFR2 phosphorylation by TSP1 is also dependent on the association between CD36 and β1-integrin (Primo et al., 2005). The inhibitory effect of TSP1 on VEGFR2 appears to decrease Akt activation (Ren et al., 2009). Other studies support this data showing that Akt signal is increased in the retinas of TSP1 knockout mice (Sun et al., 2009). Akt signalling pathway is involved in the survival and proliferation of endothelial cells (Wang et al., 2006).

1.4. Tryptophan and Indoleamine 2,3-dioxygenase 1

1.4.1 Amino acids

Amino acids are essential molecules to life as they are the building blocks of proteins and peptides and are also a source of nitrogen for the synthesis of other amino acids and other nitrogenous compounds such as nucleotide bases. These organic molecules have a basic structure which is characterised by a carbon atom (C) attached to a carboxyl group (-COOH), a hydrogen (H), an amino group (-NH2) and a side-chain specific to each amino acid (R), as schematised in Figure 5. The R group confers the unique chemical properties of the amino acid (Lodish, 2000).

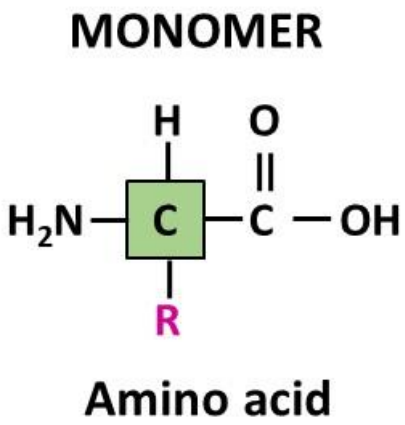


Figure 5: General structure of an amino acid (Lodish, 2000).

There are 20 standard proteinogenic amino acids. Most microorganisms, for example, E. coli, can generate all of them. However, human beings can only synthesise 11 amino acids. The amino acids that humans cannot synthesise, and therefore have to be supplied by the diet, primarily from plants, are known as essential amino acids (EAA; histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine). The remaining 9 amino acids are called nonessential amino acids (NEAA; alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine and tyrosine) (Berg JM, 2002).

The essential amino acids are synthesised by complex reactions, whereas the synthesis of nonessential amino acids requires fewer and simpler reactions, as illustrated in Figure 6. Some nonessential amino acids, e.g. alanine and aspartate, are synthesised in only one step, while the formation of essential amino acids involves 5 to 16 steps (Berg JM, 2002). The reason that humans cannot synthesise essential amino acids has been attributed to the fact that humans do not have some of the enzymes required to catalyse some of the reactions (Berg JM, 2002).

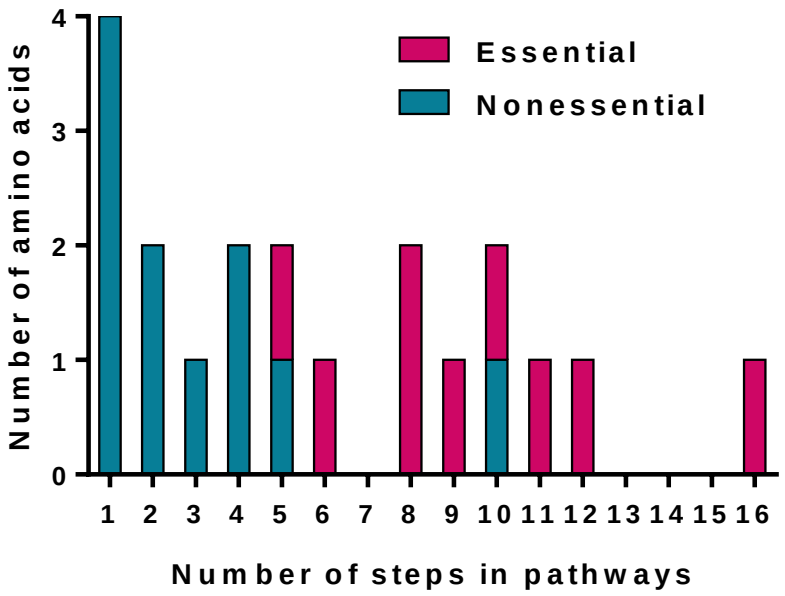


Figure 6: Number of steps required to synthesise essential and nonessential amino acids (Berg JM, 2002).

An adequate pool of amino acid is essential for growth, development, health and survival in humans and animals in general (Ren et al., 2012; Wu, 2009). For this reason, another important way of characterising amino acids has been discussed, which separates amino acids into 2 groups: nutritionally essential amino acids and nonessential.

Nutritionally essential amino acids include amino acids that cannot be synthesised by humans but also amino acids that are not synthesised in enough quantity to assure an adequate development and growth of the organism (Wu, 2010). Nevertheless, nutritionally nonessential amino acids can be produced by human beings in sufficient amounts (Le Plénier et al., 2012; Liu et al., 2012; Obayashi et al., 2012).

As mentioned before, amino acids are the building blocks of proteins which are obtained from the digestion of proteins in the intestine and the degradation of proteins within the cell. Many cellular proteins have a short half-life, being continually degraded

and for this reason cells have to resynthesize those proteins at the same rate to keep their physiological levels. Cells attach chains of small proteins, ubiquitin, to unnecessary or damaged proteins which are afterwards degraded by the proteasome, a large ATP-dependent complex (Berg JM, 2002).

Nevertheless, cells can store fatty acids and glucose; they cannot store or excrete surplus amino acids. Instead, excessive amino acids are used as metabolic fuel. The majority of surplus amino acids enter the urea cycle and their carbon skeletons are converted into a major metabolic intermediate, such as acetoacetyl-CoA, pyruvate, acetyl-CoA or a citric acid cycle intermediate. These molecules can be used to form fatty acids, ketone bodies, and glucose (Berg JM, 2002).

Proteasome degradation has been extensively studied, and is important in controlling the degradation of regulatory, short-lived, damaged or misfolded proteins (Hershko and Ciechanover, 1998; Schwartz and Ciechanover, 2009). Apart from the well-studied functions described above, the ubiquitin- proteasome system also recycles amino acids. This process has been well studied in starving cells (Nakatogawa et al., 2009). Autophagy and proteasomal degradation play a key role in the maintenance of an adequate pool of amino acids to support protein synthesis under severe nutrient deprivation conditions (Onodera and Ohsumi, 2005; Vabulas and Hartl, 2005). However, the importance of amino acid recycling by proteasomal degradation under physiological conditions is not clear.

1.3.2 Tryptophan

Tryptophan (TRP) was isolated in 1901 by Hopkins and Cole, its name derived from the isolation method employed to digest casein, called tryptic digestion (Hopkins and Cole, 1901). Rose and colleagues showed in 1954 that tryptophan is an essential amino acid (ROSE et al., 1954a), they also demonstrated that only the L isoform of tryptophan is used to synthesise protein (ROSE et al., 1954b). Adults are required to obtain 3.5 mg/Kg of L-tryptophan every day and, for example in the United States, diet provides in average 0.7- 1 g of L-tryptophan (Wildman and Medeiros, 2000).

Tryptophan circulates in the plasma and blood mostly bound to albumin (Pardridge, 1979), which is estimated that only 10- 20% is albumin-free in the plasma. It remains debatable whether tryptophan bound to albumin changes the availability of tryptophan to protein synthesis or metabolism, but it has been reported that some drugs and non-esterified fatty acids change the binding of tryptophan to albumin (Pardridge, 1979).

Once L-tryptophan is ingested, it can be used in 4 different mechanisms: protein synthesis; the serotonin pathway; the kynurenine pathway; and decarboxylation to tryptamine (Richard et al., 2009).

Tryptophan is involved in many metabolic processes, one of them being the precursor of serotonin (5-HT or 5-hydroxytryptamine). Serotonin is a neuromodulator which regulates appetite, haemodynamics, mood and gastrointestinal functions. Ninety-five percentage of the mammalian serotonin is found in the gastrointestinal tract (Sanger, 2008), and it has been estimated that only 3% of the tryptophan obtained from the diet is used for the body serotonin synthesis (van Praag HM and C, 1986). The conversion of tryptophan into serotonin is achieved in only two steps. The first step is catalysed by tryptophan hydroxylase (TPH) which converts tryptophan into 5-hydroxy-tryptophan, and this one is decarboxylated into serotonin which is dependent on vitamin B6.

Tryptamine is a molecule with several biological functions and has tryptophan as a precursor. Tryptophan is immediately decarboxylated resulting in very small amounts of tryptamine, being its primary role as a neuromodulator of serotonin (Jones, 1982). Animal studies have suggested that tryptamine can activate specific receptors, acting as an independent neurotransmitter from serotonin, however, its main function is as a modulator of serotonin function (Jones, 1982).

Tryptophan is also involved in the kynurenine pathway being the first substrate of this metabolic process where tryptophan is converted, through a series of reactions, into quinolinic acid, niacin, kynurenic and xanthurenic acid. This pathway also generates many intermediate metabolites which have been shown to have several functional activities, e.g. N-formyl kynurenine and kynurenine. The first reaction, which converts tryptophan into N-formyl kynurenine, can be catalysed by 3 different enzymes: tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase 1 (IDO1) and indoleamine 2,3-dioxygenase 2 (IDO2). IDO2 has been recently discovered and not much is known about its function or distribution, however, TDO and IDO1 are found in different tissues and also have different roles, structure, substrate specificity and cofactor requirements (Le Floc'h et al., 2011).

1.3.2.1 Protein synthesis

In human beings, the main role of tryptophan is its use in protein synthesis. Since tryptophan is an essential amino acid, the percentage of this amino acid used in cellular metabolism is lost for protein synthesis. The average proportion of essential amino acids in the body is relatively high, for instance, lysine, leucine, and threonine contribute to 7.6, 7.1 and 4.0 g per 100g of protein, respectively (Mahan and Shields, 1998). However, tryptophan only corresponds to 1.2g/ 100 g which is much lower than the other essential amino acids (Mahan and Shields, 1998). Several studies have tried to estimate the proportion of tryptophan incorporated into a protein, but the results vary considerably.

This variation has been attributed to methodology, development stage versus adulthood and physiological status. There was a study conducted by Sawadogo et al. (1997) which showed that, in growing pigs, 54% of tryptophan was incorporated into a protein, when tryptophan was supplied under the normal tryptophan requirement. However, when tryptophan was administrated above the requirement, the percentage of tryptophan retained in body protein decreased, being attributed to an increase in tryptophan metabolism (Sawadogo et al., 1997). In adults, it is still debatable how much dietary tryptophan is used for protein synthesis. Taking a few statistics from different studies, an adult human degrades and synthesises about 300 g of protein /day (Garlick et al., 1980) and as mention above, tryptophan corresponds to 1- 1.2 % of the total protein (Mahan and Shields, 1998), this way an adult would require 3 – 3.6 g of tryptophan every day. It is recommended that an adult should obtain 350 – 400 mg of tryptophan per day through diet (Lazaris-Brunner et al., 1998), which is much lower than the tryptophan necessary to resynthesize proteins, suggesting that dietary tryptophan only allows replacement of tryptophan that was metabolised.

Tryptophan is the amino acid found in the lowest concentrations being relatively less available and for that reason it is thought to play an important role in protein synthesis as a rate-limiting amino acid (Cortamira et al., 1991; Wurtman et al., 1980).

1.3.2.2 Kynurenine pathway

The degradation of L-tryptophan by the kynurenine pathway was first described in 1947 (Beadle et al., 1947) and is responsible for about 90% of the catabolism of this amino acid in humans (Richard et al., 2009). This pathway is a complex chain of reactions that depend on a series of enzymes to catalyse all the steps as represented in Figure 7. It can be initiated by 3 distinct enzymes: indoleamine 2,3-dioxygenase 1 (IDO1; (Thomas and Stocker, 1999)), indoleamine 2,3-dioxygenase 2 (IDO2; (Ball et al., 2009)) and tryptophan 2,3-dioxygenase (TDO; (Ren and Correia, 2000)). These 3 molecules cleavage the 2,3-double bond of the indole ring in the L-tryptophan resulting in N-formyl-

kynurenine. N-formyl kynurenine is converted to kynurenine, one of the key metabolites in this pathway, by formyl kynurenine formamidase.

At this point, the kynurenine pathway can then be split into two branches. Kynurenine can be irreversible transaminated by kynurenine aminotransferases (KATs) originating kynurenic acid (KYNA). Another branch, which competes with KATs, transforms kynurenine into 3-hydroxykynurenine (3-HK) and anthranilic acid, by kynurenine 3-monooxygenase (KMO) and kynureninase, respectively (Chiarugi et al., 1995). Cellular localisation, intracellular compartmentalization, and enzyme kinetics will determine the fate of kynurenine (Dang et al., 2000; Schwarcz and Pellicciari, 2002). Kynureninase and KATs can further catalyse 3-HK into 3-hydroxyanthranilic acid (3-HANA) and xanthurenic acid, respectively (Kawai et al., 1988). Anthranilic acid can also be degraded into 3-HANA by non-specific oxidases (Baran and Schwarcz, 1990). 3-HANA can be oxidised into cinnabarinic acid or it can be transformed into 2-amino-3-carboxymuconic-6-semialdehyde by 3-hydroxyanthranilic acid 3,4-dioxygenase (3-HAO; (Foster et al., 1986). The kynurenine pathway divides into two arms again, 2-amino-3-carboxymuconic-6-semialdehyde can spontaneously rearrange into quinolinic acid (QUIN), which has a short half-life, about 20 minutes. The production of QUIN can be regulated by a change in the concentration of Fe^{2+} that modulates the activity of 3-HAO (Stachowski and Schwarcz, 2012) or by changing pH and temperature to influence the non-enzymatic reaction. The second branch of this pathway is possible via the activity of 2-amino-3-carboxymuconic-6-semialdehyde decarboxylase producing picolinic acid from 2-amino-3-carboxymuconic-6-semialdehyde (Pucci et al., 2007). QUIN can be further degraded by quinolinate phosphoribosyl transferase to end up synthesising NAD+; this pathway has been shown crucial under infection diseases to prevent energy failure and apoptosis (Bellac et al., 2010; Braidy et al., 2011).

The kynurenine pathway is extensively studied. Many research projects have shown the importance of kynurenine pathway in physiological processes, such as to

degrade excess of tryptophan under normal conditions (Le Floc'h et al., 2011), and in disease settings, such as its role in modulating immunity and inflammation (Kwidzinski and Bechmann, 2007).

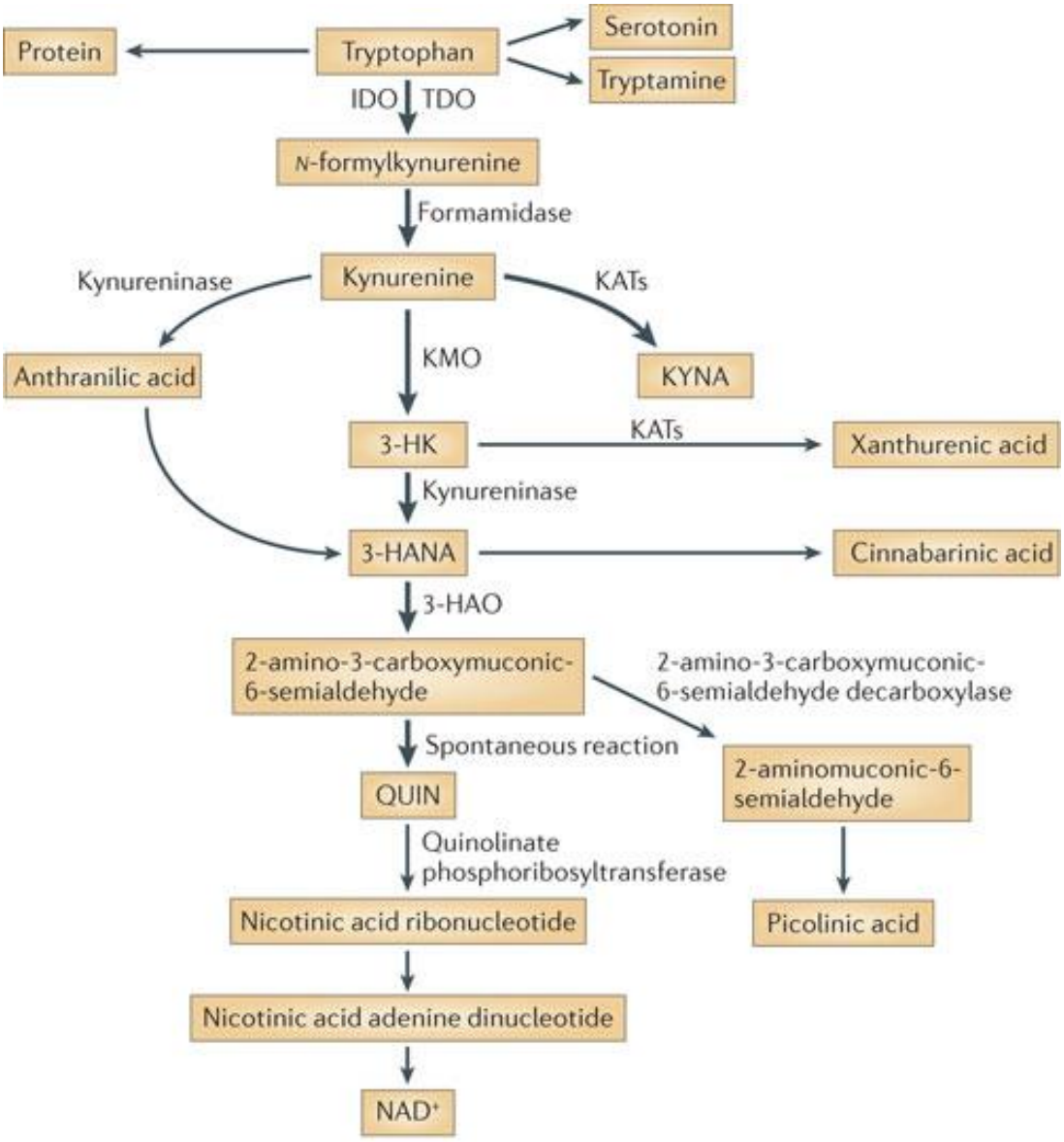


Figure 7: The kynurenine pathway (Schwarcz et al., 2012).

Tryptophan-catabolizing enzymes

Three dioxygenases are known to catalyse tryptophan into N-formyl-kynurenine and initiate the kynurenine pathway: Tryptophan 2,3-dioxygenase (TDO), Indoleamine 2,3-dioxygenase 1 (IDO1) and Indoleamine 2,3-dioxygenase 2 (IDO2). Although these enzymes have the same enzyme activity, they differ in many aspects, including function, substrate specificity, structure, cofactors requirements and tissue distribution, as summarised in the Table 2.

1.3.2.2.1 Tryptophan 2,3-dioxygenase (TDO)

In 1936, Kotake and Masayama discovered TDO in the liver of rats. Human TDO, which comprises 406 amino acids, is encoded by the TDO2 gene in the chromosome 4. The TDO2 gene comprises 12 exons and 11 introns and is composed of 65,669 bps (Comings et al., 1995). This gene has intron and exon variants that results in polymorphisms, linked to mental disorders (Miller et al., 2009b; Vasiliev et al., 1999).
TDO has only one substrate, L-tryptophan in the tetrameric form. The crystal structure of TDO has been reported in human, Ralstonia metallidurans and Xanthomonas campestris, its haeme-binding site has only been investigated in Ralstonia metallidurans (Zhang et al., 2007). TDO has an open and closed configuration, the open configuration exposes the heme-binding site to the substrate.

1.3.2.2.2 Indoleamine 2,3-dioxygenase (IDO1)

IDO1 was isolated for the first time in 1967 from the rabbit intestine (Yammotto and Hayaishi, 1967). IDO1 is an intracellular haem enzyme which converts L-tryptophan into N-Formylkynurenine. IDO1 is a monomeric protein that comprised 403 amino acids with a molecular mass of about 42- 54 kDa (Arefayene et al., 2009; De Luca et al., 2013; Tardito et al., 2013). This protein is encoded by the *IDO1* gene located on chromosome 8 with 10 exons (Burkin et al., 1993; Grohmann and Bronte, 2010; Tone et al., 1994). Several studies have identified genetic variations of IDO1 gene leading to differential expression and activity of IDO1 (Burkin et al., 1993; Grohmann and Bronte, 2010; Tone

et al., 1994). IDO1 genetic variants have been associated with the severity and outcome of some human diseases. For instance, an IDO1 variant has been correlated with a reduced risk of vulvovaginal candidiasis recurrence (De Luca et al., 2013). Also an IDO1 single nucleotide polymorphism (SNP) has been associated with autoimmune systemic sclerosis (Arefayene et al., 2009). Another type of IDO1 regulation at transcriptional level is involved in histone deacetylation by histone deacetylase (HDAC).

Interferon-gamma (IFNγ) is the most powerful stimulus of IDO1 gene expression, both in vivo and in vitro (Gough et al., 2008; Jeong et al., 2009). IFNγ activates both Janus kinase (JAK)/STAT and protein kinase Cδ (PKCδ) molecular pathways. JAK, STAT1 and IRF1 are crucial for optimal IDO1 expression (Chon et al., 1995; Jeong et al., 2009; Konan and Taylor, 1996; Robinson et al., 2003). Other molecules can be important to IDO1 expression depending on the cell type. For instance, in human cord-blood-derived mesenchymal stem cells phosphoinositide 3-kinase (PI3K) modulates STAT1 phosphorylation level, thus influencing IDO1 expression (Mounayar et al., 2015), also IFNγ- mediating IDO1 expression in murine splenic CD8α+ DCs depends on IRF8 transcription factor (Orabona et al., 2006). The promoter region of IDO1 comprises three IFNγ-activated sites (GASs), which contains binding sites to STAT1, and 2 IFNγ-stimulated response elements (ISREs), which have binding sites to IFN regulatory factor 1 (IRF1) (Robinson et al., 2006).

Tumour necrosis factor α (TNFα) cannot induce IDO1 expression on its own, but it can synergistically stimulate IDO1 with other cytokines, such as IFNγ or other stimuli. TNFα activates the nuclear factor κB (NF- κB) pathway stimulating IRF1 transcription and STAT1 phosphorylation which leads to an upregulation of the expression of IFNγ receptors (Babcock and Carlin, 2000; Braun et al., 2005; Robinson et al., 2006; Robinson et al., 2003). IDO1 expression can also be trigged or modulated by type I IFNs (IFNα and IFNβ), Toll-like receptor (TLR) 3, 4, 7, 8 and ligands, interleukin 1 (IL-1), IL-6, oestrogen, thymosin α, prostaglandin E2 (PGE2), muramyl tripeptide, glucocorticoids, α-

galactosylceramide and haemoglobin (Adams et al., 2004; Babcock and Carlin, 2000; Braun et al., 2005; Furset et al., 2008; Hissong et al., 1995; Hissong and Carlin, 1997; Jung et al., 2010; Jung et al., 2007; Robinson et al., 2005; Suh et al., 2007; Zhu et al., 2007). Bacteria and virus can also stimulate IDO1 expression (Fujigaki et al., 2006; Liu et al., 2014).

IDO1 is constitutively or upon stimulation expressed in a broad range of cell types and tissues. Under normal physiological conditions, IDO1 protein is constitutively expressed mostly in subpopulations of immune cells and mucosal tissues. In rodents, IDO1 protein expression has been identified as constitutively in the caput of the epididymis, adipose tissue, the prostate, the placenta during pregnancy, in lymphoid organs, certain lymph nodes, spleen, gastrointestinal track, central nervous system, lung, pancreas, kidney and in certain areas of the eye (Dai and Zhu, 2010). Interestingly, in adult mice, IDO1 expression in the large intestine is constitutively but it dependents on its normal flora and IFNγ which is thought to have an important homeostatic role by preventing an immune response towards the intestinal microorganisms (Harrington et al., 2008; Takikawa et al., 1999). Studies have shown that IDO1 is constitutively expressed in the eye, pancreas and placenta (Ryu and Kim, 2007; Sarkar et al., 2007; Sedlmayr et al., 2002), eosinophils (Odemuyiwa et al., 2004). IDO1 is mainly detected in the cytosol of the cell and also in the perinuclear region. Interestingly, functional IDO1 has been detected in microvesicles which were derived from human amniotic fluid stem cells (Romani et al., 2015).

IDO1 enzyme houses a haem prosthetic group within its active site and Sugimoto et al. published in 2006 the first crystal structure of human IDO1, Figure 8 A (Sugimoto et al., 2006). The crystal structure showed two large and two small α-helical domains revealing that the first ones formed a pocket for the haem group and the small domains covered the cavity, Figure 8 B (Sugimoto et al., 2006).

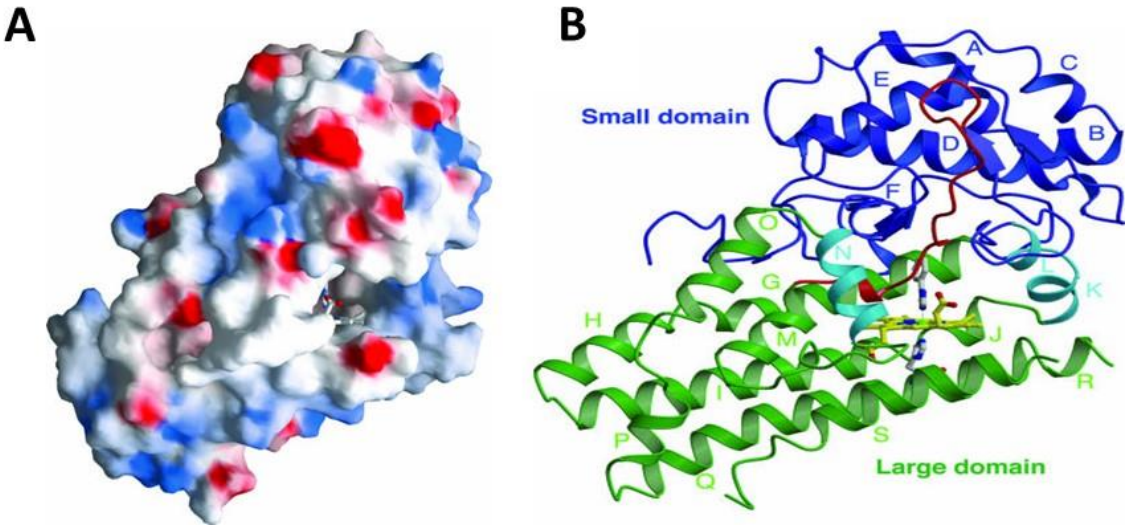


Figure 8: Crystal structure of IDO1 protein. A- Molecular module of the surface of the IDO1 protein. B- Representation of the overall IDO1 structure, small domains are represented in blue and large domains represented in green. The ball-and-stick model represent the haem molecule in both images. (Adapted from Sugimoto et al., 2006).

If IDO1 enzymatic uncontrolled activity could cause harm to the host, thus IDO1 is highly controlled through several mechanisms ahead transcriptional control. Some cell populations, which constitutively express IDO1, have been found to express low IDO1 enzymatic activity (Fallarino et al., 2002; Munn et al., 2004; Munn et al., 2002). For example, CD123+ DCs constitutively express IDO1, but requires IFNγ to activate its enzymatic activity (Munn et al., 2002). Interestingly, both mouse splenic CD8α+ DCs and CD8α- DCs express similar levels of IDO1 upon IFNγ stimulation, but only the first one degrades L-tryptophan (Fallarino et al., 2002). The mechanisms behind this phenomenon have not yet been uncovered, but DAP12, a transmembrane signalling adapts as a co-activator of immunoreceptor tyrosine-based activation motifs (ITAMs) was found to be determinant in this process (Orabona et al., 2006).

The IDO1 enzymatic activity switch has been extensively studied in the past years and a complex net of mechanisms have been identified, including: availability of the

haem group and reducing factors, redox reactions, protein-protein interactions, tyrosine phosphorylation and proteasome degradation.

IDO1 protein is only enzymatically active when the haem group is incorporated into its structure. The enzymatic IDO1 activity in monocytes is regulated by the proportion of haem-free IDO1 and haem-containing IDO1 (Thomas et al., 2001). Biosynthesis and degradation of haem is responsible for the cellular availability of haem. Haem biosynthesis is driven by δ-ALAS which initiates haem synthesis and works as a rate-limiting enzyme, while haem oxygenase 1 controls haem degradation. IFNγ-induced-Inhibition of haem synthesis in human macrophages prevented haem incorporation into IDO1 and blocked its enzymatic activity. In contrast, incorporation of a haem precursor enhanced IDO1 activity (Hill et al., 2005; Thomas et al., 2001). IDO1 activity can also be modulated by other proteins that compete for intracellular haem. For example, IDO2 has been identified as a negative regulator of IDO1 by competing for haem (Lee et al., 2014). In order to have an active IDO1, the haem group has to undergo an electron reduction from Fe^{III} to FeII, which allows O2 and L-tryptophan to form a ternary complex with the reduced haem group (Sono et al., 1980). Thus, IDO1 cofactors could involve physiological reductants. Three IDO1 cofactors have been identified such as flavin mononucleotide (FMN), tetrahydrobiopterin and O^{2-} (Hayaishi et al., 1977; Hirata and Hayaishi, 1975; Hirata et al., 1977; Ozaki et al., 1987; Ozaki et al., 1986). However, only O2 has been validated in further studies as a reducing cofactor and substrate for IDO1 (Hayaishi et al., 1977).

IDO1 can also be phosphorylated in two conserved tyrosine residues (tyr115, try253), which can trigger a signalling pathway independent from the IDO1 enzymatic activity (Pallotta et al., 2011) or can work as a signal for IDO1 protein proteasome degradation (Orabona et al., 2008) depending on the microenvironment. These two distinct signalling pathways involve interaction of IDO1 with other signalling proteins. In murine pDCs, IDO1 is phosphorylated upon TGFβ stimulation in a Fyn-dependent manner, a member of the Src family kinase (Pallotta et al., 2011). In this situation, IDO1

induces IFNs expression through activating the non-canonical NF-kB pathway by the recruitment and binding of SHP-1 and SHP-2 to IDO1. This pathway as an immunosuppressive regulatory role (Pallotta et al., 2011). IDO1 proteasomal degradation also requires phosphorylation, which in CD8α+ DCs can be stimulated by IL-6 and is dependent on the binding of suppressor of cytokine signalling 3 (SOCS3) to the IDO1 phosphorylated tyrosines that recruits the elogin/cullin/SOCS (ECS) E3 ligases that drives the proteasomal degradation of the IDO1-SOCS3 complex by ubiquitination (Pallotta et al., 2011).

IDO1 is an enzyme that has been mainly studied as a key regulator of immune and infection response through the degradation of L-tryptophan lowering its availability and producing kynurenine pathway metabolites.

Munn and collaborators described, for the first time in 1998, that IDO1 could modulate the immune response (Munn et al., 1998). This study used female mice that mated with syngeneic (same strain) or allogeneic (different strain) males. In theory, the second group of females would generate an immune response against the faetus, but it is not the case. The study showed that IDO1 was expressed in the placenta of both group of females. However, when females were treated with 1-methyl-tryptophan (1-MT), an IDO1 inhibitor, the development of the syngeneic embryos was not altered, none of the allogeneic embryos survived. Since then, the role of IDO1 in regulating the immune response has been extensively studied in order to uncover its mechanism of action. Studies have focused on the role of antigen-presenting cells (APCs), such as macrophages and dendritic cells, on mediating T-cell suppression through IDO1 expression. Interestingly, APCs can express IDO1 depending on their microenvironment and maturation status (Carlin et al., 1989a; Fallarino et al., 2002; Guillemin et al., 2005; Munn et al., 1999; Munn et al., 2004; Munn et al., 2002; Nouël et al., 2015). Other innate immune cells can also express IDO1, such as eosinophils, neutrophils and natural killer cells (De Ravin et al., 2010; Kai et al., 2003; Odemuyiwa et al., 2004).

Depending on the cell type, the IDO1 mechanism of action is different, such as on T-cells it induces Treg maturation, faulty activation of T-cell receptor or apoptosis of T-cell subpopulations, while it controls the production of cytokines by macrophages or its phenotype.

In vivo studies showed that DCs and macrophages express IDO1 and induce cell cycle arrest of T-cells, which is dependent on the IDO1 enzymatic activity (Munn et al., 1999; Munn et al., 2004; Munn et al., 2002). Interestingly, T-cell inhibition by IDO1-expressing DCs and macrophages does not necessary involve defective TCR activation. Several studies demonstrated that IDO1 or tryptophan starvation induces cell cycle arrest of T-cells via Fas signal, apoptosis and can also inhibits a T-cell response (Lee et al., 2002; Mellor et al., 2004; Mellor and Munn, 2003; Munn et al., 2004). Romani and collaborators showed that vesicles from human amniotic fluid stem cells, which were stimulated by IFNγ, contained active IDO1 and they were capable of inhibiting a T-cell response (Romani et al., 2015).

In vivo studies that the T-cell activity was restored by adding more L-tryptophan suggested that the immune regulation by IDO1 was due to a substantial decrease in tryptophan rather than any metabolite produced by the tryptophan degradation. Thus, in response to a lack of tryptophan, the levels of unchanged tRNA increase in T-cells activates the general control nonderepressible 2 (GCN2) kinase pathway, which stops most protein synthesis, but increases the synthesis of a few ones. GCN2 also inhibits the mTOR and PKCθ signalling pathway leading to T-cell anergy and autophagy (Metz et al., 2012; Munn et al., 2005; Sharma et al., 2007). In summary, the lack of L-tryptophan induced by IDO1 is responsible for some of the IDO1 immune effects on T-cells.

An intriguing question is how IDO1- expressing cells or surrounding cells survive and remain functional to the lack of L-tryptophan but not T-cells. Tryptophanyl-tRNA synthetase (TTS) is an enzyme that is constitutively expressed and catalyses the association between tRNA and tryptophan so this amino acid can be incorporated into proteins and it has been shown that TTS is primarily responsible for the survival of cells

to the lack of tryptophan (Boasso et al., 2005). This way, the level of TTS expression dictates the level of tolerance that the cells can support to an environment which lacks tryptophan preventing a cellular stress response (Boasso et al., 2005).

IDO1 plays a critical role as immune regulator in a wide range of physiological settings and diseases. IDO1 have an essential role in pregnancy (Baban et al., 2004; Mellor et al., 2001; Munn et al., 1998). Although, IDO1 gene-knockout mice do not lead to an autoimmune disorder, IDO1 knockout or inhibition has led to an unregulated and excessive immune response from the host when in contact with new or foreign antigens. IDO1 has been found to be overexpressed in autoimmune diseases, such as inflammatory bowel disease, arthritis and diabetes. The inhibition of its activity enhanced the severity of some of these diseases, suggesting that IDO1 activation might control the excessive immune response against self-antigens (Choi et al., 2006; Ciorba et al., 2010; Coquerelle et al., 2009; Grohmann et al., 2003; Gurtner et al., 2003; Ravishankar et al., 2012; Schröcksnadel et al., 2006; Szántó et al., 2007; Takamatsu et al., 2013).

IDO1 is also known to be part of the immune response against organisms such as bacteria, virus, parasites and fungi (Carlin et al., 1989b; Taylor and Feng, 1991). Even though some of these organisms can synthesise L-tryptophan, the L-tryptophan synthesis is not efficient and these organisms evolved to be tryptophan- dependent from the host (Mellor and Munn, 2004; Zegarra-Moran et al., 2004). Thus, they are sensitive to the lack of tryptophan.

Recent studies have identified IDO1 as an important molecule in different settings, such as vascular tone and blood pressure (Wang et al., 2010; Xiao et al., 2013), bone remodelling (Bozec et al., 2014; Vidal et al., 2015), and neurological disorders, such as depression and Parkinson's disease (Myint, 2012; Myint et al., 2012) and age-related cataracts (Mailankot et al., 2009).

1.3.2.2.3 IDO1 in cancer

The immune system is design to identify and eliminate cancer cells, but these cells can evolve and acquire mechanisms to be immune tolerant and escape the effects of T-cells and other immune cells. Once cancer cells reach the estate of immune resistance, tumour cells survive, including in new metastatic sites, allowing tumour progression (Prendergast et al., 2010).

Interestingly, the first in vitro studies suggested that IDO1 had an anti-tumour effect, through IFNγ in several cancer cell lines and this effect was dependent on the lack of L-tryptophan (Aune and Pogue, 1989; Ozaki et al., 1988). Also, an increase of IDO1 in stroma cells, such as human mesenchymal stem cells, which supports the growth of follicular lymphoma B-cells, leads to cell cycle arrest and apoptosis of the cancer cells (Maby-El Hajjami et al., 2009).

Despite the fact that the initial in vitro research suggests that IDO1 has an anti-tumour role, a substantial and growing human and animal data shows that IDO1 is a key molecule in the tumour cell immune escape. High expression and activity of IDO1 is a feature of a wide range of tumours, such as prostatic, breast, pancreatic and colorectal carcinomas. IDO1 is expressed by cancer cells, as well as by tumour-recruited leucocytes (e.g. macrophages and DCs) and tumour-draining lymph nodes (Munn et al., 2004; Munn et al., 2002; Uyttenhove et al., 2003). IDO1 enzymatic activity, indicated by Kynurenine and L-tryptophan levels in serum, correlates with poor prognostic in many human malignancies including ovarian, breast and lung (Astigiano et al., 2005; Brandacher et al., 2006; Chen et al., 2014; Inaba et al., 2010; Inaba et al., 2009; Ino et al., 2006; Jia et al., 2015; Okamoto et al., 2005; Pan et al., 2008; Suzuki et al., 2010; Urakawa et al., 2009; Wainwright et al., 2012; Witkiewicz et al., 2008; Yu et al., 2013; Yu et al., 2011) and in lymphomas such as myeloma (Bonanno et al., 2012; Choe et al., 2014; Lin et al., 2013; Masaki et al., 2015; Ninomiya et al., 2011; Yoshikawa et al., 2010). Also, increase IDO1 expression correlates with an increase in the number of Treg in the tumour microenvironment or in circulation (Choe et al., 2014; Curti et al., 2007b; Lin et

al., 2013; Moretti et al., 2014; Nakamura et al., 2007; Speeckaert et al., 2012; Spranger et al., 2013; Yu et al., 2013; Yu et al., 2011). *In vitro* and *in vivo* studies showed that leukaemia cells expressing IDO1 increase the number of Tregs which was prevented by 1-MT (Curti et al., 2007a; Curti et al., 2007b; Curti et al., 2010). When focused on the link between IDO1 and clinical outcome, it has been demonstrated that IDO1 overexpression correlated with poor prognosis in breast, ovarian, leukemic, colorectal, endometrial and lung cancer (Astigiano et al., 2005; Brandacher et al., 2006; Chamuleau et al., 2008; Ino et al., 2006; Okamoto et al., 2005; Suzuki et al., 2010; Takao et al., 2007).

Although these studies suggest that IDO1 expression or its enzymatic activity might indicate an adverse clinical prognosis, controversies exist in that. Some research groups have shown a complete opposite connection. For example, IDO1 expression has been shown to be correlated with a better prognosis in breast, hepatocellular and renal cell carcinoma (Ishio et al., 2004; Jacquemier et al., 2012; Riesenberg et al., 2007) or with no correlation between IDO1 expression and prognosis in melanoma and cervical cancer (Lee et al., 2003; Nakamura et al., 2007). Possible reasons for such controversy are likely to be multiple factors, including different types and subtypes of cancer, different therapeutic approaches, testing time (namely prior or post chemotherapy), the nature of test samples and test methods. Further studies clearly are needed to further clarify this important link and to better understand the precise role played by IDO1 in cancer.

Although the importance of IDO1 as a prognostic marker has still to be elucidated, animal studies have shown that IDO1-expressing tumours are more aggressive than those negative of IDO1 (Muller et al., 2005; Pilotte et al., 2012; Uyttenhove et al., 2003). The pro-inflammatory microenvironment, which is a feature of many tumour types, seems to be the driven factor for IDO1 expression by stroma cells and/or antigen-resenting cells (APCs) (Belladonna et al., 2006) resulting in a local lack of tryptophan.

Many in vivo animal data confirm the concept of tryptophan metabolism by IDO1 as an important factor in tumour immune tolerance. IDO1 expressing cancer cells

develop tumours when injected into syngeneic mice, these tumours are characterised by a lack of infiltrating T-cell, which could be reversed by 1-MT resulting in a decrease of the tumour volume (Friberg et al., 2002; Uyttenhove et al., 2003). Muller et al. showed that in a breast cancer animal model, 1-MT in conjugation with paclitaxel resulted in a decrease in 30% of the tumour volume, further suggesting that IDO1 inhibition could be an effective adjuvant therapy (Muller et al., 2005). The same group also demonstrated that the IDO1 immune effect is dependent on T cells (Muller et al., 2005).

1.3.2.2.4 Indoleamine 2,3-dioxygenase (IDO2)

Murray (2007) discovered the *ido2* gene, an *ido1* homologous gene. Meantime, three different groups independently identified that an enzyme encoded by the *ido2* gene was able to degrade tryptophan into kynurenine (Ball et al., 2007; Metz et al., 2007; Yuasa et al., 2007). This enzyme has been referred to as indoleamine 2,3-dioxygenase-like protein, indoleamine 2,3-dioxygenase-2 (IDO-2 or IDO2) and proto-indoleamine 2,3-dioxygenase (proto-IDO). Ball and collaborators showed that IDO2 degrades tryptophan in Human Embryonic Kidney 293 cells with a lower enzymatic activity (about 45% less than IDO1) (Ball et al., 2007). A different study showed similar results regarding IDO2 in T-rex cells (Metz et al., 2007).

The human genes that encode IDO1 and IDO2 proteins are adjacent to each other on chromosome 8. The IDO2 protein comprises 420 amino acids and shares 43% of its sequence with IDO1 but not with TDO. This evidence suggests that these two genes originated from gene duplication (Ball et al., 2007; Yuasa et al., 2007).

The expression patterns of IDO2 mRNA and protein have suggested some functional overlap with IDO1 but also revealed some unique aspect in its functions. IDO2 mRNA is predominantly found in the liver, kidney and cerebral cortex, while IDO1 is mostly expressed in colon and epididymis. Interestingly, mice genetically modified to have a deficiency in IDO1 expression, showed an upregulation of IDO2 in the epididymis

(Fukunaga et al., 2012), suggesting a functional redundancy of IDO2. Both IDO1 and IDO2 are expressed in antigen-presenting cells but IDO2 seems to be constitutively expressed whereas IDO1 requires inflammatory mediators (Trabanelli et al., 2014), suggesting that IDO2 might be involved in the homeostasis of these cells. IDO2 promoter has a binding site for IRF-7, a key transcription factor involved in the maturation of dendritic cells (Trabanelli et al., 2014). Intriguingly, the same study showed that IDO2 has a similar basal level of expression as IDO1 does. Like IDO1, IDO2 responds to pro-inflammatory stimuli, such as prostaglandin E2, lipopolysaccharide, IFN-γ and IL-10, with less robust (Lo et al., 2011; Metz et al., 2007; Simones and Shepherd, 2011; Sun et al., 2010; Trabanelli et al., 2014). Intriguingly, in a malaria animal model, a disease characterised by its high levels of IFN-γ, whereas levels of IDO2 mRNA and protein remain were unchanged (Ball et al., 2007).

IDO2, like IDO1, has been reported as being overexpressed in number of cancer types, such as pancreatic cancer, renal cancer and colorectal cancer (Löb et al., 2009). Interestingly, cancer cell lines only expressed IDO1 and IDO2 when stimulated by IFN-γ (Löb et al., 2009). The same study also showed that HeLa cells expressed IDO1 and IDO2 upon IFN-γ stimulation but only IDO1 was responsible for tryptophan degradation (Löb et al., 2009), suggesting that IDO2 might not have a role in the kynurenine pathway in cancer cells. IDO2 was also found to be overexpressed in basal cell carcinomas (BCC) of skin, and CXCL11 seems to be involved in the IDO2 regulation (Lo et al., 2011).

Several studies have shown that IDO2 is not a typical tryptophan enzyme (Austin et al., 2010; Meininger et al., 2011) when compared to IDO1. Pantouris and collaborators assessed the IDO1 and IDO2 kinetic properties in response to L-tryptophan, D-tryptophan and analogues (Pantouris et al., 2014). Kinetic analysis measures the behaviour of enzymes using simple chemical principles, such as Km and Kcat. Km is the substrate concentration at which the reaction is half of the maximum velocity, while Kcat is the limiting rate of any enzyme-catalysed reaction at saturation (Pantouris et al., 2014).

Among the substrates used, L-tryptophan was the second best substrate for both IDO1 and IDO2 in terms of the catalytic rate (Kcat), but L-tryptophan was second for IDO1 and fourth for IDO2 best substrate in terms of Kcat/Km. Km is defined as the concentration of substrate that the enzyme needs to reach half of the maximal velocity. The different Kcat/Km for L-tryptophan between IDO1 and IDO2 was due to a higher km for IDO2 (Pantouris et al., 2014). DO1 and IDO2 might have a different active site and substrate binding structures (Pantouris et al., 2014). Mouse recombinant IDO1 and IDO2 proteins exhibit their optimal activity occurs at pH 6.0 – 6.5 and 7.4 – 7.5, respectively (Austin et al., 2010). IDO1 and IDO2 have distinct thermal denaturation. The former was predominately denatured at 60° C, while the latter was denatured at 48° C (Austin et al., 2010). Interestingly, sperm tails express IDO2 (Ball et al., 2007). Sperm is produced at a lower temperature than the core body. Also, the seminiferous fluid has a pH of 7.2 – 8.0 (Harraway et al., 2000), which fits into the optimal IDO2 pH. As mentioned above, IDO1 and IDO2 have also been found to be overexpressed in many cancers, and tumours microenvironment is characterised as an acidic environment due to inadequate perfusion and high metabolic rates (Zhang et al., 2010). Temperature has also been reported as being increased in tumours(Chanmugam et al., 2012), suggesting that these tumour features might benefit IDO1 enzymatic activity.

Table 2: Summary of TDO, IDO1 and IDO2 characteristics

	Tryptophan 2,3-dioxygenase (TDO)	Indoleamine 2,3-dioxygenase 1 (IDO1)	Indoleamine 2,3-dioxygenase 2 (IDO2)
Substrate	L-tryptophan	L-Tryptophan, D-Tryptophan, serotonin, 5-hydroxytryptophan and tryptamine	L-Tryptophan, D-Tryptophan
Substrate specificity	Low	High	Low
Form	Tetrameric	Monomeric	Monomeric
Tissue distribution	Liver (skin and cortex)	Ubiquitous	Liver, kidney, cerebral cortex
Functions	Tryptophan homeostasis	Immune regulation	Not known
Regulation (Main stimulators)	Tryptophan, glucocorticoids and glucagon	IFN-γ, LPS, virus, bacteria	IFN-γ, IL-10, , LPS, prostaglandin E2 (less responsive than IDO1) and possible IRF-7
Non-enzymatic activity	Not known	Yes	Not known

1.3.2.2.5 Kynurenine pathway metabolites

The activation of the kynurenine pathway results in the decrease of the availability of L-tryptophan but also in the formation of metabolites from that metabolism, which has considerable biological effects.

T-cell proliferation can be inhibited *in vitro* by IDO1 and this phenomenon has been mainly attributed to a decrease in L-tryptophan at concentrations lower than 0.5-1 µM (Munn et al., 1999). However, the levels of tryptophan in plasma are between 40 and 100 µM and it would be very difficult to achieve such low tryptophan concentrations *in vivo.* Additional *in vitro* studies have shown that the treatment of activated T-cells, B-cells and NK cells with high levels of kynurenine pathway metabolites, such as kynurenine, 3-HAA, 3-HK, QA and picolinic acid can inhibit their function (Frumento et al., 2002; Lee et al., 2010a; Terness et al., 2002). Surprisingly, lower levels of these metabolites, which are closer to physiological conditions, can have the same inhibitory effects on T-cells when these cells are treated with diverse combination of these metabolites or when added to low L-tryptophan or free tryptophan medium (Fallarino et al., 2006; Frumento et al., 2002; Terness et al., 2002).

Kynurenine has been identified as a ligand of the aryl hydrocarbon receptor (AhR) transcription factor, which is involved in several physiological pathways, such as immune regulation, reproduction and vascular development (Stockinger et al., 2014). CD4+ T-cells differentiate into pro-inflammatory Th7 cells when stimulated with TGFβ, but Mezrich and collaborators reported that kynurenine modulates the differentiation of naïve CD4+ T cells when stimulated with TGFβ into FoxP3+ Treg in vitro, in an AhR dependent manner (Mezrich et al., 2010). It has also been shown that kynurenine induce the expression of IDO1 through AhR activation in DCs (Nguyen et al., 2010; Vogel et al., 2008). Other metabolites of the kynurenine pathway have been shown to activate AhR signalling, for examples, kynurenic acid induces the expression of IL-6 in MCF7 cells through AhR activation and interestingly, it synergistically stimulates IL-6 when IL-1β is

present in the medium (DiNatale et al., 2010). Also, 3-HAA plays an important role in suppressing the immunoresponse by depleting glutathione (Lee et al., 2010a) and activating NF-kB through inhibition of TCR pathway (Hayashi et al., 2007) which results in T-cell function impaired.

The studies described above show that IDO1 induce tryptophan degradation through the kynurenine pathway leading to L-tryptophan deprivation but also to the production of kynurenine pathway metabolites which have significant biological meaning that can help to explain the immune modulation role of IDO1.

1.5 Hypothesis and aims

Disseminated breast cancer cells have been detected near microvessels in a TSP1 rich microenvironment. Thus it has been speculated that the TSP1 might be one of the molecules modulating tumour dormancy. TSP1 has a higher content of L-tryptophan than the average protein. L-tryptophan is the rarest essential amino acid in mammalians and a rate limiting-step on protein synthesis. IDO1 enzyme activity controls the local availability of L-tryptophan. IDO1 is overexpressed by breast tumours.

Here, it was hypothesised that endothelial cells may reduce TSP1 protein levels in the extracellular matrix of microvessels, due to an increase of IDO1 stimulated by cancer cells. Reduction in TSP1 may be permissive to the proliferation of cancer cells and induce angiogenesis leading to development of metastasis.

Aims of this project:

- ✓ Assess TSP1 and IDO1 staining in invasive breast carcinoma, lymph node metastasis and adjacent normal tissue by immunohistochemistry. In order to evaluate their expression in each tissue and also their expression in terms of

cancer progression and survival. A correlation between vascular IDO1 and stromal TSP1 was also estimated.

- ✓ Evaluate in a series of *in vitro* assays whether endothelial cells and human recombinant TSP1 induce breast cancer dormancy. The role of TSP1 as a protective molecule against anti-tumour drugs was also investigated.

- ✓ Understand whether breast cancer cells are capable of inducing vascular IDO1 and whether tryptophan limits TSP1 synthesis and secretion by endothelial cells.

- ✓ Investigate the potential role of IFNγ as a soluble factor secreted by cancer cells to induce IDO1 in endothelial cells.

Chapter II: General Material and Methods

2.1 Materials

2.1.1 Cell lines

In this study five microvascular endothelial cell lines, one epithelial lung cell line (Table 3), one non-tumorigenic breast cell line and two breast cancer cell lines were used (Table 4). HMVECaD was obtained from Gibco (Paisley, UK). BEAS-2B, MDA-MB-231, MCF7 and MCF10A were obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA). HECV cells were kindly provided by Dr G. Di Domenico (Istituto Nazionale per la Ricerca sul Cancro, Italy). Ty09 and TY10 were kindly provided by Professor Takashi Kanda (Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi, Japan) and hCMEC/D3 was kindly provided by Professor Pierre-Olivier Couraud (Institut Cochin, Université René Descartes, Paris, France). Details of the cell lines are presented in the Tables 3 and 4 including culture conditions.

Table 3: Cell lines information, part 1.

Type	Cell line	Morphology	Sources and features	Growth conditions
Endothelial cell lines	Human Microvascular Endothelial Cells, adult dermis (HMVECad, Lonza)	Endothelial	Primary human microvascular endothelial cells isolated from adult dermis	Pre-coated flasks with Attachment Factor (AF, Lonza) in EGM™-2MV BulletKit™ (hEGF, hydrocortisone, gentamicin, amphotericin-B, 5% FBS, VEGF, hFGF-B, R3-IGF-1 and ascorbic acid; Lonza) and antibodies. Cells cultured at 37°C and 5% CO_2.
	Human endothelial vascular (HECV, provided by Dr G. Di Domenico, Centro Biotecnologie Avanzate, Italy)	Endothelial	Primary endothelial cells from umbilical cord	Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and antibodies. Cells cultured at 37°C and 5% CO_2.
	TY09 (provided by Professor Yasuteru Sano, Yamaguchi University, Japan)	Endothelial	Endothelial cells derived from the brain and immortalised with nTERT and SV40 large T antigen.	Pre-coated flasks with AF in EBM2 supplemented with SingleQuots™ Kit (hEGF, VEGF, IGF1, Ascorbic acid, Hydrocortisone, hFGF-b, Hepanin and 20% FBS; Lonza) and antibodies. Cells cultured at 34°C and 5% CO_2.
	TY10 (provided by Professor Yasuteru Sano, Yamaguchi University, Japan)	Endothelial	Endothelial cells derived from the brain and immortalised with nTERT and SV40 large T antigen.	Pre-coated flasks with AF in EBM2 containing SingleQuots™ Kit (hEGF, VEGF, IGF1, Ascorbic acid, Hydrocortisone, hFGF-b, Hepanin and 20% FBS, Lonza) and antibodies. Cells cultured at 34°C and 5% CO_2.
	Blood-Brain Barrier hCMEC/D3 (provided by Institut Cochin, France)	Endothelial	Derived from human temporal lobe microvessels and immortalised with nTERT and SV40 large T antigen.	Pre-coated flasks with AF in EBM2 supplemented with 5% FBS, 1% Penicillin-Streptomycin, Hydrocortisol (1.4 µM), ascorbic acid (5 µg/mL), 1% chemically defined lipid concentrated 1/100, HEPES (10 mM), bFGF (1ng/mL) and antibodies. (all supplements from Lonza). Cells cultured at 34°C and 5% CO_2.

Table 4: Cell lines information, part 2.

Type	Cell line	Morphology	Sources and features	Growth conditions
Lung cell line	BEAS-2B (Sigma-Aldrich)	Epithelial	Derived from normal human bronchial epithelium and immortalised by adenovirus 12-SV40 virus hybrid (Ad12SV40) and cloned	Pre-coated flasks with AF in Bronchial Epithelial Cell Growth Medium (BEGM, Lonza), BEGM Bullet Kit (Hydrocortisone solution, GA-1000, retinoic acid, BPE high protein, recombinant human insulin 0.5%, transferrin, triiodothyronine, epinephrine, hEGF) and antibiotics. Cells cultured at 37^{o}C and 5% CO_2.
Non-carcinogenic breast	MCF10A (ATCC)	Epithelial		Mammary Epithelial Basal Medium (MEBM; Lonza, Walkersville, MD, USA), supplemented with SingleQuots™ Kit (BPE, hydrocortisone, hEGF, insulin; Lonza), 0.1% Cholera toxin (Sigma-Aldrich) and antibiotics. Cells cultured at 37^{o}C and 5% CO_2.
Breast cancer cell line	MCF7 (ATCC)	Epithelial	Derived from metastatic site: pleural effusion	DMEM (Sigma-Aldrich), 10% FBS and antibiotics. Cells cultured at 37^{o}C and 5% CO_2.
	MDA-MB-231 (ATCC)	Epithelial	Derived from metastatic site: pleural effusion	DMEM (Sigma-Aldrich), 10% FBS and antibiotics. Cells cultured at 37^{o}C and 5% CO_2.

Note: all cell lines cultured under standard tissue culture conditions (37^{o}C and 5% CO2) unless otherwise specified.

2.1.2 Human tissue microarrays

Two different tissue microarrays were used in this study which comprised sections from paraffin embedded tissue. One of the tissue arrays was purchased from US BIOLAB Corporation (Gaithersburg, MD, USA) containing 52 samples of breast invasive ductal carcinoma (patient's information in Appendix I). The second tissue array was purchased from US Biomax (Rockville, MD, USA) which contained 46 cases of invasive ductal carcinoma, 1 neuroendocrine carcinoma, 3 medullary carcinoma, 40 metastatic carcinoma and 10 adjacent normal tissues (patient's information in Appendix II). Both arrays provided information about the carcinoma: grade, AJCC clinical stage and TNM classification, and also the age of the patient. Tissue was stored at 4^{o}C until use. Antibody optimisations and IgG controls were conducted using mammary tissues from the institute tissue bank at Cardiff University School of Medicine. The collection of the mammary tissue was under local ethics approval and under the Home Office Tissue Act licence.

2.1.3 Primers

Primers used in this study for quantitative PCR were designed on the primer designing tool on the Nacional Center for Biotechnology Information (NCBI, Bethesda MD, USA) website and were synthetized by Sigma (Poole, Dorset, UK). Primers were designed to have an annealing temperature of 60^{O}C. Full details of the primers are presented in Table 5 and Table 6.

2.1.4 Antibodies

Full details of primary and secondary antibodies are exhibit in Table 7.

Table 5: Primer sequences for qPCR, part 1.

Gene	Type	Primer sequence (5'-3')
IFNγ	Forward	TGTCGCCAGCAGCTAAAACA
	Reverse	**ACTGAACCTGACCGTACA**TGCAGGCAGGACAACCATTA
IDO1	Forward	AAAAGGATCCTAATAAGCCCC
	Reverse	**ACTGAACCTGACCGTACA**CAGTCTCCATCACGAAATGA
TSP1	Forward	ACCAACCGCATTCCAGAGTC
	Reverse	**ACTGAACCTGACCGTACA**TACATCAGGTTGGCATCCTCGAT
IDO2	Forward	GAGCTGCGGAGCTATCACAT
	Reverse	**ACTGAACCTGACCGTACA**CCACGTGGGTGAAGGATTGA
TDO	Forward	CCAGGTGCCTTTTCAGTTGC
	Reverse	**ACTGAACCTGACCGTACA**CTTCGGTATCCAGTGTCGGG
ABCB1	Forward	CCAGAAACAACGCATTGCCA
	Reverse	**ACTGAACCTGACCGTACA**GTGCCATGCTCCTTGACTCT
ABCC1	Forward	CCCGCTCTGGGACTGGAA
	Reverse	**ACTGAACCTGACCGTACA**GTAGAAGGGGAAACAGGCCC
ABCC2	Forward	TACTTTGGGAACTGGTGAGTCT
	Reverse	**ACTGAACCTGACCGTACA**GATGACCTTTCATCCCAACCA
ABCC4	Forward	GTGTACCAGGAGGTGAAGCC
	Reverse	**ACTGAACCTGACCGTACA**TGAGCCACCAGAAGAACACG
CFTR/ MRP7	Forward	GGCACCCAGAGTAGTAGGTC
	Reverse	**ACTGAACCTGACCGTACA**AGGCGCTGTCTGTATCCTTT

ACTGAACCTGACCGTACA represents the Z sequence

Table 6: Primer sequences for qPCR, part 2.

Gene	Type	Primer sequence (5'-3')
ABCG2	Forward	AGGCAGATGCCTTCTTCGTT
	Reverse	**ACTGAACCTGACCGTACA**ACCAACAGACCATCATAAACACA
ABCC3	Forward	GGGCTCATTGGACTCTACCC
	Reverse	**ACTGAACCTGACCGTACA**CGTACACGTACACCCAGAGG
ABCC5	Forward	CTTGTTTTGCTGCAGGGCTC
	Reverse	**ACTGAACCTGACCGTACA**GCTGGTTCTCTCCCTCACAC
ABCC6	Forward	GCTGGAACCTGGTGAAGTCT
	Reverse	**ACTGAACCTGACCGTACA**TTTGCGCATGCGTGGATTTT
ABCC10	Forward	CGGCTAGGTCTTCCAACCTC
	Reverse	**ACTGAACCTGACCGTACA**CAGGCATCCGGAACCTCAAA
CYP3 A4	Forward	CACCCCCAGTTAGCACCATT
	Reverse	**ACTGAACCTGACCGTACA**CCCCACGCCAACAGTGATTA
CYP1B1	Forward	GCAAGGGCATGGGAATTGAC
	Reverse	**ACTGAACCTGACCGTACA**AAGGAACTGGGACCTTTGCC
CYP3A5	Forward	CTCCTCTATCTATATGGGACCCG
	Reverse	**ACTGAACCTGACCGTACA**GCACAGGGAGTTGACCTTCA
GAPDH	Forward	CTGAGTACGTCGTGGAGTC
	Reverse	**ACTGAACCTGACCGTACA**CAGAGATGATGATGACCCTTTTG

ACTGAACCTGACCGTACA represents the Z sequence

Table 7: Antibodies information.

Primary antibodies						
Name	**Species**	**Technique**	**Molecular weight (KDa)**	**Supplier**	**Product code**	**Dilutions**
Anti- IDO1	Mouse	Western blotting and IHC	45	Abcam	Ab156787	WB: 1:500 IHC: 1:100
Anti- TSP1	Rabbit	IHC	NA	Thermo Scientific	MA5-13377	IHC: 1:50
Anti- Vwf	Mouse	IHC	NA	Abcam	AB6994	IHC: 1:100
Anti-GAPDH polyclonal antibody	Mouse	Western blotting	37	Santa Cruz	sc-47724	WB: 1:2000
Anti- Ki67 Alexa Fluor 488 conjugate	Rabbit	Flow cytometry	NA	Cell Signaling	11882	FC: 1:50
P21 Waf1/Cip1 Alexa Fluor 488 conjugate	Rabbit	Flow cytometry	NA	Cell Signaling	5487	FC: 1:50
IgG Isotype control Alexa Fluor 488 conjugate	Rabbit	Flow cytometry	NA	Cell Signaling	4340	FC: 1:50

Secondary antibodies					
Name		**Species**	**Supplier**	**Product code**	
Anti-mouse IgG peroxidase conjugate	NA	Rabbit	Sigma-Aldrich	A-9044	1:1000
Anti-rabbit IgG peroxidase conjugate	NA	Goat	Sigma-Aldrich	A-9169	1:1000

NA: not applicable; WB: western blot, IHC: immunohistochemistry; FC: flow cytometry

2.1.5 Specialised reagents

Recombinant human Interferon-γ (IFN-γ)

IFN-γ was purchased from Sigma-Aldrich, and diluted to 100 µg/mL with sterile BSS containing 0.1% BSA and store at -80^{o}C until use.

Recombinant human Thrombospondin 1 (TSP1)

TSP1 was purchased from Sigma-Aldrich, and diluted to 100 µg/mL with sterile BSS containing 0.1% BSA and stored at -80^{o}C until use.

L-tryptophan

Tryptophan was purchase from Sigma-Aldrich, and diluted to 20 mM with sterile H_2O and stored at -80^{o}C until use.

Tryptophan free Dulbecco's Modified Eagle's Medium (DMEM)

DMEM without tryptophan was purchased from USBiological, Life Science (Salem, MA, USA), and diluted with distilled H2O, filtered and stored at 4oC until use.

Dialyzed Fetal Bovine Serum (FBS)

Dialyzed FBS was purchased from Thermofisher Scientific (Rockford, IL, USA), and stored at -20^{o}C until use.

Docetaxel

Docetaxel was purchased from Sigma-Aldrich, and reconstituted with DMSO to a final concentration of 100 mM and stored at -80^{o}C.

Cell tracers

CellTrace™ Oregon®Green 488 Carboxylic Acid Diacetate, Succinimidyl Ester and CellTracker™ Orange CMRA Dye were purchased from ThermoFisher, reconstituted in DMSO to a final concentration of 1µg/µL and stored at -20°C.

2.1.6 Standard reagents and solutions for Western blot

Lysis Buffer

This was made up by dissolving NaCl150mM (8.76g), Tris 50mM (6.05g), Sodium azide 0.02% (200mg), Sodium deoxycholate 0.5% (5g), Triton X-100 1.5% (15ml), Aprotinin 1µg/ml (1mg), Na3VO45mM (919.5mg), Leupeptin 1µg/ml (1mg) in 1 L of distilled water. The solution was then stored at 4°C for further use.

10% Ammonium Persulphate (APS)

One gram of APS was dissolved in 10 ml distilled water and then stored at 4°C for further use.

Tris Buffered Saline (TBS)

10X TBS (0.5M Tris, 1.38 M NaCl, pH 7.4) stock solution was prepared by dissolving606g of Trisand765g of NaCl (Melford Laboratories Ltd., Suffolk, UK) in 10L distilled water. The pH was adjusted to 7.4 using HCl and stored at room temperature.

10X Running Buffer (for SDS-PAGE)

10X running buffer (0.25M Tris, 1.92M glycine, 1% SDS, pH8.3) was prepared by dissolving 303g of Tris, 1.44kg of Glycine (Melford Laboratories Ltd., Suffolk, UK) and 100g SDS (Melford LaboratoriesLtd., Suffolk, UK) in 10L distilled water. It was diluted to 1X running buffer with distilled water before use.

2.1.7 Standard solutions for immunohistochemistry

Diaminobenzidine (DAB) chromagen

2 drops (approximately 50µl) of wash buffer, 4 drops of DAB (Vector Laboratories, Inc., Burlingame, USA) and 2 drops of H2O2 were added to 5ml of distilled water and mixed well before use.

ABC Complex

The ABC complex is prepared by using a kit provided by Vector Laboratories Inc. 4 drops of each reagent A and B were added to 20ml of wash buffer before being mixed thoroughly and left at room temperature for 30 minutes before use.

2.2 Cell culture, maintenance and storage

2.2.1 Medium preparation and cell maintenance

Several growth media were used in this study. For instance, Dulbecco's Modified Eagle's medium (DMEM/ Ham's F-12 with L-Glutamine; Sigma-Aldrich, Inc., Poole, Dorset, England, UK) was supplemented with 10% heat inactivated Foetal Bovine Serum (Sigma-Aldrich, Inc., Poole, Dorset, England, UK) and antibiotics. The other growth media and respective supplements can be seen in the Table 3 and Table 4. Media was stored at 4^{o}C until used for no more than 1 month. Media and trypsin/EDTA were warmed up to 37^{o}C before use.

Cells were routinely cultured in T25 and T75 flasks and media changed every 2 days. Culture specifications of each cell line or primary cells can be found in the Table 3 and Table 4.

When cells reached 80-90% confluency medium was aspirated and cells were washed twice with sterile PBS, in order to remove any remained medium that can inhibit trypsin activity.

1 – 2 ml of Trypsin/EDTA was added to each culturing flask and incubated at 37^{o}C for 2-5 minutes to detach adherent cells. Cells were visualised under a light microscope in order to verify if cell had detached, flasks were also gently tapped to help

cells to detach from the surface of the flask. After cells detached the flask, 4-5 ml of medium was added to the cells to inhibit trypsin activity and cells were collected into a universal tube (Greiner Bio-One Ltd, Gloucestershire, UK). Cell suspension was centrifuged at 1,700 xg for 5 minutes, supernatant was aspirated and pellet re-suspended in the appropriated medium. Cells were re-cultured by transferring 1/4 of the cell suspension into a fresh culturing flask or cells were counted to be used in an experiment or to be stored at -80^{o}C.

Cells were counted using a cell counter (Tali^{TM}, Invitrogen). A slide containing 25 µl of the cell suspension was inserted into the cell counter, which allowed visualisation of the cells, in order to know if there were any cell aggregations. The cell counter calculated the cell concentration and the size of the cells in a graph.

2.2.2 Storage

Cells were stored in liquid nitrogen at low passage in order to have a stock of cells for the project.

Cells were trypsinised and counted as described above. Cell suspension was diluted to 1 million cells/ mL and 900 µl of the cell suspension was transferred into 1 mL CRYO.S^{TM} (Greiner Bio-One, Germany). One hundred µl of Dimethylsulphoxide (10% DMSO; Fisons, UK) was added to each tube and mixed gently. Tubes were closed, wrapped in tissue paper and stored at -80^{o}C overnight. Samples were then transferred to liquid nitrogen for long term storage.

In order to revive the cells, tubes were removed from the liquid nitrogen and quickly thawed in a 37^{o}C water bath. Cells were then transferred into a universal tube with 5 mL of pre-warmed medium and gently centrifuged at 1000 xg for 5 minutes. Supernatant was aspirated to remove the DMSO. Cells were re-suspended in medium and transferred into a culturing flask. Cells were then kept in an incubator.

2.3 Methods for RNA detection

2.3.1 Total RNA isolation

Total RNA was isolated from cells using the Tri Reagent kit (Sigma-Aldrich, Inc., Poole, Dorset, UK), protocol was carried out according to the manufacturer's instructions. The cell type and the conditions that cells were cultured are described in the specific result chapter. Culturing plate was placed on ice and cells were then washed twice with PBS and 1 mL of Tri Reagent was added to the cells, in order to induce cell lysis. Cells were further scrapped with cell scrapers to ensure that all cells have been lysed. The homogenate was transferred into an eppendorf and incubated on ice for 5 minutes. Two hundred µl of chloroform (Sigma-Aldrich) was added to each tube, mixed for 15 seconds and incubated on ice for 5 minutes. The solution was then centrifuged at 12 000 x g for 15 minutes at 4^{o}C. Under this conditions, the solution separated into 3 phases: a pink organic phase was at the bottom of the tube which contains proteins, a white phase which contains DNA was placed in the inter segment, and on the top there was an aqueous phase containing RNA. The top phase was carefully transferred to a fresh eppendorf, 500 µl of isopropanol (Sigma-Aldrich) was further added and samples were incubated for 10 minutes on ice. Samples were centrifuged at 12 000 x g for 10 minutes at 4^{o}C and supernatant was discarded. The pellet was washed twice with 75% ethanol in DEPC water. The RNA pellet was then air dried for 5- 10 minutes to remove any ethanol. Finally, the RNA was dissolved in DEPC water by vortexing. The DEPC water is used to inhibit the effects of RNAases as it contains histidine specific alkylating agent.

2.3.2 RNA quantification

After RNA isolation was completed, its concentration and purity was assessed by a UV1101 Biotech Photometer (WPA, Cambridge, UK). The spectrophotometer was set to read single strand RNA and the results were the difference of the absorbance between the RNA sample and the DEPC water (blank) at a wavelength of 260 nm. The purity was

assessed using the ratio of A260/A280 nm, RNA was considered of good quality when this ratio was between 1.7 and 2.0. Samples were then used for reverse transcription (RT) or stored at -80^{o}C.

2.3.3 Reverse Transcription of RNA into cDNA

Reverse transcription is a simple technique that converts mRNA into cDNA allowing further examination by PCR. The procedure employed iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories, California, USA) to convert 0.5 µg of RNA into cDNA.

Each RT reaction was set in PCR tubes (thin-walled 200 µl PCR tubes, ABgene, Surrey, UK) as described in Table 8.

Table 8: Components and volumes for the RT reaction.

Component	Volume per reaction (µl)
5x iScript Reaction Mix	4
iScript Reverse Transcriptase	1
RNA template	X (volume to have 0.5 µg)
Nuclease-free water	Up to 20
Total Volume	20

Solution was mixed, centrifuged to ensure the solution is at the bottom of the PCR tube and incubated in a T-Cy Thermocycler (Creacon Technologies Ltd, The Netherlands) through the following temperatures:

5 minutes at 25^{o}C

30 minutes at 42^{o}C

5 minutes at 85^{o}C

At the end of the reaction, cDNA was diluted 1:4 with PCR water, and used immediately as a template for PCR or stored at -20^{o}C until needed.

2.3.4 Quantitative RT-PCR (Q-PCR)

Q-PCR is a sensitive technique which allows the detection and quantification of extremely small amounts of cDNA. In this method, it is used a sequence specific DNA based fluorescence reporter probe that recognises and binds DNA containing the probe sequence, allowing its quantification. Here it was used the AmplifluorTM Universal system (Intergen company®, New York, USA) in order to quantify the DNA copies. This probe contains a 3' region that recognises the Z-sequence (ACTGAACCTGACCGTACA), which is present on the reverse target specific primers. The probe also contains a 5' hairpin structure labelled with a fluorophore (FAM). This structure does not emit any fluorescence when in its hairpin conformation, once it is linked to an acceptor moiety (DABSYL) which quenches the fluorescence. However, when the probe is incorporated and it is used as a template for DNA polymerisation, the DNA polymerase degrades and unfolds the hairpin conformation disrupting the fluorophore-quencher structure which allows the emission of fluorescence. The signal emitted during each PCR cycle is detected and it is directly correlated to the amount of DNA being amplified. The technology requires 15-20 cycles to consume the reverse Z-primer. The process described here is illustrated in Figure 9.

Table 9: Components and volumes for a Q-PCR reaction.

Component	Volume (µl)
Forward primer	0.3 (10pmol/µl)
Reverse Z primer	0.3 (1pmol/µl)
Amplifluor probe	0.3 (10pmol/µl)
2x iQTM Supermix	5
cDNA	4

Each Q-PCR reaction was set as described in the Table 9, loaded into a 96 well plate (BioRad laboratories, Hemel Hampstead, UK) and placed in an iCyclerIQ thermal cycle and detection software (BioRad) at the following conditions:

Initial denaturation: 94^{o}C for 5 minutes

Followed of 80 cycles of:

Denaturation 94^{o}C for 10 seconds

Annealing: 55^{o}C for 35 seconds

Extension: 72^{o}C for 20 seconds

Signal is quantified at the annealing stage by a camera that detects the fluorescence emitted. A threshold cycle (Ct) for each reaction is then determined which

is used to calculate the amount of the specific cDNA. The amount of a housekeeping gene (GAPDH) was also assessed in order to normalise the samples. The amount of the specific DNA is determined by ΔΔCt. For each sample, the Ct value of the gene being tested was subtracted by the GAPDH Ct value (ΔCt). Followed by the 2^ (-(ΔCt1- ΔCt2)), where ΔCt1 corresponds to a reference sample (such as control) and ΔCt2 corresponds to a tested sample (such as treated sample).

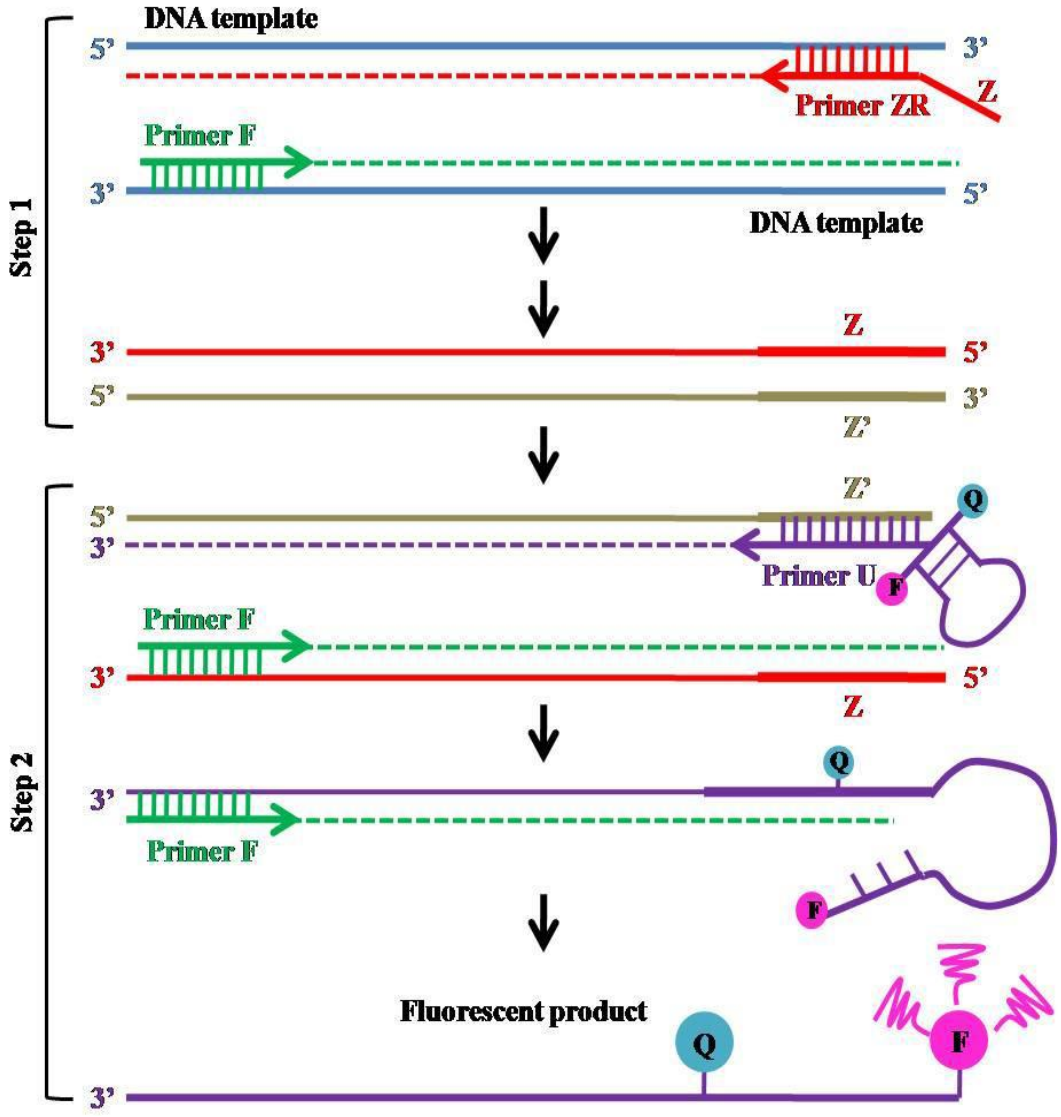


Figure 9: Diagram illustrating the incorporation and emission of the signal of the fluorescence reporter probe during the DNA amplification in Q-PCR.

2.4 Protein detection

2.4.1 Protein extraction and preparation of cell lysates

Cells were washed twice with PBS and 50 µl of lysis buffer was added to the well. Cells were then scraped off and the cell suspension was transferred into an eppendorf. The eppendorf was placed on a Labinco rotating wheel (Wolf laboratories, York, UK) for 1 hour at 4^{o}C to allow for cell lysis. In order to pellet cell debris, the cell suspension was centrifuged at 13 000 xg for 15 minutes at 4^{o}C. The supernatant was transferred into a fresh eppendorf and protein was quantified or stored at -20^{o}C.

2.4.2 Protein quantification

For an accurate analysis of the protein by western blotting, the total protein in each sample has to be standardised, for that reason the protein was first quantified. The protein quantification followed the protocol by Bio-Rad DC Protein Assay kit (Bio-Rad laboratories, Hemel Hampstead, UK). The first step was to serially dilute 50 mg/mL of bovine serum albumin (BSA) (Sigma, Dorset, UK) to a concentration of 0.78 mg/mL in order to set up a standard curve of protein concentration. In a 96 well plate, 5 µl of sample or standard was added into a well. All samples and standards were done in duplicate. The next step was to add 25 µl of working reagent A, which was prepared by adding 20 µl of reagent S/ 1 mL of reagent A, followed by 200 µl of reagent B to each well. Solution was mixed and then plate was left at room temperature for 20- 30 minutes avoiding direct light. After the period of incubation to allow the colorimetric reaction, the absorbance was read at 620 nm using the spectrophotometer (Bio-Tek, Wolf laboratories, York, UK). Using the absorbance values of the BSA dilutions, a standard curve was set and an equation was established. Protein concentration of each sample was calculated using the equation from the standard curve. Samples were then diluted in lysis buffer to a concentration of 1.0 µg/µl. Finally, samples were further diluted in 2x Lamelli sample buffer concentrated (Sigma-Aldrich, St Louis, USA), followed by a denaturation step

which was reached by boiling samples at 100ºC for 10 minutes. Samples were then loaded onto a SDS-PAGE gel or stored at -20ºC.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The system employed here was the OmniPAGE VS10 vertical electrophoresis system. Ten percentage resolving gels were prepared, as described in the Table 10, to assess IDO1 and GAPDH protein.

Table 10: Ingredients for a 10% resolving gel (enough for 2 gels)

Component	Volume (mL)
Distilled water	5.9
30% acrylamide mix (Sigma-Aldrich)	5.0
1.5M Tris (pH 8.0)	3.8
10% SDS	0.15
10% Ammonium persulphate	0.15
TEMED (Sigma-Aldrich)	0.006

Once the resolving mixture was done, it was poured in between of two glass plates that were on the loading cassette. The level of the resolving gel was until about 1.5 cm below the top of the edge of the plate. A thin layer of isopropanol (Sigma -Aldrich) was added to the top of the resolving gel to prevent oxidation. Gels were left at room temperature for 30 minutes to allow polymerisation. Isopropanol was removed and stacking gel was added on the top of the resolving gel. Stacking gel was prepared as described in the Table 11.

Table 11: Ingredients for the stacking gel

Component	Volume (mL)
Distilled water	3.4
30% acrylamide mix (Sigma-Aldrich)	0.83
1.5M Tris (pH 6.8)	0.63
10% SDS	0.05
10% Ammonium persulphate	0.05
TEMED (Sigma-Aldrich)	0.005

A well forming Teflon comb was inserted at the top of the plate. Once the stacking gel was set, the loading cassette was placed into an electrophoresis tank and covered with 1x running buffer. The comb was carefully removed, 5 µl of BLUeye Prestained Protein Ladder (Geneflow, Lichfield, UK) was added to the first well and 30 µl of each sample was added to the followed wells. In the empty wells, it was added 30 µl of 1x Lamelli sample buffer. The gels were then run at 110V, 50mA and 50W for 1.5- 2 hours.

2.4.3 Western blotting: transferring protein from gel to nitrocellulose membrane

Once the SDS-PAGE was complete, protein was transferred onto a nitrocellulose membrane by western blotting. The electrophoresis cassette was disassembled and stacking gel was discarded. Two pieces of transfer buffer pre-soaked sponges were placed on a transfer cassette, a piece of transfer buffer pre-soaked filter paper (Whatman International Ltd., Maidstone, UK) was placed on the top and the resolving gel was placed on the filter paper. On the top of the gel, it was placed a sheet of Hybond nitrocellulose membrane (Amersham Biosciences UK Ltd., Bucks, UK). An additional piece of pre-soaked filter paper and 2 pieces of pre-soaked sponges were added to the

top of the membrane, and the transfer cassette was closed and placed into a transfer tank with 1x transfer buffer. Electroblotting was carried out at 100V, 500mA and 8W for 1 hour. Once the transference was finished, the membranes were blocked at room temperature for 1 hour in 10% skimmed dry milk solution (10% milk powder and 0,1% of Tween (Sigma-Aldrich) in TBS).

After the blocking step, membranes were incubated with the primary antibody in the blocking solution for 1 hour. Membranes were then washed 3 times with 0.1% Tween-TBS (TBS-T), 5 minutes each, followed by incubation with secondary antibody for 1 hour. Membranes were washed again 3 times with TBS-T.

Protein was visualised by chemiluminescence detection. Membrane was incubated with Luminata (Millipore, Billerica, MA, USA) for 5 minutes, which consists of a chemiluminescent substrate that detects the horseradish peroxidise (HRP) in the secondary antibody. The excess solution was drained over a piece of tissue paper. The chemiluminescent signal was detected using a G:BOX Chemi XRQ imager (Syngene, Cambridge, UK).

The housekeeping gene GAPDH was used to normalise the experiments. The intensity of the bands shown by the chemiluminescent reaction was quantified by ImageJ (National Institute of Health, USA).

2.4.4 Immunohistochemistry of tissue microarray

In order to analyse IDO1 and TSP1 protein in breast cancer samples, immunohistochemistry was carried out in a tissue microarray in sequential slides. IDO1 and Von Willebrand factor (vwf) were also assessed in a second breast cancer tissue microarray, also in sequential slides. The protocol followed here is a modified protocol described previously by Jo and collaborators (Jo et al., 2011). Prior to staining, the slides were dewaxed in xylene and hydrated through alcohol gradient (100%, 95%, 70%, 50% and 30% ethanol, 5 minutes in each). For antigen retrieval, slides were immersed in citrate buffer (pH 8.0) and heated in a microwave (≥700W) for 20 minutes. Slides were

left to cool down at room temperature. Endogenous peroxidase activity was blocked with 3% H2O2 for 5 minutes and washed 3 times with TBS. Non-specific binding was blocked by incubating slides for 1 hour at 4ºC with blocking buffer (1% BSA, 1% Marvel and 5% goat serum in TBS). Samples were incubated with primary antibody (antibody specifications in Table 7) at 4ºC overnight. Mouse and rabbit IgG control antibodies were used as negative controls. On the following day, slides were washed 3 times with TBS for 5 minutes each time and incubated with secondary antibody (antibody specifications in Table 7) for 30 minutes at room temperature. The slides were then washed 3 times in TBS for 5 minutes each time and incubated with the ABC complex for 30 minutes (Vector Laboratories, Peterborough, UK). The colour reaction was developed with 3,3'-diaminobenzidine (DAB) and the sections were then counterstained with haematoxylin (Vector Laboratories, Peterborough, UK). Finally, sections were washed in tap water, dehydrated through a series of graded ethanol (30%, 50%, 70%, 95% and 100%), cleared in xylene, and mounted in DPX, followed by observation and imaging under an optical microscope. At ×200 magnification, the staining intensity was assessed in different cell types as 0 (negative), 1 (weak), 2 (intermediate) and 3 (strong) by two independent observers (FR, LJ).

2.4.4.1 Histomorphometric analysis of tissue microarray

The area fraction of staining occupied by the tumour cells, stroma and vascular ECs was evaluated. An eyepiece systemic point-sampling grid with 100 points and 50 lines was placed on the top of the section pictures at 400x magnification, as ellustrated in the Appendix III, and it was counted the number of points overlying positively-stained cells, as previously described (Ruan et al. 2011). Measurements were averaged over five microscopic fields to obtain an indexed percentage. Comparisons were performed in 20% of the staining by the two observers (LJ, JC), the coefficient of variation for the inter-observer error regarding cell count was <5%.

2.4.5 Enzyme-linked immunosorbent assay (ELISA)

The concentration of TSP1, VEGF and L-kynurenine was assessed in the conditioned medium of cells by ELISA. To perform this assay, it was used 3 different kits: TSP1 ELISA kit (R&D systems, Abingdon, UK), VEGF ELISA kit (Thermo Fisher Scientific, Runcorn, Cheshire, UK) and L-kynurenine ELISA kit (ImmuSmol, Bordeaux, France). The protocol was carried out according to the specific kit manufacturer's instructions. The generic protocol is described above.

The generation of the samples is described in the method section of each result chapter. Conditioned medium was collected and centrifuged at 17000 x g to pellet any debris and dead cells. Samples used to assess L-kynurenine had to go through an acylation step, where 20 µl of sample or standard were mixed with 500 µl of acylation buffer and 50 µl of acylation reagent and it was incubated at 37 ^{o}C for 90 minutes.

In the meanwhile, plates were washed 3 times with wash buffer and 300 µl of blocking buffer was added to each well. In the case of the TSP1, plate was coated with capture antibody the day before (100 µl/ well). One hour after incubation with blocking buffer, wells were washed 3 times with wash buffer and 100 µl of each sample or standard was added to the respective well. After 2 hours of incubation at room temperature, wells were washed again and 100 µl of detection antibody was added to each well. Two hours after, wells were washed and 100 µl of streptavidin-HRP was added to each well. Plate was incubated at room temperature for 20 minutes and direct light was avoided. Wells were once again washed and 100 µl of substrate solution was added to each well. After 10-20 minutes, 50 µl of stop solution was added to each well and mixed gently. The optical density was measured in a spectrophotometer at 450 nm. A standard curve was built using the absorbance values of the standard solutions and an equation that represents that curve was found. The concentration of TSP1, VEGF or L-Kynurenine was calculated using that equation.

2.5 Knocking down IDO1 gene expression using siRNA

In order to knockdown endothelial IDO1 expression, endothelial cells were transfected with small interference RNA (siRNA) which specifically targets IDO1 mRNA.

Ratcliff et al. discovered the RNA interference (RNAi) mechanism in plants (Ratcliff et al., 1997) and only a year later, Fire et al. demonstrated that double-stranded RNA (dsRNA), when microinjected into *Caenorhabditis elegans*, led to a transitory decrease of gene expression (Fire et al., 1998). In 2001, Elbashir et al. demonstrated that this mechanism could be applied to mammalian cells showing that siRNA could specifically knockdown a gene in several mammalian cell lines (Elbashir et al., 2001).

The siRNAs used here were purchased from Dharmacon (GE Healthcare, Lafayette, USA). Cells were transfected with a SMARTpool siRNA which contains 4 different siRNA sequences to increase the knockdown effect. As a negative control, cells were transfected with a non-targeting negative control. All information on the siRNA used here are presented in the Table 12.

Since HMVECad cells do not constitutively express IDO1, to the cells were stimulated by IFNγ or breast cancer conditioned medium. Cell number and protocol to stimulate IDO1 expression is described in the respective result chapters.

All knockdown experiments were conducted in 24 well plates and cells were transfected with Lipofectamine 3000 (Invitrogen, Paisley, UK) as described by the manufacturer's instructions and described below. Cells were cultured in antibiotics free medium prior to transfection and cells were at the confluency of 60 – 80% at the time of transfection.

First, the transfection solution was prepared (volumes correspond to one reaction). One hundred µl of DMEM (no FCS or antibiotics) was transferred to an eppendorf, followed by 5 µl of siRNA (20 µM) and solution was carefully mixed. In a

different eppendorf, 100 µl of DMEM (no FCS or antibiotics) and 5 µl of Lipofectamine were mixed. The content of the second eppendorf was then mixed to the first one and left to incubate at room temperature for 30 minutes. An extra 800 µl of DMEM (no FCS or antibiotics) was added to the eppendorf. Cells were washed twice with PBS and the transfection mixture was added to the well and plate was placed in a standard incubator at 37ºC. After 6- 8 hours, 1 mL of full endothelial medium was added to the cells, and 24 hours after transfection, medium was aspirated, cells washed with PBS and fresh medium was added to the cells. Knockdown confirmation was done 48 and 72 hours after transfection.

Table 12: siRNA information (Dharmacon, GE Healthcare, Lafayette, USA).

	siRNA code number	siRNA sequence
IDO1 pool	J-010337-09	UCACCAAAUCCACGAUCAU
	J-010337-10	UUUCAGUGUUCUUCGCAUA
	J-010337-11	GUAUGAAGGGUUCUGGGAA
	J-010337-12	GAACGGGACACUUUGCUAA
Non-targeting	D-001810-10-05	UGGUUUACAUGUCGACUAA
		UGGUUUACAUGUUGUGUGA
		UGGUUUACAUGUUUUCUGA
		UGGUUUACAUGUUUUCCUA

2.6 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The MTT assay is a common method to assess proliferation, viability and cytotoxicity of health cells in response to a stimulus. MTT can be cleavage by dehydrogenase enzymes in the cell into an insoluble purple substrate. The insoluble

formation can be dissolved in proper solvents and measured spectrophotometrically and the proliferation/viability/toxicity can be quantified.

After a set amount of time of treatment or culture, 10% of the culture volume of MTT (5 mg/mL in PBS) was added to the cells, plate was shaken for 5 minutes and then cells were incubated at 37^{o}C for 4 hours. Medium was discard and 100 µl of isopropanol was added to each well. Plate was then shaken for 30 minutes to ensure that all precipitate is dissolved by the isopropanol. The absorbance of the converted dye was measured at 540 nm in a spectrophotometer.

2.7 Flow cytometry

This technique was used to analyse the expression of Ki67 and p21 in cells under different conditions. Cells were washed with PBS and harvested at 17000 x g for 5 minutes. Supernatant was discarded and cell pellets were re-suspended in 100 µl of 4% formaldehyde for 10 minutes on ice for 1 minute. Nine-hundred µl of ice-cold ethanol was added to the cell suspension, vortexed and left on ice for 30 minutes. Following incubation, cells were then counted and aliquoted 1 x 10^{6} cells/ tube. Cells were washed twice with incubator buffer (0.5% BSA in PBS) by centrifugation. Cells were incubated for 1 hour with relevant antibodies diluted in incubator buffer. Then, cells were washed twice with incubator buffer. At the end of the second wash, cells were re-suspended in PBS. Samples were analysed using the flow cytometer (BD FACSCANTO II, Beckman Coulter (UK) Ltd., High Wycombe, UK) and the FCS Express 4 Flow Research Edition software (DeNovo, Glendale, CA, USA), measuring any fluorescence emission at 530 nm and 575 nm. A range of controls were used to assure the quality of the results, including non-stained cells and cells incubated with the respective IgG control.

2.8 Apoptosis analysis by flow cytometry

Annexins are a family of calcium-dependent phospholipid-binding proteins, which bind to phosphatidylserine (PS) indicating apoptotic cells. In healthy cells, PS is

predominantly located along the cytosolic side of the plasma membrane. Upon initiation of apoptosis, PS loses its asymmetric distribution in the phospholipid bilayer and translocates to the extracellular membrane, which is detectable with fluorescently labelled Annexin V. In early stages of apoptosis, the plasma membrane excludes viability dyes such as propidium iodide (PI) and 7-AAD, therefore cells which display only Annexin V staining (PI/7-AAD negative) are in early stages of apoptosis. During late-stage apoptosis, loss of cell membrane integrity allows Annexin V binding to cytosolic PS, as well as cell uptake of PI and 7-AAD. Annexin V staining, paired with 7-AAD or PI is widely used to identify apoptotic stages by flow cytometry.

For this technique, it was used an Annexin V apoptosis kit (eBioscience, San Diego, US). The protocol was carried out according to the manufacturer's instructions as follows. Both adherent cells and cells floating in the medium were harvested, centrifuged and washed once in PBS. Cells were re-suspended in 1 X binding buffer at a concentration of 1 x 10^6 cells/mL. 200 µl of the cell suspension was transferred into a new eppendorf. Five µl of Annexin V- FITC was added to each eppendorf and mixed well for 10 minutes. Cells were then centrifuged, washed in binding buffer and re-suspended in 190 µl of the same buffer. Ten µl of Propidium iodide (20 µg/mL) was added to the cell suspension. Stained cells were analysed immediately or kept on ice and in the dark until required, but no more than 4 hours. Apoptosis analysis was procedure using the BD FACSCANTO II flow cytometer (Beckman Coulter (UK) Ltd., High Wycombe, UK) and FCS Express 4 Flow Research Edition software (DeNovo, Glendale, CA, USA). Fluorescence was read at 530 nm and 575 nm. In order to have a positive control for apoptosis, cells were treated with staurosporine (Sigma, 1 µg/mL) for 1.5 hours before harvesting. Negative and single staining was also carried out using apoptotic cells in order to calculate compensation settings and define gates.

2.9 Cell sorting

This technique was used to separate two different cell lines that were co-cultured for a period of time. One of the cell lines was pre-labelled with a red dye (CellTrackerTM Orange CMRA Dye) by incubating the cells with the dye (1 µg/mL) diluted in FCS free DMEM for 20 minutes.

At the end of the co-culture time, cells were washed twice with PBS and harvested. Cells were re-suspended in ice cold PBS at the density of $1x10^6$ cells/100 µl. The two different cell lines were separated on the basis of the size and labelled fluorescent dye (emission at 575 nm) in the MoFlo™ XDP (Beckman Coulter (UK) Ltd., High Wycombe, UK). A range of controls were used to define proper gates for the sorting, including non-labelled cells and only labelled cells.

2.10 statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc, San Diego, CA). Data were represented as mean± S.E.M. Student's t- test was used to evaluate the statistical difference of two groups, while One-Way ANOVA was employed to calculate the difference between more than 2 groups. Linear regression was also used in this study to find a correlation between two dependent variables. The sample size of each experiment was at least 3. The legend of each figure clearly informs of the statistical test used and the sample size of each experiment. P value < 0.05 was considered statistical significant.

Chapter III: IDO1 and TSP1 expression in breast cancer tissue

3.1 Introduction

Indoleamine-2,3- dioxygenase (IDO1) is an intracellular enzyme that degrades L-tryptophan in mammalians (Austin and Rendina, 2015). IDO1 is overexpressed in many types of tumour, which has been shown to confer immune tolerance to cancer cells (Munn et al., 2004; Munn et al., 2002; Uyttenhove et al., 2003). Although the IDO1 mechanism of action is not fully understood, research suggests that IDO1 leads to a depletion of tryptophan in the tumour microenvironment, which induces apoptosis of T-cells (Friberg et al., 2002; Uyttenhove et al., 2003). IDO1 is expressed in tumours by many cell types, including cancer cells, macrophages, dendritic cells and endothelial cells (Vigneron et al., 2015). IDO1 expression has been correlated with a poor prognosis in several tumours, including breast cancer (Astigiano et al., 2005; Brandacher et al., 2006; Chen et al., 2014; Inaba et al., 2010; Inaba et al., 2009; Ino et al., 2006; Jia et al., 2015; Okamoto et al., 2005; Pan et al., 2008; Speeckaert et al., 2012; Suzuki et al., 2010; Urakawa et al., 2009; Wainwright et al., 2012; Witkiewicz et al., 2008; Yu et al., 2013; Yu et al., 2011), however more studies are needed to clarify this assumption.

Thrombospondin 1 (TSP1) is an extracellular matrix protein that belongs to the thrombospondin family (Adams and Lawler, 2004, 2011). TSP1 was the first protein to be identified as an endogenous anti-angiogenic molecule (Good et al., 1990). In tumours, decreased level of TSP1 has been linked with tumour growth and angiogenesis (Kawakami et al., 2001). Interestingly, TSP1 has also been shown to promote of metastasis (Incardona et al., 1995; Yee et al., 2009). In the tumour microenvironment TSP1 is secreted by stromal fibroblasts, endothelial cells and immune cells (Lawler, 2002).

This chapter aimed to evaluate the expression of IDO1 and TSP1 in human invasive breast cancer, lymph node metastasis and adjacent normal tissue. It also aimed to assess whether stromal TSP1 and vascular IDO1 correlate with the TNM stage or with the grade of the primary tumours. As this thesis hypothesises that the enzymatic activity

of vascular IDO1 limits the synthesis of TSP1, it was also logic to determine if there was a correlation between these two molecules.

3.2 Material and methods

3.2.1 Tissue microarrays

Two human tissue microarrays were used in this study. One of the tissue arrays (USBIOLAB, Maryland, US) contained 52 sections of tissue (breast cancer) while the second one (Biomax, Inc. Rockville, MD, USA) had 101 sections of tissue (tissue from 50 primary tumours, 40 lymph node metastasis, 10 adjacent tissues and an adrenal gland section). For each tissue array, there were 2 sequential slides. The 52 samples tissue array was used to stain for IDO1 and vwf. The other tissue array was used to assess the expression of IDO1 and TSP1.

3.2.2 Immunohistochemistry

IDO1, TSP1 and vwf expression was assayed in the tissue arrays by immunohistochemistry. Description of the full protocol and antibody details can be found in the sections 2.4.4 and 2.1.4. Observation and imaging of the staining were done under an optical microscope. At ×200 magnification, the staining intensity was assessed as 0 (negative), 1 (weak), 2 (intermediate) and 3 (strong). The area fraction of staining occupied by the tumour cells, stroma and vascular cells was evaluated using an eyepiece systemic point-sampling grid as described in the section 2.4.4.1, a representative image with the grid system can be seen in the Appendix III. The area of the staining of IDO1 and TSP1 was also evaluated according to the same method.

3.3 Results

3.3.1 Differential expression of TSP1 and IDO1 in breast cancer tissue

The tissue microarray used here contained samples from patients with invasive breast carcinoma. The primary tumour samples comprised forty-six cases of invasive duct carcinoma, one neuroendocrine carcinoma and three medullary carcinomas. The microarray also contained forty metastatic lymph node, ten adjacent normal tissues and a pheochromocytoma (male) which could be used as a control. IDO1 and TSP1 expression was assessed by immunohistochemistry in sequential sections of the tissue in 50 primary breast cancer samples, 40 lymph node metastasis and 10 adjacent normal tissues.

IDO1 and TSP1 expression were assessed by immunohistochemistry and the intensity of the signal was scored from 0-3 (weak to strong). The TSP1 staining was very week in the invasive breast carcinoma tissue, while stronger in adjacent normal tissue, as shown in the representative images in Figure 10 (All pictures shown in Appendix IV). TSP1 staining was also weak in the lymph node metastasis (Figure 10 and Appendix IV). TSP1 was observed as typical fine fibrillary stromal staining as well as in the basement membrane of the ductal space (Figure 10). As represented in Table 13, the average intensity of the TSP1 signal in the invasive breast carcinoma was significantly weaker when compared to the adjacent normal tissue (p= 0.021). The intensity of the TSP1 staining was also found to be statistically weaker in the lymph node metastasis (p= 0.035). On the other side, there was no statistical differences between adjacent normal tissue and invasive breast carcinoma or lymph node metastasis in terms of IDO1 signal (Table 13).

To further evaluate the signal of TSP1 and IDO1 in the primary tumour, a histomorphometry analysis was adopted. This method allowed estimating the area of the TSP1 and IDO1 signal by cancer cells, stromal cells and vascular cells in the primary

tumour. In this analysis, the distinction between the different cell types was done taking into consideration the morphology of the cells. The TSP1 signal was strong in the adjacent normal tissue but it was weak in the primary tumour and lymph node metastasis (Figure 11 and Table 14). In the primary tumour the TSP1 signal was mostly observed in the stromal tissue. In average, 6.576% of the signal was observed in the stromal cells, while positive tumour cells corresponded to 2.12% and vascular cells to 0.016% (Table 14).

IDO1 signal was detected in the tissues of the primary tumour but also in the lymph node metastasis and adjacent normal tissue (Figure 11). In the primary tumour, IDO1 positive tumour cells accounted for 43.068% of the total IDO1 staining, while stroma accounted for 44.012 % and vasculature for 1.112% (Table 14).

Although TSP1 is a protein that is secreted by many cell types, it is constitutively synthesised by endothelial cells under physiological conditions (Ghajar et al., 2013). Since the main hypothesis of this work is that endothelial IDO1 limits the synthesis of TSP1, it is important to determine whether IDO1 is expressed in the tumour vasculature. As mention above, IDO1 staining showed that the vasculature does expresse IDO1. The microvasculature of the tumour was first identified only by its morphology. A vascular marker, vwf, were also used to determine the tumour vasculature. In a second tissue microarray, the IDO1 and vwf signal was detected by immunohistochemistry in sequential sections of primary breast cancer, allowing clearly identification of microvessels and assessment of endothelial/vessel staining of IDO1. In 23 breast cancer tissue sections it was possible to detect vessels, of which 21 were positive for IDO1 (Figure 12), corresponding to 91.3% of samples with vessels that exhibited vascular IDO1.

Table 13: Summary of the IHC intensity results in the adjacent normal tissue, invasive carcinoma and lymph node metastasis. Statistical significance of each group to the adjacent normal tissue was determined by One-way ANOVA.*p<0.05

		Staining intensity (SI)				Average ±SEM
		0	1	2	3	
Adjacent normal (n=10)	TSP1	0	9	1	0	1.100±0.100
	IDO1	1	5	4	0	1.3000±0.2134
Invasive carcinoma (n=50)	TSP1	26	23	0	1	0.5200±0.08685*
	IDO1	1	22	23	4	1.560±0.09545
Lymph node metastasis (n=40)	TSP1	17	18	3	2	0.7500±0.1279*
	IDO1	0	26	13	1	1.375±0.08539

Table 14: Summary of the IHC morphometric results in the primary tumour. The units "% of points" indicate the number of points overlying the structure of interest divide by total number of points overlying the tissues.

		Mean	minimum	maximum
Tumour Cells (%)	TSP1	2.12	0.00	16.50
	IDO1	43.068	5.00	98.60
Stromal (%)	TSP1	6.576	0.00	42.20
	IDO1	44.012	0.00	76.60
Vasculature (%)	TSP1	0.016	0.00	0.08
	IDO1	1.112	0.00	4.60

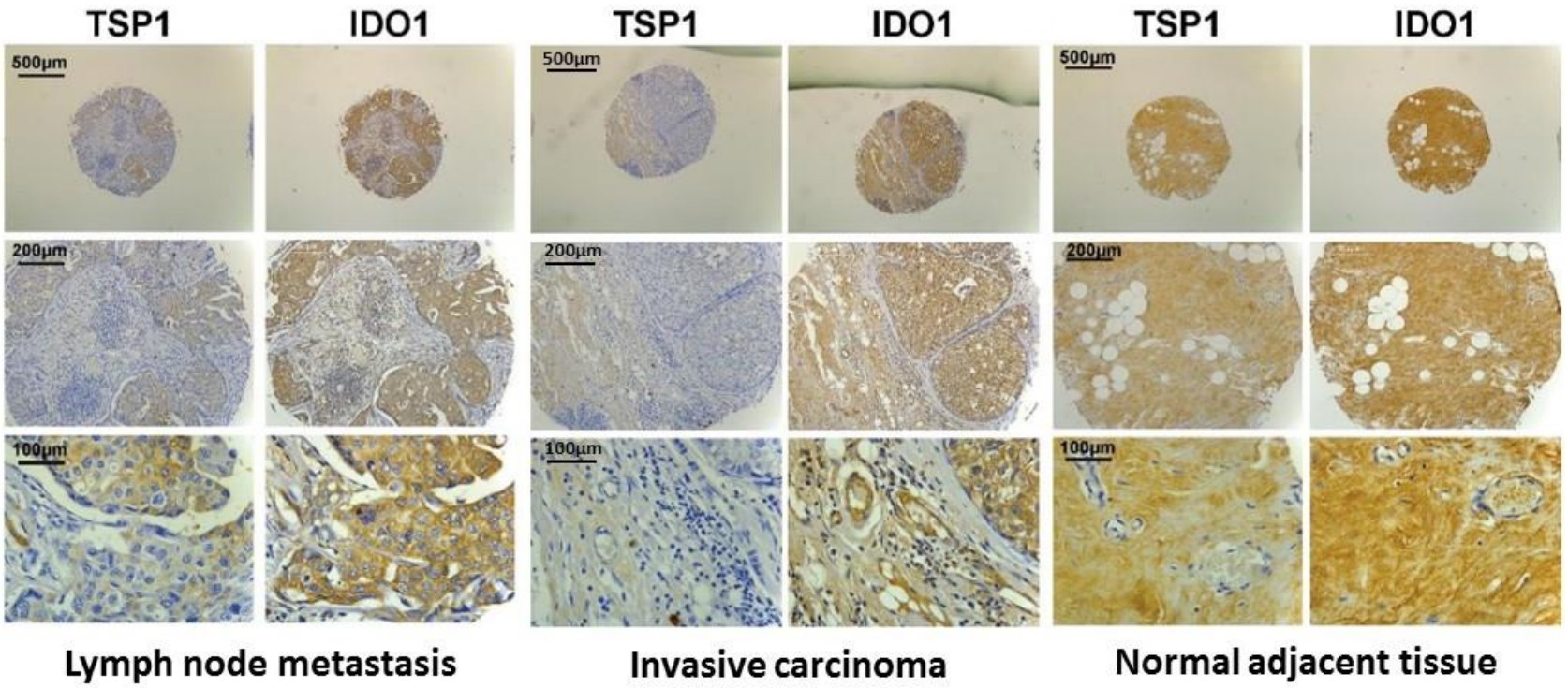


Figure 10: Representative immunohistochemistry images of TSP1 and IDO1 in tissue sections from breast cancer patients which englobes lymph node metastasis, invasive carcinoma and normal adjacent tissue.

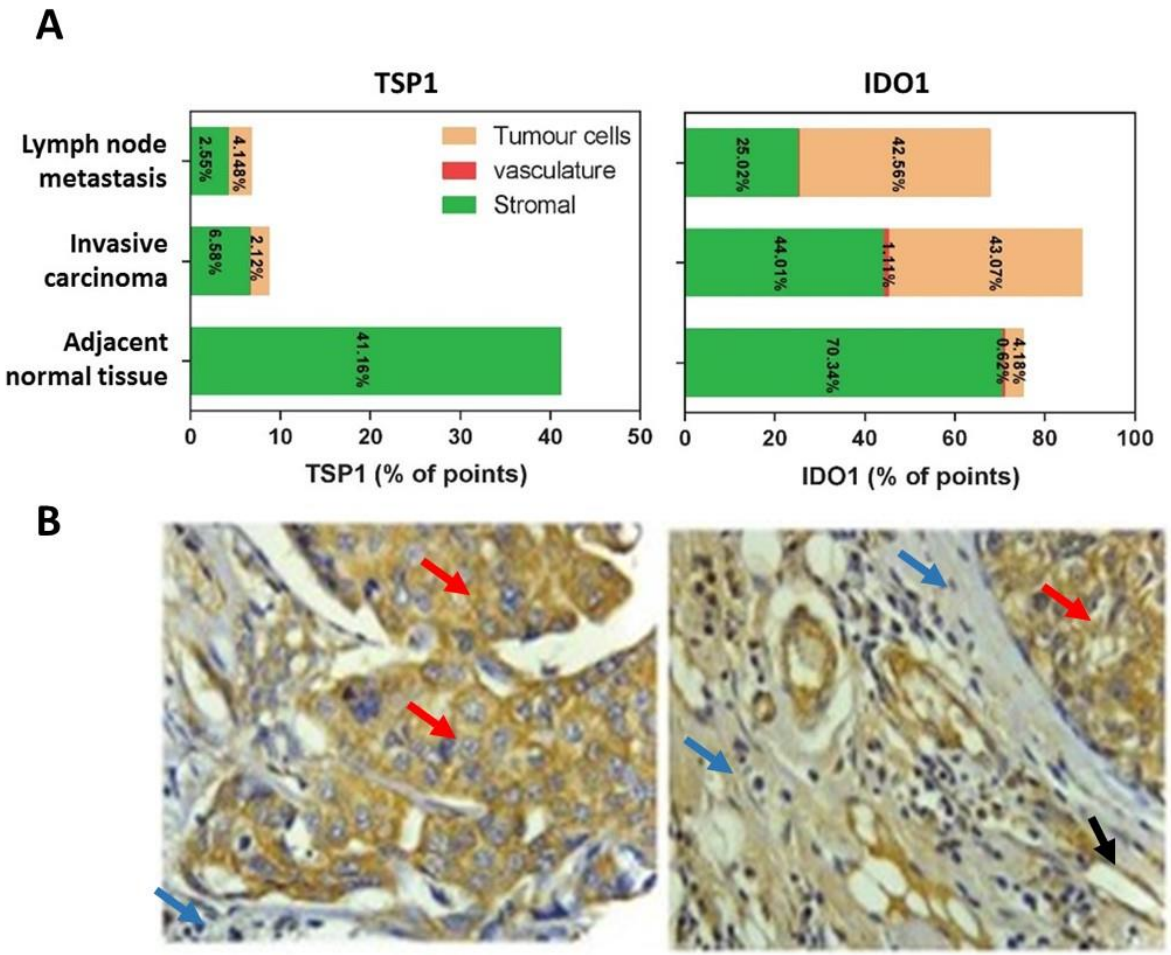
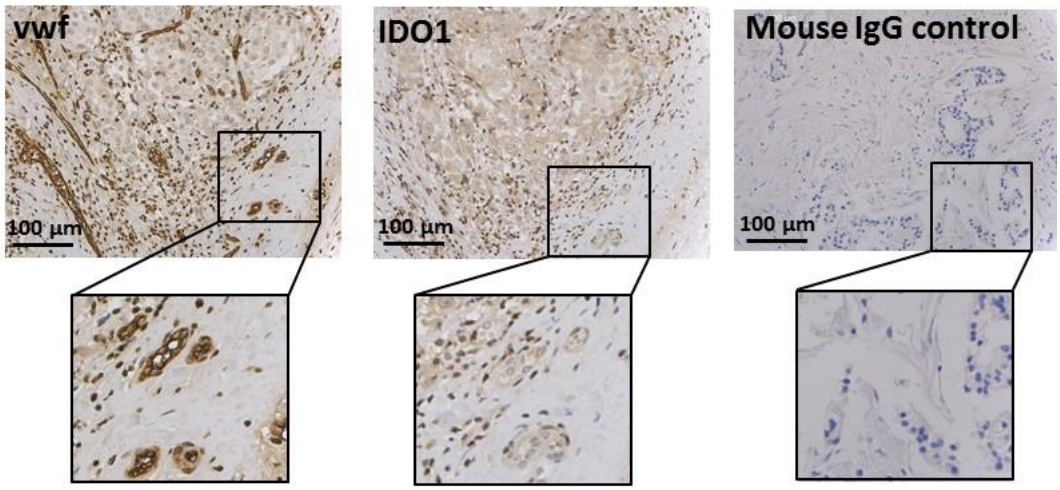


Figure 11: A- Percentage of TSP1 and IDO1 immunostaining area, in lymph node metastasis, invasive carcinoma and adjacent normal tissue, by tumour cells, vasculature and stromal cells. B- Two representative images of tumour sections stained by IDO1 where it is possible to see tumour cells (red arrow), stromal cells (blue arrow) and vessels (black arrow).

A



B

N. of samples with detectable vessels (vwf staining)	N. of samples where IDO1 was detected in vessels
23	21 (91.3%)

Figure 12: Detection of vascular IDO1 in invasive carcinoma by immunohistochemistry. A- Representative images of vwf and IDO1 immunostaining of sequential sections of invasive breast carcinoma. Representative image of their IgG control staining. B- Quantification of the total number of samples with detectable vessels and the number of samples with IDO1 expressing endothelial cells.

3.3.2 Stromal TSP1 and vascular IDO1 in invasive breast cancer

As disseminated breast cancer cells have been found dormant near microvessels (Ghajar et al., 2013), it has been speculated that the perivascular niche is responsible for inducing and maintaining the dormancy of those cells. The same study identified TSP1, an extracellular matrix protein that is secreted by endothelial cells, as a possible protein involved in this process. Thus, in the present study only stromal TSP1 has been analysed. The present study also hypothesised that TSP1 synthesis can be limited by vascular IDO1, therefore only vascular IDO1 has been considered in this section.

Breast cancer can be classified according to the TNM stage system, which evaluates the tumour on the basis of the primary tumour (T), lymph node (N) and metastasis (M). For the analysis of the TSP1 and IDO1 signal, it was only taken into account the primary tumour classification (T) of the TNM stage system because the tissue array did not have enough samples for some of the groups in the lymph node and metastasis classification. The T1 group (n=6) represents patients with a tumour not bigger than 2 cm (mean stromal TSP1: 9.90% of total TSP1), the T2 (n=27) contains patients with a tumour between 2 and 5 cm of dimension (mean stromal TSP1: 12.31% of total TSP1), the T3 patients (n=9) had tumours bigger than 5 cm (mean stromal TSP1: 2.089% of total TSP1), whereas T4 group (n=10) contains tumours of any size with direct extension to the chest wall or skin increasing the probability of metastasis (mean stromal TSP1: 1.900% of total TSP1). Although the stromal TSP1 staining is higher in the T2 stage, compared to T1, it was found an inverse correlation between the level of stromal TSP1 staining and the TNM grading (Figure 13 A; p=0.0207).

Breast cancer can also be classified according to the degree of differentiation of the tissue and cells. It can be graded 1- well differentiated, 2- moderately differentiated and 3- poorly differentiated. In terms of aggressiveness, grade 3 is the most aggressive phenotype. Stromal TSP1 staining was also negatively correlated to the grading of breast cancer (Figure 13 B; p=0.0375). The difference in the stromal TSP1 staining between

the grade groups was even more apparent when compared to the TNM classification, grade 1 tumours presented 17.43% of TSP1 positive- stromal cells (n=6), grade 2 tumours corresponded to 5.738% (n=32), and grade 3 tumours had 4.333% (n=6).

As mention above, IDO1 staining was strong in the primary tumour and only one sample was negative for this enzyme (Table 13). In terms of primary tumours, vascular IDO1 occurred in most cases (49/50). Although there was a trend towards a decrease in the vascular IDO1 with high T staging (1.300% for T1, 1.278% for T2, 0.320% for T3 and 0.640% for T4), it did not reach statistically significant (Figure 13 C; p=0.7481). On the other hand, vascular IDO1 expression was significantly decreased in the group of high grade tumours (Figure 13 D; p= 0.0247). The immunohistochemistry results showed that vascular IDO1 corresponded to 2.633% of the total IDO1 in grade 1 tumours, which is reduced to 0.9438% in grade 2 and it was very low in grade 3.

As TSP1 is secreted by endothelial cells, the vascular IDO1 might work as a negative regulator of the tumour stromal TSP1. Interestingly, a significant negative correlation between stromal TSP1 and vascular IDO1 (r^2=0.1125, p=0.000456) was found in the primary tumour tissues (Figure 13 E).

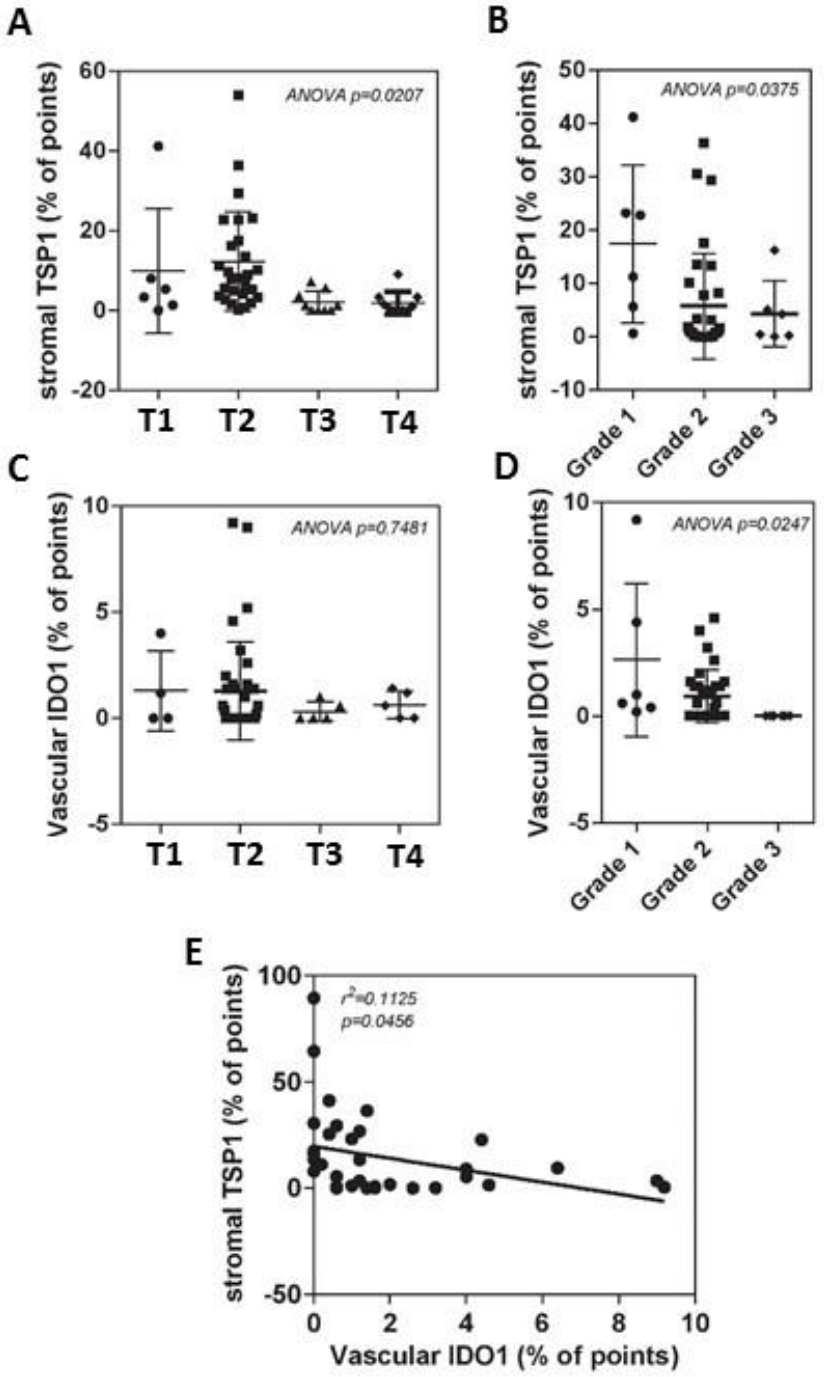


Figure 13: Stromal TSP1 and vascular IDO1 in invasive carcinoma.
Histomorphometric scores of stromal TSP1 in invasive carcinoma by A- the TNM stage system and by B- the grading system. Histomorphometric scores of vascular IDO1 in invasive carcinoma by C- the TNM stage system and by D- the grading system. E- Linear regression between the histomorphometric scores of stromal TSP1 and vascular IDO1 in invasive carcinoma. A-D, One-Way ANOVA was used to detect any differences between the groups.

3.3.3 Both stromal TSP1-medium/high and vascular IDO1-low groups exhibit a trend towards survival superiority of patients with breast cancer

Although both stromal TSP1 and vascular IDO1 in primary breast cancer correlated negatively with the progression of the tumour, it was found that these two molecules were negatively correlated between themselves. To further evaluate a possible role of these two molecules in the aggressiveness of the tumour, their signal was correlated with the overall survival of the patients. There was no statistical difference between the TSP1-low and TSP1-medium/high groups (p=0.3587), but a trend in overall survival was observed where survival was higher in the group of patients with low stromal TSP1 (Figure 14). On the other hand, overall survival of patients did not show a statistical difference between vascular IDO1-low and vascular IDO1-medium/high groups (Figure 14). However, the total survival curve also exhibited a trend of superiority for the vascular IDO1-low group compared to the vascular IDO1-medium/high group (Figure 14). These two trends observed here might reach significance if the sample size is increased.

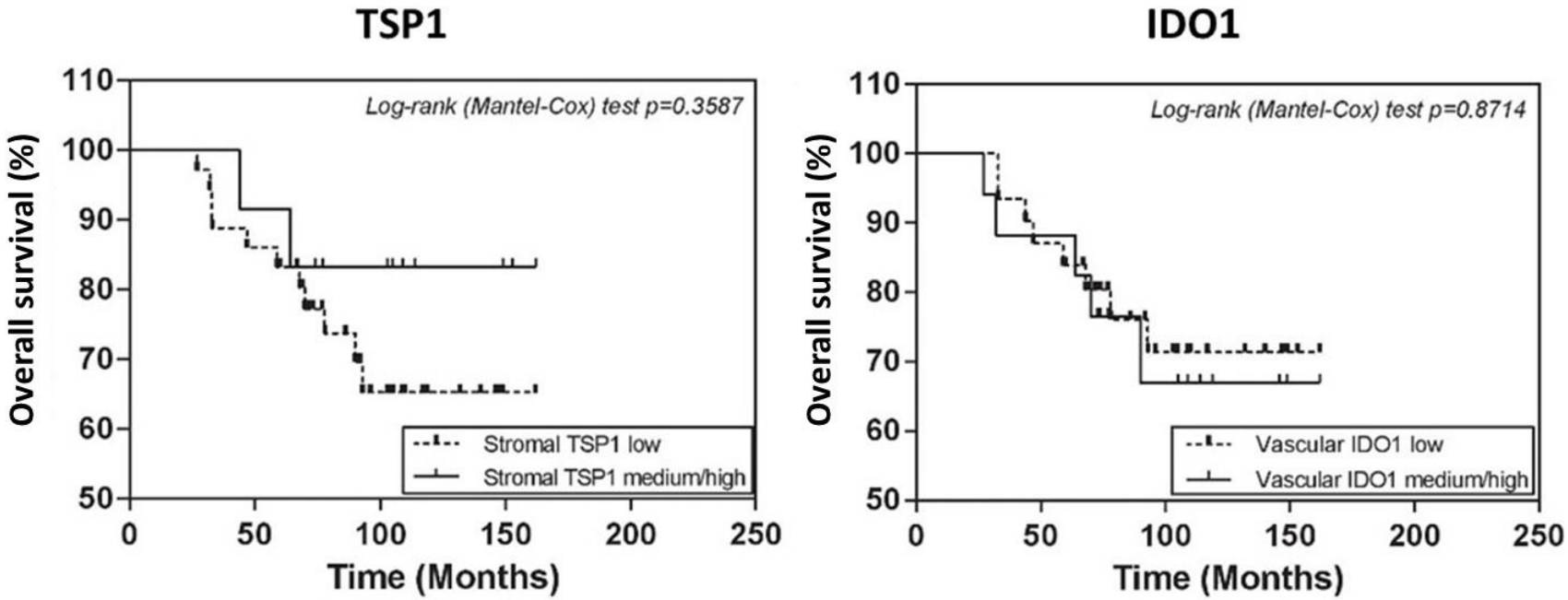


Figure 14: Stromal TSP1 and vascular IDO1 might be potential breast cancer survival markers. Kaplan-Meier invasive breast carcinoma patient's survival curves stratified by two groups of histomorphometric scores of stromal TSP1 or by two groups of histomorphometric scores of vascular IDO1. N (stromal TSP1 low) = 38, N (stromal TSP1 medium/high) = 12, N (vascular IDO1 low) = 37, N (stromal IDO1 medium/high) =13.

3.4 Discussion

Angiogenesis is essential for tumour growth and progression, once tumours cannot grow beyond 2-3 mm in diameter without the formation of new blood vessels (Vasudev and Reynolds, 2014). Angiogenesis is controlled by the ratio of anti- and pro-angiogenic factors in the microenvironment of the tumour. These angiogenic factors are secreted by the cells within the tumour microenvironment, such as cancer cells, macrophages, fibroblasts and endothelial cells (Lopes-Bastos et al., 2016). TSP1 was the first endogenous anti-angiogenic molecule to be identified (Good et al., 1990). In tumours, TSP1 has been correlated to tumour growth and vascularisation (Kawakami et al., 2001), and TSP1 inhibits angiogenesis and limits the growth of the primary tumour (Yee et al., 2009). Controversially, TSP1 has also been shown to promote metastasis (Incardona et al., 1995; Yee et al., 2009), probably due to the ability of TSP1 in promoting cell migration and invasion (Albo et al., 1997; Albo et al., 2000). However, there is a study showing that TSP1 expression in the primary tumour was inversely correlated with lymph node metastasis (Ioachim et al., 2012).

Here, 26 out of 50 invasive breast carcinoma cases showed the absence of TSP1 expression. The intensity of the TSP1 immunohistochemistry signal was significantly decreased in the primary tumour and the lymph node metastasis when compared to the adjacent normal tissue, suggesting a role of this extracellular matrix protein in the primary tumour but also at the metastatic site. Interestingly, TSP1 signal was mainly observed in the stroma of the primary tumour, and was inversely correlated with the size of the primary tumour, assessed by the tumour (T) stage. This finding confirmed the findings from the others that TSP1 limits tumour growth (Kawakami et al., 2001). Stromal TSP1 was also inversely correlated with tumour grade of the primary tumour, classified based on the degree of differentiation of cancer cells and poor differentiated cells indicate a more aggressive phenotype. There was a trend in the survival of the patients and the stromal TSP1, which suggested a protective role of stromal TSP1 in the primary tumour.

Taking together, these results indicated that stromal TSP1 might be a good marker for tumour progression and a good tool to assess the patient's survival. However, a larger cohort of samples is required to clarify whether TSP1 can be used as a predictive tool in breast cancer.

IDO1 is an intracellular enzyme that degrades tryptophan causing a local deprivation of that amino acid (Richard et al., 2009). IDO1 has been identified as an immune protective enzyme in physiological and pathological settings (Munn et al., 1998) once T cells are sensitive to the lack of tryptophan and initiate apoptosis (Carlin et al., 1989a; Fallarino et al., 2002; Guillemin et al., 2005; Munn et al., 1999; Munn et al., 2004; Munn et al., 2002; Nouël et al., 2015). During the metastatic process, cancer cells have to overcome the immune system in order to colonise a new organ (Aguirre-Ghiso, 2007). IDO1 has been increasingly recognised as one of the mechanisms employed by cancer cells to evade the immune system (Lee et al., 2002; Munn et al., 1999). A wide range of solid tumours express IDO1 and the level of IDO1 activity has been correlated with a poor prognosis (Astigiano et al., 2005; Brandacher et al., 2006; Chen et al., 2014; Inaba et al., 2010; Inaba et al., 2009; Ino et al., 2006; Jia et al., 2015; Okamoto et al., 2005; Pan et al., 2008; Speeckaert et al., 2012; Suzuki et al., 2010; Urakawa et al., 2009; Wainwright et al., 2012; Witkiewicz et al., 2008; Yu et al., 2013; Yu et al., 2011). Immunohistochemistry analysis of IDO1 protein I showed that the overall IDO1 protein is present at the same level in the invasive carcinoma and lymph node. Interestingly, the adjacent normal tissue also expressed the same level of IDO1. One of the plausible explanations for this observation is the fact that IDO1 expression is stimulated by cytokines, such as IFNγ (Gough et al., 2008; Jeong et al., 2009), resulting in easily diffusion of IDO1 to the adjacent tumour tissue. The tumour cells adapted the strategy to immunise the surroundings of the tumour. In the primary tumour, the IDO1 protein was expressed across the tissue and cancer cells, stromal cells and endothelial cells.

Disseminated breast cancer cells have been localised in the perivascular niche of several organs, including bone marrow, lung and brain (Ghajar et al., 2013). These cells were in a dormant state and one of the molecules present in the perivascular niche was TSP1, suggesting that TSP1 might be involved in inducing and/or maintaining the dormancy of single breast cancer cells (Ghajar et al., 2013).

This project hypothesised that vascular IDO1 might regulate the TSP1 synthesis and therefore cancer dormancy. Thus, this research focused on the vascular IDO1.

Interestingly, the vascular IDO1 levels did not correlate with the tumour staging, but it inversely correlated with the grading of the primary tumour. The vascular IDO1 might be more important in the early development of the tumour. Others group reported that IDO1 was only expressed in newly formed microvessels (Riesenberg et al., 2007). Although an inverse correlation between vascular IDO1 and the tumour grading, patients with low vascular IDO1 signal in the primary tumour showed a weak trend towards an improved survival rate. Lastly, an inverse correlation between vascular IDO1 and stromal TSP1 was found supporting the idea that vascular IDO1 might limit TSP1 synthesis.

Taking together, these results support the hypothesis that vascular IDO1 might regulate TSP1 synthesis in endothelial cells. Thus, the dormant cancer cells, near microvessels, may induce IDO1 expression in nearby endothelial cells in order to escape dormancy by reducing TSP1 synthesis.

Although the results above are encouraging, they have to be validated in a bigger cohort and in an in vivo dormancy model.

Chapter IV: Endothelial cells slow down MDA-MB-231 proliferation via TSP1

4.1 Introduction

Most tumour-related deaths are due to metastasis that grows in the host organs/tissues. Metastasis leads to the development of cachexia and subsequently weakens the functions of vital organs (Pantel and Brakenhoff, 2004). Disseminated tumour cells are cells that colonise distant tissues and organs and, upon successful survival in the new host environment, are the source of metastatic lesions. These cells often experience a period of dormancy (Schmidt-Kittler et al., 2003). About 20- 45% of breast and prostate cancer patients suffer relapses years or even decades after therapy (Karrison et al., 1999; Pfitzenmaier et al., 2006; Weckermann et al., 2001). A study detected disseminated tumour cells in 17% and 27% of breast cancer patients, depending on the cancer type, after treatment (Hall et al., 2010). It has been difficult and even impossible in most cases to determine whether and when metastases will occur (Aguirre-Ghiso, 2007). Although the recently proposed concept of metastatic niche can be used to explain the earlier relapses, the late relapsing population of cancer cells has yet to be studied and understood.

In vivo studies and the analysis of human tumour sample have unveiled that single and small cluster of tumour cells can remain dormant for a long period of time (Goss and Chambers, 2010; Naumov et al., 2002; Pantel et al., 1993; Suzuki et al., 2006). Because it is very challenging to identify disseminated tumour cells, it is not known much about them and key questions, such as the location that these cells reside and the inducing and awakening factors for their dormancy state, are still under investigation. Tumour dormancy can be divided into two major groups (Osisami and Keller, 2013): 1- tumour mass dormancy, in which cancer cells still divide but the lesion does not grow due to the lack of blood vessels or the action of the immune system, 2- cellular dormancy, in which cancer cells are in a quiescence state.

Ghajar and collaborators have demonstrated in a mouse model that disseminated breast cancer cells are found near microvessels in a brain, bone marrow and lung

(Ghajar et al., 2013). The extracellular matrix, in particularly the basement membrane, has shown to provide a microenvironment that allows survival, quiescence and resistance to cytotoxic molecules of mammary epithelial cells, key features common to dormant cancer cells (Bissell et al., 1982; Boudreau et al., 1995; Petersen et al., 1992; Spencer et al., 2011; Weaver et al., 2002; Weaver et al., 1997). Endothelial cells have been shown to induce and sustain the quiescent state of breast cancer cells and pre-treated lung carcinoma cells with endothelial cell conditioned medium resulted in a less metastatic phenotype (Franses et al., 2011; Ghajar et al., 2013). TSP1 was present in stable microvascular niches at high concentrations, which resulted in cellular quiescence. TSP1 was absent in the microenvironment of sprouting vessels, which did not stop cellular division, suggesting that TSP1 might be involved in inducing and maintaining cellular quiescence in cancer (Ghajar et al., 2013).

In this chapter, it was aimed to confirm that endothelial cells and TSP1 induce breast cancer dormancy. It was also aimed to evaluate whether TSP1 confers any resistance to breast cancer chemotherapy.

4.2 Material and Methods

4.2.1 Cell lines

Breast cancer cell lines MCF7 and MDA-MB-231, non- tumorigenic breast cell line MCF10A and primary endothelial cells HMVECad were used in this chapter. MDA-MB-231 and MCF7 cells were continuously maintained in DMEM with 10% FBS and antibiotics. MCF10a were continuously cultured in MEBM supplemented with MEGM kit and cholera toxin (100 ng/mL). HMVECad were continuously cultured in pre-coated flasks with attachment factor in EGM with endothelial supplements and antibiotics.

4.2.2 Fluorescence microscopy

HMVECad cells were seeded in pre-coated 6 well plates with attachment factor at the density of 500,000 cells/well. On the following day, MDA-MB-231, MCF7 and MCF10A cells, which are at 70- 80% confluency, were washed twice with PBS and incubated with serum free DMEM containing a red dye (1µg/mL; CellTracker™ Orange CMRA, life technologies) for 20 minutes. Cells were then trypsinized and 500,000 MDA-MB-231, MCF7 or MCF10A cells were seeded on the top of the endothelial monolayer. Forty- eight hours after co-culture, cells were visualised under a fluorescence microscope with a 40x objective. Cells were imaged using bright field and under FITC filter.

4.2.3 Flow cytometry

Flow cytometry was used to analyse several aspects: percentage of each cell line after a period of co-culture, percentage of MDA-MB-231 cells expressing ki67 and p21-waf1-cip1 after co-culture with endothelial cells, treatment with endothelial conditioned medium or TSP1 treatment.

For the co-culture *in vitro* model, HMVECad cells were incubated with serum free DMEM containing a red dye (1µg/mL; CellTrackerTM Orange CMRA, life technologies) for 20 minutes. Cells were then trypsinized and seeded in pre-coated 6 well plates with

attachment factor at the density of 500,000 cells/well. On the following day, 500,000 MDA-MB-231, MCF7 or MCF10A cells were seeded on the top of the endothelial monolayer. Twenty-four or 48 hours after the initiation of the co-culture, cells were trypsinized and re-suspended in ice-cold PBS at the confluency of $1x10^6$ cells/100 µl. Cells were then run on a flow cytometer and the percentage of each cell lines, or the percentage of ki67 or p21-waf1-cip1 positive MDA-MB-231 cells were evaluated. Full protocol for ki67 and p21-waf1-cip1 staining is described in section 2.7. In order to analyse the MDA-MB-231 cell cycle by PI staining after co-culture with endothelial cells, the endothelial cells were traced with a green dye (1µg/mL; CellTrace™ Oregon®Green 488 Carboxylic Acid Diacetate, ThermoFisher). PI staining protocol is described in section 2.9.

For the endothelial conditioned medium experiment, HMVECad were seeded in 6 well plates pre-coated with attachment factor at the density of 500,000 cells/well. On the following day, the medium was changed and conditioned medium after 48 hours culture was collected and centrifuged at 1,700 xg for 5 minutes to pellet any dead cells and debris. MDA-MB-231 cells were seed in 6 well plates at the density of 200,000 cell/mL. On the following day medium was aspirated, MDA-MB-231 cells were washed twice with PBS and 250 µl of DMEM and 750 µl of the endothelial conditioned medium was added into each well. As a control, 1 mL of DMEM was added to the cells. The percentage of Ki67 positive cells was evaluated by flow cytometry.

In order to evaluate the effect of TSP1 on the proliferation of MDA-MB-231 cells, MDA-MB-231 cells were seeded in 6 well plates at the confluency of 200,000 cells/mL. On the following day medium was aspirated, cells washed twice with PBS and 1 mL of medium with TSP1 (100ng/mL) or PBS (control) was added to the cells. Twenty-four hours after treatment, the percentage of Ki67 positive cells or the percentage of live, early apoptosis or late apoptosis was evaluated by flow cytometry. Apoptosis was evaluated by annexin V/propidium iodide apoptosis assay, full protocol in section 2.8.

4.2.3 ELISA

TSP1 concentration was assessed in the medium of several endothelial cell lines by ELISA. HMVECad, HECV, CMEC, TY09 and TY10 were seeded in 24 well plates at the density of 200,000 cells/mL. Media from each well was collected and centrifuged at 1,700 xg for 5 minutes before being used in the assay. The ELISA protocol for TSP1 is fully described in the section 2.4.5.

4.2.4 Proliferation /cell survival

MDA-MB-231 cell proliferation and cell survival was assessed by MTT.

MDA-MB-231 cells were seeded in 96 well plates at the density of 5,000 cells/mL. On the following day, the medium were aspirated, cells washed twice with PBS and 135 µl of medium containing 0, 10, 50 or 100 ng/mL of TSP1 was added to each well and left for 48 hours.

The second experiment aims to evaluate the effect of TSP1 on cell survival to a breast cancer drug, Docetaxel. First the percentage of cell survival of MDA-MB-231 cells to a range of concentrations of docetaxel was evaluated. MDA-MB-231 cells were seeded in 96 well plates at the density of 5,000 cells/mL. On the following day, the medium was aspirated, cells washed twice with PBS and 135 µl of medium containing docetaxel was added to the wells for 24 hours. The mitochondria activity was measured with MTT solution and read at 540 nm in a plate reader. The full MTT protocol is described in section 2.6. The next step of the experiment was to seed MDA-MB-231 cells as described above and treated them with 0, 10, 50 or 100 ng/mL of TSP1 for 48 hours. The medium was aspirated, and medium containing 100 µM of docetaxel was added to each well and left for 24 hours. The mitochondria activity was also measured with MTT at 540 nm in a plate reader.

4.2.5 RNA isolation, cDNA synthesis and qPCR

The expression of 13 genes (SLCO1B3, CYP3A4, CYP1B1, CYP3A5, ABCB1, ABCC1, ABCC2, ABCC4, CFTR, ABCG2, ABCC5, ABCC6 and ABCC10) was analysed by qPCR in the MDA-MB-231 cells. MDA-MB-231 cells were seeded in 6 well plates at the density of 200,000 cells/mL. In the first experiment, MDA-MB-231 cells were treated with TSP1 (100 ng/mL) or BSA (control) for 48 hours. In the second experiment, MDA-MB-231 cells were treated with TSP1 for 48 hours followed by 24 hours of docetaxel treatment (100 µM) or DMSO (control). RNA was isolated using the TRI reagent kit; the detailed protocol is described in the section 2.3.1, followed by the generation of cDNA by reverse transcription PCR, as described in the section 2.3.3. Quantitative PCR was then performed as described in the section 2.3.4 and data was normalised to GAPDH.

4.3 Results

4.3.1 Endothelial cells induce MDA-MB-231 cell cycle arrest

In order to study the influence of the endothelial extracellular matrix on breast cancer cells, a two-dimension *in vitro* model was developed. Stable microvessels are composed of non-proliferating endothelial cells (Ghajar et al., 2013), for this reason, a large number of endothelial cells were seeded to reach high confluency to mimic a stable endothelium niche (Ghajar et al., 2013).

To determine whether endothelial cells are able to induce breast cancer dormancy, cells from two different breast cancer cell lines, a non-aggressive cell line (MCF7) and an aggressive cell line (MDA-MB-231) and a non-tumorigenic breast cell line (MCF10A) were co-cultured on the top of an endothelial monolayer. Co-culture models can be challenging and medium and medium supplements may influence the results. To overcome this limitation, cells were cultured with half endothelial medium with its supplements and half breast cell medium and respective supplements. Breast cells were stained with a red dye before co-culture. The breast cells could be visualised by fluorescence microscopy and they also could be separated by flow cytometry. Fluorescence microscopy showed that both breast cancer cell lines, MDA-MB-231 and MCF7 cells, were well spread along the endothelial monolayer but they were still not confluent after 48 hours after seeding (Figure 15 A). Interestingly, MCF10A cells, a non-tumorigenic breast cell line, appeared to be much more confluent than MCF7 and MDA-MB-231 cells as they do in a single culture (Figure 15 A). However, to have a better understanding of the influence of the endothelial cells on the confluency and growth patterns of the breast cells used in this experiment, it should have been added proper controls. These controls would be each breast cell line cultured individually in the same culturing conditions. In order to have a more analytical evaluation of the proliferation of each cell line, the percentage of each breast cell line in co-cultured cell mixture with the endothelial monolayer was assessed by flow cytometry. Results show that MCF7 cell

line was the one that was in a smaller percentage in comparison to the endothelial cells (Figure 15 B), about 13.1 %. MDA-MB-231 cells were about 30.6 % of the cells after 48 hours of co-culture with endothelial cells (Figure 15 B). Surprisingly, MCF10A cells were almost 80% of the cells after 48 hours of co-culture (Figure 15 B). Results show that there is a significant difference in the percentage of MDA-MB-231 and MCF7 in comparison to MCF10A. MCF7 cells are also in a smaller percentage than MDA-MB-231 cells. The proliferation marker Ki67 was also evaluated on MDA-MB-231 cells of co-culture with endothelial cells, revealing that a decreased proportion of MDA-MB-231 cells express this molecule (Figure 16 B and C, p=0.0021). The senescence marker p21-waf1-cip1 was also assessed, showing that the percentage of MDA-MB-231 cells expressing this marker significantly decreased after co-culture with endothelial cells (Figure 16 D and E, p=0.0025). Cell cycle analysis by propidium iodide showed that there is a significant accumulation of MDA-MB-231 cells in the G0/G1 phase (Figure 17 A, p<0.0001) and a decrease in the percentage of MDA-MB-231 cells in the S phase after 24 hours of co-culture with endothelial cells.

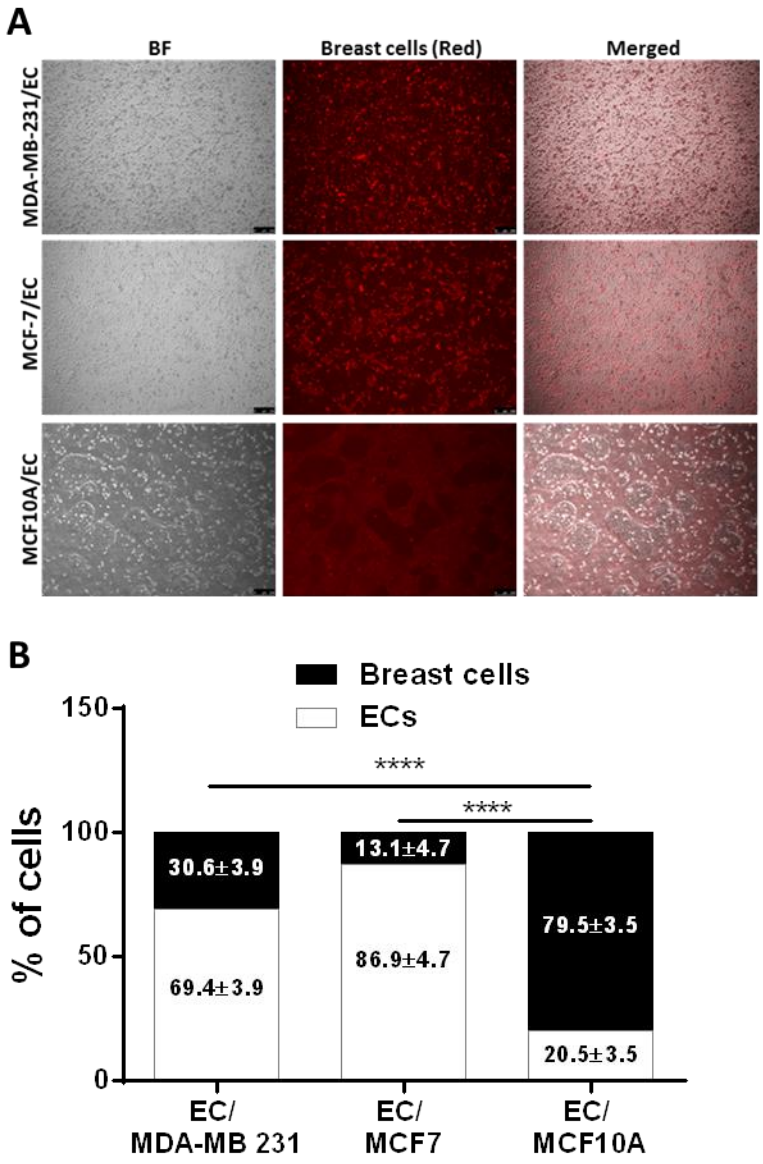


Figure 15: Endothelial cells reduce breast cancer proliferation but not normal breast cell proliferation. A) Representative images of the co-culture of MDA-MB-231, MCF7 or MCF10A cells with endothelial cells (ECs, in red). B) Percentage of endothelial cells and breast cells 48 hours after co-culture was initiated, 1 million of each cell type were seeded in each well. N=3. One-way ANOVA was used to analyse any statistical differences between the groups. ****p< 0.0001.

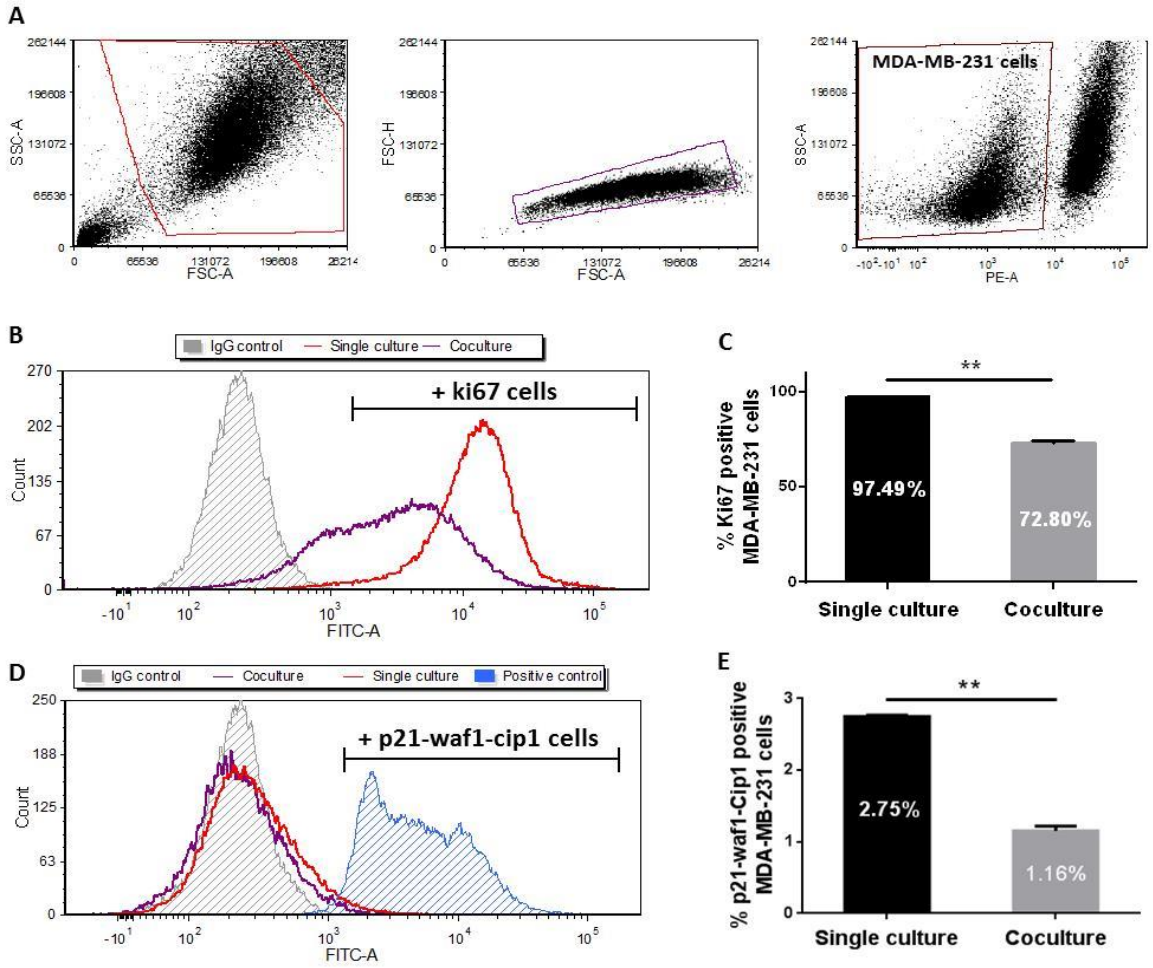


Figure 16: MDA-MB-231 cells reduce Ki67 and p21 expression when in co-culture with endothelial cells for 24 hours. A- Representative gates used to select live cells, single cells and MDA-MB-231 cells, respectively. B, C- percentage of Ki67 positive MDA-MB-231 cells. D, E- percentage of p21-waf1-Clp1 positive MDA-MB-231 cells. N=3, bars are mean±S.E.M. Student t-test was used to identify any statistical differences between the groups. **p<0.01.

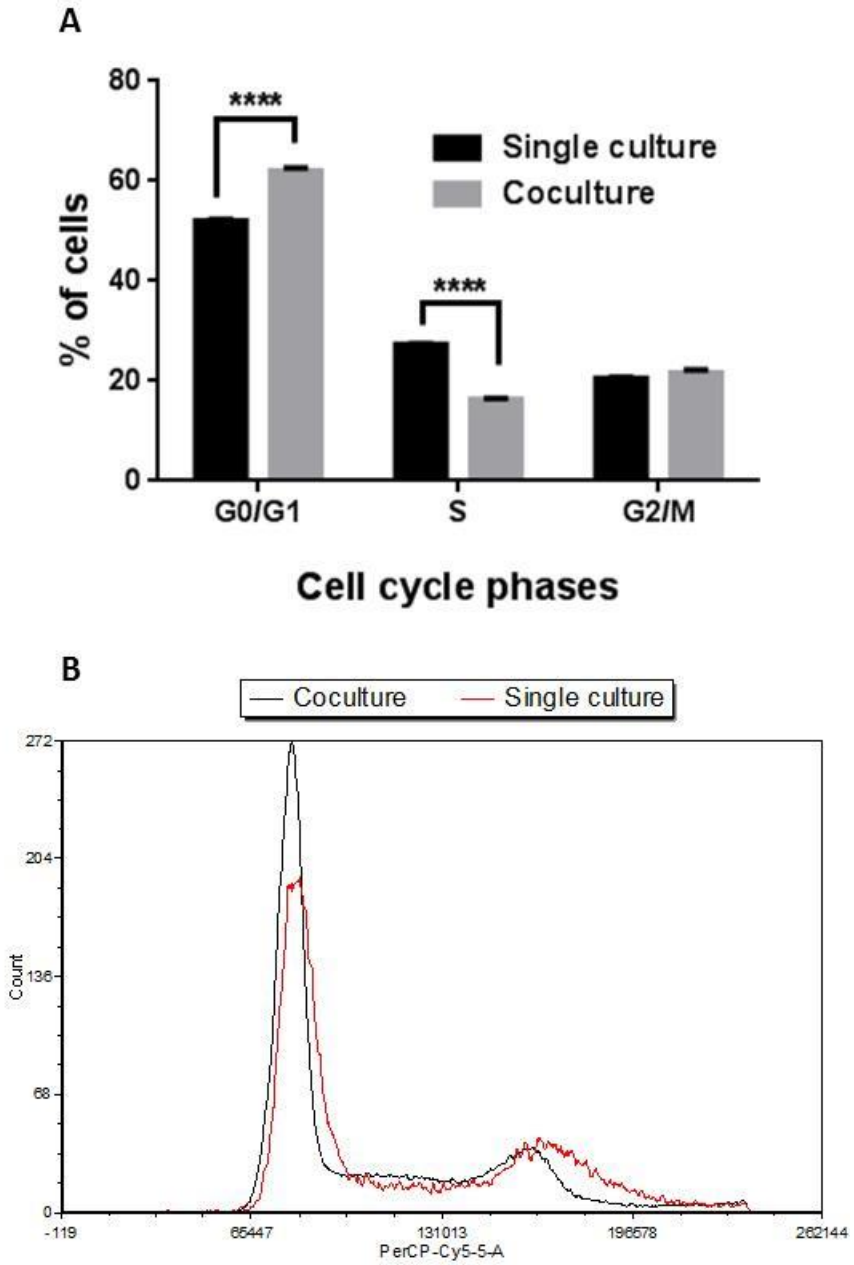


Figure 17: The proportion of MDA-MB-231 cells in G0/G1 increases, and S decreases, when in co-culture with endothelial cells for 24 hours. A, B- percentage of cells in each cell cycle phase in a bar graph and a representative flow cytometry histogram, respectively. N=3, bars are mean±S.E.M. One-way ANOVA was used to detect any statistical differences between the groups. ****p<0.0001.

4.3.2 TSP1 reduces MDA-MB-231 proliferation

To determine whether endothelial cells regulate breast cancer cell proliferation through soluble factors, MDA-MB-231 cells were cultured with the endothelial conditioned medium. This study intended to investigate the influence of endothelial cells and TSP1 on breast cancer cells that later will eventually form a tumour. Once MCF-7 is a non-aggressive cell line and doesn't form tumours when injected into mice, only MDA-MB-231 cells were used in these experiments. Flow cytometric analysis of Ki67 expression showed that conditioned medium induced a significant reduction in MDA-MB-231 cell proliferation (Figure 18, p=0.0425). The TSP1 concentration of several EC conditioned medium was evaluated by ELISA including those from microvascular endothelial cells (HMVECad), endothelial cells from the umbilical cord (HECV) and microvascular endothelial cells from the brain (TY09 and TY10). All the endothelial cells in this experiment secreted TSP1 and its concentration ranged from 100 to 50 ng/mL (Figure 19 A).

Culturing MDA-MB-231 cells with different TSP1 concentrations (0, 10, 50 and 100 ng/mL) showed that TSP1 inhibited MDA-MB-231 cell proliferation with a significant decrease at 100 ng/mL (Figure 19 B, p=0.0325). Flow cytometry also showed a significant decrease in the percentage of MDA-MB-231 cells expressing Ki67 when cultured with TSP1 (100 ng/mL, Figure 19 D, p=0.0269). Since TSP1 induced a decrease in MDA-MB-231 proliferation of just over 10%, it was logic to determine whether TSP1 also triggers apoptosis in breast cancer cells. To assess apoptosis, the presence of annexin Vin the outer side of the cellular membrane, a marker of apoptosis, and the infiltration of propidium iodide into the cells, a marker of necrosis, was evaluated. Flow cytometric analysis of annexin V revealed that comparing MDA-MB-231 cells cultured with and without TSP1 (100 ng/mL), TSP1 did not change the percentage of live, apoptotic or necrotic cells (Figure 20 B).

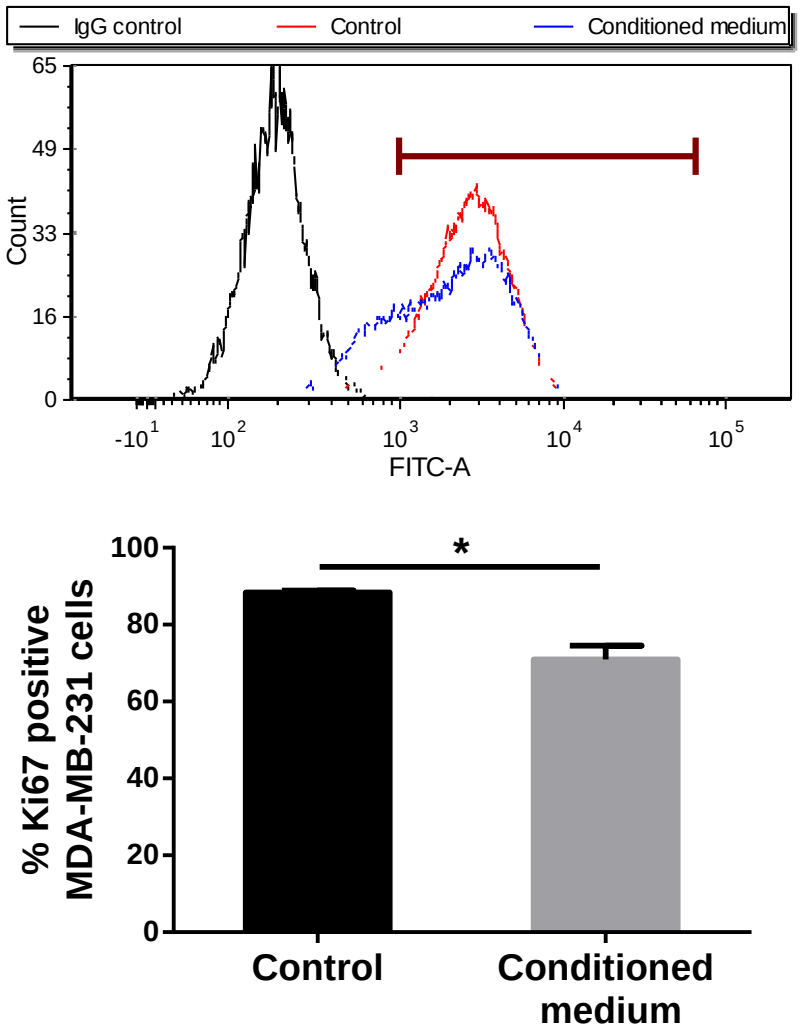


Figure 18: MDA-MB-231 cells decrease Ki67 expression when cultured with endothelial cell conditioned medium for 24 hours. A, B- Representative flow cytometry histogram and bar graph, respectively. N=3, bars are mean±S.E.M. Student t-test was used to detect any statistical differences between the groups. *p<0.05.

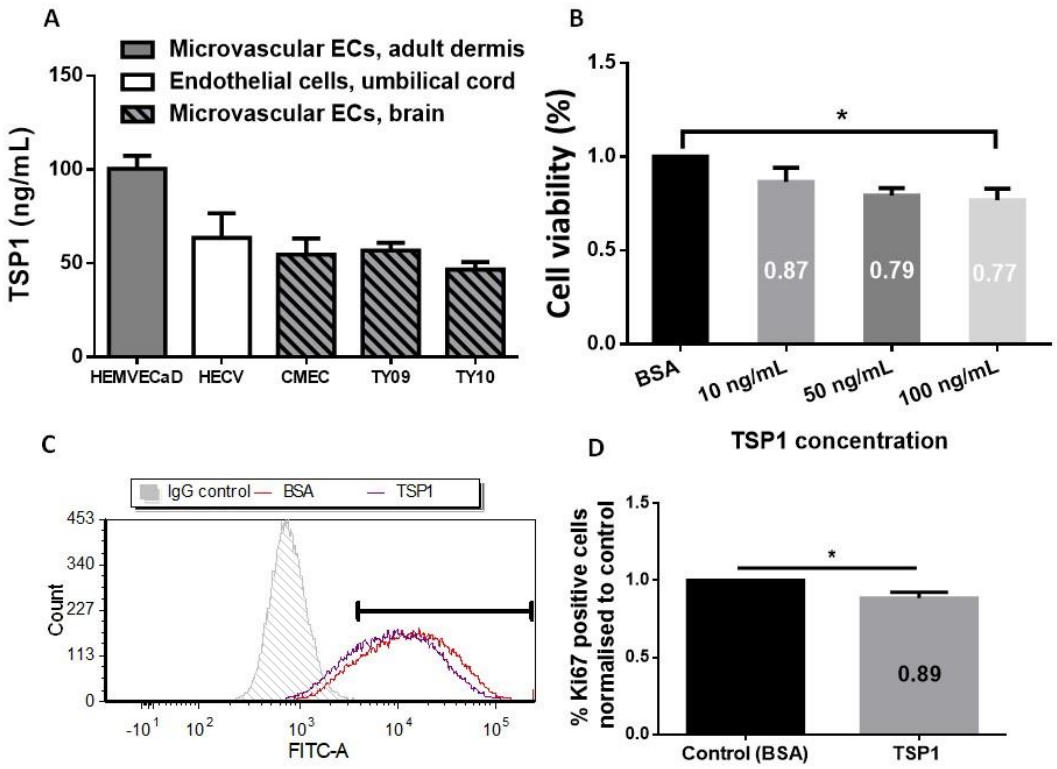


Figure 19: A: TSP1 expression in different endothelial cells and the effect of TSP1 on MDA-MB-231 proliferation. A- TSP1 concentration in the medium of several endothelial cell types. B- MDA-MB-231 cell proliferation when cultured with TSP1 normalised to the control group (BSA). C,D: Percentage of Ki67 positive MDA-MB-231 cells when cultured with TSP1 normalised to the control group (BSA). N=4, bars are mean±S.E.M. One-way ANOVA and Student t-test were used to detect any statistical differences between the groups. *p<0.05.

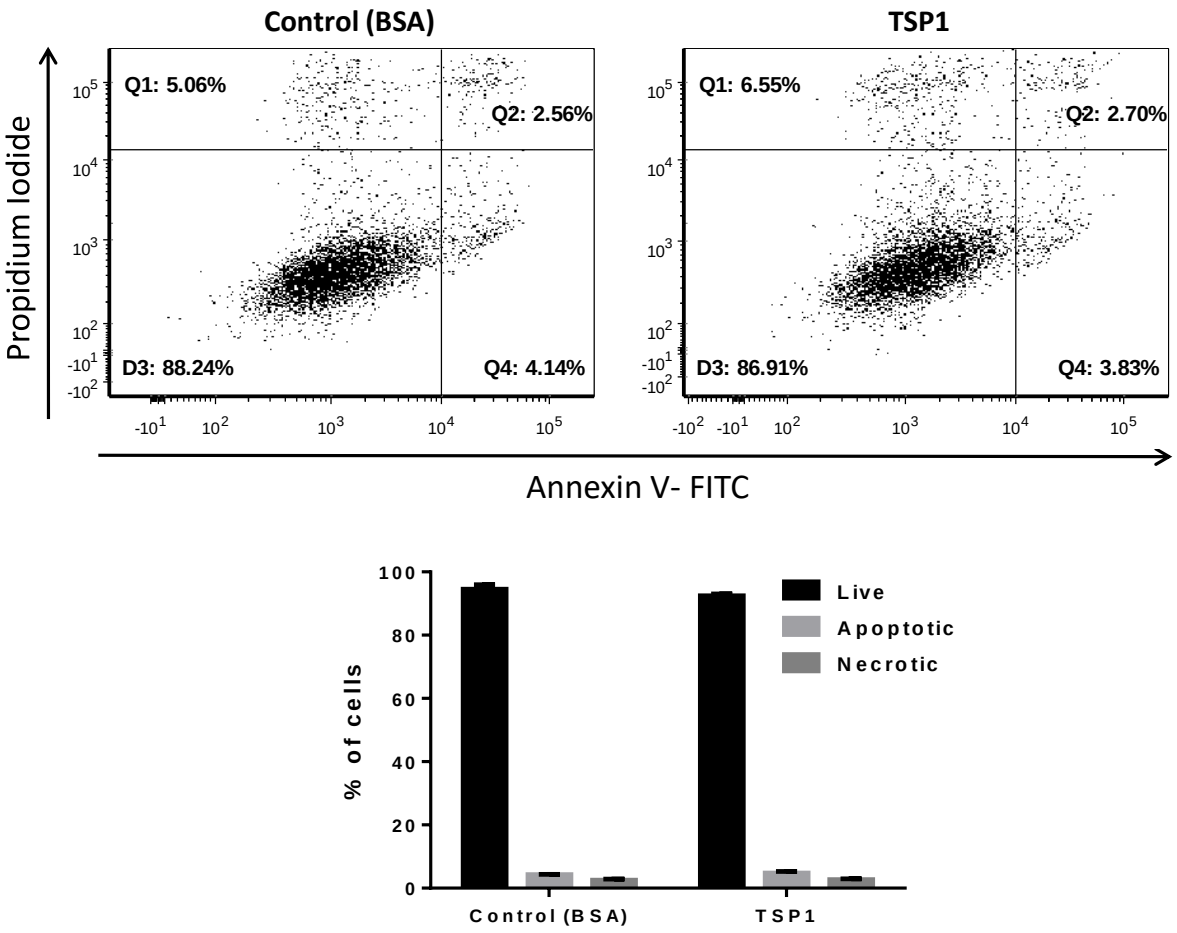


Figure 20: The percentage of live, apoptotic and necrotic MDA-MB-231 cells were analysed 24 hours after TSP1 or BSA treatment by flow cytometer. Segment Q1 of the quadrants refers to non-specific PI staining which are alive cells; Q2 indicates late apoptotic and necrotic cells which are stained with both annexin V and PI; Q3 includes healthy and alive cells with low staining of both PI and annexin V; and Q4 to early apoptotic cells with high annexin V but low PI staining. N=3, bars are mean±S.E.M. One-way ANOVA was used to detect any statistical differences between the groups.

4.3.3 TSP1 increases MDA-MB-231 resistance to docetaxel

An essential characteristic of disseminated dormant cancer cells is their ability to resist cytotoxic drugs (Hall et al., 2010). For that reason, it was assessed whether TSP1 confers MDA-MB-231 cells any cytotoxic resistance to a breast cancer drug, docetaxel.

First, a range of docetaxel concentrations was tested on MDA-MB-231 cell viability, measured by MTT assay, following a 24 hour drug treatment. Figure 21 A shows that the docetaxel EC50 is 83 µM. It was decided to treat MDA-MB-231 with 100 µM docetaxel, once it is an easier concentration to prepare, to evaluate the effect of TSP1 pre-treatment. MDA-MB-231 cells were pre-treated with 0, 10, 50 or 100 µM of TSP1 for 48 hours, followed by docetaxel treatment for 24 hours. There is a positive trend between TSP1 concentration and cell survival (Figure 21 B). Pre-treatment with 100 µM of TSP1 increased significantly cell survival to docetaxel (p=0.0423) from 43% to 58%. In this experiment, it should have been added a control group where MDA-MB-231 cells were cultured only with TSP1 (no docetaxel) to have an idea of the influence of this protein on the viability of the MDA-MB-231 cells.

Docetaxel is a taxane drug that prevents cell division by stabilising microtubules which leads to cell death (Huizing et al., 1995; Jordan and Wilson, 2004). It is thought that slowly proliferating or non-proliferating cancer cells are resistant to therapy, mainly due to that fact that cancer drugs act on cell division. For that reason, qPCR was used to evaluate whether TSP1 increases gene expression of any of the genes that have been involved in the uptake (SLCO1B3), metabolism (CYP3A4, CYP1B1 and CYP3A5) or withdrawal (ABC transporters) of docetaxel. As shown in Figure 21 C, CYP3A5, an enzyme that is involved in the metabolism of docetaxel, is upregulated in MDA-MB-231 cell treated with TSP1 (100 ng/mL, p=0.062) for 48 hours. Gene expression analysis was conducted to evaluate the same group gene after TSP1 pre-treatment for 48 hours followed by docetaxel treatment for 24 hours. In order to determine whether the drug itself could induce the expression of those genes and TSP1 could have a synergetic role.

In Figure 21 D it is shown that CYP3A5 is again the only gene that is significantly up-regulated when MDA-MB-231 cells are pre-treated with TSP1 (p=0.0023).

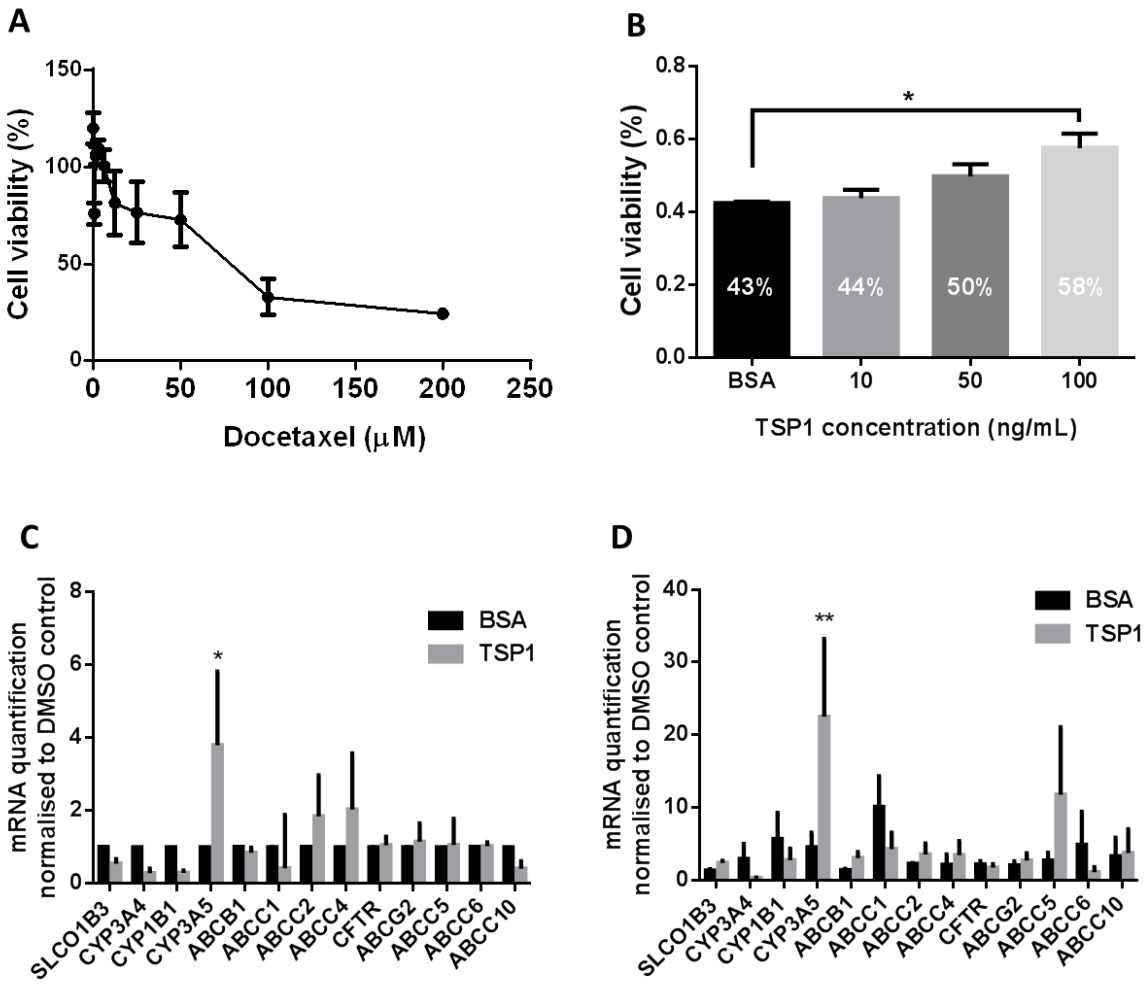


Figure 21: TSP1 pre-treatment for 48 hours, significantly improved cell survival against docetaxel. A- Dose-dependent curve for docetaxel in MDA-MB-231 cells. B- MDA-MB-231 cell survival to 24-hour docetaxel treatment when pre-cultured with or without TSP1 for 48 hours. C, D- mRNA quantification of genes involved in docetaxel metabolism in MDA-MB-231 cells C- before and D- after docetaxel treatment when cells were pre-cultured with TSP1 or BSA (control). N=3, bars are mean±S.E.M. One-way ANOVA was used to detect any statistical differences between the groups. *p<0.05, **p<0.01.

4.4 Discussion

Disseminated breast cancer cells have been found near microvessels in lung, brain and bone marrow in cell cycle arrest (Ghajar et al., 2013). The extracellular matrix has already been shown to induce quiescence and provide cytotoxic resistance to mammary epithelial cells (Bissell et al., 1982; Boudreau et al., 1995; Petersen et al., 1992; Spencer et al., 2011; Weaver et al., 2002; Weaver et al., 1997). Ghajar and collaborators demonstrated in a series of in vivo and in vitro assays that a stable endothelium induces cell cycle arrest of breast cancer cells, identifying TSP1 as a possible contributor (Ghajar et al., 2013). Using a two- dimensional co- culture model and endothelial conditioned medium, the study of this chapter demonstrated that endothelial cells induce a quiescence state in breast cancer cells TSP1, an extracellular matrix protein secreted by endothelial cells, reduces breast cancer cell proliferation and increases cell survival to a breast cancer drug. TSP1 has been described as a central ECM protein in cancer progression and metastasis initiation (Cinatl et al., 1999; Harada et al., 2003; Kawakami et al., 2001; Yee et al., 2009), but its anti-tumour role has been mainly attributed to its anti-angiogenesis effect. The results of this chapter provided further evidence to support the previous findings by Ghajar and collaborators (Ghajar et al., 2013) that TSP1 role in metastasis initiation has been studied, not just as a protein that modulates angiogenesis, but also as having a direct effect on the cancer cells.

Vascular endothelial cells can directly modulate the phenotype of other cells in the perivascular niche of non-tumorigenic tissues (reviewed in (Butler et al., 2010). Stable endothelium inhibits proliferation of smooth muscle cells (Dodge et al., 1993), maintains neural, haematopoietic and mesenchymal stem cell pluripotency (Butler et al., 2010; Crisan et al., 2008; Ding et al., 2012; Kobayashi et al., 2010; Shen et al., 2004) and induces differentiation of pancreatic cells (Lammert et al., 2001). However, lung alveoli regeneration and liver growth and morphogenesis are stimulated by endothelial cells, which possess a neovascular tip cell phenotype (Matsumoto et al., 2001). In

cancer, stablished endothelium inhibits proliferation of breast cancer cells, whereas the sprouting endothelium stimulates tumour growth (Ghajar et al., 2013).

Using an *in vitro* stablished endothelium model developed in this project, which is characterized by low levels of cell proliferation, suggests that endothelial cells can modulate breast cancer dormancy. Endothelial cells reduced MDA-MB-231 cell proliferation, but this effect was stronger in the proliferation of MCF7 cells. Previous *in vivo* studies have demonstrated that MDA-MB-231 cells have a high metastatic potential while MCF7 have no metastatic potential (Adams et al., 2011; Ziegler et al., 2014). Disseminated breast cancer cells have been found near microvessels (Ghajar et al., 2013), suggesting that endothelial cells might have a role in inducing and maintaining breast cancer cells in a quiescence status (Ghajar et al., 2013). Surprisingly, endothelial cells did not show to have any inhibitory effect on the proliferation and growth pattern of MCF10A cells, a non-tumorigenic breast cell line with no metastatic potential (Sanchez-Garcia et al., 2014).

Taking together, these results suggest that endothelial cells modulate breast cancer cell proliferation. Additionally, endothelial cells decreased the expression of Ki67 in MDA-MB-231 cells, a proliferation marker, and increased the percentage of MDA-MB-231 cells in the G0/G1 phase indicating that MDA-MB-231 cells are proliferating slower or they are in cell cycle arrest. Ki-67 is expressed in all cell cycle phases apart from the G0 phase (Scholzen and Gerdes, 2000). These group of cells can be in quiescence or senescence. Quiescent cells are in cell cycle arrest but can enter G1 phase and initiate proliferation, while senescence cells are not able to proliferate again (Blagosklonny, 2011). Dormant cancer cells are characterised by cell cycle arrest, but they can proliferate after a period of dormancy. For that reason, it is important to evaluate whether endothelial cells induce senescence or quiescence of breast cancer cells. Interestingly, endothelial cells reduced the percentage of MDA-MB-231 cells expressing the senescent marker, p21-waf1-cip1 (Romanov et al., 2010), suggesting that MDA-MB-231 cells are

not in a senescence status. The endothelial conditioned medium also reduced Ki67 expression in MDA-MB-231 demonstrating that the inhibitory effect of endothelial cells on breast cancer cell proliferation is due to an extracellular molecule.

TSP1 was one of the proteins present at high concentration in the perivascular niches of lung, bone marrow and brain, where disseminated breast cancer cells where found (Ghajar et al., 2013). Endothelial cells secrete TSP1. In several endothelial cell lines, the TPS1 concentrations in the conditioned medium were between 50 and 100 ng/mL. However, it is important to bear in mind that TSP1 bind to structural extracellular proteins, resulting in deposition and retention of TSP1 in the matrix (Sottile and Hocking, 2002). Taking this into consideration, it is likely more closely to the endothelial cells, much higher of TSP1 concentration. TSP1 can bind to fibronectin and other structural proteins. For this reason, the subsequent experiments used the highest concentration of TSP1 (100 ng/mL) measured in the conditioned medium of endothelial cells, a much lower concentration showed to affect tubule formation in vitro (1 µg/mL; (Qin et al., 2014).

Recombinant TSP1 showed to reduce ki67 expression in MDA-MB-231 cells as a proliferative marker, but not increase apoptotic markers, suggesting that the endothelial cells secreting TSP1 might involve in the dormancy process of breast cancer. Even though TSP1 can exert some degree of proliferative effects on cells under physiological conditions, pathological TSP1 concentration is much higher with different post-translational modifications and a stronger anti-proliferative effect. An *in vivo* study showed that RenCa renal carcinoma cells formed less metastasis in the liver which overexpressed TSP1, but not in the lung (Lee et al., 2010b). Also, a lack of TSP1 has been associated with an increase in spontaneous tumours, which was attributed to an inhibition of angiogenesis, but it could also be explained in part by the inhibitory effect of TSP1 on the proliferation of tumour cells (Gutierrez et al., 2003; Lawler, 2002; Rodriguez-Manzaneque et al., 2001).

Another important characteristic of dormant cancer cells is their resistance to therapy, which may lead to the disseminated tumour cells forming metastasis many years or even decades after cancer therapy and patient declared disease-free (Hall et al., 2010). Dormant cancer cells are at cellular arrest. Non-proliferating cells or slow proliferative cells have been found to be more resistant to therapy (Addla et al., 2008; Dembinski and Krauss, 2009; Hadnagy et al., 2006; Ho et al., 2007; Nishimura et al., 2002; Roesch et al., 2010; Scharenberg et al., 2002). TSP1 treatment increased 15% survival of MDA-MB-231 cells upon to docetaxel. Docetaxel is a common cancer drug that blocks cell division leading to cell death (Huizing et al., 1995; Jordan and Wilson, 2004). Many tumours acquire resistance to this drug, and for that reason, the mechanism behind it has been extensively studied. There is a set of proteins involved in the uptake, metabolism and efflux of docetaxel in cancer cells. Gene analysis revealed that TSP1 up-regulates the expression of CYP3A5 in MDA-MB-231. CYP3A5 is an enzyme involved in the metabolism of docetaxel. Docetaxel treatment further enhanced CYP3A5 expression in the TSP1 pre-treated MDA-MB-231 cells. Knockdown CYP3A5 has been applied to validate the TSP1- CYP3A5 pathway in docetaxel resistance. Two independent studies, one in gastric cancer (Bi et al., 2014) and another one in prostate cancer (Lih et al., 2006) showed that a decrease in TSP1 increased resistance to oxaliplatin and taxane, respectively. The results here suggest a protective role of TSP1 in breast cancer cells to docetaxel, by increasing CYP3A5, but a knockdown of this gene is essential to clarify this assumption.

The results in this chapter support notion that the stable endothelium induces breast cancer dormancy via TSP1 modulating breast cancer cell proliferation. Interestingly, this study suggested that TSP1 might have a protective role in breast cancer against docetaxel. TSP1 up-regulated CYP3A5 expression in MDA-MB-231 cells, which is an enzyme that metabolises docetaxel, but further study is required to assess the importance of this novel pathway.

Chapter V: Breast cancer cells induce endothelial tryptophan degradation via IDO1 causing a possible decrease in TSP1 secretion by endothelial cells

5.1 Introduction

Tryptophan is an essential amino acid and therefore has to be obtained from the diet. This amino acid is also one of the lowest concentrations in mammalians, and for that reason is relatively less available than other essential amino acids and it is thought to be a rate-limiting amino acid for protein synthesis (Cortamira et al., 1991; Wurtman et al., 1980). Although most tryptophan is used to synthesise protein, it can also be used for serotonin and tryptamine synthesis or enter the kynurenine pathway (Figure 7) (Richard et al., 2009). The kynurenine pathway is responsible for about 90% of the tryptophan metabolism (Richard et al., 2009), and it can be initiated by three enzymes, TDO (Ren and Correia, 2000), IDO1 (Thomas and Stocker, 1999) and IDO2 (Ball et al., 2009). An important consideration is that the tryptophan used in cellular metabolism is lost for protein synthesis. The first step of the kynurenine pathway is dependent on the concentration and activity of these three enzymes (Austin and Rendina, 2015). IDO1 has been reported as being overexpressed in some diseases, including several types of cancer (Munn et al., 2004; Munn et al., 2002; Uyttenhove et al., 2003). IDO1 overexpression and increased tryptophan degradation have been linked to more aggressive tumours in some studies (Chen et al., 2014; Inaba et al., 2010; Inaba et al., 2009; Okamoto et al., 2005; Yu et al., 2013; Yu et al., 2011).

The lack of tryptophan in the microenvironment of IDO1 expressing tumours has been shown to be a strategy to confer immune protection to cancer cells against T-cells, which are very sensitive to a low concentration of tryptophan trigging T-cell anergy and autophagy (Munn et al., 2005; Munn et al., 2004; Szántó et al., 2007; Wang et al., 2010). While cancer cells and other stromal cells seem not to be affected by the lack of tryptophan, this situation has been attributed to a higher expression of TTS in those cells than in T-cells preventing them from activating autophagy (Boasso et al., 2005). IDO1 overexpression which leads to a decrease in tryptophan concentration in the cancer

microenvironment has been extensively studied in an immune protective perspective, but other effects, such as on protein synthesis, has not been assessed.

TSP1 is a well-known anti-angiogenic ECM protein that has been shown to play an important role in cancer progression. TSP1 protein levels were found to be lower in malignant tumours than in adenomas in gliomas, human adrenocortical carcinomas, thyroid carcinomas and non-small lung carcinomas (Bunone et al., 1999; de Fraipont et al., 2000; Hsu et al., 1996; Kazuno et al., 1999; Oshika et al., 1998; Tenan et al., 2000). In the thyroid, colon and bladder carcinomas, TSP1 expression was also reported to correlate inversely with the survival rate and with the grade of the tumour (Bunone et al., 1999; Grossfeld et al., 1997; Tokunaga et al., 1999). In animal models of melanoma, lung and breast carcinomas, cancer cells that expressed an increased level of TSP1 were correlated with a reduced metastatic potential (Zabrenetzky et al., 1994). Endothelial TSP1 has also been involved in contributing for breast cancer dormancy (Ghajar et al., 2013). Additionally, the current study as presented in the previous chapter has shown that TSP1 slows down MDA-MB-231 cell proliferation.

The amino acid sequence of TSP1 showed that this protein is composed of 1170 amino acids of which 22 are tryptophan, as represented in Figure 22, revealing that TSP1 contains a higher percentage of tryptophan (1.88% of tryptophan) than the average protein – 1.2% (Mahan and Shields, 1998).

In the previous two result chapters it was shown that endothelial cells exert an inhibitory effect on the proliferation of breast cancer cells, and TSP1, which is secreted by endothelial cells, seems to have a similar effect. It was also shown that IDO1 is expressed in the tumour vasculature and an inverse correlation between vascular IDO1 and stromal TSP1 was found in the breast cancer tissue. Therefore, this chapter aims to evaluate whether cancer cells are able to induce enzymatically active endothelial IDO1

and whether tryptophan can limit TSP1 synthesis. We also aim to evaluate the effect of low tryptophan on VEGF secretion by MDA-MB-231 cells.

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   1 mglawglgvl flmhvcgtnr ipesggdnsv fdifeltgaa rkgsgrrlvk gpdpsspafr
  61 iedanlippv pddkfqdlvd avraekgfll laslrqmkkt rgtllalerk dhsgqvfsvv
 121 sngkagtldl sltvqgkqhv vsveeallat gqwksitlfv qedraqlyid cekmenaeld
 181 vpiqsvftrd lasiarlria kggvndnfqg vlqnvrfvfg ttpedilrnk gcssstsvll
 241 tldnnvvngs spairtnyig hktkdlqaic giscdelssm vlelrglrti vttlqdsirk
 301 vteenkelan elrrpplcyh ngvqyrnnee wtvdsctech cqnsvtickk vscpimpcsn
 361 atvpdgeccp rcwpsdsadd gwspwsewts cstscgngiq qrgrscdsln nrcegssvqt
 421 rtchiqecdk rfkqdggwsh wspwsscsvt cgdgvitrir lcnspspqmn gkpcegeare
 481 tkackkdacp inggwgpwsp wdicsvtcgg gvqkrsrlcn nptpqfggkd cvgdvtenqi
 541 cnkqdcpidg clsnpcfagv kctsypdgsw kcgacppgys gngiqctdvd eckevpdacf
 601 nhngehrcen tdpgynclpc pprftgsqpf gqgvehatan kqvckprnpc tdgthdcnkn
 661 akcnylghys dpmyrceckp gyagngiicg edtdldgwpn enlvcvanat yhckkdncpn
 721 lpnsgqedyd kdgigdacdd dddndkipdd rdncpfhynp aqydydrddv gdrcdncpyn
 781 hnpdqadtdn ngegdacaad idgdgilner dncqyvynvd qrdtdmdgvg dqcdncpleh
 841 npdqldsdsd rigdtcdnnq didedghqnn ldncpyvpna nqadhdkdgk gdacdhdddn
 901 dgipddkdnc rlvpnpdqkd sdgdgrgdac kddfdhdsvp diddicpenv disetdfrrf
 961 qmipldpkgt sqndpnwvvr hqgkelvqtv ncdpglavgy defnavdfsg tffinterdd
1021 dyagfvfgyq sssrfyvvmw kqvtqsywdt nptraqgysg lsvkvvnstt gpgehlrnal
1081 whtgntpgqv rtlwhdprhi gwkdftayrw rlshrpktgf irvvmyegkk imadsgpiyd
1141 ktyaggrlgl fvfsqemvff sdlkyecrdp
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Figure 22: TSP1 amino acid sequence, tryptophan highlighted in yellow (NP_003237.2, National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/).

5.2 Material and methods

5.2.1 Cell lines

MDA-MB-231 breast cancer cells and HMVECad, primary endothelial cells, were used in this chapter. MDA-MB-231 cells were continuously maintained in DMEM media with 10% FBS and antibiotics. HMVECad were continuously cultured in pre-coated flasks with attachment factor in EGM with endothelial supplements and antibiotics.

5.2.2 Two-dimensional co-culture and cell sorting

HMVECad cells were seeded in pre-coated T75 flasks with attachment factor and left to proliferate to 70- 80% confluency. Endothelial cells were then washed with PBS twice and incubated with serum free DMEM containing a red dye (1µg/mL; CellTracker^{TM} Orange CMRA, life technologies) for 20 minutes. Cells were then trypsinized and seeded in pre-coated 6 well plates with attachment factor at the density of 500,000 cells/well. On the following day, 500,000 MDA-MB-231 cells were seeded on the top of the endothelial monolayer and left for 48 hours (co-culture). Wells with just endothelial cells or MDA-MB-231 cells were also left for 48 hours (single culture). After 48 hours, cells were trypsinized and re-suspended in cold PBS at the confluency of $1x10^6$ cells/100 µl. Then, both cell lines were sorted and collected using the BD FACSAriaTM III (BD Biosciences, US). This was achieved by plotting the data onto a dot plot SSC-A vs PE (575 nm) that allowed gating each cell line according to the presence of absence of the red dye followed by its sorting.

5.2.3 Generation of conditioned medium

Two types of conditioned media, namely that with low and that with high glucose, were generated from MDA-MB-231 cells. MDA-MB-231 cells were seeded in 6-well plates at the density of 200,000 cells/well. The day after seeding, the medium was aspirated, cells washed twice with PBS and 1 mL of standard DMEM, which contains about 5.5 mM of glucose, or 1 mL of low glucose DMEM (1 mM glucose) was added to

each well. After 48 hours, conditioned media were collected and centrifuged at 1,700 ×g for 5 minutes to pellet any dead cells and debris. The supernatant was used to treat endothelial cells.

4.2.4 Conditioned media treatment

Endothelial cells (HMVECad) were seeded in pre-coated 24-well plates with attachment factor at the density of 200,000 cells/well. On the next day, the medium was aspirated, cells were washed twice with PBS, and 250 µl of the standard endothelial medium was added to each well. To each well an additional 750 µl of standard endothelial medium (control group), standard DMEM (DMEM control), MDA-MB-231 conditioned medium generated with standard DMEM, low glucose DMEM (low glucose DMEM control) or MDA-MB-231 conditioned medium generated with low glucose medium was added. Cells were cultured in these conditions for 24 hours.

5.2.5 Generation of IDO1 Knockdown

HMVECad cells were seeded in pre-coated 6-well plates with attachment factor at the density of 200,000 cells/well. On the following day medium was removed, cells were washed twice with PBS and 1 mL of the siRNA/ Lipofectamine mix containing the IDO1 targeting siRNA or the non-targeting siRNA (control), was added to the respective well (full protocol described in the section 2.5). An additional 1 mL of endothelial medium was added to each well 6 hours later. 24 hours after transfection cells were trypsinized and seeded onto pre-coated 24-well plates at the density of 200,000 cells/ well. On the next day, 750 µl of MDA-MB-231 conditioned medium generated under low glucose conditions and 250 µl of the standard endothelial medium was added to each well. Cells were cultured for 24 hours.

5.2.6 RNA isolation, cDNA synthesis and qPCR

The expression of TDO, IDO1 and/or IDO2 was analysed by qPCR in the endothelial samples generated as described in the sections 5.2.3, 5.2.4 and 5.2.5. RNA

was isolated using the TRI reagent kit, the detailed protocol is described in the section 2.3.1, followed by the generation of cDNA by reverse transcription PCR, as described in the section 2.3.3. Quantitative PCR was then performed as described in the section 2.3.4 and data was normalised to GAPDH.

5.2.7 Differential tryptophan culturing conditions

DMEM with different tryptophan concentrations was used in this experiment to culture HMVECad and MDA-MB-231 cells. The different tryptophan concentration media was generated using tryptophan-free DMEM (supplemented with Dialyzed FBS) and tryptophan was added to it from a stock solution of 50 mM in order to reach the intended concentration.

HMVECad were seeded onto pre-coated 24-well plates at the density of 200,000 cells/well in the standard endothelial medium. On the next day, the medium was removed, cells washed twice with PBS and 1 mL of the different tryptophan concentration DMEM was added to the respective well. Media were collected at 24, 48 or 72 hours.

MDA-MB-231 cells were seeded in 24-well plates at the density of 200,000 cells/ well. On the next day, the medium was removed, cells washed twice with PBS and 1 mL of the different tryptophan concentration DMEM was added to the respective well. Media were collected after 48 hours of culturing.

In both experiments, cells were trypsinised and the number of cells/ well was assessed by an automatic cell counter, full protocol in section 2.2.

5.2.8 ELISA

TSP1, kynurenine and VEGF concentrations were assessed in the medium of endothelial cells or MDA-MB-231 cells which were generated as described in the sections 5.2.4, 5.2.5 and 5.2.7. The media from those experiments were collected and

centrifuged at 1,700 × g for 5 minutes before being used in the assay. The ELISA protocol for TSP1, kynurenine and VEGF is fully described in the sections 2.4.5.

5.3 Results

5.3.1 MDA-MB-231 cells induce endothelial tryptophan degradation via IDO1

In chapter III, it was shown that the vasculature of breast cancer could express IDO1, in this subchapter, it was aimed to evaluate whether cancer cells can directly induce IDO1 expression in endothelial cells without the recruitment of immune cells known to secrete cytokines, such as IFNγ. To assess this question, MDA-MB-231 cells were co-cultured on the top of an endothelial monolayer for 48 hours, before separating by way of cell sorting. IDO1 mRNA was significantly increased (p=0.0484), about 20-fold increase, in endothelial cells when co-cultured with MDA-MB-231 cells compared to endothelial single culture control (Figure 23, A). While MDA-MB-231 cells did not change IDO1 mRNA level.

IDO1 expression can be trigged by several soluble molecules, such as IL-6 and IFNγ (Adams et al., 2004; Braun et al., 2005; Fallarino et al., 2006). To determine whether breast cancer cells can induce endothelial IDO1 expression via soluble factors, an endothelial monolayer was cultured with MDA-MB-231 conditioned medium and endothelial IDO1 was assessed by qPCR and western blot. IDO1 enzymatic activity was also evaluated by quantifying kynurenine concentration, the first product of tryptophan degradation by IDO1, in the medium by ELISA. The expression of TDO and IDO2, the other two enzymes able to catabolise tryptophan, was also evaluated. In the context of tumour dormancy, as described in the previous chapter, the results suggest that the vascular microenvironment down regulates breast cancer cell proliferation. Thus, when the micrometastasis reaches the point that requires new vessels to support growth, cancer cells are in an equilibrium of proliferating and apoptotic cells due to the lack of oxygen and nutrients (Bergers and Benjamin, 2003). For this reason, endothelial cells were treated with MDA-MB-231 conditioned medium generated under standard

conditions, but also under low glucose conditions to mimic the context of the cancer cells in the centre of the micrometastasis.

The MDA-MB-231 conditioned medium, generated under normal conditions (~5.5 mM glucose), significantly increased IDO1 (p=0.02116) but not TDO (p=0.4521) nor IDO2 (p=0.1147) gene expression in endothelial cells in comparison to the control group (Figure 23 B-D). Interestingly, MDA-MB-231 conditioned medium, which was generated under low glucose conditions (1mM), further increased IDO1 expression in endothelial cells (p=0.0001) in comparison to standard conditioned medium (Figure 23 B). TDO and IDO2 mRNA expression did not significantly change (Figure 23, C and D). Endothelial IDO1 protein was also evaluated by western blot which did not detect IDO1 under any of the above conditions after 48 hours of culture (data not shown). Even though IDO1 protein was not detected, kynurenine concentration, which indicates tryptophan degradation and it is used as a way to assess IDO1 enzymatic activity, showed that the standard MDA-MB-231 conditioned medium induced a significant increase in tryptophan degradation (p=0.0005, Figure 23 E) but not the control treatment with normal DMEM (p=0.1416) in which the conditioned medium was generated. Although an increase in endothelial IDO1 mRNA was observed when endothelial cells were cultured with the low glucose MDA-MB-231 conditioned medium it was not detected an increase in tryptophan degradation, compared to standard MDA-MB-231 conditioned medium.

In order to evaluate whether MDA-MB-231 conditioned medium induced an increase in kynurenine through IDO1, endothelial cells were transfected with IDO1 siRNA before culturing these cells with MDA-MB-231 conditioned medium generated under low glucose conditions. This way, IDO1 mRNA was knockdown as shown in Figure 24 A (p< 0.0001), which led to a significant decrease in kynurenine concentration (Figure 24 B, p=0.0002). TDO and IDO2 gene expression was also evaluated showing that neither of these genes significantly changed their expression upon IDO1 knockdown as represented in Figure 24 C and D (p= 0.0626, p= 0.2664, respectively).

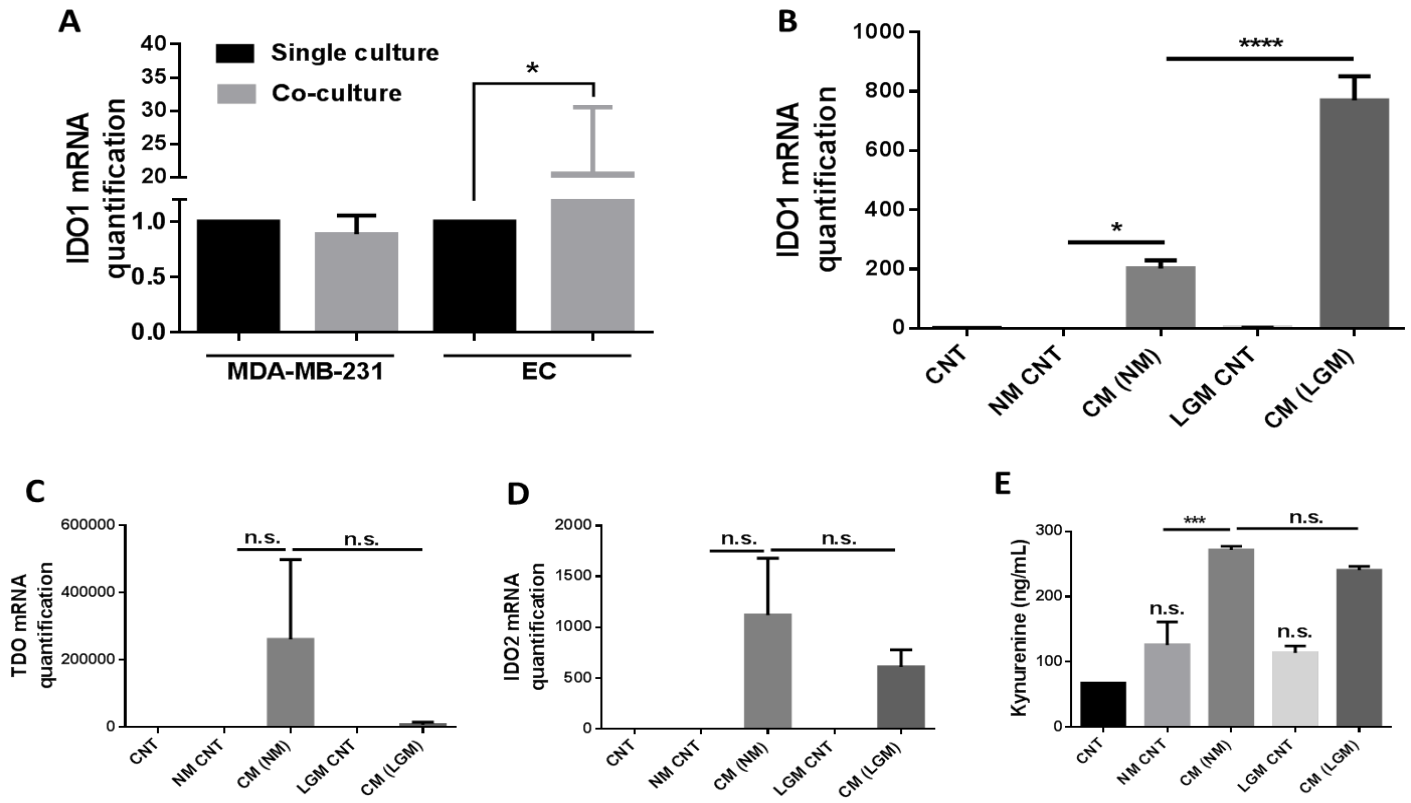


Figure 23: MDA-MB-231 cells induce an increase in tryptophan degradation by stimulating endothelial IDO1 expression. A- MDA-MB-231 cells were cultured for 48 hours on the top of an endothelial monolayer, which was previously labelled with a red dye. Each cell type was separated by cell sorting and RNA was extracted. The relative IDO1 mRNA was assessed by qPCR. (B, C and D) Conditioned media were generated by culturing MDA-MB-231 cells in normal medium (NM) or low glucose medium (LGM) for 48 hours. Endothelial cells were cultured with standard endothelial medium (CNT), DMEM (NM CNT), MDA-MB-231 conditioned medium [CM (NM)], low glucose medium (LGM CNT) or MDA-MB-231 conditioned medium generated with low glucose medium [CM (LGM)] for 24 hours. The relative mRNA expression of IDO1 (B), TDO (C) and IDO2 (D) in endothelial cells was assessed by qPCR. Kynurenine concentration was also measured by ELISA in the conditioned medium (E). Data are represented as mean ± S.E.M. (n=3), One-way ANOVA was used to analyse any statistical differences between the different groups. n.s., non-significant, **P<0.01, ***P<0.001 compared to control cells (CNT) unless represented otherwise.

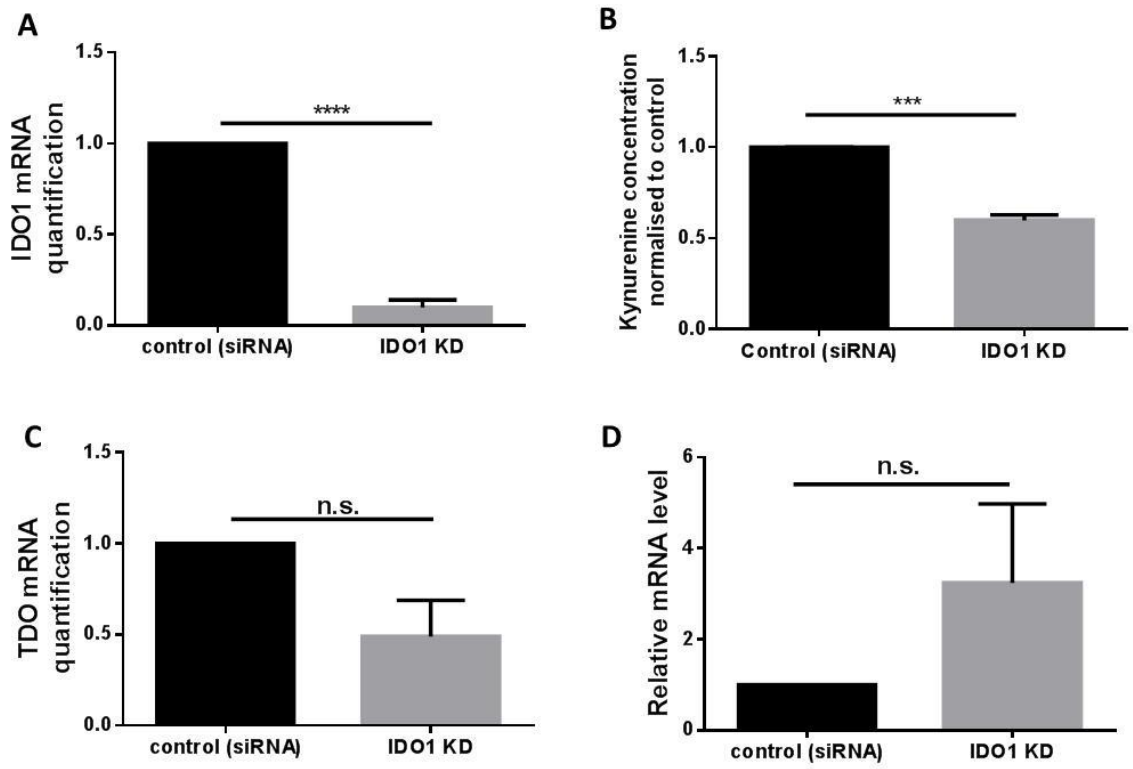


Figure 24: MDA-MB-231 conditioned medium induces endothelial tryptophan degradation via IDO1. Endothelial cells were transfected with IDO1 siRNA (IDO1 KD) or with nonsense siRNA sequence (control) and then cultured, for 24 hours, with MDA-MB-231 conditioned medium which was under low glucose conditions. The relative mRNA expression of IDO1 (A), TDO (C) and IDO2 (D) was assessed in these samples by qPCR. Kynurenine (B) concentration was also evaluated in the medium by ELISA. Data are represented as mean ± S.E.M. (n=3), student-t test was used to analyse any statistical differences between the groups. n.s., non-significant, ***P<0.001, ****P<0.0001.

5.3.2 Low tryptophan limits TSP1 secretion

In vivo studies have shown that the levels of tryptophan can influence protein and serotonin synthesis (Cortamira et al., 1991; Wurtman et al., 1980). In this chapter, it was aimed to assess whether tryptophan concentration can determine TSP1 synthesis and therefore its concentration in the ECM. In an *in vivo* ovarian cancer mouse model, it was shown that tryptophan concentration could vary in xenografts tumours from just below 50 μM by day 14 in IDO1 negative tumours and 20 μM after a 7 day IFNγ treatment to induce IDO1 expression (Burke et al., 1995). For that reason, endothelial cells were cultured with 8 tryptophan serial dilutions of 1:2, with the highest being 100 μM. TSP1 concentration in the medium was measured at 24, 48 and 72 hours by ELISA.

Different tryptophan concentrations did not significantly affect endothelial TSP1 secretion at 24 hours and 48 hours, Figure 25 A and C, respectively. However, there appears to be a trend that low tryptophan concentrations limited TSP1 secretion. At 72 hours, there was a significant decrease in TSP1 secreted by endothelial cells at the lowest tryptophan conditions (3.125, 1.5625 and 0.78125 μM) as shown in Figure 25 E, (p=0.0327, p=0.0051, p=0.0253, respectively) when compared to 100 μM of tryptophan. The number of cells in each day was also assessed showing no significant difference between any of the tryptophan concentration when compared to the highest tryptophan concentration group, in any of the days, as represented in Figure 25 B, D and F.

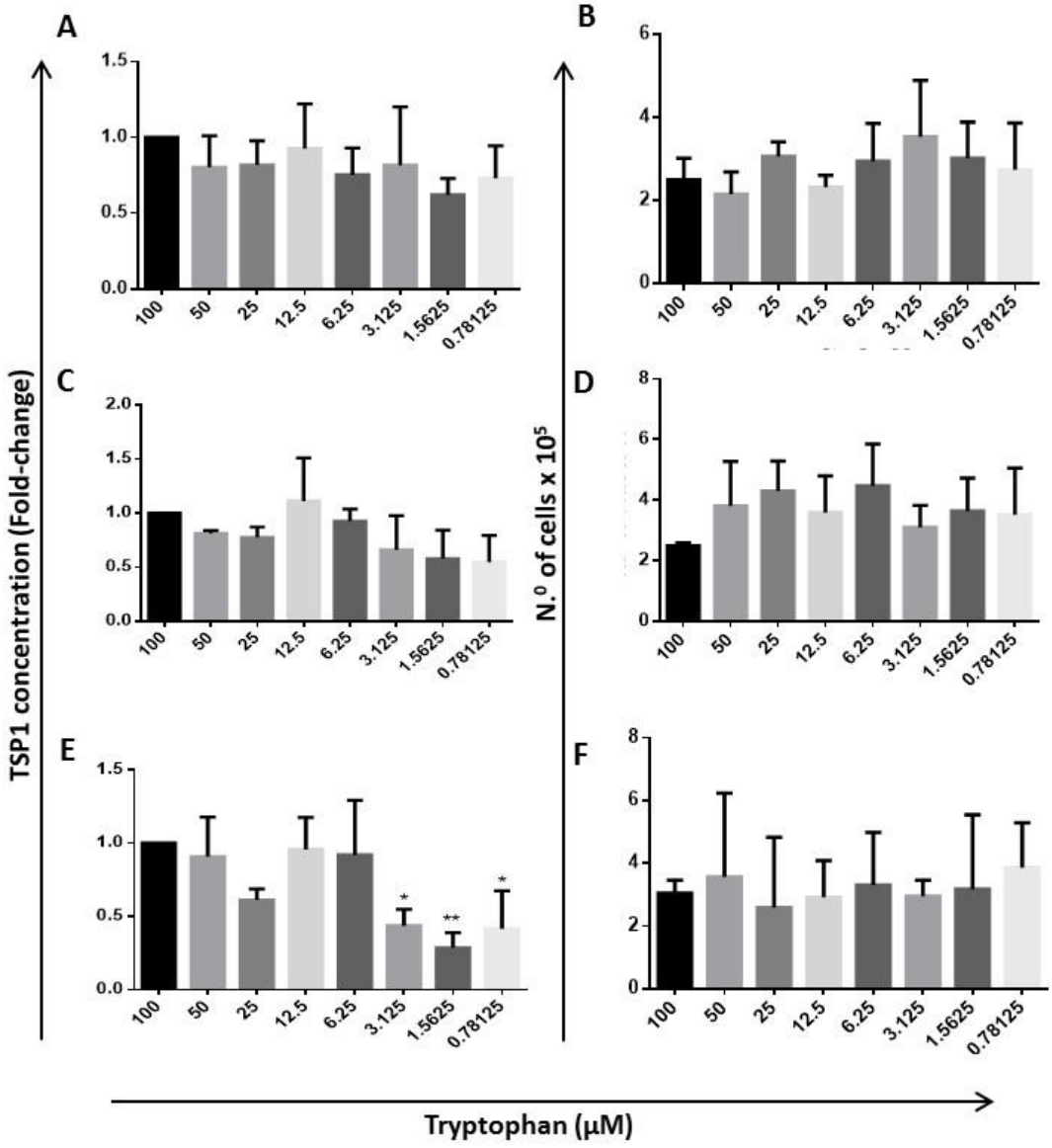


Figure 25: Tryptophan concentration affects TSP1 secretion by endothelial cells. Endothelial cells were cultures with different tryptophan concentrations for 24 hours (A, B), 48 hours (C, D) and 72 hours (E, F). TSP1 concentration in the medium was assessed by ELISA (A, C, D) and the number of cells was also determined by an automatic cell counter (B, D, F). Data are represented as mean ± S.E.M. (n=3), One-way ANOVA was used to analyse any statistical differences between the groups. *P<0.05, **P<0.01 compared to the highest tryptophan concentration group.

5.3.3 Low tryptophan induces VEGF secretion by MDA-MB-231 cells

The present study so far has shown that human breast tumours overexpress IDO1 in both cancer cells and stromal cells, including endothelial cells. It has also shown that cancer cells can induce IDO1 expression in endothelial cells and therefore tryptophan degradation. Thus, cancer cells have to survive and somehow overcome the lack of tryptophan. One way that cancer cells could overcome this situation would be to induce angiogenesis, such as by increasing VEGF secretion.

For this reason, the effect of low tryptophan concentration on the secretion of VEGF by MDA-MB-231 cells was evaluated. The experiment showed, the levels of VEGF secretion by MDA-MB-231 cells did not differ, when treated with 10 µM and 20 µM of tryptophan (Figure 26 A). Strikingly, when MDA-MB-231 cells were cultured with lower tryptophan concentration, 1 µM, it significantly increased the level of VEGF secretion, when compared with the cells treated with high concentrations (p=0.0174, Figure 26 A). The number of cells at the end of the experiment significantly increased (Figure 26 B, p=0.0039) when the concentration of tryptophan decreased from 20 to 10 µM, but the number of cells significantly decreased when cells were cultured with 1 µM (Figure 26 B, p<0.0001). Even though the lowest tryptophan concentration decreased the number of MDA-MB-231 cells, a 2.6- fold increase of VEGF secretion/ 1000 cells was observed at 1 µM of tryptophan when compared to 20 µM of tryptophan.

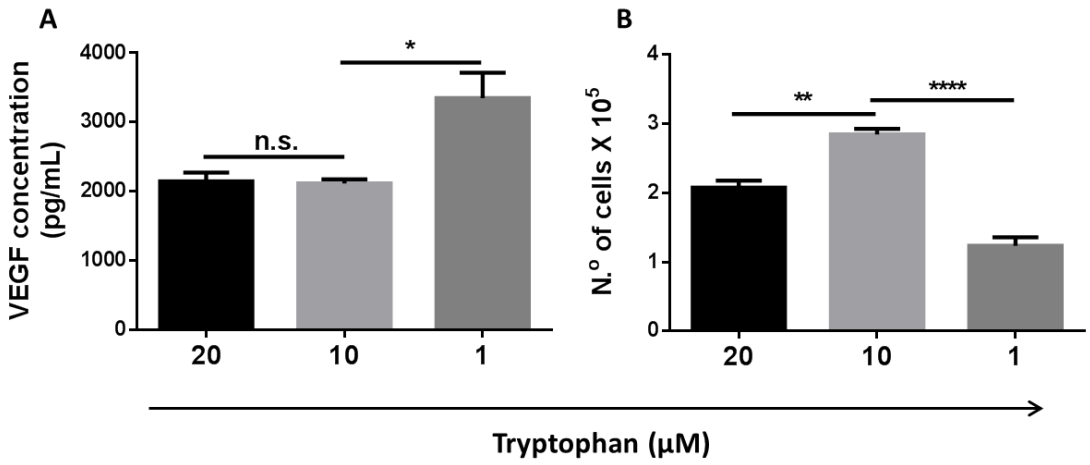


Figure 26: VEGF secretion by MDA-MB-231 cells is enhanced under low tryptophan conditions. MDA-MB-231 cells were cultured with 3 different tryptophan concentrations (20, 10 and 1 µM) for 48 hours and VEGF was measured in the medium by ELISA (A) and the number of cells was assessed at the end of the experiment (B). Data are represented as mean ± S.E.M. (n=3), One-way ANOVA was used to analyse any statistical differences between the groups. ***P<0.001, ****P<0.0001.

5.4 Discussion

TSP1 is an extracellular matrix protein that inhibits endothelial cell motility and growth and therefore stabilises the microvascular endothelium and inhibits angiogenesis (Roberts, 1996). Endothelial secreted TSP1 has also been suggested as a possible key molecule in inducing sustained breast cancer cell dormancy (Ghajar et al., 2013). In the previous chapter, my findings support this claim that the endothelium and human TSP1 recombinant protein reduce breast cancer cell proliferation. In a two- dimensional co-culture model and a conditioned medium model, breast cancer cells induce an increase in tryptophan degradation in endothelial cells via IDO1. My study also showed that a lack of tryptophan limits TSP1 synthesis by endothelial cells and enhances VEGF secretion by breast cancer cells. Studies on tumours have focused on the role of IDO1 as an immune modulator, but my study has suggested a possible new function of this tryptophan-catabolizing enzyme. Taken together with data by others, my data suggest that IDO1 might be involved in the outgrowth of dormant breast cancer cells and angiogenesis.

Several tumour types overexpress IDO1, including prostatic, breast, pancreatic and colorectal, and most studies indicate that IDO1 overexpression is correlated with a poor prognosis (Astigiano et al., 2005; Bonanno et al., 2012; Brandacher et al., 2006; Chamuleau et al., 2008; Ino et al., 2006; Okamoto et al., 2005; Suzuki et al., 2010; Takao et al., 2007). IDO1 expression in tumours have been attributed mainly to cancer cells and infiltrating leukocytes (Ishio et al., 2004; Uyttenhove et al., 2003). In cervical carcinoma, T-cells and tumour cells- expressing IDO1 are both located in the periphery of the tumour (Théate et al., 2015) and IDO1 expression was correlated with T-cell infiltration in melanoma (Spranger et al., 2013), suggesting that the IDO1 driven factor in tumours is the generic inflammation process. However, some tumours expressed IDO1 in the absence of any inflammatory response (Théate et al., 2015; Uyttenhove et al., 2003), which leads to speculation that there might be additional mechanisms that

upregulates IDO1 expression in tumours. My study (shown in Chapter III) and the results from other groups suggested that the tumour vasculature can also express IDO1 (Riesenberg et al., 2007).

The origin of factors that regulate endothelial IDO1 is not entirely clear nor its biological role. Endothelial IDO1 could be driven by inflammatory mediators, such as IFNγ secreted by T-cells. *In vivo* studies have shown that the immune system is able to target and induce cytolysis of most tumour cells (Sosa et al., 2014), but some cancer cells scape this process and remain clinically dormant under the immune system pressure (Sosa et al., 2014). Thus, the inflammatory process might induce endothelial IDO1 expression during cancer dormancy. As dormant breast cancer cells have been found in close association with the microvasculature of lung, bone marrow and brain (Ghajar et al., 2013), it was hypothesised that breast cancer cells are able to directly induce endothelial IDO1. In my study, co-culture and conditioned medium experiments demonstrated that breast cancer cells could directly induce endothelial IDO1 via soluble factors and increase tryptophan degradation. It has been demonstrated that IDO1 present in microvesicles can degrade tryptophan (Romani et al., 2015). The data of the endothelial IDO1 knockdown experiments provided evidence that breast cancer-mediated tryptophan degradation in endothelial cells occurs via IDO1 and not through other tryptophan-catabolizing enzyme nor via breast cancer vesicles containing IDO1 protein in the conditioned medium. Although there is no information whether IDO1 is expressed within the dormant breast tumour microenvironment, blocking CTLA-4, a potent negative regulator of T-cell response, or blocking IDO1 in BALB/c mice implanted with breast cancer cells only resulted in a transient delayed tumour growth, but combined CTLA-4/IDO1 blockage resulted in sustained growth delay and prolonged survival (Holmgaard et al., 2013) demonstrating that IDO1 is a key immune regulator, not just in the primary tumours, but also in the early phases of metastasis.

Once the micrometastasis status is reached, there is an equilibrium of proliferating cells at the edge and apoptotic cells in the centre of the micrometastasis (Gelao et al., 2013). Apoptosis is occurring due to a lack of a vascular system to supply nutrients and oxygen to the cells and removing toxic molecules resulted from the cellular metabolism (Gelao et al., 2013). Conditioned medium from breast cancer cells cultured under low glucose, to mimic those cells in the centre of the micrometastasis, induced a stronger endothelial IDO1 expression than standard conditions. This suggests that breast cancer cells directly induce endothelial IDO1, but this effect is enhanced when cells are under stress. Although endothelial IDO1 activity was not increased with low glucose cancer cell conditioned medium treatment, in comparison to standard conditioned medium, it is likely to be due to the short-term conditioned medium treatment of 24 hours.

As mentioned above, in order for the micrometastasis to grow, cells have to trigger angiogenesis. In renal cell carcinoma, IDO1 is predominantly expressed by endothelial cells, and mostly present in carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM) positive endothelial cells, suggesting that IDO1 is expressed in newly formed vessels (Riesenberg et al., 2007). The authors also demonstrated that endothelial IDO1 positive tumours had a higher microvascular density (Riesenberg et al., 2007). Interestingly, IDO1 is expressed in the normal lung vasculature and IDO1 knockout mice had a reduced lung vascular density (Smith et al., 2012). IDO1 also exhibits other effects in the vasculature, such as vasodilatation and reduced blood pressure (Wang et al., 2010). Despite the fact that there are a few studies indicating an association between IDO1 and angiogenesis, there is no report so far regarding the related molecular mechanism. In chapter III, I described an inverse correlation between endothelial IDO1 and stromal TSP1 in breast cancer tissue. The analysis of the TSP1 amino acid sequence from the National Center for Biotechnology Information website revealed that TSP1 is composed of 1.88% of tryptophan, a higher percentage of

tryptophan than the average protein (1.2%). This fact suggests that TSP1 synthesis might be affected by a decrease in tryptophan than most proteins. Low tryptophan concentration showed to significantly decrease TSP1 secretion by endothelial cells after 72 hours, without changing the number of cells. Interestingly, my results showed that TSP1 secretion did not decreased in a tryptophan dose-dependent manner, instead TSP1 secretion is stable between 100 and 6.25 µM and drops between 6.25 µM and 3.125 µM suggesting that cells activate a mechanism that modulates TSP1 synthesis between those concentrations. To my knowledge, my result is the first time to show that the concentration of tryptophan can limit TSP1 synthesis and its concentration in the extracellular matrix. In an *in vivo* study, Burke and colleagues used ovarian cancer xenografts, which did not constitutively expressed IDO1, demonstrated that by IFNγ treatment induced IDO1 expression (Burke et al., 1995). They observed the total concentration of tryptophan in these tumours would go from 90 µM in IDO1 negative tumours and 20 µM in IDO1 positive tumours in only 2 days after IFNγ/BSA treatment (Burke et al., 1995). At day 7, tryptophan concentrations were stable at about 40 and 20 µM in IDO1 negative and positive tumours, respectively (Burke et al., 1995). The total tryptophan concentration in the IDO1 positive tumours was higher than the concentration required to affect TSP1 secretion, shown in this chapter. IDO1 is an intracellular enzyme, and therefore is likely that the uptake of tryptophan does not compensate its degradation leading to a much lower intracellular tryptophan concentration in IDO1 positive cells. A study by Kane et al. demonstrated that stimulating IDO1 expression in human cervical epithelial cells (ME-180) by IFNγ (1 ng/mL) led to such low levels of intracellular tryptophan that it was not possible to detect the intracellular tryptophan concentration after only 24 hours, while IDO1 negative cells demonstrated to have a stable level of intracellular tryptophan (Kane et al., 1999). A study previously described a synergetic role of CTLA-4 and IDO1 in cancer immune protection (Holmgaard et al., 2013), in another study, a CTLA-4/VEGF pathway has been found to be implicated in immune regulation but also in angiogenesis (Ott et al., 2015). Thus, CTLA-4 is overexpressed in

cells that are under low tryptophan conditioned and interestingly, CTLA-4 is composed of only 0.57% tryptophan (NCBI website).

Low tryptophan has been reported to induce T-cell anergy and autophagy (Metz et al., 2012; Munn et al., 2005; Sharma et al., 2007) but such conditions seem not to affect other cell types within the tumour microenvironment (Boasso et al., 2005). Newly formed microvessels express IDO1 in renal cell carcinoma and an inverse correlation between those microvessels, and Ki67-positive cancer cells were found (Riesenberg et al., 2007), suggesting that the lack of tryptophan induces cell cycle arrest in tumour cells. Indeed, my study suggested that culturing breast cancer cells under low tryptophan resulted in less proliferative cells, but also resulted in an increase in VEGF secretion by cancer cells. A molecular mechanism has been described to explain the resistance of some cell types to a low tryptophan microenvironment (Boasso et al., 2005), but it does not explain the ability of cells to synthesise proteins with a high content of tryptophan. VEGF is a protein that has many isoforms and most of them contain between 2.2 and 1.3% of tryptophan in its structure, except isoform s that does not contain any tryptophan (Appendix V). It would be interesting to investigate whether this particular isoform is upregulated under low tryptophan conditions and its role in angiogenesis.

The results presented in this chapter shed light on the origin of the regulation force in endothelial IDO1 in the context of cancer, demonstrating that cancer cells may trigger IDO1 expression and tryptophan degradation in endothelial cells without the recruitment of T-cells or other immune cells. My findings also showed that a lack of tryptophan leads to a decrease in the secretion of endothelial TSP1 at the same time that induces an increase in VEGF secretion by cancer cells. A lack of tryptophan seems to tilt the balance of anti-angiogenic/ angiogenic factors, benefiting the latter, thus suggesting that endothelial IDO1 might be involved in a novel angiogenic pathway.

Although the findings presented here could be supported by *in vivo* studies published by other authors, it is necessary to investigate this pathway in a cancer dormancy context to evaluate the significance of this study. It remains to be determined whether micrometastasis is able to induce an IDO1 signal in endothelial cells strong enough to decrease tryptophan concentration to such low levels that limits TSP1 synthesis.

Chapter VI: MDA-MB-231 cells increase IFNγ expression due to nutrient deprivation, which may induce a local decrease in endothelial TSP1 secretion via IDO1

6.1 Introduction

Tumours are constituted of cancer cells but also of stromal cells, such as fibroblasts, macrophages and endothelial cells. Stromal cells are a key component of the tumour and play an essential role in initiation, growth and progression of the tumour (Lin et al., 2013; Tang et al., 2016). Studies have attributed other functions to stromal cells, such as protection of cancer cells to the immune system (Harper and Sainson, 2014) and against chemotherapy drugs (Shree et al., 2011; Tiago et al., 2014).

Tumour cells have to send signals to attract and modify stromal cells. It has been shown that tumours are not able to grow beyond 2-3 mm in diameter without inducing the formation of new blood vessels. A crucial process called tumour angiogenesis is to build a network of microvessels capable of sustaining the growth of the tumour. Since cell-to-cell communication is crucial for cancer cells to induce angiogenesis, the disruption of cellular communication has been demonstrated to reduce both growth and aggressiveness of the tumour (Vasudev and Reynolds, 2014).

Many molecules are responsible for mediating the cell-to-cell communication between cancer cells and endothelial cells can be through many means, including soluble factors, adhesion receptors, gap junctions or vesicles (Lopes-Bastos et al., 2016).

Soluble factors are one of the most common means of cell communication, which are secreted into the extracellular space and targets adjacent cells or those in a distant site (Lopes-Bastos et al., 2016). IFNγ is a soluble factor mainly produced by natural killer and natural killer T cells and mediates the immune response of the innate and adaptive immune system. IFNγ has a key role in protecting the host against bacteria, protozoa and virus infection (Carlin et al., 1989b; Taylor and Feng, 1991), but also in preventing the formation of tumours in mice challenged with chemical carcinogens (Kaplan et al., 1998; Shankaran et al., 2001). In the last decade, IFNγ has emerged as a promising anti-

tumour molecule. Several therapeutic strategies to increase the expression of this cytokine have been developed and proven its efficacy in *in vitro* experiments (Xie et al., 2013; Zhao et al., 2007; Zuo et al., 2011), in pre-clinical *in vivo* and clinical trials (Dummer et al., 2004; Miller et al., 2009a). IFNγ is also the strongest stimulator of IDO1 expression *in vitro* and *in vivo* (Gough et al., 2008; Jeong et al., 2009).

In the previous chapter, my study showed that cancer cells or cancer cell conditioned medium could induce IDO1 expression in endothelial cells, suggesting that the cancer cells may communicate with endothelial cells via soluble factors. For that reason, this chapter aims to evaluate whether IFNγ is a possible mediator between cancer cells and endothelial cells. It was also aimed to verify the impact of this cytokine on other tryptophan degrading enzymes and whether IFNγ is capable of modulating TSP1 secretion. Lastly, this chapter intended to evaluate the impact of IFNγ on other stromal cells and cancer cells.

6.2 Material and methods

6.2.1 Cell lines

Breast cancer cell lines MCF7 and MDA-MB-231, non- tumorigenic breast cell line MCF10A, primary endothelial cells HMVECad and epithelial lung cell line BEAS-3B were used in this chapter. BEAS-2B cells were continuously cultured in collagen pre-coated flasks with BEBM, supplemented with BEGM kit and antibiotics. MDA-MB-231 and MCF7 cells were continuously maintained in DMEM with 10% FBS and antibiotics. MCF10A were continuously cultured in MEBM supplemented with MEGM kit and cholera toxin (100 ng/mL). HMVECad were continuously cultured in pre-coated flasks with attachment factor in EGM with endothelial supplements and antibiotics.

6.2.2 Low glucose experiment

HMVECad and MDA-MB-231 cells were seeded in 6-well plates at the density of 200,000 cells/well. On the next day, the medium was removed, cells washed twice with PBS, and 1 mL of standard MEBM medium was added to MCF10A cells, and 1 mL of standard DMEM supplemented with FBS or 1 mL of low glucose DMEM (1 mM glucose) supplemented with FBS was added to MDA-MB-231 cells. Cells were cultured under those conditions for 48 hours.

6.2.3 IFNγ treatment

HMVECad, MDA-MB-231, MCF7, MCF10A and lung cells were seeded in 6-well plates at the density of 500,000 cells for HMVECad and 200,000 for the other cells lines. On the next day, medium was removed, cells washed twice with PBS and 1 mL of fresh medium was added, supplemented with IFNγ (10 ng/mL) or BSA. Cells were cultured for 24 hours and RNA or protein was extracted.

To investigate the expression and the enzymatic activity of IDO1 in endothelial cells upon IFNγ, HMVECad cells were seeded in pre-coated 24-well plates with attachment factor at the density of 200,000 cells/ well. On the next day, the medium was

removed, cells washed twice with PBS and fresh medium supplemented with 0, 10, 125, 250, 500 or 1000 ng/mL of IFNγ was added to cells. Cells were cultured for 24 hours. RNA and protein were extracted and medium collected for further analysis.

6.2.4 Two-dimensional co-culture and cell sorting

HMVECad cells were seeded in pre-coated T75 flasks with attachment factor and left to proliferate to 70- 80% confluency. Endothelial cells were then washed with PBS twice and incubated with serum free DMEM containing a red dye (1µg/mL; Cell Tracker™ Orange CMRA, life technologies) for 20 minutes. Cells were then trypsinised and seeded in pre-coated 6 well plates with attachment factor at the density of 500,000 cells/well. On the following day, 500,000 MDA-MB-231 cells were seeded on the top of the endothelial monolayer and left for 48 hours (co-culture). Wells with just endothelial cells or MDA-MB-231 cells were also left for 48 hours (single culture). After 48 hours, cells were trypsinised and re-suspended in cold PBS at the confluency of $1x10^6$ cells/100 µl. Then, both cell lines were sorted and collected using the BD FACSAria™ III (BD Biosciences, US). This was achieved by plotting the data onto a dot plot SSC-A vs PE (575 nm) that allowed gating each cell line according to the presence of absence of the red dye followed by its sorting.

6.2.5 Generation of IDO1 Knockdown

HMVECad cells were seeded in pre-coated 6-well plates with attachment factor at the density of 200,000 cells/well. The medium used here was supplemented with IFNγ (10ng/mL). After 16 hours, the medium was removed, cells were washed twice with PBS and 1 mL of the siRNA/ Lipofectamine mix containing the IDO1 targeting siRNA or the non-targeting siRNA (control), was added to the respective well (full protocol described in the section 2.5). An additional 1 mL of endothelial medium was added to each well 6 hours later. 24 hours after transfection cells were trypsinised and seeded onto pre-coated 24-well plates at the density of 200,000 cells/ well. Cells were cultured for 24 hours, medium and RNA was extracted for subsequent analysis.

6.2.6 TSP1 experiments

In order to assess whether TSP1 in the medium regulates its own expression in endothelial cells, 200,000 HMVECad cells were seeded in pre-coated 24-well plates with attachment factor. In the first experiment, the medium was removed in half of the wells, cells washed with PBS and fresh medium was added. RNA was extracted at 0.5, 1, 3, 6, 12 and 24 hours after the medium was renovated (fresh medium group), RNA was also extracted at the same time points in wells that the medium was not renovated (unchanged medium group).

In the second experiment, the medium was removed, cells washed twice with PBS and 1 mL of fresh medium supplemented with TSP1 (100 ng/mL) or BSA was added to the wells. RNA was extracted 3 hours after treatment.

6.2.7 RNA isolation, cDNA synthesis and qPCR

The expression of TDO, IDO1, IDO2, TSP1 and IFNγ was analysed by qPCR in the samples generated as described in the sections 6.2.2, 6.2.3, 6.24, 6.2.5 and 6.2.6. RNA was isolated using the TRI reagent kit, the detailed protocol is described in the section 2.3.1, followed by the generation of cDNA by reverse transcription PCR, as described in the section 2.3.3 Quantitative PCR was then performed as described in the section 2.3.4 and data was normalised to GAPDH.

6.2.7 Protein extraction and Western blotting

IDO1 protein levels were assessed in the samples generated in the section 6.2.3 and 6.2.5. Protein was isolated as described in the section 2.4.1 and protein detection was assessed by western blotting, the full protocol described in the section 2.4.3. The intensity of the signal was measured by ImageJ software ((National Institutes of Health, USA). Data was normalised to GAPDH.

6.2.8 ELISA

Kynurenine and TSP1 concentrations were assessed in the media generated in the sections 6.2.3. The media from those experiments were collected and centrifuged at 1,700 ×g for 5 minutes before being used in the assay. The ELISA protocol for TSP1 and kynurenine is fully described in the sections 2.4.5.

6.3 Results

6.3.1 Nutrient deprivation induces IFNγ expression in MDA-MB-231 cells potentially leading to endothelial IDO1 expression

In chapter V it was demonstrated that breast cancer cells could directly induce endothelial IDO1 expression, which was further enhanced when breast cancer cells were under low glucose conditions. These experiments also showed that IDO1 expression in endothelial cells was stimulated by cancer cells via soluble factors. This subchapter aims to evaluate whether IFNγ plays a role in this molecular mechanism.

To assess this question, I investigated IFNγ expression in MDA-MB-231 cells under standard conditions, namely with glucose at 5.5 mM and under low glucose conditions (glucose at 1 mM). IFNγ expression was also assessed in MCF10A cells as a non-aggressive mammary epithelial cell control. While there was no difference in the IFNγ expression between MCF10A cells and MDA-MB-231 under standard conditions, IFNγ was significantly increased in MDA-MB-231 cells when cultured in low glucose, as shown in Figure 27 A.

IFNγ treatment induced a significant increase of IDO1 expression in endothelial cells, but did not change the expression of TDO and IDO2 (Figure 27 B). Endothelial cells also significantly increased IDO1 protein level when treated with IFNγ (Figure 27 C).

My data and others have shown that endothelial cells induce breast cancer dormancy (Ghajar et al., 2013). This lead to additional hypothesis in this study, that IFNγ may be involved in the molecular mechanism that breast cancer cells use to overcome this situation. For that reason, IFNγ mRNA was assessed in MDA-MB-231 cells after 48 hours of co-culture with endothelial cells. Quantitative analysis of gene transcript showed that IFNγ expression was significantly decreased in MDA-MB-231 cells when cultured with endothelial cells (Figure 27 D).

In order to analyse whether IFNγ concentration induces a dose-dependent IDO1 expression and IDO1 enzymatic activity, endothelial cells were treated with a range of IFNγ concentration for 48 hours. IFNγ treatment induced endothelial cells to synthesise IDO1 protein, but the level of protein induced by the different IFNγ concentrations was not statistically different (Figure 28 A). The same result was observed when IDO1 activity was evaluated by measuring kynurenine concentration in the medium (Figure 28 B).

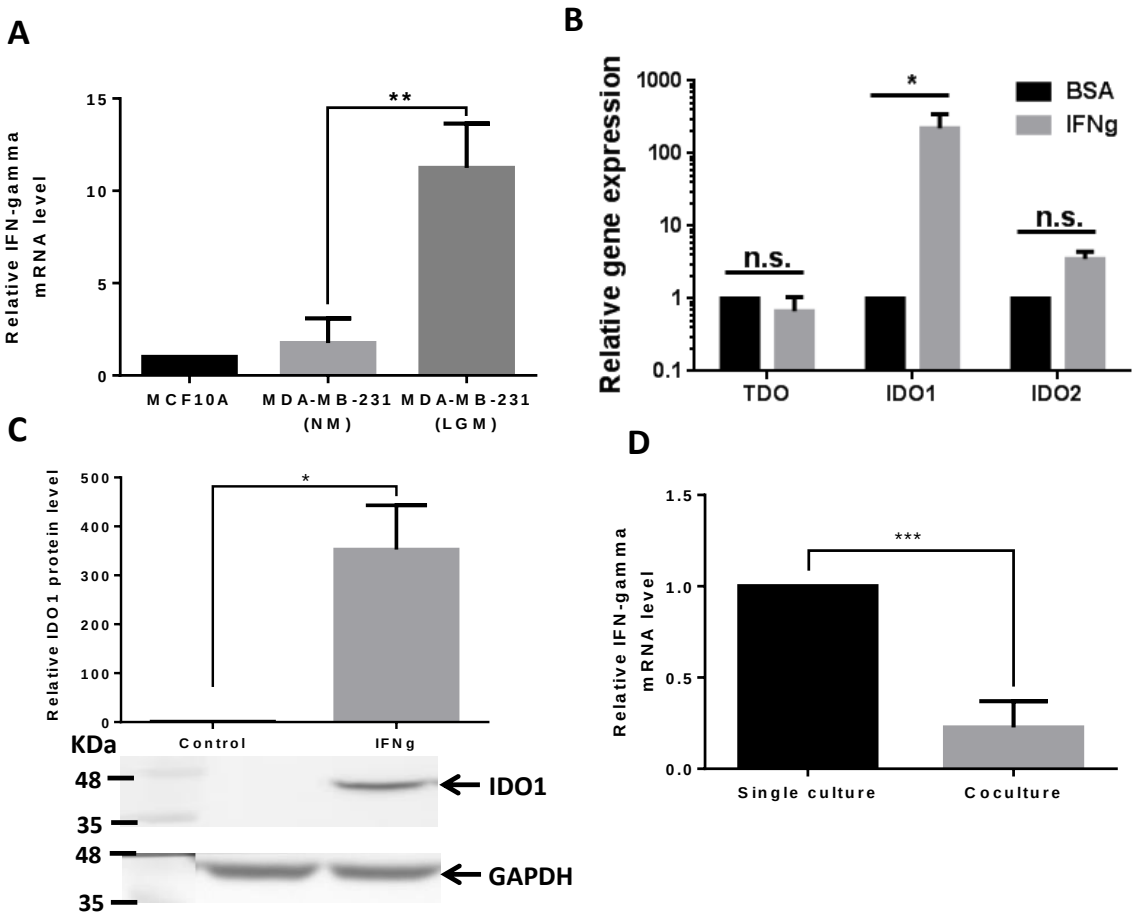


Figure 27: IFNγ indices IDO1 expression in endothelial cells. A- MDA-MB-231 significantly increased IFNγ expression when cultured in low glucose medium (LGM). B- Endothelial IFNγ (10 ng/mL) treatment induced an increase in IDO1 mRNA, but not TDO nor IDO2. C- IFNγ (10 ng/mL) treatment also induced an increase in IDO1 protein by endothelial cells. D- Interestingly, IFNγ expression by MDA-MB-231 cells was reduced when in co-culture with endothelial cells. N=3, bars are mean±S.E.M. Student t-test and One-way ANOVA were used to analyse any statistical differences between the groups. *P<0.05, **P<0.01, ***P<0.001.

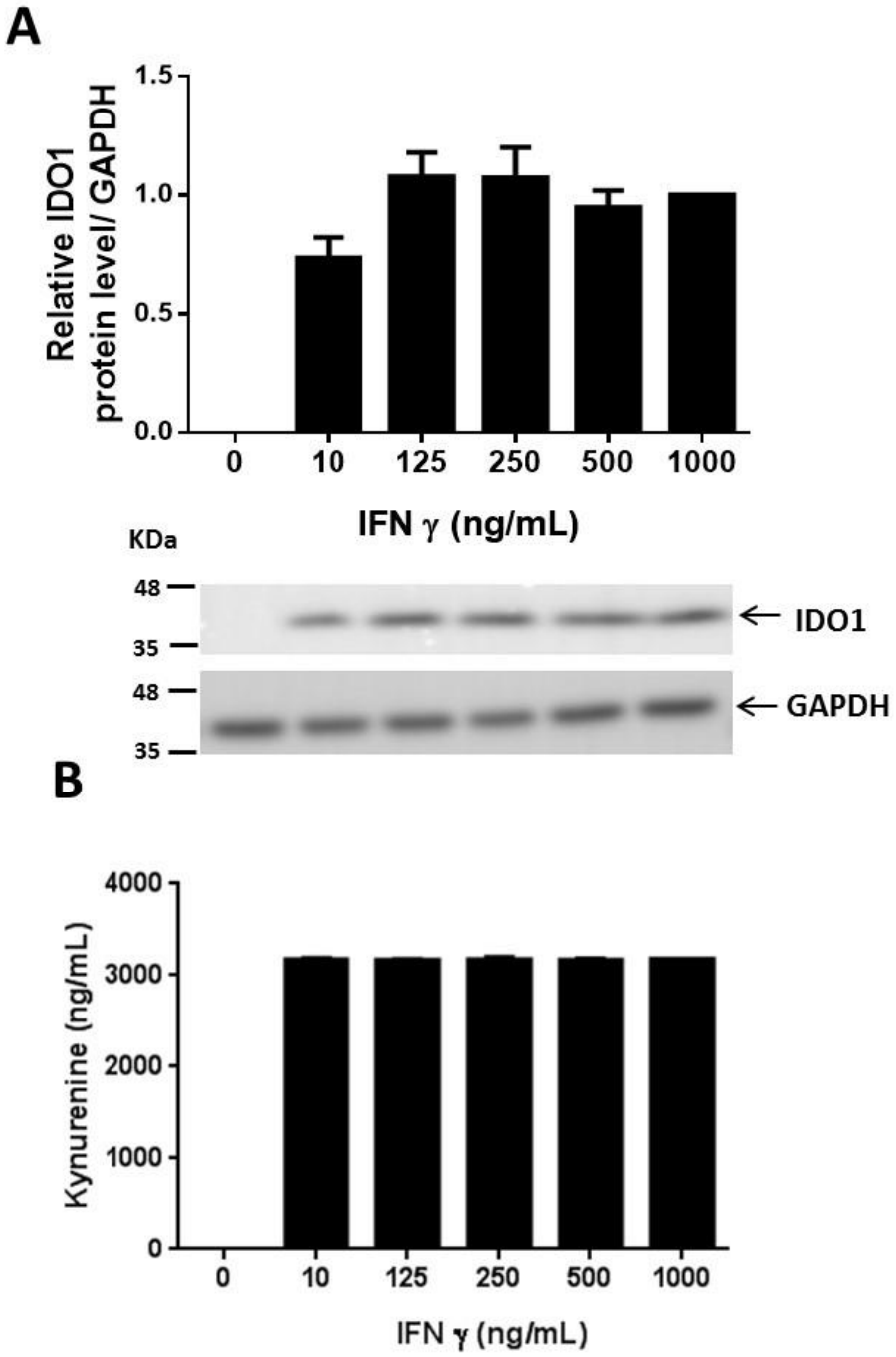


Figure 28: IFNγ induces endothelial IDO1 which is enzymatically active. A- IFNγ stimulated IDO1 expression in endothelial cells. B- IFNγ induced an increase in kynurenine in the endothelial medium which was assessed by ELISA. IFNγ treatment was for 48 hours. N=3, bars are mean±S.E.M. One-way ANOVA was used to analyse any statistical differences between the groups. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

6.3.2 Endothelial IDO1 decreases TSP1 secretion

The main part of my hypothesis is that breast cancer cells induce a decrease in endothelial TSP1 secretion via IDO1. My data suggested that IFNγ is a possible intermediate between cancer cells and endothelial cells. IFNγ is upregulated in cancer cells under low glucose, leading to induction of IDO1 expression and tryptophan degradation in endothelial cells. In the chapter V I presented that tryptophan limits endothelial TSP1 synthesis/secretion. It was logical for me to evaluate whether IFNγ is able to induce a decrease in TSP1 secretion by endothelial cells.

When endothelial cells were cultured with IFNγ (10 ng/mL) for 48 hours, the amount of secreted TSP1 were reduced by about 50%, compared with that by control endothelial cells with IFNγ (Figure 29 A). The next step of my investigation was to determine whether IFNγ induced a decrease in TSP1 via IDO1. IDO1 expression stimulated by IFNγ was successfully knockdown by siRNA (Figure 29 B), but kynurenine concentration was not decreased (Figure 29 C), showing that the degradation of tryptophan was not inhibited. Thus, I went to assess the expression of TDO and IDO2 in the IDO1 knockdown endothelial cells. My data showed that TDO was significantly increased, but not IDO2 (Figure 29 D), suggesting that TDO might be increased to compensate the lack of IDO1 in endothelial cells upon IFNγ stimulation. TDO compensation during IDO1 knockdown is an interesting novel finding. However, this finding leads to another layer of complexity for dissection of whether IFNγ induces a decrease in endothelial TSP1 protein levels via IDO1 enzyme activity. In order to circumvent this difficulty, I performed QPCR analysis of TSP1 expression in endothelial cells treated by IFNγ. Intriguingly, TSP1 mRNA was found to significantly increase in the endothelial cells after IFNγ treatment when compared to untreated counterparts (Figure 30 A). Taking together, IFNγ induces a decrease in TSP1 protein secretion but an increase in the TSP1 mRNA, suggesting that the effect of IFNγ on TSP1 protein is at the

translational level. This hypothesis is also supported by the previous chapter that showed that tryptophan limits TSP1 secretion in endothelial cells.

Further experiments investigated whether the TSP1 protein in the medium could regulate TSP1 expression in endothelial cells. Endothelial cells were cultured for 16 hours, and then the medium was changed or left unchanged. The purpose of this experiment was to remove TSP1 from the medium and verify the absence of the activation of receptors by TSP1 on TSP1 expression. Endothelial TSP1 mRNA was significantly increased 1, 3 and 6 hours after the medium was changed, when compared to cells which the medium was not changed (Figure 30 B). In another experiment, TSP1 mRNA was significantly reduced in endothelial cells that were cultured with human recombinant TSP1 (100 ng/mL) for 3 hours. Taking together, this set of experiments, suggest that extracellular TSP1 negatively regulates its own expression in endothelial cells. This finding explains the increase in TSP1 mRNA upon IFNγ treatment which led to a decrease in TSP1 protein.

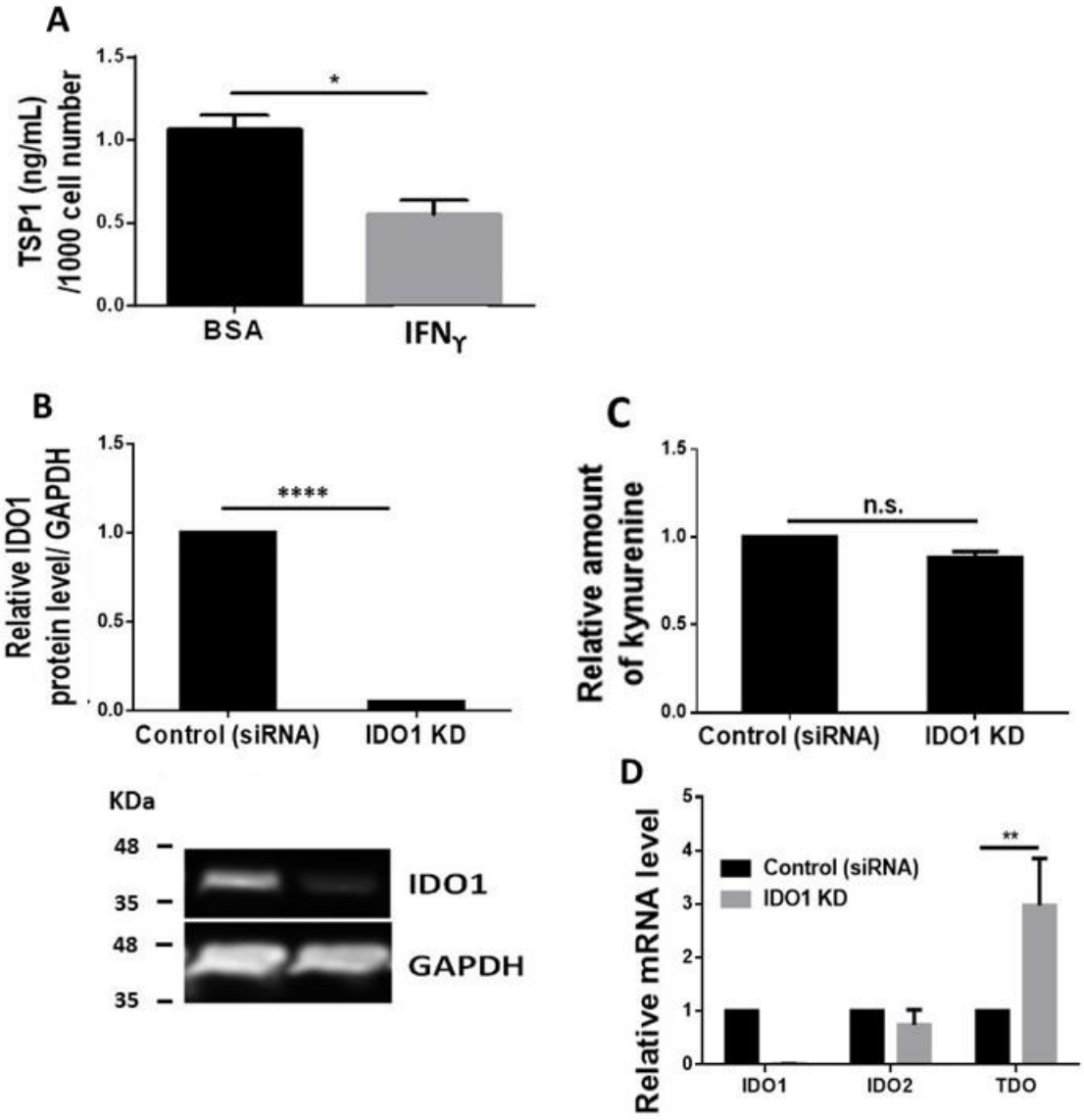


Figure 29: IFNγ decreases endothelial TSP1 synthesis. A- IFNγ induced a decrease in TSP1 synthesis by endothelial cells. B- IDO1 knockdown proof by western blot. C- L-Kynurenine level in IDO1 knockdown endothelial cells. D- IDO1, IDO2 and TDO mRNA quantification in endothelial cells stimulated with IFNγ (10 ng/mL) upon IDO1 knockdown. N=3, bars are mean±S.E.M. Student t-test and One-way ANOVA were used to analyse any statistical differences between the groups. *P<0.05, **P<0.01, ****P<0.0001, n.s. non-significant.

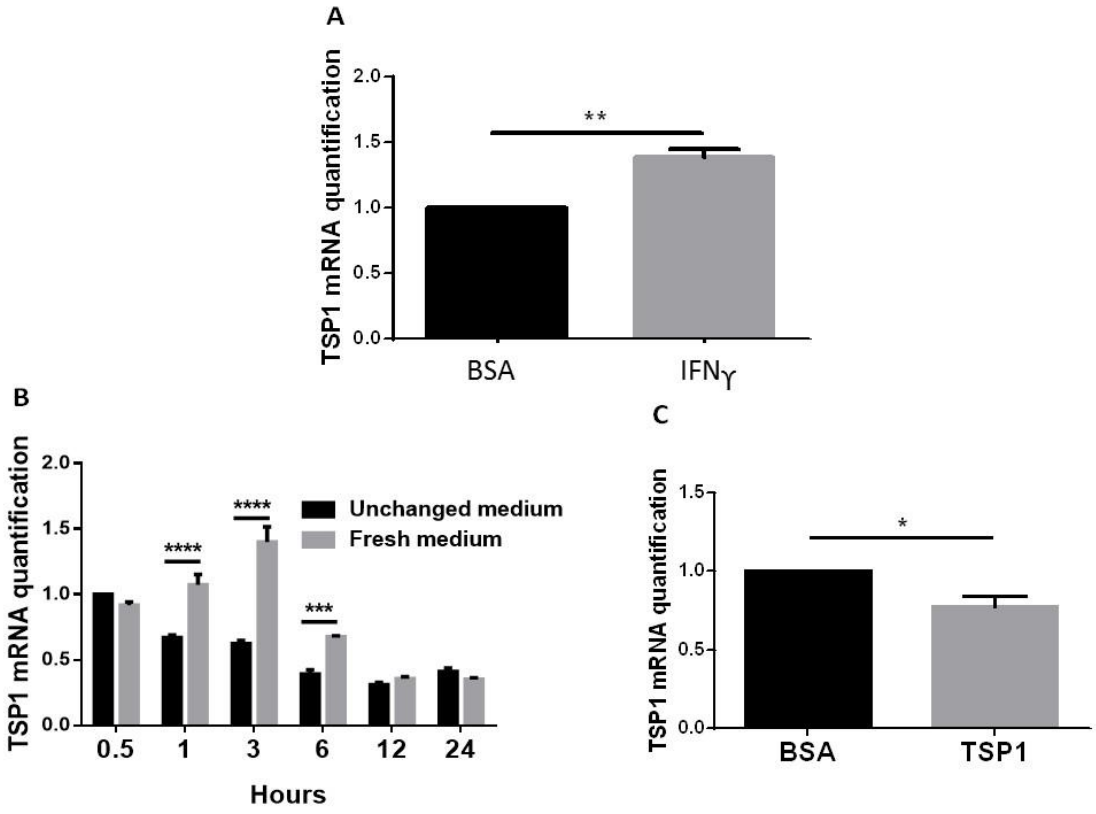


Figure 30: Endothelial cells upregulate TSP1 mRNA when treated with IFN-γ for 48 hours. TSP1 protein inhibited TSP1 mRNA expression by endothelial cells. Endothelial cells were cultured for 24 hours and then in one group, medium was not changed and in the second group, medium was changed. TSP1 mRNA was measured over-time. Also TSP1 mRNA was measured 3 hours after cultured with TSP1 (100 μM) or BSA. N=3, bars are mean±S.E.M. Student t-test and One-way ANOVA were used to analyse any statistical differences between the groups. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

6.4 IFNγ also triggers IDO1 protein synthesis in a non-tumorigenic breast cell line and in a lung epithelial cell line

The above experiments suggest that IFNγ might be the molecule secreted by breast cancer cells that induce IDO1 expression in endothelial cells. It is worth to note that a cytokine secreted by cancer cells might not just target endothelial cells. For that reason, it is important to assess the impact of IFNγ on other cells within the tumour microenvironment or micrometastasis niche, especially in the induction of IDO1 expression.

IFNγ did not increase IDO1 protein level in breast cancer cell lines, MCF7 and MDA-MB-231 cells (Figure 31 A). Interestingly, the IDO1 protein was significantly increased upon IFNγ treatment in a non-tumorigenic breast cells line, MCF10A (Figure 31 A). IFNγ also induced an increase in the IDO1 protein level in an epithelial lung cell line (Figure 31 B).

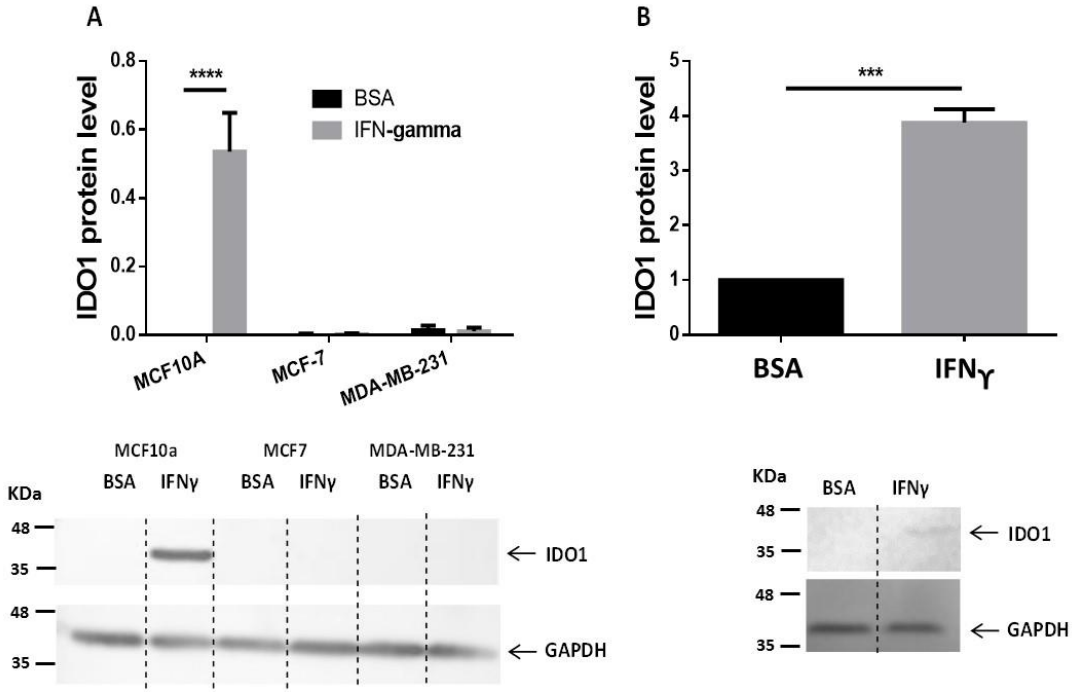


Figure 31: The effect of IFNγ on breast cells and lung epithelial cells. A- IFNγ (10ng/mL) induced IDO1 expression in MCA-10A but not in MCF7 nor MDA-MB-231 cells. B- IFN-γ also induced IDO1 expression in lung epithelial cells. IFNγ treatment was over 48 hours. N=3, bars are mean±S.E.M. Student t-test was used to analyse any statistical differences between the groups. ***P<0.001, ****P<0.0001.

6.4 Discussion

Once cancer cells escape the quiescence state called cancer cell dormancy, it reaches another dormancy phase called tumour mass dormancy. This estate is characterised by proliferating cells and apoptotic cells that are highly dependent on oxygen and nutrients availability. For the micrometastasis to overcome the lack of nutrients and oxygen, it has to trigger angiogenesis (Aguirre-Ghiso, 2007).

It was shown in the previous chapter that the medium generated by breast cancer cells under low glucose induced a higher IDO1 expression in endothelial cells, suggesting that a soluble factor secreted by the cancer cells might be in the origin of the endothelial IDO1. The promoter region of IDO1 contain 3 IFNγ-activated sites (Robinson et al., 2006) and this cytokine has been described to be the most powerful inductor of IDO1 *in vitro* and *in vivo* (Gough et al., 2008; Jeong et al., 2009). The present study has clearly demonstrated that expression of IFNγ is significantly increased in the breast cancer cells cultured under low glucose conditions. Another independent study by Voss and colleagues supports this finding. It showed that breast cancer cell lines, MCF7 and MDA-MB-435, release IFNγ under hypoxia, although at very low levels (Voss et al., 2011). The same study showed that MDA-MB-231 secrete IL-8 and IL-6, also both stimuli of IDO1 expression, in much higher concentrations (Voss et al., 2011). Interestingly, IFNγ inhibited proliferation of MCF7 and MDA-MB-231 cells when growing in monolayer, but it did not affect MDA-MB-231 anchorage-independent growth in an agar growth assay (Gooch et al., 2000). This suggests that this cytokine is secreted by a range of breast cancer cell lines under hypoxia conditions, but might have an adverse effect on less aggressive breast cancer cell lines.

Although IFNγ is a strong stimulus of IDO1, the IDO1 driven factor(s) in tumours is still debatable. IDO1 expression is thought to be due to the generalised inflammation that is present in many tumours (Spranger et al., 2013; Taube et al., 2012; Théate et al., 2015; Tumeh et al., 2014), but there are a few subtypes of tumours that express IDO1 in

the absence of inflammation (Théate et al., 2015; Uyttenhove et al., 2003). The expression pattern and the expression regulation of IDO1 in endothelial cells are even less understood. In some tumours, such as kidney cancer, IDO1 is almost restricted to endothelial cells (Riesenberg et al., 2007), suggesting a tissue- specific molecular mechanism to induce endothelial IDO1.

It was shown here that IFNγ could induce IDO1 expression in endothelial cells, a finding supported by other authors (Wang et al., 2010). Importantly, IFNγ induced a similar level of IDO1 protein and enzymatic activity at low (10 ng/mL) and high (1000 ng/mL) concentration in endothelial cells. This suggests that IFNγ is a potent stimulus of endothelial IDO1 and very low levels of this cytokine might be enough to trigger IDO1 expression and tryptophan degradation.

As shown in Chapter V, tryptophan concentration limits TSP1 secretion by endothelial cells and it was also demonstrated that breast cancer cells can directly induce IDO1 expression and tryptophan degradation in endothelial cells. Furthermore, IFNγ induced a decrease in TSP1 secretion by endothelial cells, supporting the initial hypothesis that IDO1, by degrading tryptophan, restricts endothelial TSP1 synthesis and secretion. Although IFNγ did not change the expression of the other 2 tryptophan degrading enzymes, TDO and IDO2, the expression of TDO was significantly increased when IDO1 was knockdown, which would allow sustained the level of tryptophan degradation in the cells. A previous study has shown that IDO2 was upregulated in the epididymis of IDO1 deficient mice, a tissue that would otherwise constitutively expresses IDO1 (Fukunaga et al., 2012), indicating that IDO2 might have a functional redundancy with IDO1. It suggests that TDO might compensate for an IDO1 deficiency in IFNγ stimulated- endothelial cells.

As demonstrated in Chapter IV endothelial cells induce breast cancer cell cycle arrest, and here it was shown a possible molecular mechanism led by IFNγ to overcome

that situation. Interestingly, endothelial cells induced a decrease in IFNγ expression by breast cancer cells, which might be another way of endothelial cells to maintain breast cancer cells under dormancy.

Here, it was established that extracellular TSP1 protein modulates TSP1 expression in endothelial cells, working as an auto-inhibitory feedback possibly to control the level of TSP1 in the extracellular matrix. It is worth noting that IFNγ increased endothelial TSP1 mRNA level, which can be explained by the TSP1 negative feedback. It also suggests that the IFNγ effect on TSP1 secretion is not at the transcriptional level corroborating the idea that TSP1 decrease is due to a lack of tryptophan followed an IDO1 increase.

As IFNγ is secreted into the extracellular matrix it can target many cell types. In Chapter III, IDO1 was visualised in stromal cells but also in cancer cells, however, IFNγ did not induce a detectable amount of IDO1 protein in MCF7 and MDA-MB-231 cells. This result could indicate that IDO1 in breast cancer cells is stimulated by other cytokines, such as IL-6, or it requires a genetic mutation and Bin1 inactivation has been implicated in overexpression of IDO1 in a tumour (Jia et al., 2015). Surprisingly, IFNγ induced a strong IDO1 signal in a non-carcinogenic breast cell line (MCF10A) and a weak signal in an epithelial lung cell line. This could suggest an important role of IDO1 expression in the normal tissue surrounding the cancer cells or even stablishing pre-metastatic niches, which could lead to a decrease of tryptophan in the tumour microenvironment. Clearly, the importance of this interesting observation would have to be further evaluated in larger and more sophisticated studies. However, as it stands, this data has shed light on the immune resistance of cancer cells and the induction of angiogenesis during cancer progression.

Taken together, this chapter shows that IFNγ is involved in the upregulation of endothelial IDO1 induced by breast cancer cells under low glucose. Here it was also

demonstrated that IFNγ is capable of decreasing TSP1 synthesis in a transcriptional independent manner, supporting the results from the previous chapter that showed that tryptophan concentration limits TSP1 secretion. In the context of cancer dormancy, IFNγ appears to be a central cytokine in the molecular mechanism employed by cancer cells to overcome dormancy.

This study thus indicates that when breast cancer cells are under stress, they secrete IFNγ, which in turn induces a decrease in endothelial TSP1 via IDO1. Although this mechanism has to be further validated in *in vivo* studies, these early findings have an alarming implication once IFNγ is considered as a potential therapeutic tool in cancer treatment. Although IFNγ has been successfully used during viral infection, its application in cancer therapy has been lagging. This is largely due to the significant concerns raised in recent years that whilst IFNγ is able to induce certain anti-cancer immune response, it has demonstrated effect on the stimulation of cancer cells to grow, regrowth and metastasise (Zaidi and Merlino, 2011). The findings from the present study certainly add more weight to this growing concern.

Chapter VII: General conclusion

Metastasis remains the main challenge for patients and oncologists alike (Uhr and Pantel, 2011). For patients with breast cancer, their prognosis is a great deal better when the tumour is detected at an early stage, however their clinical outcome become poorly when either diagnosed at a later stage or with metastasis (Cardoso et al., 2009). Another challenge is that about 10% of breast cancer patients who did not present metastasis at the diagnosis stage develop metastasis years or decades after cancer treatment (Noh et al., 2011). This phenomenon can be explained by the fact that metastatic cancer cells from the primary lesion undergo a period of dormancy before the manifestation of metastasis (Aguirre-Ghiso, 2007). Current cancer therapies seem inefficient to eliminate these cells (Goss and Chambers, 2010).

The dissemination of cancer cells from the primary tumour to other parts of the body can begin at an early stage and in many cases micrometastasis and/or metastasis would already occur at the diagnosis stage (Klein, 2011; Klein et al., 2002). No current therapeutic strategy is available to prevent it from happening. Therefore, a better understanding of tumour dormancy and the mechanism behind it will allow scientists to develop better drugs to eliminate dormant cancer cells or to keep them dormant.

This study aimed to evaluate the role of TSP1 in breast cancer dormancy and a potential molecular mechanism via IDO1, which might be employed by dormant cancer cells to trigger the outgrowth of metastasis.

7.1 TSP1 induces breast cancer dormancy

TSPs are extracellular matrix proteins that do not work as structural proteins, rather exert their functions by interacting with and modulating other proteins in the extracellular matrix, or activating cell surface receptors (Chen et al., 1996; Cockburn and Barnes, 1991; Greenaway et al., 2007; Gupta et al., 1999). TSP1 was first detected in platelets (Lawler et al., 1978). Sequentially, TSP1 has been found synthesised and secreted by non-platelet cells including endothelial cells, cancer cells, smooth muscle

cells, macrophages, fibroblasts and others (Naganuma et al., 2004; Wight et al., 1985). In early years, TSP1 was recognised as an anti-angiogenic protein controlling tumour growth and progression (Good et al., 1990). Moreover, the levels of TSP1 in the lung or bone are correlated with the metastatic potential of melanoma and prostate cells (Catena et al., 2013; El Rayes et al., 2015), suggesting that TSP1 might be a key molecule in metastasis initiation.

Ghajar et al. showed that the dormant disseminated tumour cells were located in the perivascular niche of the organs (lung, bone marrow and brain) and that *in vitro* stable endothelial tubules exerted an anti-proliferation effect on breast cancer cells (Ghajar et al., 2013). These authors concluded that the endothelium induces dormancy of individual disseminated cancer cells with TSP1, found rich in tissue (including tumour tissue) microenvironments. In the present study, immunohistochemistry analysis of a tissue microarray revealed that the stromal TSP1 expression is inversely correlated with the increased malignance of the invasive breast carcinoma. Higher stromal TSP1 also suggests a possible survival benefit, but further confirmation is needed. Another study showed that colorectal patients that presented high levels of TSP1 in the primary tumour were less likely to have lymph node metastasis (Iddings et al., 2007).

Here, *in vitro* experiments further demonstrated by that endothelial cells induce cell cycle arrest in the G0/G1 phase of breast cancer cells. The cell cycle arrest induced by endothelial cells was not due to senescence, suggesting that endothelial cells might induce a quiescent state which is a better fit for cellular dormancy. An increased concentration of TSP1 had also an anti-proliferative effect on cancer cells, which is consistent with the findings by Ghajar and colleagues that TSP1 induce breast cancer dormancy. The majority of chemotherapeutic drugs fails in eliminating individual dormant cell (Mitchison, 2012). Pre-culturing breast cancer cells with TSP1 conferred an advantage of survival in the presence of docetaxel, a commonly used druf in breast cancer treatment. Chemoresistance of the dormant cancer cells has been attributed

mainly to the fact that these drugs target high proliferative cells and have little or no effect on slow and non-proliferative cancer cells (Mitchison, 2012). Interestingly, TSP1 induced breast cancer cells to express CYP3A5, an enzyme involved in the metabolism of docetaxel. This would suggest that TSP1 has a direct protective effect against the toxicity of a chemotherapy drug, such as docetaxel. Weng et al. also showed that dendritic cells efficiently protected cancer cells against the immune system via secretion of TSP1 (Weng et al., 2014).

Taken together, TSP1 may be involved in all types of cancer dormancy: cellular dormancy, angiogenic dormancy and immune-mediated dormancy, making TSP1 a potential key molecule in spread and regrowth of metastatic breast cancer. Even though the elevation of TSP1 levels in organs such as lung, brain and bone seems to be a good strategy of preventing the relapse of breast cancer, the story has not been a straight forward one, as with many other molecules involved in cancer. In this case, it has been recognised that TSP1 exerts diverse effects on a wide range of cell receptors, including CD47 (integrin associated protein, IAP), CD36 (FAT) and Gabapentin receptor alpha2delta-1. For example, TSP1 has been shown to promote tumour progression (Incardona et al., 1995; Yee et al., 2009), Identification of molecular pathways related to the diversity of TSP1 cellular functions, including cancer dormancy, drug resistance or the escape from the immune system are essential for targeting the TSP1 downstream signalling pathway to reduce unwanted adverse-effects.

7.2 The role of IFNγ-IDO1- TSP1 pathway in the outgrowth of metastasis from dormant breast cancer cells

IDO1 is an intracellular enzyme that degrades L-tryptophan and overexpressed in many tumour types, such as breast cancer, prostatic cancer and colorectal cancer (Munn et al., 2004; Munn et al., 2002; Uyttenhove et al., 2003). Within a solid tumour, IDO1 has been detected in cancer cells, but also in macrophages (Munn et al., 2004; Munn et al., 2002; Uyttenhove et al., 2003) and endothelial cells (Riesenberg et al.,

2007). The main function of IDO1 in tumours is believed to protect cancer cells against the immune system by producing a lack of L-tryptophan in the tumour microenvironment (Prendergast et al., 2010). Thus, high IDO1 enzymatic activity correlates with poor prognosis in many cancer types, including breast cancer (Astigiano et al., 2005; Brandacher et al., 2006; Chen et al., 2014; Inaba et al., 2010; Inaba et al., 2009; Ino et al., 2006; Jia et al., 2015; Okamoto et al., 2005; Pan et al., 2008; Speeckaert et al., 2012; Suzuki et al., 2010; Urakawa et al., 2009; Wainwright et al., 2012; Yu et al., 2013; Yu et al., 2011).

L-tryptophan is the rarest essential amino acid in mammalians and it is thought to work as a rate-limiting amino acid in protein synthesis (Cortamira et al., 1991; Wurtman et al., 1980). Tryptophan availability is mainly regulated by its absorbance from the blood and its catabolism, for instance by IDO1.

Here, the immunohistochemistry analysis of a tumour tissue array of invasive breast carcinomas revealed the presence of vascular IDO1 staining in the majority of the samples. Interestingly, the vascular IDO1 staining was negatively correlated with the stage of the tumour, which might suggest that IDO1 is more important in the early development of the tumour. This assumption agrees with another study that shows that vascular IDO1 was only present in newly formed vessels in renal cell carcinoma (Riesenberg et al., 2007).

The analysis of the amino acid sequence of TSP1 revealed a higher percentage of tryptophan than the average protein. Interestingly, *in vitro* co-culture experiments and conditioned medium experiments showed that breast cancer cells induce an increase in IDO1 expression in endothelial cells. The conditions obtained from low glucose culture of breast cancer cells enhanced endothelial IDO1 expression. These findings are potentially related to cancer dormancy, since disseminated breast cancer cells have been found near microvessels (Ghajar et al., 2013). Disseminated tumour cells might be

able to induce vascular IDO1 and cause a local decrease in L-tryptophan. Low tryptophan limited the amount of TSP1 secreted by endothelial cells, which was further supported by the immunohistochemistry data that showed an inverse correlation between vascular IDO1 and stromal TSP1. Furthermore, low vascular IDO1 in the primary tumour was found to have a potential trend towards an improved overall survival. Interestingly, low tryptophan increased VEGF secretion by breast cancer cells.

Taking together, these results suggest that disseminated dormant tumour cells near microvessels might be capable of inducing vascular IDO1, leading to a decrease in the extracellular concentration of TSP1 alongside with an increase in VEGF. Although these findings need further confirmation, the results of this thesis provide a potential mechanism that dormant breast cancer cells use to overcome the inhibitory effect of the vascular TSP1.

IFNγ is known to be the most potent stimulus of IDO1 (Gough et al., 2008; Jeong et al., 2009). This connection was confirmed in the present study that IFNγ induces a strong IDO1 signal in endothelial cells. Although IDO1 signal was observed in the cancer cells of the primary tumour, in the *in vitro* assay in the present study, IFNγ did not increase IDO1 protein in breast cancer cells. This might indicate that endothelial cells are more responsive to IFNγ and that IDO1 observed in cancer cells in the primary tumour might be induced by other molecules. As described above, the low glucose-conditioned medium from breast cancer cells induced a stronger IDO1 signal in endothelial cells. The analysis of gene expression in breast cancer cells showed that IFNγ mRNA was upregulated under low glucose condition. The results here suggest that IFNγ is the molecule responsible for the increase of vascular IDO1 in this situation. It was also shown here that IFNγ induced a weak signal in epithelial lung cells, indicating a possible role of IDO1 in the normal tissue during the metastatic process. Interestingly, IFNγ induced a strong IDO1 signal in non-tumorigenic breast cells (MCF10A), which agrees with the tissue array analysis that showed a strong IDO1 staining in the adjacent

tissue. This might suggest that the adjacent normal breast tissue might help tumour cells to create a lack of tryptophan in the tumour microenvironment in order to increase the immune protection or even to increase angiogenesis.

7.3 Summary – the proposed mechanism

As a whole, the work presented here provides evidences for a non-canonical role of the IFNγ-IDO1-TSP1 pathway for breast cancer cells to evade from tumour dormancy, as shown in Figure 32. First, breast cancer cells, in part via IFNγ, induce vascular IDO1 expression leading to tryptophan degradation. Second, the reduction in tryptophan limits TSP1 synthesis and secretion, which gradually results in a reduction of TSP1 in the perivascular niche. The deprivation of TSP1 in the microenvironment permits cancer cells to evade cellular dormancy. Once a micrometastasis is formed, a stress conditions, such as lack of nutrients and oxygen, would induce cancer cells secreting more IFNγ, which in turn results in high vascular IDO1 expression and tryptophan degradation. A significant reduction in TSP1 synthesis alongside an increase in VEGF secretion by cancer cells may be sufficient to trigger angiogenesis. These collectively trigger the outgrowth of the metastasis.

7.3 Future work

Although the findings in this thesis are novel, there are a number of shortfalls and new directions that can be tested in future studies.

1. In vivo tumour models: some of the key findings from the study would benefit from validation experiments using in vivo tumour models. Key questions to answer include whether vascular IDO1 is induced near dormant breast cancer cells, whether the vascular IDO1-inducedthe deprivation of L-tryptophan is sufficient, to reduce TSP1 in the dormant niche. Another important point is to determine whether this TSP1 regulation pathway occurs in the all organs vulnerable to breast cancer metastasis and is employed by all breast cancer subtypes. Several experimental strategies can be valuable to get

answers to the questions raised above, such as IFNγ knockout breast cancer cells, IDO1/TSP1 knockout mice or drugs that modulate the expression or activity of those molecules.

2. The analysis of human samples in a larger and most focused cohort is also a useful approach. This would include a large number of the patients supported by a longer follow up and potentially primary/secondary tumour pairs. This would allow a firm conclusion drawn between IDO1/TSP1/IFNγ and patient's outcome including metastasis and analysis of organ trophic nature of the microenvironment associated these molecules, namely if those tumours destined for a specific organ have a specific pattern of these molecule expression.

3. The study of dormant single cancer cells is challenging and requires the development of new technology and techniques. The single-cell sequencing may be a powerful tool in investigating the importance of rare cells in tumour progression (Navin, 2015).

Finally, the current work also showed that TSP1 increased the survival of cancer cells to docetaxel, which might be due to an increase in the expression of CYP3A5, an enzyme involved in the metabolism of docetaxel. This finding is promising but needs further *in vitro* and *in vivo* confirmation.

7.4 Clinical implications

The identification of a non-canonical IFNγ-IDO1-TSP1 pathway that drives breast cancer cells to evade tumour dormancy might contribute to better understand this phenomenon. The manipulation of the expression or activity of any of key molecules in this pathway might help to delay or prevent the relapse of metastasis. It might also allow to develop strategies to sensitise dormant cancer cells to therapy.

IFNγ was initially identified to play a significant role in the detection and elimination of tumour cells as well as enhancing tumour cell immunogenicity (Pestka et

al., 2004). Thus, IFNγ has been tested as an anti-cancer therapy. However, this cytokine has also demonstrated to have pro-tumorigenic effects (Bernabei et al., 2001; Bröcker et al., 1988; Gorbacheva et al., 2002) and has caused some significant concerns (Zaidi and Merlino, 2011). Here, the results have firmly demonstrated that IFNγ induces vascular IDO1. Together with the concerns in the literature, this strongly argues that IFNγ may indeed trigger the development of metastasis from disseminated and dormant tumour cells. For this reason, it is strongly suggested that the efficacy and safety of IFNγ for cancer treatment must be carefully considered and counter measures for the unwanted side effect will have to be sought if IFNγ is to be used in these patients.

Another medical challenge in oncology is to detect metastasis at the beginning of their development or to evaluate at diagnosis stage whether that patient will develop late metastasis. Currently, it is not possible to predict the risk of late recurrence or to know which dormant cancer cells or micrometastasis are going to generate a full scale metastasis. Thus, there is a medical need to find markers that help to predict metastasis formation. Two proteins, MYC and Ras, have shown to affect dormancy by regulating genes involved in angiogenesis, which make them promising dormancy markers (Watnick et al., 2003).

If the molecular mechanism described in this study is further validated, it would open the prospect of using the levels of L-tryptophan or L-kynurenine in the blood as a marker for metastasis initiation or even as an early sign of cancer dormancy evasion. This would of course need a rather large and carefully devised clinical trial.

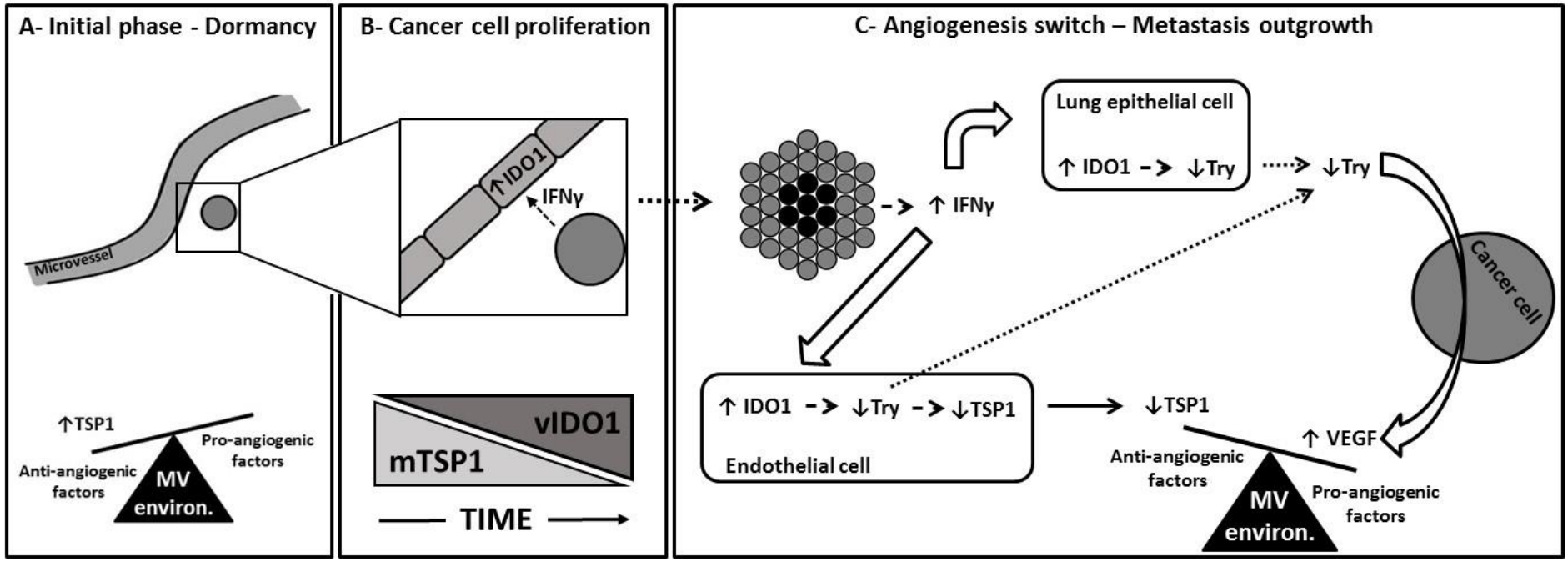


Figure 32: Proposed molecular mechanism for breast cancer dormancy evasion. A- Breast cancer cells remain near microvessels, a TSP1 rich microenvironment which induces cell cycle arrest of cancer cells. B- Breast cancer cells induce vascular IDO1 expression, which leads to a decrease in TSP1 synthesis and secretion overtime, allowing cancer cells to proliferate. C- Once cancer cells form a micrometastasis, the cells in the centre of the micrometastasis are under stress due to the lack of oxygen and nutrients. Those cells increase the secretion of IFNγ increasing even further vascular IDO1 leading to a more dramatic decrease of TSP1 in the microenvironment. IFNγ also induces IDO1 expression in other cell types, such as lung epithelial cells, within the tumour microenvironment. The lack of L-tryptophan caused by IDO1 induces also an increase in the secretion of VEGF by cancer cells. The decrease of TSP1 and an increase in VEGF may trigger angiogenesis allowing the outgrowth of the metastasis. (MV environm.: microvessel environment; vIDO1: vascular IDO1; mTSP1: microenvironment TSP1).

Appendix I: Breast cancer patient's clinical data.

Number of sample	Pathology diagnosis	Age	TNM	Grade
1	Invasive ductal carcinoma	64	T1N0M0	G2
2	Invasive ductal carcinoma	48	T1N0M0	G3
3	Invasive ductal carcinoma	56	T1N0M0	G2-G3
4	Invasive ductal carcinoma	58	T1N0M0	G2
5	Invasive ductal carcinoma	36	T1N0M0	G3
6	Invasive ductal carcinoma	42	T1N1M0	G3
7	Invasive ductal carcinoma	48	T1N1M0	G3
8	Invasive ductal carcinoma	59	T1N1M0	G2
9	Invasive ductal carcinoma	70	T2N0M0	G3
10	Invasive ductal carcinoma	37	T2N0M0	G2
11	Invasive ductal carcinoma	70	T2N0M0	G2
12	Invasive ductal carcinoma	50	T2N0M0	G2
13	Invasive ductal carcinoma	42	T2N0M0	G2
14	Invasive ductal carcinoma	63	T2N0M0	G2
15	Invasive ductal carcinoma	50	T2N0M0	G2
16	Invasive ductal carcinoma	49	T2N0M0	G3
17	Invasive ductal carcinoma	68	T2N0M0	G2
18	Invasive ductal carcinoma	53	T2N0M0	G2
19	Invasive ductal carcinoma	46	T2N0M0	G3
20	Invasive ductal carcinoma	77	T2N0M0	G3
21	Invasive ductal carcinoma	63	T2N0M0	G3
22	Invasive ductal carcinoma	52	T2N0M0v	G3
23	Invasive ductal carcinoma	71	T2N1M0	G1
24	Invasive ductal carcinoma	72	T2N1M0	G2
25	Invasive ductal carcinoma	56	T2N1M0	G2
26	Invasive ductal carcinoma	56	T2N1M0	G2
27	Invasive ductal carcinoma	34	T2N1M0	G3
28	Invasive ductal carcinoma	55	T4N0M0	G3
29	Invasive ductal carcinoma	52	T2N0M0	G2
30	Invasive ductal carcinoma	60	T3N0M0	G3
31	Invasive ductal carcinoma	47	T2N2M0	G3
32	Invasive ductal carcinoma	41	T3N1M0	G3
33	Invasive ductal carcinoma	42	T3N2M0	G3
34	Invasive ductal carcinoma	77	T4N2M0	G2
35	Invasive ductal carcinoma	46	T3N3M0	G2
36	Invasive ductal carcinoma	53	T3NxM0	G2
37	Invasive ductal carcinoma	46	-	G2
38	Invasive ductal carcinoma	42	T2NxM0	G2
39	Invasive ductal carcinoma	-	-	G2
40	Invasive ductal carcinoma	52	-	G2
41	Invasive ductal carcinoma	53	-	G2
42	Invasive ductal carcinoma	-	T?N0M?	G2-G3
43	Invasive ductal carcinoma	-	-	G3
44	Invasive ductal carcinoma	-	-	G2-G3
45	Invasive ductal carcinoma	54	T1NxMx	G3
46	Invasive ductal carcinoma	79	T1NxMx	G2
47	Invasive ductal carcinoma	74	T2NxMx	G3
48	Invasive ductal carcinoma	-	-	G3
49	Invasive ductal carcinoma	52	T2NxMx	G3
50	Invasive ductal carcinoma	57	T2NxMx	G3
51	Invasive ductal carcinoma	50	T2NxMx	G2
52	Ductal carcinoma and lobular carcinoma	43	-	G2

Appendix II: Breast cancer patient's clinical data.

IDC: invasive ductal carcinoma, NC: neuroendocrine carcinoma, MC: medullary carcinoma, MIDC: metastasis invasive ductal carcinoma, LN: lymph node, NAT: normal adjacent tissue

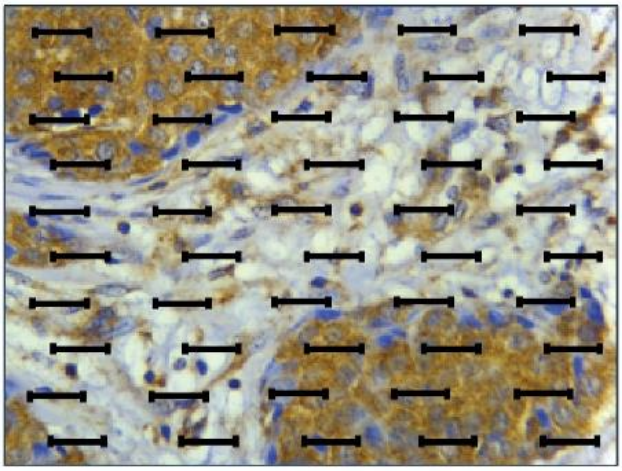
Number of sample	Age	Organ	Pathology diagnosis	TNM	Grade	Stage	Type
1	53	Breast	IDC	T3N0M0	2	IIB	Malignant
2	44	Breast	IDC	T2N0M0	1	IIA	Malignant
3	63	Breast	IDC	T4N0M0	2	IIIB	Malignant
4	41	Breast	IDC	T2N0M0	1--2	IIA	Malignant
5	43	Breast	IDC	T1N0M0	2	I	Malignant
6	44	Breast	IDC	TicA0M0	1	I	Malignant
7	39	Breast	IDC	T2N0M0	1	IIA	Malignant
8	54	Breast	IDC	T3N0M0	2	IIB	Malignant
9	40	Breast	IDC	T4N0M0	1	IIIB	Malignant
10	44	Breast	IDC	T2N1M0	2	IIB	Malignant
11	55	Breast	IDC	T2N0M0	2	IIA	Malignant
12	63	Breast	IDC	T2N0M0	2	IIA	Malignant
13	70	Breast	IDC	T2N0M0	2	IIA	Malignant
14	50	Breast	IDC	T2N1M0	2	IIB	Malignant
15	60	Breast	IDC	T2N0M0	2	IIA	Malignant
16	38	Breast	IDC	T2N0M0	2	IIA	Malignant
17	48	Breast	IDC	T2N0M0	2	IIA	Malignant
18	75	Breast	IDC	T2N1M0	2	IIB	Malignant
19	40	Breast	IDC	T2N0M0	2	IIA	Malignant
20	49	Breast	IDC	T4N1M0	2	IIIB	Malignant
21	43	Breast	IDC	T2N0M0	2	IIA	Malignant
22	61	Breast	IDC	T2N1M0	2	IIB	Malignant
23	55	Breast	IDC	T2N0M0	2	IIA	Malignant
24	58	Breast	IDC	T1N0M0	2	I	Malignant
25	57	Breast	IDC	T4N2M0	2	IIIB	Malignant
26	45	Breast	IDC	T2N0M0	2	IIA	Malignant

27	53	Breast	IDC	T2N1M0	2	IIB	Malignant
28	47	Breast	IDC	T2N0M0	2	IIA	Malignant
29	34	Breast	IDC	T2N0M0	2	IIA	Malignant
30	53	Breast	IDC	T3N1M0	2	IIB	Malignant
31	39	Breast	IDC	T2N0M0	1	IIA	Malignant
32	36	Breast	IDC	T1N0M0	2	I	Malignant
33	60	Breast	IDC	T2N0M0	3	IIA	Malignant
34	60	Breast	IDC	T2N0M0	2	IIA	Malignant
35	29	Breast	IDC	T2N0M0	3	IIA	Malignant
36	50	Breast	IDC	T2N0M0	-	IIA	Malignant
37	66	Breast	IDC	T2N0M0	2	IIA	Malignant
38	37	Breast	IDC	T2N0M0	2	IIA	Malignant
39	46	Breast	IDC	T2N0M0	2	IIA	Malignant
40	43	Breast	IDC	T2N0M0	2	IIA	Malignant
41	62	Breast	IDC	T4N0M0	3	IIIB	Malignant
42	46	Breast	IDC	T2N0M0	3	IIA	Malignant
43	45	Breast	IDC	T2N0M0	2	IIA	Malignant
44	49	Breast	IDC	T2N0M0	3	IIA	Malignant
45	52	Breast	IDC	T2N1M0	3	IIB	Malignant
46	32	Breast	NC	T2N3M0	-	IV	Malignant
47	51	Breast	IDC	T2N0M0	2	IIA	Malignant
48	40	Breast	MC	T3N0M0	-	IIB	Malignant
49	58	Breast	MC	T3N0M0	-	IIB	Malignant
50	64	Breast	MC	T2N0M0	-	IIA	Malignant
51	49	LN	MIDC	T1N1M0	-	-	Metastasis
52	41	LN	MIDC	-	-	-	Metastasis
53	50	LN	MIDC	T2N1M0	-	-	Metastasis
54	61	LN	MIDC	T2N1M0	-	-	Metastasis
55	57	LN	MIDC	T4N2M0	-	-	Metastasis
56	38	LN	MIDC	T2N1M0	-	-	Metastasis
57	52	LN	MIDC	T4N1M0	-	IIIB	Metastasis

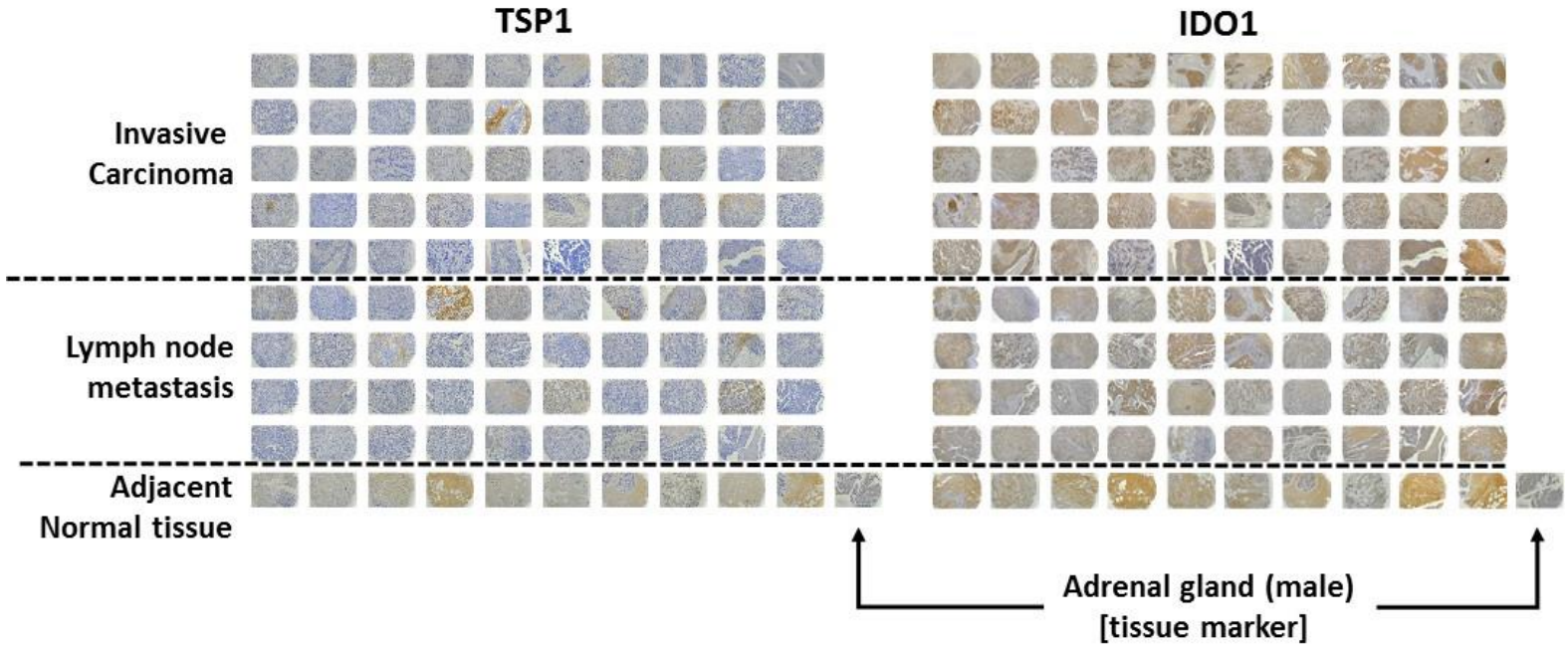
58	46	LN	MIDC	-	-	-	Metastasis
59	39	LN	MIDC	T4N1M0	-	-	Metastasis
60	48	LN	MIDC	T3N1M0	-	-	Metastasis
61	50	LN	MIDC	T2N1M0	-	-	Metastasis
62	38	LN	MIDC	-	-	-	Metastasis
63	46	LN	MIDC	T4N2M0	-	IIIB	Metastasis
64	52	LN	MIDC	T2N1M0	-	-	Metastasis
65	57	LN	MIDC	T2N2M0	-	-	Metastasis
66	50	LN	MIDC	T2N2M0	-	-	Metastasis
67	45	LN	MIDC	T4N1M0	-	-	Metastasis
68	58	LN	MIDC	T4N1M0	-	-	Metastasis
69	57	LN	MIDC	T2N1M0	-	-	Metastasis
70	33	LN	MIDC	-	-	-	Metastasis
71	76	LN	MIDC	-	-	-	Metastasis
72	48	LN	MIDC	T2N1M0	-	-	Metastasis
73	57	LN	MIDC	T3N1M0	-	IIIA	Metastasis
74	46	LN	MIDC	-	-	-	Metastasis
75	56	LN	MIDC	-	-	-	Metastasis
76	42	LN	MIDC	-	-	-	Metastasis
77	49	LN	MIDC	-	-	-	Metastasis
78	51	LN	MIDC	-	-	-	Metastasis
79	47	LN	MIDC	T3N2M0	-	IIIA	Metastasis
80	36	LN	MIDC	T2N2M0	-	-	Metastasis
81	41	LN	MIDC	T2N2M0	-	-	Metastasis
82	39	LN	MIDC	-	-	-	Metastasis
83	53	LN	MIDC	-	-	-	Metastasis
84	29	LN	MIDC	-	-	-	Metastasis
85	34	LN	MIDC	T2N1M0	-	-	Metastasis
86	35	LN	MIDC	T2N1M0	-	-	Metastasis
87	42	LN	MIDC	-	-	-	Metastasis
88	47	LN	MIDC	-	-	-	Metastasis

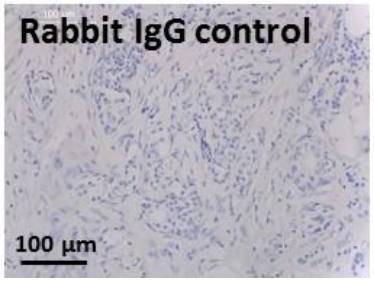
89	47	LN	MIDC	-	-	-	Metastasis
90	53	LN	MIDC	T2N1M0	-	-	Metastasis
91	40	Breast	-	-	-	-	NAT
92	43	Breast	-	T2N1M0	-	-	NAT
93	42	Breast	-	T1N1M1	-	-	NAT
94	44	Breast	-	-	-	-	NAT
95	51	Breast	-	-	-	-	NAT
96	43	Breast	-	T2N1M0	-	-	NAT
97	43	Breast	-	-	-	-	NAT
98	35	Breast	-	-	-	-	NAT
99	42	Breast	-	-	-	-	NAT
100	42	Breast	-	-	-	-	NAT

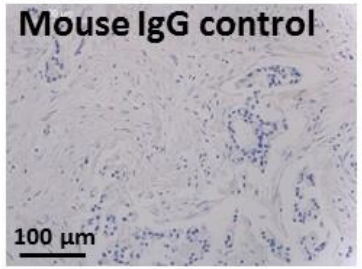
Appendix III: Representative image of a tissue section visualised under an optical microscope at 400x magnification with the grid used for the eyepiece systemic quantification.



Appendix IV: Images of all invasive carcinoma, lymph node metastasis and adjacent normal tissue sections stained for TSP1 and IDO1. Representative images of TSP1 (rabbit) IgG control and IDO1 (mouse) IgG control staining.







Appendix V: Tryptophan composition of the different VEGFA isoforms (NCBI website)

Isoform	NCBI code	N.º of amino acids	N.º of tryptophans	% of tryptophan
a	NP_001020537.2	412	7	1.7
c	NP_001020538.2	389	5	1.3
d	NP_001020539.2	371	5	1.4
e	NP_001020540.2	354	5	1.4
f	NP_001020541.2	327	5	1.5
g	NP_001028928.1	371	5	1.3
h	NP_001165093.1	317	5	1.6
i	NP_001165094.1	232	5	2.2
j	NP_001165095.1	215	4	1.9
k	NP_001165096.1	209	3	1.4
l	NP_001165097.1	191	3	1.6
m	NP_001165098.1	174	3	1.7
n	NP_001165099.1	147	3	2.0
o	NP_001165100.1	191	3	1.6
p	NP_001165101.1	137	3	2.2
q	NP_001191313.1	171	4	2.3
r	NP_001191314.1	351	6	1.7
s	NP_001273973.1	163	0	0
x	NP_001303939.1	213	3	1.4
b	NP_003367.4	395	6	1.5

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