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UNDERSTANDING SURVIVAL IN BREAST CARCINOMA: THE ROLE OF CHEMOKINE ASSOCIATED RECEPTORS

TREBALL DE FI DE GRAU

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INDEX

1 Abstract:

BACKGROUND: Breast cancer is one of the most common cancers in the world. Chemokine (CC-motif) ligand 2 (CCL2) is an inflammatory chemokine highly expressed in the tumour microenvironment and has been correlated with adverse prognosis in breast cancer patients. It exerts its biological function by binding to CCR2, but its signalling can be attenuated by decoy receptors like Duffy Antigen Receptor for Chemokines (DARC) and D6. Indeed, CCL2 mediates the migration of macrophages to tumour sites, which promotes a tumourgenic microenvironment. Nowadays there is emerging support for a tumour promoting role of CCL2 in breast cancer.

OBJECTIVE: This study aimed to understand the interactions between the chemokines involved in the development of invasive ductal breast carcinoma and to find associations between their expression and different patient outcomes.

METHODS: Histological cuts from 286 women with this type of cancer were evaluated by immunochemical staining in the protein levels of CCL2, CCR2, DARC, D6, IL-10 and CD68.

RESULTS: This study has shown that there is no association between CCL2 and adverse patient outcome nor with macrophage infiltration, evaluated by CD68 immunostaining, did not correlate with any patient outcome. More importantly, D6 and CCR2 have been shown to have potential value as breast cancer predictor factors for developing a secondary neoplasia and survival respectively.

CONCLUSIONS: Further studies are needed to validate D6 and CCR2 as predictor factors and also to better understand the interactions between the proteins of the CCL2 network.

2 Introduction:

2.1 Cancer statistics:

Cancer is an uncontrolled growth and spread of cells originated from a change in one single cell. This change may be started by external and inherent genetic factors which can affect almost any part of the human body. The transformation of a normal cell into a tumour cell is a multistage process where the tumour often invades surrounding tissues and can metastasize to distant sites. Many cancers can be prevented by avoiding exposure to common risk factors like tobacco smoking. Besides, a significant proportion of cancers can be cured, either by surgery, radiotherapy or chemotherapy, if their detection is early[1].

Cancer is considered to be the second cause of death worldwide. Its incidence has increased in both developed and developing countries in the world due to increased exposure to risk factors[2]. Among these risk factors we find alcohol, tobacco and obesity. With more than 3 new million cases and 1.7 million deaths per year, cancer represents the second most important cause of death and morbidity in Europe. On a global perspective, cancer accounted for 7.4 million deaths in 2004, which represent around 13% of the total. Although more than 40% of deaths caused by cancer can be prevented, cancer is the leading cause of dead, being responsible of 20% of the total in the European Region[1]. According to SEOM (Sociedad Española de Oncologia Médica) cancerôs incidence and mortality ratios in Spain were, respectively, 241.4 and 109.5 out of 100.00 inhabitants in 2008 for the general population[3].

Lung, stomach, liver, colon and breast cancers cause the most cancer deaths a year[1]. Breast cancer is, particularly, one of the most common cancers with more than 1,300,000 cases and 450,000 deaths a year in the world[4]. In 2012, 521.000 women succumbed to this disease[5]. Representing a 28% of all the womenôs cancers is the most common cancer among women in the WHO European Region[1]. It has been estimated that, approximately, one in eight women in the USA will develop breast cancer throughout their lifetime[6]. According to SEOM breast cancerôs incidence and mortality represented, respectively, a 28.5% and a 15.6% of the overall total of cancers registered in women during the year 2008 in Spain[7]. The majority of patients suffering from breast

cancer succumb to this disease due to caner invasion and metastasis. Treatment of breast cancer invasion and metastasis is difficult and the survival rate for this kind of patients is low[8].

2.2 Breast cancer:

In order to understand breast cancer knowing the normal anatomy of the breast may be useful. The breast is composed by glands that produce milk (lobules), small tubules that carry this milk from the lobules to the nipple (ducts), fatty and connective tissue and blood and lymph vessels. Most breast cancers are, indeed, originated from the cells that line the duct (Figure 1)[9].

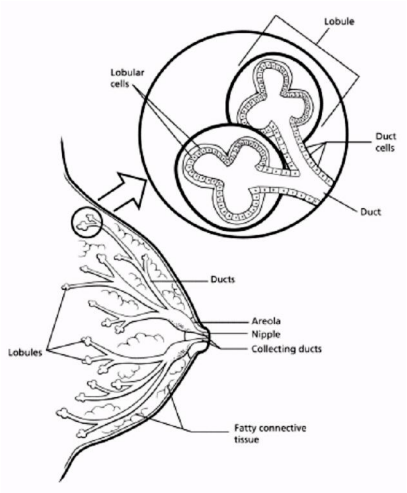

Figure 1: Scheme of the normal anatomy of the breast. Lobular cells form lobules that converge into ducts. These ducts converge to form bigger ducts that carry the milk outside the body. Surrounding this structures there are fatty and connective tissues[9]. [Picture extracted from reference 9].

Carcinoma is the medical term used to describe a cancer that has its origins in the epithelial cells of an organ, such as the breast. There are two types of carcinoma: ***ñin situò*** and invasive carcinomas. On the one hand, ñ*in situ****ò*** carcinomas are characterized for showing cancer cells where the cancer originated. In the context of breast cancer ñ*in situ****ò*** carcinomas are the ones where cancer cells are only present in the breast ducts. That means that this cells have not invaded the depth of the breast nor other organs. On the other hand, invasive carcinomas, also called infiltrating carcinomas, occur when the cancer cells have spread beyond the place in which they were originated[9].

Ductal carcinoma ñ*in situ*ò (DCIS) happens when cancer is still concerned in the milk ducts and has not invade any other area yet, whereas infiltrating ductal carcinoma (IDC) is a cancer begun in the duct which is currently invading the surrounding tissues[10]. IDC, representing eight out of ten breast infiltrating tumours, starts at breast milk ducts, then penetrates the duct wall and grows in the breast adipose tissue. At this point it can have the capacity to spread (metastasize) to other tissues through the lymphatic system and the bloodstream. In fact, the lymph system is one of the main ways through which breast cancer cells can spread out the mama. These cells travel in the lymph and grow in the lymph nodes. If this happens, thereôs a greater likelihood that these cells have also spread to other places in the body. In fact, the more lymphatic nodes invaded by cancer cells, the stronger chance of cancer relapse and presence of metastasis (Figure 2)[9].

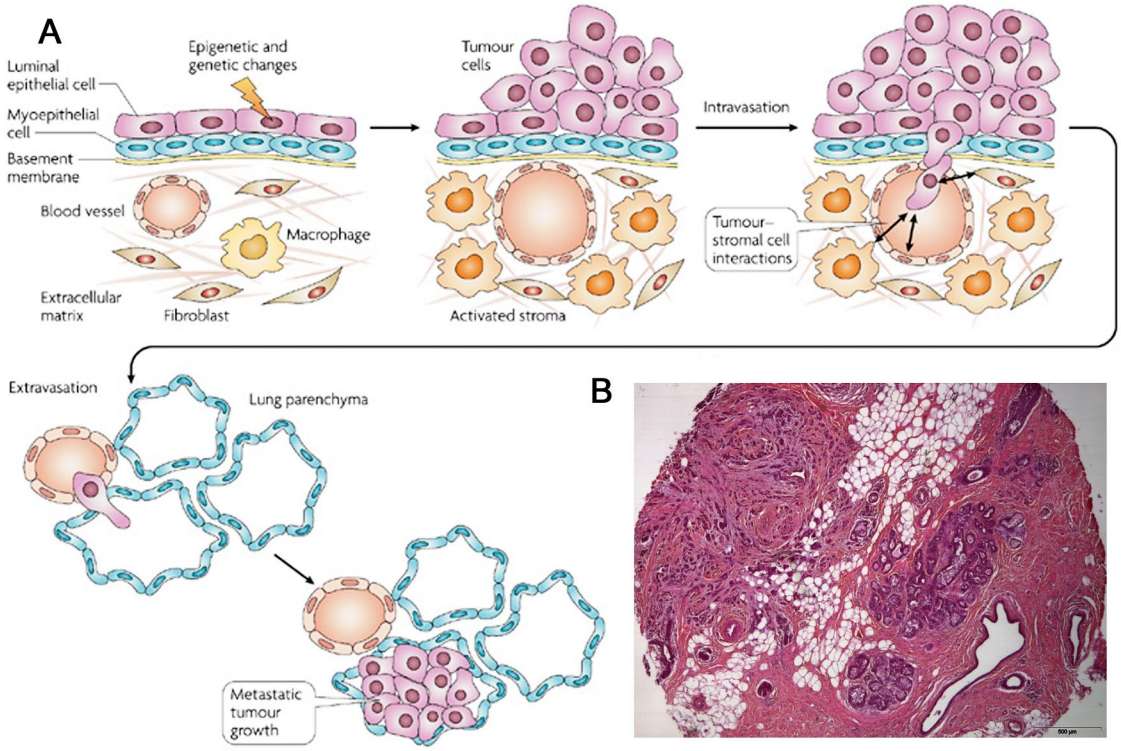

Figure 2: (**A**) Scheme of the breast cancer progression. The transformation of breast epithelial cells to metastatic breast cancer is an amalgamation of epigenetic and genetic changes and aberrant interaction with the microenvironment. This transformation requires a multistage process during which the control of proliferation, survival, differentiation and migration of the cells are deregulated. To form metastases cells must: Invade through the basement membrane. Then intravasate, which is the entry of this cells into the vasculature. After that, survive without adhesion. Next, extravase, which means to exit the vasculature. Finally, establish a new tumour in a foreign microenvironment[64]. [Modified figure extracted from reference 64] (**B**) Representative picture (X2 magnification) taken with optical microscope of human invasive breast carcinoma subjected to hematoxylin and eosin staining.

Breast cancer is divided into 5 different stages, form stage 0 to 4 (Figure 3). This division helps to determine the best way to contain and eliminate the illness. This classification actually indicates the size of the tumour of abnormalities and whether or not cancer cells are contained to the place of origin. In stages 0 and 1 cancer cells are confined to a very limited area of the breast. More specifically, in stage 0 only some abnormal cells are found whereas in stage 1 the cancer is evident. In stage 2 cancer is still growing but it is confined in the breast or has only extended to the near lymph nodes. Stage 3 presents extension to beyond the immediate region of the tumour and may have invaded nearby lymph nodes and muscles. Nevertheless no invasion to distant organs has happened yet. In stage 4 cancer has spread to other places of the body, such as the brain, bone, lungs and liver. Indeed, this advanced phase is considered to be incurable[10].

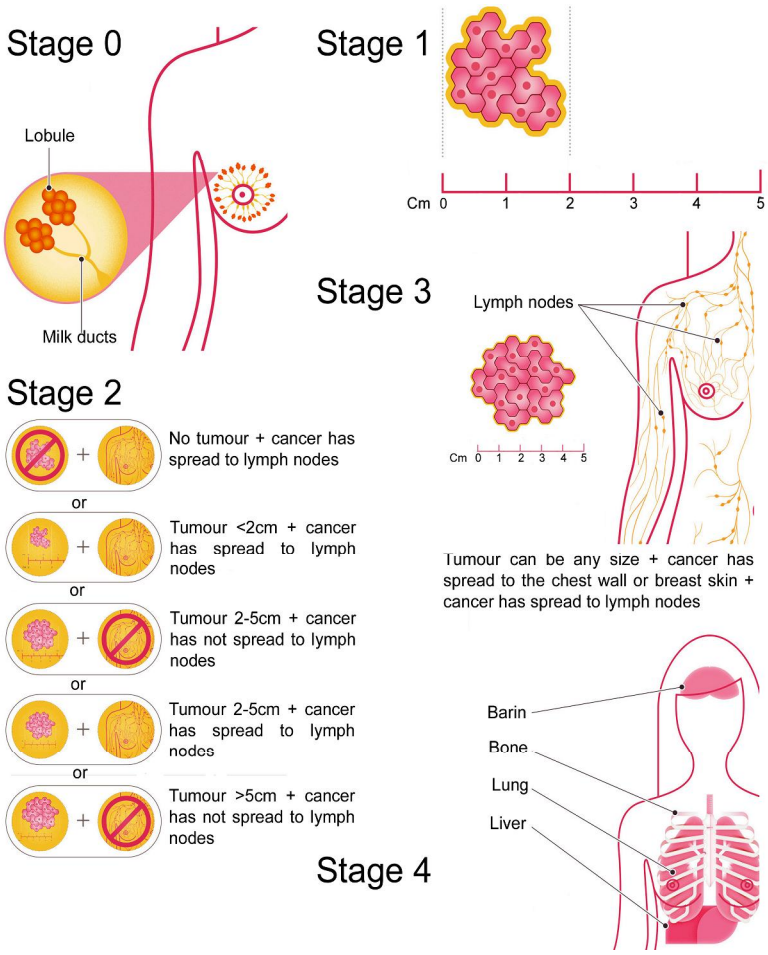

Figure 3: Scheme of the stages of breast cancer. In stage 0 the cancer only presents few cancer cells. In stage 1 the cancer is not bigger than 2cm in diameter. Cancer in stage 2 can present different features. On stage 3 cancer has spread to the chest wall or the breast skin and tumour can be any size. In stage 4 cancer has invaded other parts of the body such as brain, bone, lungs or liver[10]. [Modified figure extracted from reference 10]

2.3 Relationship between cancer and immunity:

The link between tumourgenesis and chronic inflammation was first proposed in 1863 because of the observation that infiltrating leukocytes were a distinctive peculiarity of tumours. Since then, a huge amount of studies have contributed to the characterization of tumour microenvironment and have further complicated the challenging task of understanding and treating cancer. In fact, tissues subjected to chronic inflammation generally show a higher cancer incidence. The start of tumourgenesis in these tissues is supported by a despaired inflammatory response in which various stromal cells types accumulate, become activated, and their normal function of maintaining homeostasis turns maladaptive. As a result, a pro-tumourgenic environment is set. Indeed, tumourgenesis is modulated by the alteration of the tissueôs homeostasis and despaired immune response. In cancer, the regulation of the normal adult tissue is altered as the microenvironment changes in order to promote tumour growth (Figure 4)[11].

Controversially, some retrospective analyses indicate that adequate immune function may be protective against specific types of cancer. Although in some cases the nature of the immune response can be paradoxical, impaired immune response can correlate with high cancer incidence[11].

Involved in the function of the immune system, chemokines are a diverse group of molecules with important implications for the development of solid tissues. Changes in this complex system can have important and dangerous consequences leading to diseases. The specific implications of the various chemokines in diseases have been elucidated in the last few years, prompting hope of manipulating this system for therapy or prevention of diseases[12].

The knowledge of the inflammation offers new an novel candidate targets for therapeutic intervention. The functional relevance of the immune network in tumours may provide new prognostic and/or predictive tools useful for doctors. A better understanding of tumour-specific inflammation will allow a more personalized medicine. In fact, the targeting of inflammation can bring us closer to successful therapy for this dreaded and deadly disease[13].

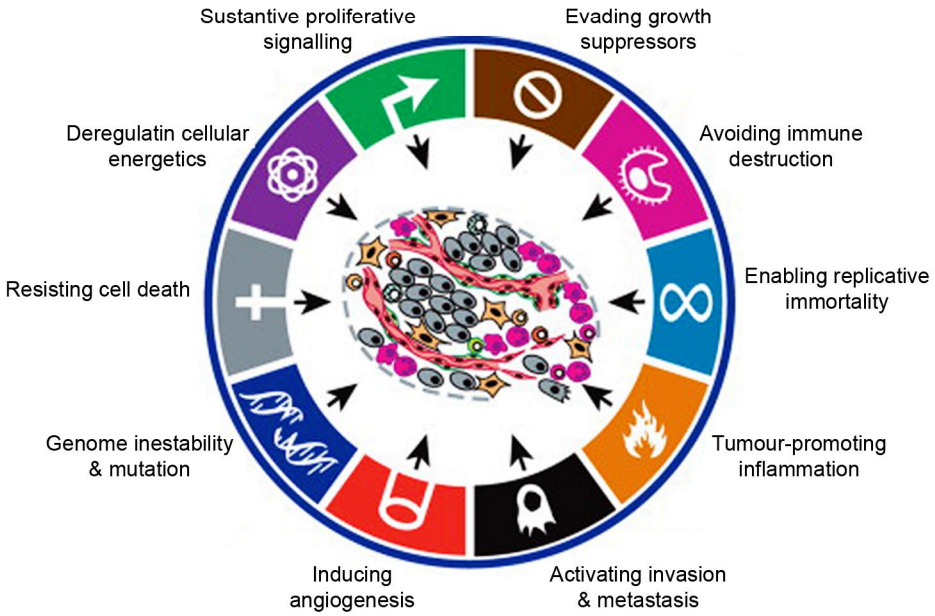

Figure 4: Cancer hallmarks. This figure shows the six originally proposed hallmarks of cancer, which are: the capacity to enable replicative immortality, the activation of invasion and metastasis, the evasion of growth suppressors, a sustained proliferative signalling, the cell death resistance and the induction of angiogenesis. Nowadays the research seems to suggest that the ability to avoid immune destruction, the deregulation of cellular energetic, the genome instability and a tumour-promoting inflammatory microenvironment are additional hallmarks of cancer[39]. [Modified figure extracted from reference 39]

2.4 Tumour-associated macrophages:

First, cancer was seen as a heterogeneous illness that involved aberrant mutations in tumour cells. Now it is clear that tumours are also diverse in their microenvironmental nature instead[11]. Such microenvironment is composed by tumour cells as well as an heterogeneous population of cells like fibroblasts, endothelial cells and infiltrating immune cells. The whole of these cells produce different products, like chemokines, cytokines, growth factors, enzymes and metabolites that are also considered as a part of the tumour microenvironment. Among these cells, tumour-associated macrophages (TAMs) are considered to be the most powerful inhibitors of anti-tumour immunity and the greatest obstacle to accomplish a successful immunotherapy. TAMs are a large component of such microenvironment, ranging from 50% to 80% of the tumour mass, and can be detected with the CD68 marker[11,14]. Macrophages show a great heterogeneity and functional plasticity in response to microenvironmental stimuli. In a context of inflammation they contribute to the initiating phase as well as to the resolution phase[15].

TAMs can be polarized into mainly two phenotypes: proinflamatory M1 TAMs, arising from exposure to Th1 cytokines, which have a high bactericidal and tumouricidal capacity, whereas anti-inflamatory M2 TAMs, arising from exposure to Th2 cytokines like IL10, which have mostly pro-tumour functions (Figure 5) [14,16]. Although M1 and M2 can infiltrate to tumour sites, TAMs generated under this circumstances are polarized to the M2 phenotype, promoting tumour progression, inducing tumour-angiogenesis and lowering anti-tumour response. Patients with high-infiltration of TAMs are supposed to show poor prognosis. Specifically in breast cancer, high infiltration of TAMs correlates with tumour cell invasion, increased vascularization and axillary lymph node involvement. Also, patients with higher TAMs infiltration present significantly worse relapse-free survival and overall survival[14].

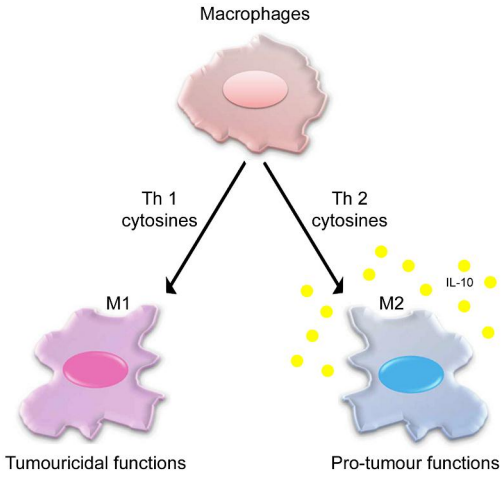

Figure 5: Macrophage polarization. M1 macrophages, with tumoricidal functions, arise from exposure to Th2 cytocines whereas M2 macrophages, with pro-tumour functions, arise from Th2 cytosines like IL-10[14].

2.5 IL-10 in breast cancer:

Interleukin 10 (IL-10) is known to be present in the tumor microenvironment, promoting the polarization of the macropgahes to the M2 phenotipe[14,17]. Human IL-10 is a 35KD homodimer composed by two monomers not covalently bonded. Within each monomer there are two essential disulfide bridges that maintain the structure and the biological activity of the cytokine. Its synthesis is characteristical of almost all leuckocytes an can occur in a wide range of cells, including dendritic cells, mast cells, and neutrophilic and eosinophilic granulocytes. Nevertheless, the main sources of IL-10

ñin vivoò are monocytes, Th cells and macrophages. Monocytes and macrophages release IL-10 after activation by both endogenous (e.g. catecholamines) and exogenous (e.g. bacterial lipololysaccharide) mediators through induction of gene transcription. Moreover, they can also secret IL-10 when stimulated during apoptotic cell clearance by CD36 and p38 mitogen-activated protein (MAP) kinase. Interestingly, macrophages and monocytes are the main target cells for the inhibitory effects of IL-10 cytokine. In fact, it influences three crucial functions of both cellular types, which are: The release of immune mediators, the antigen presentation and the phagocytosis. In plain English, IL-10 suppresses all the functions of monocytes and macrophages that are responsible for a positive role of this cells in both innate and adaptative immunity[18].

In diseases with an overproduction of IL-10, immunosuppressive effects of IL-10 and growth of some tumours is reported. As a result, some of cells are not able to control tumour growth[18]. IL-10 is a well known anti-inflammatory and immunosuppressive cytokine that promotes macrophage polarization towards the M2 phenotype. Thus, IL-10 promotes the tumour-supporting phenotype of macrophages[15]. Moreover, IL10 also helps to maintain an immunosuppressive cytokine environment within the tumour[19]. It is important to highlight that IL-10 the most important cytokine with anti-inflammatory properties besides TGF-ß and IL-35[18].

2.6 The chemokine family:

As mentioned before, several chemokines are present in the tumour microenvironment. These molecules and their receptors belong to a superfamily which regulates the migration of a vast array of immune cells types, including macorphages[20]. Being small soluble molecules, ranging from 8 to 17kDa, they regulate cell migration through the formation of concentration gradients. Currently, 47 chemokine ligands and 23 chemokine receptors have been identify[21.] These molecules play a pivotal role in the development, homeostasis and function of the immune system and are essential for hostôs defence mechanisms. This superfamily of small molecules, is classified into, at least, four different groups: C, CC, CXC and CX_3C, depending on the relative position of the first two residues of cysteine on the NH_3 terminus (Figure 6) [22].

Chemokine biological activities are regulated at several levels and their production can be constitutive or induced by environmental stimuli. Based on this, chemokines are usually divided into two different subgroups in concordance to their biological activity. On the one hand, the inflammatory chemokines promote leukocyte infiltration and their expression is inducible, whereas the homeostatic chemokines are constitutively expressed and are involved in hematopoiesis and lymphoid organ development[20]. Seven-transmembrane G-protein coupled chemokine receptors are a expressed on the surface of various cells and are involved with chemokines in the process of tumourgenesis and malignant progression in breast cancer[23,24].

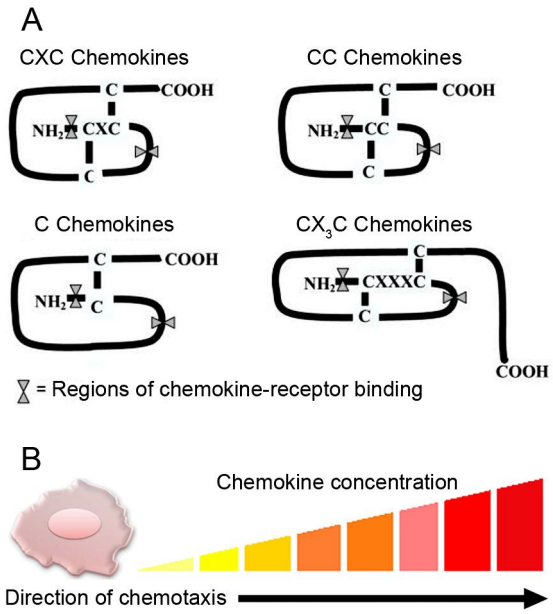

Figure 6: (**A**) The chemokine family is classified according the position of the cysteine residues. C chemokines only present one disulfide bridge. Between the cysteines forming disulfide bridges one can find no residues (CC members), one (CXC members) or three residues (CX3C members)[39]. [Modified figure extracted from reference 39] (**B**) Chemokines mediate chemotaxis by creating concentration gradients. Cells able to respond to their signalling migrate from the zones with low concentration of chemokines to the zones with higher concentration[18].

Infiltrated immune cells and stromal cells can secret chemokines. Moreover cancer cells can both secret chemokines and express their receptor on its surface, enabling the cell to respond to those stimuli. Consequently, a complex chemokine network is formed, which influences tumour cell growth, survival, migration, angiogenesis and immune cell infiltration[25].

Chemokine (CC-motif) ligand 2 (CCL2), also named as Monocyte chemotactant protein 1 (MCP-1), is a chemokine with chemotactic functions which is not only expressed by immune cells but also by several types of malignant cells and stromal cells[26,27]. It belongs to the CC chemokine class and has been shown to play an important role in the modulation of inflammation, regulation of macrophages recruitment during wound healing, infections, and autoimmune diseases[21]. It exerts a potent chemotactic, stimulatory and mitogenic effects on mononuclear cells. Besides, CCL2 has the capacity to attract tumour-promoting cells, which supports tumour progression and mediate the recruitment of specific monocyte populations that support the establishment of metastatic disease[27]. It has been demonstrated that tumour epithelial regions express CCL2 with higher levels than other regions of the tumour. Furthermore, several tumours express CCL2 ***ñ****in vivo****ò***, including gliomas, melanomas and carcinomas[28].

CCL2 has been shown to be the major factor for macrophagesô recruitment into tumours. I fact, CCL2 expression has been positively associated with TAM infiltration in breast and ovarian cancers among others. Furthermore, several studies with animal models also support this association by demonstrating the correlation between CCL2 levels and TAM accumulation in tumours[28]. Other studies report this evidence[21]. Blocking CCL2 activity in mammary tumour-bearing mice decreases tumour growth and metastasis accompanied by decreased macrophage recruitment and angiogenesis. These studies point out that CCL2 regulates tumour progression via a macrophage-dependent mechanism that is sustained through a positive feedback loop. Also, elevate CCL2 expression has been shown to correlate with tumour grade and poor patient prognosis demonstrated in breast tumours immunohistochemical studies[21]. In addition, high circulating concentrations of CCL2 have been associated with poor prognosis in breast cancer patients[27].

CCL2 is expressed in a low grade by normal tissues but is highly expressed by certain types of tumours. As mentioned before, CCL2 is produced by a vast array of cells, including epithelial cells, fibroblasts, endothelial cells, smooth muscle cells, astrocytes, macrophages, microglia cells and certain types of cancerous cells. Despite being produced by such an amount of distinct cells its distribution in the tumour is not uniform. In fact, it is expressed at significantly higher grade in the epithelial region of various tumours, including breast cancer[28].

The biological effects of chemokines are mediated via G-proteins coupled receptor. Most of these chemomikes receptors can be activated by several chemokines. There are five pro-inflammatory chemokines that can bind with high affinity to chemokine C-C motif Receptor 2 (CCR2), being CCL2 its most potent activator. Indeed, CCL2 can only bind to CCR2. The binding of CCR2 with CCL2 leads to the activation of a signal transduction pathway that ends in promoting monocyte transmigration. The signalling axis of CCR2 can involve phosphatidylinositol-3-OH kinase (PI3K), mitogen activated protein kinases (MAPK) and protein kinase C[29].

CCR2 is expressed on peripheral blood monocytes, activated T cells, B cells and immature dendritic cells[30]. Also, TAMs express CCR2 in their cellular surface. In fact, TAMs expressing CCR2 facilitate cancer cell extravasation, seeding and growth[31]. Nevertheless, there exist two different isoforms of CCR2, named CCR2A and CCR2B, differing only in their carboxy-terminal tail. Although activating different signalling cascades and, hence, exerting different actions, CCR2B is the dominant form, accounting 90% of the CCR2 protein expressed on the cell surfac[29].

2.7 Promiscuous chemokine decoy receptors:

Typical receptors bind to its ligand(s) with both high affinity and specificity. By this union it initiates a signalling cascade which ends in a cellular response. Nevertheless, there is a group of receptors among the chemokine system that, although binding to its ligand, do not transmit any signal and, hence, do not exert cellular response. Therefore, they are indicated as nonsignalling chemokine-binding proteins, promiscuous decoy receptors, silent receptors, interceptors (internalizing receptors), chemokine sinks, spongers or scavengers. Duffy antigen receptor for chemokines (DARC) and chemokine binding protein 2 (D6) are one of these molecules capable of binding chemokines with high affinity and without signalling function[20]. Therefore, some chemokines are subjected to modulation of their concentrations by proteins to which they bind without any signalling response[8].

DARC is a typical decoy receptor that binds with angiogenic CXC chemokines presenting the glutamine-leucine-agrinine motif (ELR^+) in the N-terminus and some CC chemokines, but neither with ERL^- CXC chemokines nor C chemokines. Among this

ligands, one with high affinity to DARC is CCL2. Chemokine regulation by DARC may be essential since DARC is widely expressed on the surface of endothelial cells[20]. Besides endothelial cells, it is also expressed in red blood cells and neuronal cells. DARC is involved in the neutralization of chemokines at endothelial barriers and on erythrocytes, acting as a regulator of plasma chemokine levels[8].

Going deeper into the DARC function, when chemokines able to bind to DARC are released in the bloodstream during inflammation they will be quickly retained by DARC. Then, chemokines are rapidly removed from the blood via liver clearance. As this takes place, DARC-retained chemokines will free themselves from DARC and restore the dynamic equilibrium of chemokine concentration. Thus, DARC can act as a reservoir of chemokines and can maintain the equilibrium of free versus bounded chemokines. Through this system, DARC can act as a buffer against large rises and precipitous drops in chemokine abundance in plasma (Figure 7) [32].

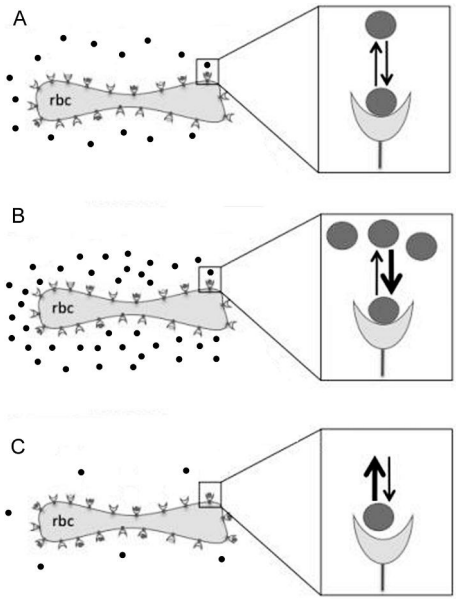

Figure 7: Duffy Antigen Receptor for Chemokines (DARC) function. In homeostasis (**A**) some chemokines are occupy available DARC molecules on red blood cells (rbc). This chemokines are in dynamic equilibrium with the free chemokines in plasma. When the concentration of chemokines rises (**B**) DARC acts as a buffer and tends to capture more chemokines. When the concentration of chemokines lowers (**C**) DARC releases the chemokines previously captured in order to maintain their concentration[32]. [Modified figure extracted from reference 32]

DARC expression in tumour or endothelial cells downregulates tumour progression via controlling the levels of inflammatory chemokines. In addition, it can

transduce a signal of senescence to the cells through interacting with certain types of molecules. Overexpression of DARC protein in human breast cancer cells result in a significant inhibition of tumourgenesis and/or metastasic potential as well as lung metastasis ñ*in vivo*ò due to its ability to clear angiogenic chemokines and a consequently insufficient neovasculartity[20].

D6, also named as chemokine binding protein 2, chemokine binding receptor 2, CC chemokine receptor 9 (CCR-9) and CCR10, is another receptor without signalling activity. Despite that, it does not mean that it has not any function. It actually shuttles chemokines from the plasma membrane to endocytic compartments where they are targeted to be degraded via a ligand-independent internalization. It can bind to almost any inflammatory CC chemokine, such as CCL2, but not with homeostatic chemokines. In fact, D6 is a potent CC chemokine scavenger that acts as a negative regulator of inflammatory response[33,34]. When D6 binds to chemokines they rapidly enter the cell through endosoma compartments and dissociate quickly form the receptor in order to be targeted to degradation. At the same time, D6 is recycled back to the cell surface for further chemokine capture. One property of this system is that is not influenced by chemokine exposure, so chemokine-induced signalling is not needed (Figure 8)[8]. Therefore, D6 can be recycled without a reduction of cell-surface D6 levels. Due to repeated rounds of chemokine internalization D6 can destroy large quantities of proinflammatory chemokines. Thus, D6 regulates continuously the level of chemokines[8].

In humans, D6 is expressed widely on lymphatic endothelial cells, the gut, the lungs and may be found on trophoblasts, leukocytes, macrophages and dendritic cells. Moreover, it has been proven to be expressed in human breast cancer cells[8]. It has been demonstrated that D6 inhibits tumourgenesisi and metastasis *in vivo*. This inhibition is associated with decreased vessel density, TAM infiltration and intratumour chemokines. In fact, D6 inhibits the growth and metastasis of breast cancer by inhibiting chemokine-mediated angiogenesis and TAM infiltration due to its ability on scavenging tumour-supporting chemokines[20].

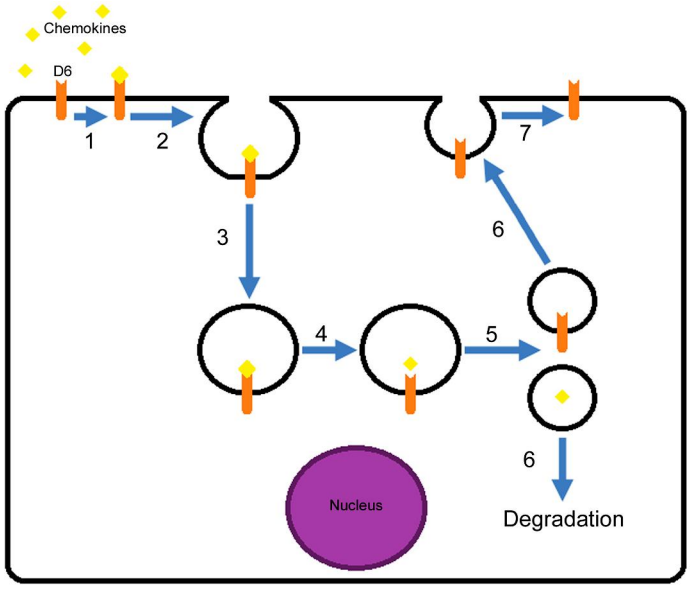

Figure 8: (A) Chemokine binding protein 2 (D6) function. When chemokines are bound to D6 (1) the ligand-receptor complex is internalized (2) and forms a endocytosis vesicle (3). When the pH lowers the chemokines free themselves form D6 (5). Then, the chemokines are send to degradation and D6 back to the cell membrane (6). Finally D6 becomes expressed again in the cellular surface (7)[32].

DARC and D6 are proteins crucial in inflammation as well as in chemokine-associated diseases, for instance cancer, since they can capture chemokines and block their signalling function (Figure 9). In the context of cancer the chemockine-blocking function of this receptors has been widely described[8].

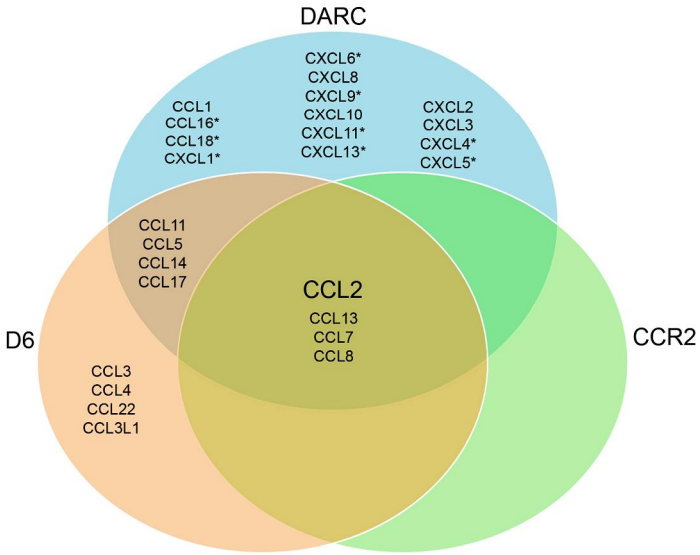

Figure 9: Vennôs diagram for Duffy Antigen Receptor for Chemokines (DARC), chemokine binding protein 2 (D6) and chemokine C-C motif Receptor 2 (CCR2) ligands. This figure shows the chemokines that can bind with DARC, D6 and/or CCR2. Note that CCL2 can bind with all three receptors. Chemokines that have not been tested on human D6 are denoted by an asterisk (*)[32,34].

2.8 Chemokines as future biomarkers:

A biomarker is a characteristic objectively measured and evaluated as an indicator of a biological process. In the context of cancer, it refers to a substance or process that indicates the presence of the cancer. It could be secreted by the tumour or produced due to a specific response of the body to the presence of the tumour. Despite its utility, nowadays there are few high-sensitive and specific markers for cancer detection[35]. Hormone receptors (HR), Human Epidermal Growth Factor Receptor 2 (HER-2), Ki 67 antigen, tumour protein p53 and alterations on the genes BRCA1 and BRCA2 are some of the well-established prognostic and/or therapeutic breast cancer markers[36]. However, thereôs a vast array of patients developing breast cancer that do not present currently known risk factors[37]. Many studies have suggested the involvement and role of inflammatory chemokines, such as CCL2, in breast malignancy. The study of the network in cancer of this protein is supposed to play a significant role as an effective breast cancer marker[36].

3 Hypothesis & Objective:

The cellular and molecular heterogeneity of breast cancer added to the large number of molecular events involved in controlling cell growth, differentiation, proliferation, invasion and metastasis accentuate the importance of studying multiple molecular alterations in concert[38]. Inflammation can be considered as an enabling characteristic for the acquisition of tumour characteristics[39]. Hence, immunity and inflammation play a pivotal role in cancer development[11]. Monocyte chemotactant protein 1 (CCL2) is a protein involved in the migration of immune cells, including macrophages, to inflammation sites[27].

Thus, our hypothesis is that CCL2 and other proteins related to the chemokine network, including DARC, D6, CCR2, IL-10 and macrophage infiltration, have a crucial role in the development of cancer and have a potential future as breast cancer predictor factors.

In order to verify this hypothesis this study aims to find the relationship between CCL2, CCR2, DARC, D6, IL-10 and macrophage infiltration in the context of infiltrating ductal breast carcinoma and describe the differences of these proteins levels between women with bad and better outcomes. Specifically, the outcome features that will be subjected to study are presence of metastasis, survival, presence of secondary neoplaias and cancer relapse. Thus, this is a descriptional study of the inflammation process involved in cancer.

4 Materials and Methods:

4.1 Participants:

This trial includes 286 women, aged between 26 and 91 years old, diagnosed with infiltrating ductal breast carcinoma, including those accompanied by ductal carcinoma ñ*in situ*ò, at Hospital Universitari Joan XXIII of Tarragona between 1st January 1994 and 31st December 2006. The excluding criteria was the following:

- Women operated on for a primary tumour relapse.
- Women with special ductal carcinoma diagnose.
- Women with less than 1mm of infiltration of ductal carcinoma, called microinfiltrating ductal carcinoma.
- Women diagnosed with collapsing tumour with a ductal carcinoma part.
- Women diagnosed or treated in the hospital but operated on in an external centre.

Breast cancer tissue was obtained from patients suffering from the established criteria. Pathologists diagnostically examined the tumour breast tissue which were removed from the patients. Samples were collected in the moment of the histological study and were fixed with formalin 4% during 18-24 hours. Then the biopsies were embedded with paraffin according to the Patològica Anatomyôs standard procedure form Hospital Universitari Joan XXIII for later usage. This whole procedure was carried out at Hospital Universitari Joan XXIII[40].

This study was approved by the Ethics Committee of Hospital Universitari Joan XXIII. All patientsô agreement was needed to include them in the study. Their anonymity was guaranteed at all times.

4.2 Relevant data:

Relevant clinical data were extracted from clinical records provided by Hospital Universitari Joan XXIII. This collection of clinical information is summarized in table

(Table 2). Also, tumour size and age was extracted from clinical records. Once the data was collected, samples were anonimalized, eliminating both clinical record and biopsy numbers.

4.3 Immunohistochemical detection:

Formalin-fixed, paraffin-embedded sections from human breast cancer biopsies were subjected to immunohistochemical staining for CCL2, DARC, D6, IL-10, CD68 and CCR2. CD68 was used in order to evaluate macrophage infiltration[11]. The paraffin-included material was sectioned at a thickness between 3-5µm and was collected on slides coated with poly-L-Lysine. The slides were deparaffinized with xylen and rehydrated through a descending ethanol series to distilled water before microwave antigen retrieval was carried out by placing the slides in 0.15mol/L sodium citrate buffer at pH=6 (DakoCytomation, Dako, Glostrup, Denmark) in a microwave oven until reaching 95ºC. After waiting 30 minutes for tempering the slides were washed using phosphate-buffered saline (PBS) solution, followed by the step of quenching endogenous peroxidase activity with 3% hydrogen peroxide for 25 minutes. Then sections were incubated in 2% bovine serum albumin to block non-specific binding. Then the sections were incubated overnight at 4ºC with primary polyclonal antibody (Table 1). Primary antibodyôs signal was amplified for 90 minutes with a biotinylated secondary antibody (Table 1) followed by incubation with Vectastain ABC kit (ABC Complex, Vectastain ABC Kit Standard, Vector, Burlingame, California) for 45 minutes. Colorimetric detection with 3,3ô-diaminbenzidine (D.A.B, Dako, Glostrup, Denmark) was carried out to detect the signal. The sections were counterstained with Mayerôs hematoxylin (Sigmaï Aldrich Inc., Steinheim, Germany) for 2 minutes and then dehydrated through ethanol and xylol series. Finaly, they were fixed in D.P.X. mounting medium (VWR International, Llinars del Vallés, Spain).

4.4 Evaluation of immunostaining:

Sections were evaluated with an optical microscope (Nikon, Eclipse E600, Madrid, Spain) by using objectives with x2 magnifications. Sectionôs area and colour intensity were measured by using the software AnaliSIS (Soft Imaging System, Münster, Germany).

Table 1: Primary and secondary antibodies and dilution used in the immunohistochemical assay.

Antigen	Primary antibody	Dilution	Secondary antibody	Dilution
CCL2	Rabbit anti-human MCP-1 (Abcam, Cambridge, United Kingdom, ab9669)	1:100	Goat anti-rabbit IgG (Vector, Burlingame, CA, USA, BA-1000)	1:200
CCR2	Rabbit anti-human CCR2 (Abcam, Cambridge, United Kingdom, ab21667)	1:50	Goat anti-rabbit IgG (Vector, Burlingame, CA, USA, BA-1000)	1:200
DARC	Rabbit anti-human DARC (Abnova, Taipei, Taiwán, PAB13254)	1:200	Goat anti-rabbit IgG (Vector, Burlingame, CA, USA, BA-1000)	1:200
D-6	Goat anti-human D-6 (Abcam, Cambridge, United Kingdom, ab1658)	1:200	Rabbit anti-goat IgG (Vector, Burlingame, CA, USA, BA-5000)	1:200
IL-10	Rabbit anti-human IL-10 (Abcam, Cambridge, United Kingdom, ab34843)	1:500	Goat anti-rabbit IgG (Vector, Burlingame, CA, USA, BA-1000)	1:200
CD68	Mouse anti-human CD68 (Dako, Glostrup, Denmark, MCA497R)	Ready to use	Horse anti-mouse IgG (Vector, Burlingame, CA, USA, BA-2000)	1:200

4.5 Statistical analysis:

In order to evaluate our hypothesis different statistical tests were applied. Kolmogorov-Smirnov tests was used to assess the normality distribution of our variables. After applying Kolmogorov-Smirnov test, all the variables showed a p value <0.001 (Data not shown). Hence, the non-normal distribution nature of our variables was highlighted. Once known this information, non-parametric tests were used for the statistical treatment of our data. Spearman rho values were used to determine the existent correlations between CCR2, DARC, D6, CCR2, CCL2 IL-10 and CD68 protein levels one another. These results are expressed with proteinôs median and 95% interval of confidence. Mann-Whitney U test was used to assess the differences between protein

levels grouped in different patient outcome (e.g. positive presence of metastasis versus negative presence of metastasis).These results are expressed in proteinôs median and 95% interval of confidence. All the statistical analyses were carried out by using the SPSS 18.0 package (IBM, Madrid, Spain). P values <0.05 were considered statistically significant in all of the analysis.

5 Results:

In this study immunohistochemical staining of DARC, IL-10, D6, CCL2, CCR2 and CD68 was carried out in order to establish the relationship between these proteins and link their expression with several adverse outcomes in women suffering from invasive ductal breast carcinoma. This may lead to find potential prognosis factors for several outcomes in breast cancer patients.

5.1 Patient description:

The age of the patients ranged from 26 to 91 years old and the tumour average was 2.6 ± 1.8 (cm ± standard deviation). Patient characteristics of the 286 included in the study are shown in table (Table 2).

Table 2: Patientsô clinical data extracted from clinical records.

Clinical data (n=286)		Percentage (%)
Breast cancer location	Unspecified mama	1.7
	Right mama	41.3
	Left mama	56.3
	Both mamas	0.7
Histological grade	Well differentiated	20.6
	Partially differentiated	40.6
	Not differentiated	38.8
Survival	Alive	74.5
	Dead	24.8
	Lost cases	0.7
First relapse	Yes	27.6
	No	72.4
Second relapse	Yes	7.3
	No	92.7
Third relapse	Yes	1.4
	No	98.6
Secondary neoplasia	Yes	12.3
	No	87.7
Metastasis	Yes	40.6
	No	55.9
	Lost cases	3.5

The location of the cancer varies from one patient to another. In our study, 118 (41.3%) women presented the cancer in the right mama whereas 161 (56.3%) had it on the left one. Moreover, 2 (0.7%) women had the cancer in both right and left mama. For several reasons, 5 (1.7%) clinical records lacked of this information.

The histological grade of a cancer is a description of how abnormal the cells of a tumour are. It indicates how quickly the tumour is expected to grow and spread. When the cells of the tumour and the organization of the tissue of the tumour are close to normal cells and tissues, the tumour is called well-differentiated. When the tumour is called undifferentiated (or not differentiated) abnormal-looking cells are present in the tumour. Between these two forms we find the partially differentiated form[41]. The biopsies subjected to this study presented different histological grades, 59 (20.6%) of them had a well differentiated cancer, 116 (40.6%) partially differentiated and 111 (38.8%) not differentiated.

In our study the variable survival is defined as the state of the patient, meaning if she was dead or alive by the time the study ended. In our study, 213 (74.5%) of the women were alive at the end of the study whereas 71 (24.8) of them had already died. This information was missing in the clinical records of 2 (0.7%) women for several reasons.

When cancer relapses it means that the cancer is formed again in a breast that was, supposedly, cured. From the total of women 79 (27.6%) of them had a cancer relapse, 21 (7.3%) of which had a second relapse, 4 (1.4%) of which had a third relapse. 207 (72.4%) of the women did not suffer from any cancer relapse.

In our study it is established that a women has a secondary neoplasia when suffers from a cancer originated from a different cell that originated the breast cancer. In our study 35 (12.3%) women presented a secondary neoplasia whereas 257 (87.7%) did not.

Metastasis was defined as the spread of the cancer cells from the breast to other parts of the body. 116 (40.6) of the women in our study showed cancer metastasis while

160 (55.9%) of them did not. This information was missing in the clinical records of 10 (3.5%) women for several reasons.

Lost cases were not included in the study. Also, since the number of the samples of women that had suffered from a second and a third cancer relapse was too small (n Ò 30) it was decided not to include this information in our analyses.

5.2 Protein correlations:

In order to accomplish our objective, which was to find the relationship between the proteins and their influence on the patientôs outcome, chemokines involved in cancer, macrophage infiltration, determined through CD68 immunostaining, and IL-10 protein were quantified by immunochemical staining. Once the values were obtained, correlations between these proteins were evaluated in order to find associations between one another.

5.2.1 CCL2 is associated with CD68 and DARC:

CCL2 protein levels detected by immunohistochemistry showed a high direct significant association with CD68 and a significant association with DARC (Figure 10) protein levels. CCL2 was not found to be significantly correlated with D6, IL-10 or CCR2.

In the Figure 10A there is shown the association between CCL2 and CD68. This graphic shows different circles that are representing the quantification of DARC and CD68 proteins from a single patient. Once the circles are represented in the graphic, a tendency line, with the 95% of confidence interval, was defined and represented. As it can be seen, the tendency is that the more CCL2 is expressed the more CD68 is found in tumour sites. This association is represented in Figure 10B, which shows representative histological cuts stained for CCL2 and CD68 form two different patients. When there is a high concentration of the chemokine or the decoy receptor the histological cut is presented with a dark brown colour. Oppositely, when the concentrations of these proteins are low the staining results in a pale brown colour. Note that the histological cuts from the patient on the right present a higher intensity of brown in the staining when compared with the cuts from the patient on the left. These two representative patients show clearly the relationship between CCL2 and CD68. When the staining for

CCL2 is strong brown, the staining for CD68 is also very brown (case on the right). This link between the proteins also works the other way round, presenting pale staining for CCL2 and DARC at the same time (case on the left).This association between CCL2 and CD68 implies that when CCL2 is expressed it attracts macrophages to tumour sites since CD68 is a macrophage marker[11].

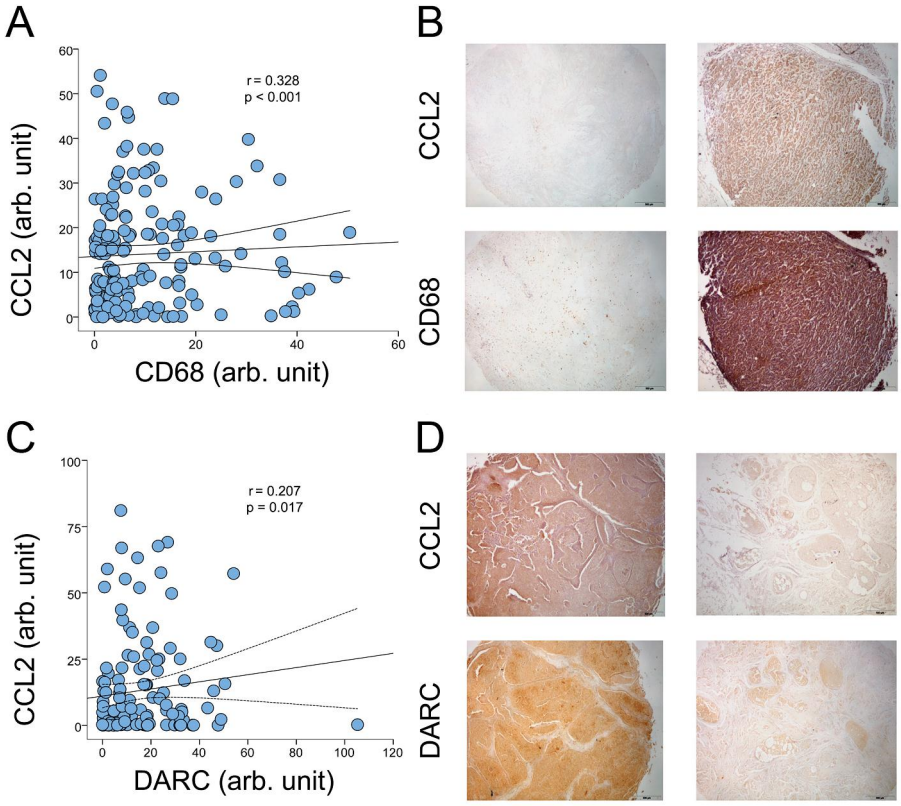

Figure 10: (**A**) Relationship between CCL2 and CD68 protein levels detected by immunohistochemical assays. (**B**) Immunohistochemistry examination of CCL2 protein (x2) and CD68 protein (x2) in infiltrating ductal breast carcinoma sections. (**C**) Relationship between CCL2 and DARC protein levels detected by immunohistochemical assays. (**D**) Immunohistochemistry examination of CCL2 protein (x2) and DARC protein (x2) in infiltrating ductal breast carcinoma sections. Pictures in B on the left represents a single patient and the one the right another one. The same happens in pictures in D. Spearman rho values were used to evaluate the correlations. Correlations were considered to be statistically significant if pÒ0.05. A total of 126 patients were evaluated in A and 132 in C.

The association between DARC and CCL2 is shown in Figure 10C. Note that the tendency is that when CCL2 levels rise, DARC rises as well. This can easily be seen in Figure 10D, where two different histological cuts form two different patients are stained for CCL2 and DARC. Note that if one patient has strong brown staining for one protein the staining for the other is also strong (case on the left). On the contrary situation, the tendency is also corroborated (case on the right). This association suggests

that when CCL2 is expressed DARC is also expressed and, hence, the response to CCL2 may not be as strong as it should be due to the scavenging effects of DARC.

5.2.2 DARC is associated with CCR2, D6 and IL-10:

DARC protein levels correlate with CCL2 levels but also strongly correlated directly with CCR2 protein levels. Furthermore, DARC showed a positive association with D6 and IL-10 as well (Figure 11). No significant association between DARC and CD68 was found.

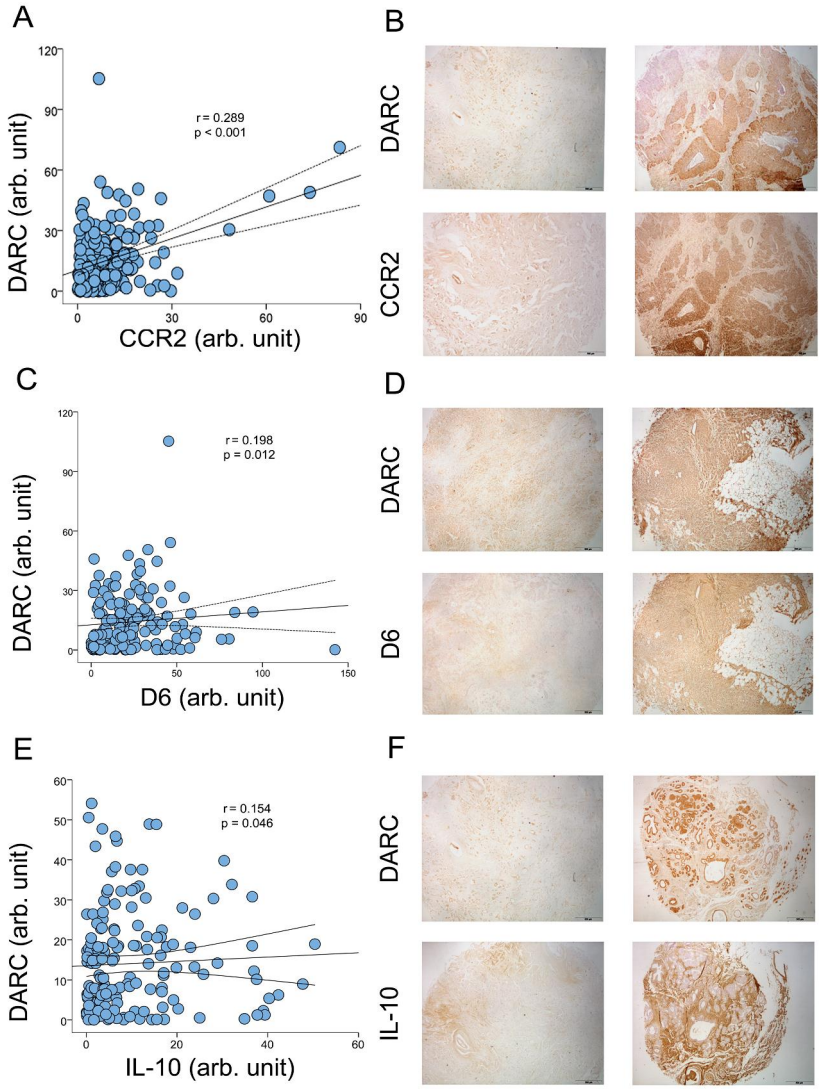

Figure 11: (**A**) Relationship between DARC and CCR2 protein levels detected by immunohistochemical assays. (**B**) Immunohistochemistry examination of DARC protein (x2) and CCR2 protein (x2) in infiltrating ductal breast carcinoma sections. (**C**) Relationship between DARC and D6 protein levels detected by immunohistochemical assays. (**D**) Immunohistochemistry examination of DARC protein (x2) and D6 protein (x2) in infiltrating ductal breast carcinoma sections. (**E**) Relationship between DARC and IL-10 protein levels detected by immunohistochemical assays. (**F**) Immunohistochemistry examination of DARC protein (x2) and IL-10 protein (x2) in infiltrating ductal breast carcinoma sections. Spearman rho values were used to evaluate the correlations. Correlations were considered to be statistically significant if pÒ0.05. A total of 184 patients were evaluated in A, 160 in C and 167 in E.

Shown in Figure 11A and 11B there is the association between DARC and CCR2. This association is positive, which means that when DARC levels are high CCR2 tend to be as well high. The representative examples of the staining in pale and dark brown from DARC match with the staining for CCR2, which show plainly the association between these two proteins.

In addition to the previously described association, DARC has been correlated with D6 protein levels, which, interestingly, seem to vary together. These results in the graphic shown in Figure 11C and the representative examples in Figure 11D. Note that the patient in the left has low staining for both DARC and D6 whereas the patient on the right has high staining for them. Hence, our study reveals that when DARC is expressed D6 is expressed too, suggesting that both mechanisms of chemokine sequestering are activated at the same time.

Finally, DARC has been linked to IL-10 expression. In Figure 11E the values of the quantification of DARC and IL-10 proteins from each patient are represented. This association in their expression can be seen macroscopically. Note the histological cuts stained for DARC and IL-10 in Figure 11F. On the one hand, the ones in the left correspond to a patient with a low levels of both proteins whereas, on the other hand, the ones on the right are dark brown when stained specifically for DARC and IL-10.

5.2.3 D6 and CCR2 are associated with IL-10:

IL-10 protein levels were directly associated with DARC and CCL2, but were also associated with D6 and CCR2 (Figure 12). Nevertheless, no significant association was found between IL-10 and CD68 protein levels. D6 did not correlate significantly with CCR2 nor with CD68. Moreover no statistical association was found between CD68 and CCR2.

CCR2 has been associated with DARC, but it also shows a relationship with IL-10 protein levels. Indeed, as shown in Figure 11A, CCR2 tends to be low when IL-10 is high, and the same happens the other way round. In fact, their relationship is inversely proportional. This relationship could bee seen macroscopically when the samples were stained for CCR2 and IL-10. Note in Figure 11B that the patient in the left had high IL-

10 but low CCR2 expression whereas the patient on the right had high CCR2 levels but low in IL-10. This, indeed, results form the inverse relationship that is found between CCR2 and IL-10.

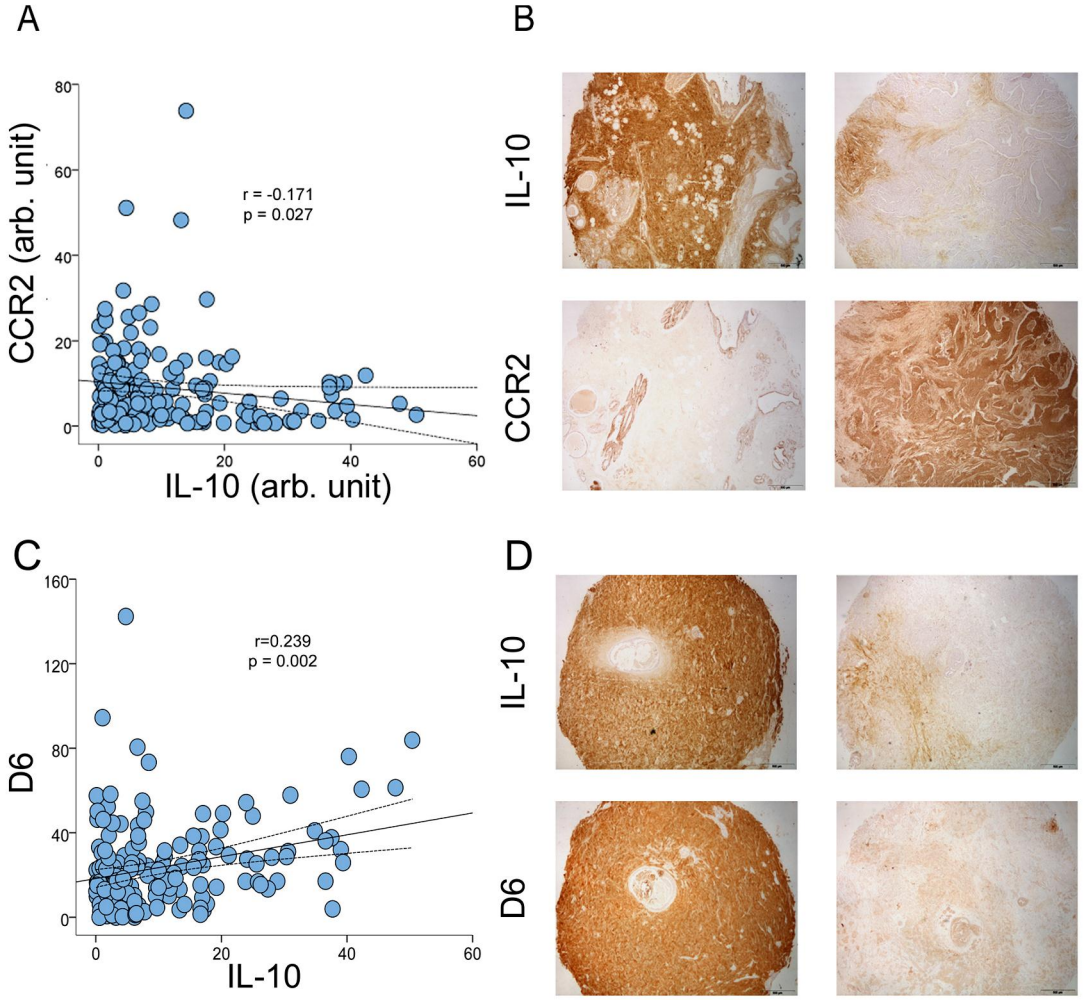

Figure 12: (**A**) Relationship between CCR2 and IL-10 protein levels detected by immunohistochemical assays. (**B**) Immunohistochemistry examination of CCR2 protein (x2) and IL-10 protein (x2) in infiltrating ductal breast carcinoma sections. (**C**) Relationship between D6 and IL-10 protein levels detected by immunohistochemical assays. (**D**) Immunohistochemistry examinations of D6 protein (x2) and IL-10 protein (x2) in infiltrating ductal breast carcinoma sections. Spearman rho values were used to evaluate the correlations. Correlations were considered to be statistically significant if pÒ0.05. A total of 163 patients were evaluated in A and 167 in C.

IL-10 also correlates with D6. A representation of their association is shown in Figure 12C and 12 E. With this graphic it is understood that the higher levels of D6 the higher levels of IL-10 are reported. Patients with high IL-10 expression, which results in a very dark brown staining, have also dark D6 staining. Besides, patients with low brown intensity in the staining of IL-10 also have low D6 expression.

5.3 Relationship between proteins and patientôs outcome:

Once protein levels were determined, data was grouped according to patient outcome. The established groups were: first cancer relapse (yes, no), presence of metastasis (yes, no), presence of second neoplasia (yes, no) and survival (dead, alive). Note that the percentage of women suffering from these conditions is summarized in Table 1. Protein median was compared within each individual group to have a clue of the potential value of these proteins as predictor factors.

Only two significant associations were found (Figure 13). D6 protein median was significantly lower (p=0.022) in those patients who had had a secondary neoplasia compared with those who had not had. Also, CCR2 protein median was found to be significantly lower (p=0.017) in those patients alive by the end of the study compared with those who had died. Unexpectedly, no strong associations were found between CCL2 and any of the features described above. In addition, CD68 did not show any association either.

Figure 13A represents the differences in the median between women suffering from a secondary neoplasia and women free of it. The black bar in the middle of the box-plot represents the median of the women, which corresponds to the percentile 50% of the distribution. The top of the blue box corresponds to the percentile 75% whereas the bottom to the percentile 25%. Finally, the thin black bars indicate the minimum and maximum values of the distribution. Note that women free of secondary neoplasias have a higher median in D6 protein levels with a significance of p=0.022. Figure 13B also gives clues about what happens in women suffering from a secondary neoplasia. Note that the women with high brown in the staining for D6, which is translated as a high D6 protein expression in the tumour, are the ones who had a secondary neoplasia. Moreover, the ones with low brown-colour do not present this adverse feature. Thanks to this information it is know that women with higher levels of D6 protein expressed in the tumour may have fewer chances to develop a secondary neoplasia.

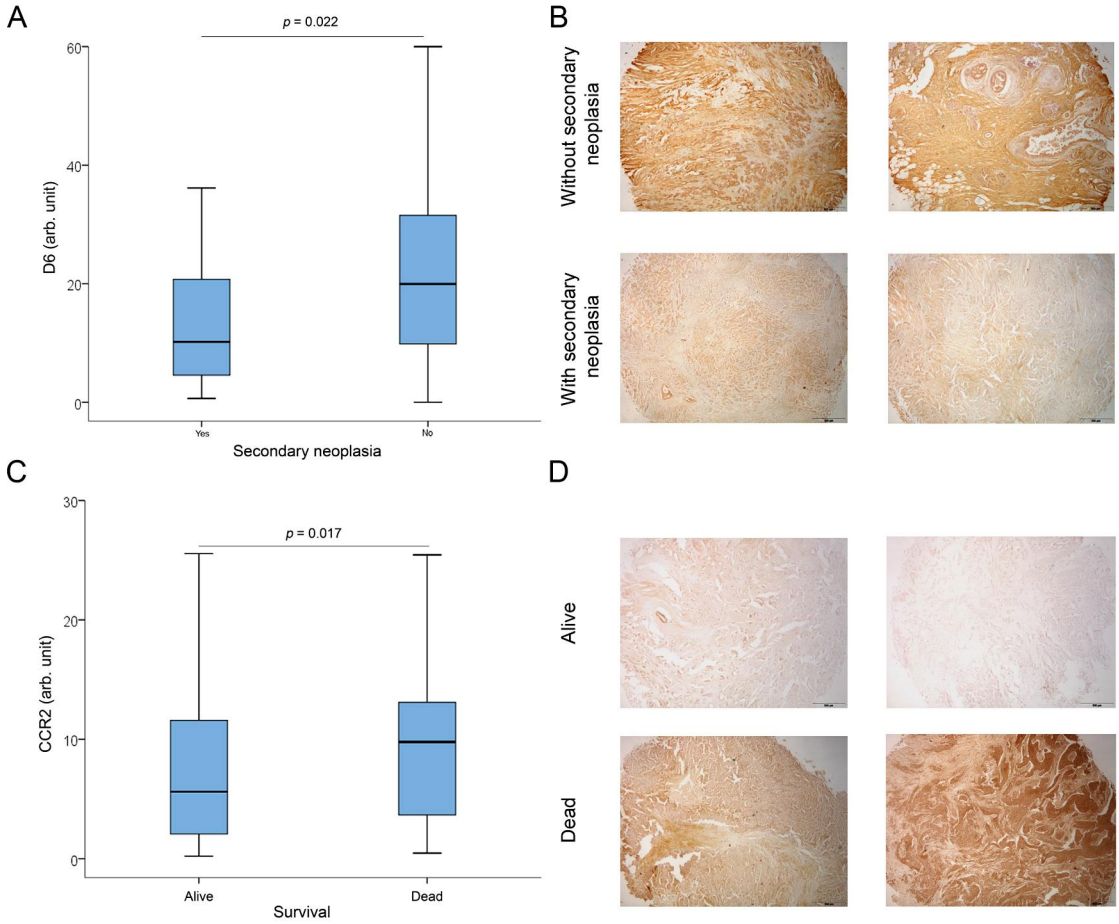

Figure 13: (**A**) Differences in D6 protein levels between women having a secondary neoplasia and women without it. Women suffering from a secondary neoplasia presented significative (p=0.022) D6 levels than the ones free of it. (**B**) Immunohistochemical examination of D6 protein in histological cuts (X2) from patients with and without secondary neoplasia. (**C**) Differences in CCR2 protein levels between dead and alive women. Women who died as a result of the illness showed higher CCR2 levels than those who survived. The differences were statistical significant with p=0.017. (**D**) Immunohistochemical examination of CCR2 protein in histological cuts (X2) from patients alive and dead. Mann-Whitney U test was used to evaluate the differences in the median. Differences were considered to be statistically significant if pÒ0.05. A total of 196 patients were evaluated in A and 215 in C.

Figure 13C and 13D also give us information about the chemokine network in the breast tumour. Women who have died due to the progression of the cancer showed a higher median in CCR2 levels compared with those who had not succumbed to it (*p*=0.017). Note that representative histological cuts stained for CCR2 from women alive and dead present different brown intensity. Indeed, women dead because of the illness have a browner intensity, which means higher CCR2 expression in the tumour, whereas women alive showed pale brown colours. This means that when CCR2 is highly expressed in the tumour microenvironment women have fewer chances to beat the disease and survive to it.

6 Discussion:

Inflammation is considered to be as a key process in the development of breast cancer[39]. The study chemokine network, which is involved in inflammation, may lead to find new molecular prognostic factors[36]. Hence, the interaction between these proteins and the differences in their levels in different outcomes should be discussed.

The experiments carried out by Qian BZ at al. indicate that CCL2 is a critical molecule for the recruitment of monocytes expressing CCR2. In fact, CCL2 continually recruits monocytes to tumour sites, and these differentiate into macrophages[42]. Macrophages can be detected with anti-CD68 antigens, since CD68 has been established as a molecular marker for TAM[11]. Thereby, CCL2 could be associated with CD68 expression. Given this information it is not strange that a strong positive relationship between CD68 and CCL2 (p<0.001) protein levels has been found. In the context of infiltrating ductal breast carcinoma this positive association is highlighting the chemotactant functions of CCL2. If it is interpreted, the effect of requiting macrophages into tumour sites performed by CCL2 is now demonstrated in this type of human breast cancer. Once this relationship is clear, it makes the consideration of the relationship between CCL2 and IL-10 and between CD68 and IL-10 logical. Nevertheless, no significant association was found between IL-10 and CCL2 nor with CD68. It has to be pointed out that macrophages are a main source of IL-10 ñ*in vivo*ò [18]. Furthermore, M2 macrophages have been reported to produce high levels of IL-10[43]. Despite that, there are different ñ*In vivo*ò sources of IL-10, which had not been taken into consideration[18]. Cancer cells, for example, also secret significant levels of IL-10[44]. This may explain why CD68 and IL-10 did not correlate. In addition to that, CCL2 not only mediates the migration of macrophages to tumour sites but also the migration of several different cellular types[27]. This, besides the fact that there are different sources of IL-10, is a reasonable theory that explains why CCL2 and IL-10 levels did not correlate[18]. Being logical, these results may indicate that, although macrophages are requited in invasive ductal breast carcinoma, these cells are not due to express IL-10.

Several receptors for CCL2 were also evaluated. This chemokine mediates the migration of the immune cells that express its biological receptor, CCR2[29]. Unexpectedly, no significant association was found between these two proteins of the

chemokine network. Thereby, this fact suggests that if CCL2 is expressed it does not imply CCR2 to be expressed simultaneously. Moreover, it also does not seem to work the other way round either. In fact, in the tumour microenvironment there coexist different subpopulations of macrophages, which can express CCL2 and CCR2 in different rates[45]. Among the other receptors for CCL2 another association was found. Indeed, it was observed that DARC and CCL2 protein levels were directly and significantly (p=0.017) associated. In their preliminary studies Wang J *et al.*[46] revealed that CCL2 and DARC were simultaneously and constitutionally expressed in several human breast cancer cell lines[46]. Besides, Hou T *et al.*[47] reported a negative association between DARC and CCL2 in cervical squamous cancer[47]. So, given the fact that when CCL2 is expressed also is DARC, a mechanism of control of the levels of this chemokine seems to be promoted in the type of cancer that is being studied. Despite that, the other promiscuous receptor for CCL2 evaluated in this study, D6, did not correlate with CCL2. A negative association between both proteins seemed logical, since D6 lowers the levels of several chemokines via degradation[32]. In fact, other types of cancer have been shown to report this association[46]. More specifically, overexpression of D6 in human breast cancer cells has several inhibitory effects that are associated with decreased chemokines levels, such as CCL2 and others[20]. Indeed, Cancellieri C *et al.*[47] reviewed that the over-expression of D6 in such cell lines is associated with decreased chemokine levels, including CCL2[47]. Moreover, Hou T *et al*[46]. reported a case in which they found a negative association between D6 and CCL2[46]. A hypothetical explanation for our negative results is that D6 has high specificity for several chemokines a part from CCL2, which may result into not lowering sufficiently the levels of CCL2 to have statistical significant relationship[32]. Hence, in the tumours caused by infiltrating ductal breast carcinoma it seems that DARC, but not D6, is expressed in other to maintain the levels of CCL2.

In addition to the links described above, the receptors of CCL2 have been also shown to correlate with different protein. Indeed, DARC and D6 protein levels have been directly correlated with IL-10 in our study. Both promiscuous receptors DARC and D6 have the ability to block chemokine signalling pathways, including CCL2/CCR2 signalling axis[8]. In addition, CCL2 has been reported to be a major factor for macrophages recruitment in tumour sites[28]. Macrophages are, indeed, one of main sources of IL-10 ***ñin vivoò***, which is synthesized when IL-10 gene transcription is

induced[18]. Consequently, it has sense that when DARC and D6 protein levels rise, less macrophages are recruited in the tumour and, therefore, less IL-10 is produced. Controversially, our results suggest the opposite. That means that the more D6 or DARC is expressed, the more IL-10 levels will be found in the tumour, which do not concord with the literature found. It has to be pointed out that the association between DARC and IL-10 is not very strong (p=0.046), but it is surprisingly strong between D6 and IL-10 (p=0.002). Carefulness is required when extracting conclusions because there exist a complex chemokine network that influences the infiltration of immune cells in the tumour and the production of IL-10[25]. Moreover, the role of IL-10 in cancer remains unclear due to the contradictory activities of the cytokine[49]. For one reason or another, IL-10 and the decoy receptors have been shown to correlate in this type of carcinoma, which may suggest that IL-10 has a protective effect when enhancing the expression of those receptors that attenuate the signalling of CCL2. In order to clarify this results, further studies should be taken into consideration.

DARC and D6 have also been shown to present a positive relationship between each other. These chemokine scavengers mediate two different mechanisms by which the concentration of pro-inflammatory chemokines is controlled[8]. Hence, the regulation of their expression could be similar. Indeed, inflammation seems to substantially up-regulate the expression of DARC while inflammatory CC chemokines are able to up-regulate D6 expression[20,32,50]. Therefore, both D6 and DARC seem to be regulated by inflammatory process. Since cancer is actually an inflammatory process, it is not rare that a positive association in the expression of DARC and D6 was found. Hence, it likely that in the type of cancer that we are dealing with the presence of DARC and D6 in the tumour microenvironment is simultaneously regulated. Also, since DARC is correlated with CCL2 but D6 is not, it is likely that there exist a major factor that is regulating their expression. Once the decoy receptors are known to vary together, it is logical that DARC and D6 are to be inversely associated with CD68. Although their biological function in attenuating pro-inflammatory chemokine signalling, neither DARC nor D6 have been correlated with macrophageôs infiltration[8]. Macrophages, which can be detected with the CD68 marker, tend to migrate to places with high concentration of CCL2[28]. This was also corroborated in our study by the association between CCL2 and CD68. In fact, the overexpression of D6 in breast cancer cell lines is associated with decreased chemokine levels (e.g. CCL2 and CCL5), and tumour-

associated macrophage infiltration[48]. Moreover Rundle CH *et al.*[51] reported that DARC knocked out mice presented lower macrophages infiltration in inflammatory microenvironments. Despite that, neither DARC nor D6 associated with CD68 marker of macrophage infiltration[51]. Thereby, this suggests that in breast cancer DARC and D6 are not determinant factors for lowering macrophageôs infiltration. The chemokine network is very complex, and further studies that include the whole chemokine molecules should be taken into consideration to better understand these results[51].

Linking now with CCR2 expression, only one of the two decoy receptors has been associated with their levels. Indeed, CCR2 and DARC have a strong relationship in the tumours caused by infiltrating ductal breast carcinoma. In a study similar to ours no association between CCR2 and DARC was found. Nevertheless, precaution is needed since their results were obtained in blood samples and the patients had either benign disease, ***ñ****in situ****ò*** carcinoma or invasive breast carcinoma[52]. In spite of the results fund by Wang J *et al.*[52] our results seem to indicate again that DARC is expressed in the tumour microenvironment of invasive ductal breast carcinomas to attenuate the signalling axis mediated by CCL2/CCR2, but not D6. Note that DARC was also associated with CCL2, which makes this supposition more plausible.

Other associations have been reported with CCR2 protein levels. In our study CCR2 and IL-10 protein levels are directly correlated. TAM express CCR2 and, hence, are able to migrate to sites with high concentrations of CCL2. Also, TAM secret IL-10[18]. More precisely, TAM have been reported to highly express IL-10 by Wang R *et al.*[16] This study seems to be the first one in establishing a direct relationship between CCR2 and IL-10 in a cancer context. Indeed, our results indicated that CCR2 and IL-10 levels vary together. In spite of that, more studies should be proposed to fully understand this interaction. Continuing with the relationships showed by CCR2, it seems logical that, since macrophages express CCR2 and produce IL-10, the higher infiltration of TAM in the tumour the larger production of IL-10 should be reported. Despite that, no association between CCR2 and macrophage infiltration, evaluated with CD68 staining, was found. Wyler L *et al.*[53] reported an association between CD68 and CCR2 expression in brain cancer[53]. Also, Zhang T el al. reported that infiltrating cells expressing CCR2 were mainly macrophages in cardiac myoxoma cancer[54]. Nevertheless, there are different cellular types that are not macrophages that can secret

IL-10 and migrate to tumour sites, one of which is cancer cells themselves[18,44]. Hence, our results seem to indicate that, in a context of infiltrating ductal breast carcinoma, IL-10 levels are not determined by macrophage-mediated synthesis.

Now attention is going to be paid to the positive and negative results in the differences in the median when comparing different patientôs outcome.

Surprisingly, no associations between CCL2 and any patient outcome feature were found. Several authors have found associations between CCL2 and tumour growth, macrophages requirement, poor patient prognoses and/or tumour grade[21,28]. It seemed reasonable to find that women who had succumbed to the disease should present high CCL2 proteins levels compared with those alive. Also, since CCL2 is the major recruiter for macrophages, which are an important factor in promoting tumour cell metastasis in breast carcinomas, the logical results were to find that women suffering from metastasis should have had higher levels of CCL2 when compared with those without metastasis[55,56]. Even though CCL2 correlates with CD68, CCL2 was not associated with adverse patient outcome. These controversial results may be explained by the fact that CCL2 has multifunctional activities that might conflict with one another[56]. Our results suggest that CCL2 has not a great potential in determining the patient outcome by itself. Hence, in invasive ductal breast carcinoma, CCL2 has turned out not to present value as a predictor marker for the features analysed.

After the disappointing results of CCL2, CCR2 seems to show light into the effects of the chemokine network. In our study it was revealed that women with low CCR2 protein levels are more likely to live than the ones with high CCR2 levels. Eferl R *et al.*[56] reported that the CCL2/CCR2 signalling axis had a relevant role in extravassation of tumour cells ***ñin vivoò*** by inducing vascular permeability[57]. Qian BZ *et al.*[42] also funded that the inhibition of CCL2/CCR2 signalling inhibits metastasis ***ñin vivoò*** and prolongs the survival of tumour-bearing mice[42]. Moreover, it has been demonstrated that only macrophages expressing CCR2 can respond to CCL2 stimuli. Confirming this fact, CCR2 KO mice are not capable of promoting tumour cell trans-endothelial migration[46]. Supporting this evidence, Fang WB *et al*[21]. concluded that CCR2 is essential for CCL2 induction of breast cancer cell motility and survival[21]. In addition to these results women who had died because of cancer may also show higher

levels of CCR2 protein compared with those with better outcome since CCR2 is the only receptor for CCL2[29]. Hence CCR2 levels may have predictor value of survival for patients suffering from invasive ductal breast carcinoma. Although the results with CCR2 are encouraging, CCR2 did not present differences in the other outcome features. Yao Y *et al.*[29] also reported that women suffering from metastasis or cancer relapse had higher levels of CCR2[29]. Despite that, our results do not suggest CCR2 as a predictor of cancer relapse nor metastasis but for a survival predictor.

Unlike CCR2, CD68 did not show association with any of the patientôs features. High TAM infiltration has been associated with low survival[14,59]. TAM can increase the survival and proliferative capacity of cancer cells and promote other adverse features, including cancer cell motility, invasiveness, drive angiogenesis and mediate immunosuppression[58]. In fact, Ong C *et al.*[59] concluded that in mice breast tumours CD68 infiltration associates with increased malignicity[59]. Furthermore, Kapplan-Meier plots obtained by Tymoszuk P *et al.*[60] indicate that low CD68 patients have a better survival rate[60]. Despite that, any differences between dead and alive women in the levels of CD68 protein were found. Additionally, CD68 protein levels were not higher in those women suffering from metastasis compared with the metastasis-free ones. Although literature and our results do not match caution is required when interpreting ours since CD68 is a marker for both M1 and M2 macrophages, which have opposite functions in cancer development[14,59]. Thereby, our results suggest that CD68 macrophage marker, independently of the polarization of the macrophages towards M1 or M2, is not useful as a predictor of survival, metastasis, cancer relapse or development of a secondary neoplasia.

Besides CD68 and CCL2, more negative results were found. No association between IL-10 protein levels and outcome features studied was found in our study. IL-10 has been reported to be expressed by both tumour and stromal cells. High IL-10 expression by tumour cells has been associated with good patient outcome, like low histological grade and negative vascular invasion. Also, higher levels of IL-10 are associated with better disease-free survival and overall survival. Logically, low expression of IL-10 is associated with poor survival outcome. Moreover, Li Y *et al.*[61] concluded that IL-10 can be used as a predictor of breast cancer prognosis[61]. Nevertheless, according to Wang Y el al. the role of IL-10 in cancer remains unclear.

IL-10 is a multifunctional cytokine that shows anti-inflammatory (potentially cancer-promoting) and antiangiogenic (potentially cancer-inhibiting) functions. As a result, IL-10 plays a controversial role in human carcinogenesis, as a tumour-promoting and -inhibiting factor[49]. This may be a possible explanation for our non-significant results. Our study did not seem to support any of the theories above. More importantly, IL-10 levels are not suggested to have potential predictor value in invasive ductal breast carcinoma.

The promiscuous receptors also showed controversial results. On the one hand, Galzi JL *et al.*[62] observed that D6 inhibits metastasis ñ*in vivo***ò** due to its capacity to sequester chemokines[62]. Also, breast cancer cell lines over-expressing D6 have been shown to decrease metastasis[20,63]. Our study has found a significant association between D6 protein levels of women having a secondary neoplasia compared with those who have not. In fact, women without a secondary neoplasia showed a higher significant level of D6 protein. That founding correlates with several literature exposed above[20,62,63]. Moreover, Hou T *et al.*[47] reported that D6 positive expression was correlated with favourable overall survival and recurrence-free survival[47]. In addition, Wu FY *et al.*[20] discovered that D6 expression was positively correlated to disease-free survival in breast cancer[20]. The reason for this may be that D6 has a physiological role in the clearance of chemokines, binding to 12 different chemokines, 8 of which have pro-inflammatory functions. The function of D6 is to prevent an excessive response to chemokines and eventually terminates the inflammatory response[62]. Although in our study any association was found between D6 levels and cancer relapse other studies suggest the contrary[46]. Hence, D6 should be better studied to be considered as a predictor marker for cancer relapse, metastasis and survival. Despite that, our results support the value of D6 as a predictor of development of secondary neoplasias.

On the other hand, DARC overexpression was not found to be correlated with any of the studied patient outcomes. Hou T *et al.*[47] also reported negative results when they found that there was no significant difference between DARC positive and negative groups with respect to clinical survival time[47]. Nevertheless, studies into the expression of the atypical chemokine receptors in breast cancer suggest that DARC is significantly associated with disease-free survival[55]. Also, over-expressing DARC breast cancer cell lines have been shown to suppress their growth and metastasis ***ñ****in*

*vivo***ò**. Moreover, low DARC levels in human breast cancer samples have been associated to lymph node metastasis and low survival[32]. In spite of all the literature found, DARC seems not to have potential value as a prognosis factor for adverse nor beneficial invasive ductal breast carcinoma patientôs outcome. Our negative results may be because DARC functions as a chemokine buffer system and does not degrade chemokines as D6 does. Also, DARC has an essential role in the regulation of chemokines in the blood, but its effects on tumour microenvironment may vary.

7 Conclusion:

The results in CCL2 expression in this study seem not to have a very high clinical relevance. Despite that, CCL2 has been verified to be a key factor for macrophages requirement to infiltrating ductal breast carcinoma tumour sites. Although this, CD68 macrophage marker turned out not to be a good biomarker for adverse outcome. The association between DARC and CCL2 may suggest that DARC is overexpressed when the concentrations of CCL2 rise, but not D6. This would imply that when CCL2 levels increase DARC would be expressed to control such increased concentration. To corroborate that, further studies should be taken into consideration.

In spite of that, we believe that D6 and CCR2 expression may provide valuable clinical information as predictor factors for the development of a secondary neoplasia and chances of survival respectively. However, they should be better evaluated by including different co-variables, such as histological grade, disease-free time, tumour size, etc. in the statistical analysis to better understand and have a stronger association in the patientôs outcome.

Obviously, extensive research is needed in order to asses the impact of protein interactions on invasive ductal breast carcinoma and further statistical analysis should be carried out to better understand their interaction.

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9 References:

[1] World Health Organization. http://www.euro.who.int/en/health-topics/non communicable-diseases/cancer/data-and-statistics.htm [Consulta: 16 abril 2014].

[2] Banin Hirata BK, Oda JM, Losi Guembarovski R, Ariza CB, de Oliveira CE, Watanabe MA. Molecular markers for breast cancer: prediction on tumour behavior. Dis Markers. 2014 Jan;2014:513158-513158.

[3] Sociedad Española de Oncolgía Médica. El cáncer en España 2013. http://www.seom.org/es/prensa/el-cancer-en-espanyacom/104018-el-cancer-en-espana-2013.htm [Consulta: 16 abril 2014].

[4] Koboldt DC, Fulton RS, McLellan MD, Schmidt H, Kalicki-Veizer J, McMichael JF, Fulton LL, Dooling DJ, Ding L, Mardis ER,Wilson RK, Ally A, Balasundaram M, Butterfield YS, Carlsen R, Carter C, Chu A, Chuah E, Chun HJ, Coope RJ, et al. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature. 2012 Oct;490:61-70.

[5] World Health Organization. http://www.who.int/mediacentre/factsheets/fs297/en/ [Consulta: 16 abril 2014].

[6] Patel JD, Krilov L, Adams S, Aghajanian C, Basch E, Brose MS, Carroll WL, de Lima M, Gilbert MR, Kris MG, Marshall JL, Masters GA, O'Day SJ, Polite B, Schwartz GK, Sharma S, Thompson I, Vogelzang NJ, Roth BJ. Clinical Cancer Advances 2013: Annual Report on Progress Against Cancer from the American Society of Clinical Oncology. J Clin Oncol. 2014 Jan;32(2):129-160.

[7] Sociedad Española de Oncolgía Médica. El cáncer en España 2013. http://www.seom.org/es/prensa/el-cancer-en-espanyacom/104018-el-cancer-en-espana-2013.htm [Consulta: 16 abril 2014].

[8] Chew AL, Tan WY, Khoo BY. Potential combinatorial effects of recombinant atypical chemokine receptors in breast cancer cell invasion: A research perspective. Biomed Rep. 2013 Mar;1(2):185-192.

[9] American Society of Cancer. Breast Cancer Overview. http://www.cancer.org/ cancer/breastcancer/overviewguide/breast-cancer-overview-what-is-breast-cancer [Consulta: 16 abril 2014].

[10] National Breast Cancer Fundation, INC. Invasive Ductal Carcinoma (IDC). http://www.nationalbreastcancer.org/invasive-ductal-carcinoma.htm [Consulta: 16 abril 2014].

[11] Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013 Nov;19(11):1423-1437.

[12] Rolin J, Maghazachi AA. Implications of chemokines, chemokine receptors, and inflammatory lipids in atherosclerosis. J Leukoc Biol. 2014 Apr;95(4):575-585.

[13] Coussens LM, Zitvogel L, Palucka AK. Neutralizing tumor-promoting chronic inflammation: a magic bullet? Science. 2013 Jan;339:286-291.

[14] Zhang Y, Cheng S, Zhang M, Zhen L, Pang D, Zhang Q, Li Z. High-infiltration of tumour-associated macrophages predicts unfavorable clinical outcome for node-negative breast cancer. PLoS One. 2013 Sep;8(9):e76147-e76147.

[15] Jung M, Weigert A, Tausendschön M, Mora J, Ören B, Sola A, Hotter G, Muta T, Brüne B. Interleukin-10-induced neutrophil gelatinase-associated lipocalin production in macrophages with consequences for tumour growth. Mol Cell Biol. 2012 Oct;32(19):3938-3948.

[16] Wang R, Zhang T, Ma Z, Wang Y, Cheng Z, Xu H, Li W, Wang X. The interaction of coagulation factor XII and monocyte/macrophages mediating peritoneal metastasis of epithelial ovarian cancer. Gynecol Oncol. 2010 Jun;117(3):460-466.

[17] Jung M, Weigert A, Tausendschön M, Mora J, Ören B, Sola A, Hotter G, Muta T, Brüne B. Interleukin-10-induced neutrophil gelatinase-associated lipocalin production in macrophages with consequences for tumor growth. Mol Cell Biol. 2012 Oct;32(19):3938-3948.

[18] Sabat R, Grütz G, Warszawska K, Kirsch S, Witte E, Wolk K, Geginat J. Biology of interleukin-10. Cytokine Growth Factor Rev. 2010 Oct;21(5):331-344.

[19] Domínguez-Soto A, Sierra-Filardi E, Puig-Kröger A, Pérez-Maceda B, Gómez-Aguado F, Corcuera MT, Sánchez-Mateos P, Corbí AL. Dendritic cell-specific ICAM-3-grabbing nonintegrin expression on M2-polarized and tumour-associated macrophages is macrophage-CSF dependent and enhanced by tumour-derived IL-6 and IL-10. J Immunol. 2011 Feb;186(4):2192-2200.

[20] Wu FY, Ou ZL, Feng LY, Luo JM, Wang LP, Shen ZZ, Shao ZM. Chemokine decoy receptor d6 plays a negative role in human breast cancer. Mol Cancer Res. 2008 Aug;6(8):1276-1288.

[21] Fang WB, Jokar I, Zou A, Lambert D, Dendukuri P, Cheng N. CCL2/CCR2 chemokine signaling coordinates survival and motility of breast cancer cells through Smad3 protein- and p42/44 mitogen-activated protein kinase (MAPK)-dependent mechanisms. J Biol Chem. 2012 Oct;287(43):36593-36608.

[22] Zlotnik A, Yoshie O. The chemokine superfamily revisited. Immunity. 2012 May;36(5):705-716.

[23] Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verástegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. Nature. 2001 Mar;410(6824):50-56.

[24] Rollins BJ. Inflammatory chemokines in cancer growth and progression. Eur J Cancer. 2006 Apr;42(6):760-767.

[25] Balkwill F. Chemokine biology in cancer. Semin Immunol. 2003 Feb;15(1):49-55.

[26] Steiner JL, Murphy EA. Importance of chemokine (CC-motif) ligand 2 in breast cancer. Int J Biol Markers. 2012 Oct;27(3):e179-e185.

[27] Li M, Knight DA, A Snyder L, Smyth MJ, Stewart TJ. A role for CCL2 in both tumour progression and immunosurveillance. Oncoimmunology. 2013 Jul;2:e25474-e25474.

[28] Lee HW, Choi HJ, Ha SJ, Lee KT, Kwon YG. Recruitment of monocytes/macrophages in different tumour microenvironments. Biochim Biophys Acta. 2013 Apr;1835(2):170-179.

[29] Yao Y, Tsirka SE. Monocyte chemoattractant protein-1 and the blood-brain barrier. Cell Mol Life Sci. 2014 Feb;71(4):683-697.

[30] Vande Broek I, Asosingh K, Vanderkerken K, Straetmans N, Van Camp B, Van Riet I. Chemokine receptor CCR2 is expressed by human multiple myeloma cells and mediates migration to bone marrow stromal cell-produced monocyte chemotactic proteins MCP-1, -2 and -3. Br J Cancer. 2003 Mar;88(6):855-862.

[31] Ryder M, Gild M, Hohl TM, Pamer E, Knauf J, Ghossein R, Joyce JA, Fagin JA. Genetic and pharmacological targeting of CSF-1/CSF-1R inhibits tumor-associated macrophages and impairs BRAF-induced thyroid cancer progression. PLoS One. 2013 Jan;8:e54302-e54302.

[32] Hansell CA, Hurson CE, Nibbs RJ. DARC and D6: silent partners in chemokine regulation? Immunol Cell Biol. 2011 Feb;89(2):197-206.

[33] Fra AM, Locati M, Otero K, Sironi M, Signorelli P, Massardi ML, Gobbi M, Vecchi A, Sozzani S, Mantovani A. Cutting edge: scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6. J Immunol. 2003 Mar;170(5):2279-2282.

[34] Borroni EM, Buracchi C, de la Torre YM, Galliera E, Vecchi A, Bonecchi R, Mantovani A, Locati M. The chemoattractant decoy receptor D6 as a negative regulator of inflammatory responses. Biochem Soc Trans. 2006 Dec;34(6):1014-1017.

[35] Mishra A, Verma M. Cancer biomarkers: are we ready for the prime time? Cancers (Basel). 2010 Mar;2(1):190-208.

[36] Banin Hirata BK, Oda JM, Losi Guembarovski R, Ariza CB, de Oliveira CE, Watanabe MA. Molecular markers for breast cancer: prediction on tumor behavior. Dis Markers. 2014 Jan;2014:e513158-e513158.

[37] Arveux P, Bertaut A. [Epidemiology of breast cancer]. Rev Prat. 2013 Dec;63(10):1362-1366.

[38] D.M. Fletcher, C. Diagnostic Histopathology of Tumours, 3rd ed.; CHURCJILL LIVINGATONE ELSEVIER: Boston, 2007.

[39] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar;144(5):646-674.

[40] GARCÍA FONTGIVELL, Joan Fransesc. Contribució a lêstudi molecular de cáncer de mama. Dirigida per Dr. Juan José Sirvent Calvera. Tésis inèdita. Universitat Rovira i Virgila, Departament de ciències mèdiques bàsiques, 2011.

[41] Nacional Cancer Institute. http://www.cancer.gov/cancertopics/factsheet/ detection/tumor-grade [Consulta: 16 abril 2014].

[42] Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, Kaiser EA, Snyder LA, Pollard JW. CCL2 recruits inflammatory monocytes to facilitate breast-tumor metastasis. Nature. 2011 Jun;475(7355):222-225.

[43] Zhang Y, Sime W, Juhas M, Sjölander A. Crosstalk between colon cancer cells and macrophages via inflammatory mediators and CD47 promotes tumour cell migration. Eur J Cancer. 2013 Oct;49(15):3320-3334.

[44] Zhang Y, Sime W, Juhas M, Sjölander A. Crosstalk between colon cancer cells and macrophages via inflammatory mediators and CD47 promotes tumour cell migration. Eur J Cancer. 2013 Oct;49(15):3320-3334.

[45] Sierra-Filardi E, Nieto C, Domínguez-Soto A, Barroso R, Sánchez-Mateos P, Puig-Kroger A, López-Bravo M, Joven J, Ardavín C, Rodríguez-Fernández JL, Sánchez-Torres C, Mellado M, Corbí AL. CCL2 shapes macrophage polarization by GM-CSF and M-CSF: identification of CCL2/CCR2-dependent gene expression profile. J Immunol. 2014 Apr;192(8):3858-3867.

[46] Wang J, Ou ZL, Hou YF, Luo JM, Shen ZZ, Ding J, Shao ZM. Enhanced expression of Duffy antigen receptor for chemokines by breast cancer cells attenuates growth and metastasis potential. Oncogene. 2006 Nov 16;25(54):7201-7211.

[47] Hou T, Liang D, Xu L, Huang X, Huang Y, Zhang Y. Atypical chemokine receptors predict lymph node metastasis and prognosis in patients with cervical squamous cell cancer. Gynecol Oncol. 2013 Jul;130(1):181-187.

[48] Cancellieri C, Caronni N, Vacchini A, Savino B, Borroni EM, Locati M, Bonecchi R. Review: Structure-function and biological properties of the atypical chemokine receptor D6. Mol Immunol. 2013 Aug;55(1):87-93.

[49] Wang Y, Liu XH, Li YH, Li O. The paradox of IL-10-mediated modulation in cervical cancer. Biomed Rep. 2013 May;1(3):347-351.

[50] Savino B, Borroni EM, Torres NM, Proost P, Struyf S, Mortier A, Mantovani A, Locati M, Bonecchi R. Recognition versus adaptive up-regulation and degradation of CC chemokines by the chemokine decoy receptor D6 are determined by their N-terminal sequence. J Biol Chem. 2009 Sep;284(38):26207-26215.

[51] Rundle CH, Mohan S, Edderkaoui B. Duffy antigen receptor for chemokines regulates post-fracture inflammation. PLoS One. 2013 Oct;8(10):e77362-e77362.

[52] Wang J, He Q, Shao YG, Ji M. Chemokines fluctuate in the progression of primary breast cancer. Eur Rev Med Pharmacol Sci. 2013 Mar;17(5):596-608.

[53] Wyler L, Napoli CU, Ingold B, Sulser T, Heikenwälder M, Schraml P, Moch H. Brain metastasis in renal cancer patients: metastatic pattern, tumour-associated macrophages and chemokine/chemoreceptor expression. Br J Cancer. 2014 Feb;110(3):686-694.

[54] Zhang T, Koide N, Wada Y, Tsukioka K, Takayama K, Kono T, Kitahara H, Amano J. Significance of monocyte chemotactic protein-1 and thymidine phosphorylase in angiogenesis of human cardiac myxoma. Circ J. 2003 Jan;67(1):54-60.

[55] Kalatskaya I, Berchiche YA, Gravel S, Limberg BJ, Rosenbaum JS, Heveker N. AMD3100 is a CXCR7 ligand with allosteric agonist properties. Mol Pharmacol. 2009 May;75(5):1240-1247.

[56] Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood. 2000 Jul;96(1):34-40.

[57] Eferl R. CCL2 at the crossroad of cancer metastasis. JAKSTAT. 2013 Apr;2(2):e23816-e23816.

[58] Su S, Liu Q, Chen J, Chen J, Chen F, He C, Huang D, Wu W, Lin L, Huang W, Zhang J, Cui X, Zheng F, Li H, Yao H, Su F, Song E. A Positive Feedback Loop between Mesenchymal-like Cancer Cells and Macrophages Is Essential to Breast Cancer Metastasis. Cancer Cell. 2014 May;25(5):605-60.

[59] Ong C, Brandenberger C, Kiupel M, Kariagina A, Langohr IM. Immunohistochemical Characterization and Morphometric Analysis of Macrophages in Rat Mammary Tumors. Vet Pathol. 2014 May. In Press.

[60] Tymoszuk P, Charoentong P, Hackl H, Spilka R, Müller-Holzner E, Trajanoski Z, Obrist P, Revillion F, Peyrat JP, Fiegl H, Doppler W. High STAT1 mRNA levels but not its tyrosine phosphorylation are associated with macrophage infiltration and bad prognosis in breast cancer. BMC Cancer. 2014 Apr;14:257-257.

[61] Li Y, Yu H, Jiao S, Yang J. [Prognostic value of IL-10 expression in tumor tissues of breast cancer patients]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2014 May;30(5):517-520.

[62] Galzi JL, Hachet-Haas M, Bonnet D, Daubeuf F, Lecat S, Hibert M, Haiech J, Frossard N. Neutralizing endogenous chemokines with small molecules. Principles and potential therapeutic applications. Pharmacol Ther. 2010 Apr;126(1):39-55.

[63] Mantovani A, Savino B, Locati M, Zammataro L, Allavena P, Bonecchi R. The chemokine system in cancer biology and therapy. Cytokine Growth Factor Rev. 2010 Feb;21(1):27-39.

[64] Vargo-Gogola T, Rosen JM. Modelling breast cancer: one size does not fit all. Nat Rev Cancer. 2007 Sep;7(9):659-672.

[65] Vargo-Gogola T, Rosen JM. Modelling breast cancer: one size does not fit all. Nat Rev Cancer. 2007 Sep;7(9):659-672.