UNIVERSITA' DEGLI STUDI DI VERONA

DIPARTIMENTO DI

Neuroscienze, biomedicina e Movimento

SCUOLA DI DOTTORATO DI

Scienze Ingegneria Medicina

DOTTORATO DI RICERCA IN

Imaging Multimodale in biomedicina

CICLO /ANNO

28°- 2013

Characterization of cancer–associated adipose tissue

S.S.D. BIO/16

Coordinatore: Prof. Carlo Zancanaro

Firma

Tutor: Prof. Andrea Sbarbati

Firma

Dottorando: Dott./ssa Asmaa Chakir

Firma

Sommario

Tanti studi hanno contribuito a stabilire una stretta interazione tra il tessuto adiposo e il cancro in presenza di malattie metaboliche come l'obesità. Studi epidemiologici hanno dimostrato che i pazienti obesi hanno un rischio drasticamente più elevato di sviluppare il cancro. Si ritiene che questo effetto pro-tumorale in pazienti obesi sia dovuto all'aberrante rilascio sistemico e paracrino di citochine pro-infiammatorie da parte delle cellule adipose, che collabora ad aumentare la proliferazione delle cellule tumorali e la diffusione metastatica.

Inoltre, gli adipociti associati al cancro (CAA) possono contribuire allo sviluppo tumorale, fornendo un supporto metabolico all'incrementata richiesta bioenergetica delle cellule tumorali.

Tuttavia, resta ancora da stabilire se un rilevante effetto pro-infiammatorio possa essere esercitato dal tessuto adiposo peritumorale nei pazienti non-obesi con cancro gastrointestinale.

Il cancro gastrointestinale è responsabile di più morti di qualsiasi altro tipo di cancro. Come per altri tipi di cancro, molti studi hanno dimostrato la forte relazione tra neoplasie gastrointestinali e le malattie sistemiche del tessuto adiposo, come l'obesità e il diabete, ma quasi nulla si sa riguardo l'interazione locale fra le cellule tumorali gastrointestinali e il tessuto adiposo peritumorale in pazienti non-obesi/non-diabetici.

Per iniziare a colmare questo vuoto abbiamo studiato se il tessuto adiposo che circonda tumori gastrointestinali umani possa essere alterato in termini di morfologia e infiltrazione infiammatoria. Specificamente abbiamo confrontato il tessuto adiposo peritumorale e non peritumorale (grasso viscerale distante dalla lesione tumorale) degli stessi soggetti per dimensioni e morfologia delle adipociti, numero di linfociti (cellule positive CD3, CD3 +) e macrofagi (cellule positive CD68, CD68 +) infiltranti.

Abbiamo trovato che il tessuto adiposo peritumorale mostra adipocitici di dimensioni significativamente ridotte, e un maggiore numero di linfociti e macrofagi attivati. I nostri risultati forniscono evidenze cliniche a supporto del concetto secondo cui gli adipociti all'interfaccia tumore-stroma siano coinvolti in un complesso circolo vizioso organizzato dalle cellule tumorali per promuovere la

progressione del tumore. In particolare i nostri risultati suggeriscono che gli adipociti peritumorali potrebbero fornire un contributo allo sviluppo tumorale alimentando le richieste metaboliche di cellule cancerose e fornendo segnali mitogenici attraverso il rilascio paracrino di citochine pro-infiammatorie.

Abstract

A large body of evidences has contributed to establish a strict association between adipose tissue and cancer in the contest of metabolic disorders such as obesity. Epidemiological studies have shown that obese patients have a drastically higher risk to develop cancer. This tumor-promoting role in the context of obesity is thought to rely mainly on the aberrant systemic and paracrine release of pro-inflammatory cytokines by fat cells, which ultimately cooperate to boost cancer cell proliferation and metastatic dissemination.

In addition, cancer associated adipocytes (CAAs) may contribute in tumor progression by providing a metabolic support to the aberrant bioenergetics demand of cancer cells.

However, it still remains to be established whether a relevant pro-inflammatory effect might be exerted by peritumoral adipose tissue in non-obese patients with gastrointestinal cancer.

Gastrointestinal cancers are responsible for more deaths than any other cancer in the body. As for other types of cancer, many studies have revealed the strong relationship between gastrointestinal neoplasms and systemic disorders of adipose tissue, such as obesity and diabetes, but almost nothing is known about the local interaction between gastrointestinal cancer cells and peritumoral adipocytes in non-obese/non-diabetic patients.

In order to start bridge this gap we assess whether adipose tissue surrounding human gastrointestinal tumors might be altered in terms of adipocyte morphology and inflammatory infiltration. Specifically we compare peritumoral and non-peritumoral adipose tissue (visceral fat distant from tumor lesion) for the adipocyte size and morphology, cell count of lymphocytes (CD3 positive cells, CD3+) and macrophages (CD68 positive cells, CD68+).

We found that peritumoral adipose tissue exhibit significantly reduced adipocyte size and increased number of activated lymphocytes and macrophages. Our results provide clinical evidences in support of the emerging notion that adipocytes at the tumor-stroma interface participate in a highly complex vicious cycle organized by cancer cells to promote tumor progression. Specifically our results suggest that peritumoral adipocytes might provide a significant contribution to enhance tumor

burden by fueling cancer cell's metabolic demands and providing mitogenic signals via the paracrine release of pro-inflammatory cytokines.

Index

Chapter 1

Introduction

1.1 Tumor definition

An irregular progress of tissue is called Neoplasm, and when it forms a mass, is usually referred to as a tumor (Birbrair et al, 2014) (Figure1).

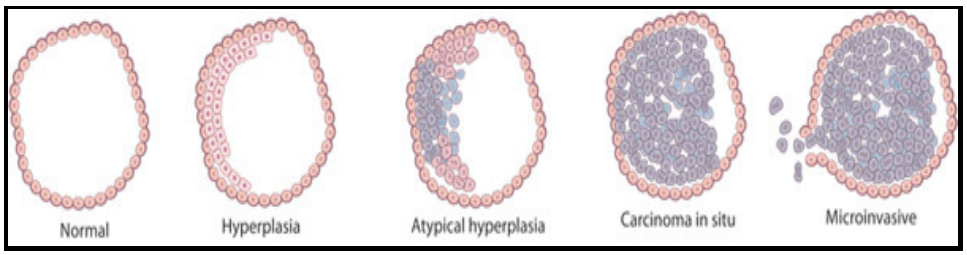


Figure 1: The step modifications of a normal tissue to cancerous tissue. A normal cell population can change into a population of invasive cancerous cells. The diagram shows five different groups of cells, which are labeled: *normal, hyperplasia, atypical hyperplasia, carcinoma in situ, and microinvasive*. The *normal* cells are arranged in a single layer to form a closed, hollow, ring. The *hyperplasia* is a group of cells that begins to divide and increase in number, forming a second and third layer of cells within the ring. In *atypical hyperplasia*, the three layers of cells have expanded to form a small mass of purple and blue, rapidly proliferating cells. The cells are no longer organized in rows, and some are even piled up on top of one another. In *carcinoma in situ*, the small mass of cells has expanded further to form a dense population of blue and purple, mutant cancer cells that occupies most of the space within the ring of normal healthy cells. In the final cell population, *microinvasive*, a rupture in the left side of the ring of healthy cells has appeared, and several purple, mutant cancer cells are shown spilling out from the dense mass of cancer cells inside. (Nature. Cell division and cancer. 2010).

The World Health Organization classifies neoplasms into four important groups (WHO, 2014):

- benign neoplasms: they are limited and localized and do not develop a cancer,
- in situ neoplasms: possibly malignant neoplasms include carcinoma in situ, they are localised, do not exceed and devastate but in time may develop a cancer,
- malignant neoplasms, are cancers, they exceed and devastate the surrounding tissue, can form metastases and when they are untreated or unresponsive to treatment, will became mortal,
- Neoplasms of unknown factor.

1.2 Tumor Classification

In clinical practice multiple protocols of tumor classification have been developed, which help optimize the choice of therapeutic approaches. These rely on different parameters that describe different anatomical, histological and molecular features of tumor parenchyma and its surrounding microenvironment. The most traditionally used protocols of tumor classification are based on the definition of three major parameters that are *stage*, *grade* and *histological* aspect of a tumor.

1.2.1 Stage of tumor

The TNM classification of malignant tumors, made and affirmed by The Union for International Cancer Control (UICC), describes the stage of a cancer by alphanumeric codes (Gospodarowicz et al, 2003) based on the size and extension of the primary tumor, its lymphatic involvement, and the presence of metastases (Jeffrey et al, 2013):

- **T**: describes the size or the direct extent of the primary tumor (the original tumor):

  Tx: tumor cannot be evaluated

  Tis: carcinoma in situ

  T0: no signs of tumor

  T1, T2, T3, T4: size and/or extension of the primary tumor
- **N**: degree of spread to regional lymph nodes that are involved:

  Nx: lymph nodes cannot be evaluated

  N0: tumor cells absent from regional lymph nodes

  N1: regional lymph node metastasis present

  N2: tumor spread to an extent between N1 and N3

  N3: tumor spread to more distant or numerous regional lymph nodes
- **M**: presence of distant metastasis (spread of cancer from one part of the body to another):

  M0: no distant metastasis

  M1: metastasis to distant organs (Haggstrom et al, 2009)

1.2.2 Grade of Tumor

The grade of the cancer is based on the resemblance of the tumor to the tissue of origin (Abrams et al, 2010); they are low grade if they appear similar to normal cells and high grade if they appear poorly differentiated.

The grade score (G1-G4) increases with the lack of cellular differentiation, it reflects how much the tumor cells differ from the cells of the normal tissue they have originated from.

Grading systems are also different for many common types of cancer, though following a similar pattern with grades being increasingly malignant over a range of 1 to 4. If no specific system is used, the following general grades are most commonly used:

- G1 Well differentiated (Low grade)
- G2 Moderately differentiated (Intermediate grade)
- G3 Poorly differentiated (High grade)
- G4 Undifferentiated (High grade)

1.2.3 Histology

Cancer types can be grouped into histological categories, which include (Lemoine et al, 2001) (Figure 2):

Epithelial cells ⇨ carcinoma:

- Adenocarcinoma: refers to a carcinoma featuring microscopic glandular-related tissue cytology, tissue architecture, and/or gland-related molecular products.
- Squamous cell carcinoma: refers to a carcinoma with observable features and characteristics indicative of squamous differentiation.
- Adenosquamous carcinoma: refers to a mixed tumor containing both adenocarcinoma and squamous cell carcinoma, wherein each of these cell types comprises at least 10% of the tumor volume.

- Undifferentiated carcinomas: refers to a heterogeneous group of high-grade carcinomas that feature cells lacking distinct histological or cytological evidence of any of the more specifically differentiated neoplasms.

Mesenchymal cells ⇨sarcoma:

- Liposarcoma is a cancer that arises in fat cells in deep soft tissue, such as that inside the thigh or in the retroperitoneum (Bell, 2012). Liposarcoma is a rare type of cancer that bears a resemblance to fat cells when examined under a microscope.
- Chondrosarcoma is a cancer composed of cells derived from transformed cells that produce cartilage (Gelderblom H, 2008). About 30% of skeletal system cancers are chondrosarcomas. It is resistant to chemotherapy and radiotherapy. Unlike other primary bone cancers that mainly affect children and adolescents, chondrosarcoma can present at any age. It more often affects the axial skeleton than the appendicular skeleton.
- Angiosarcoma derives from neoplastic transformation of endothelial cells of blood (hemangiosarcomas) or lymphatic (lymphangiosarcomas) vessels.

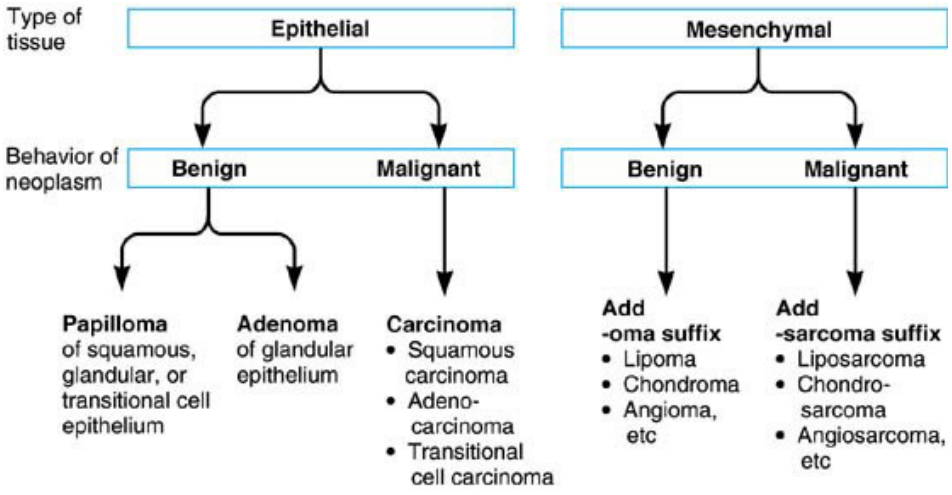


Figure 2: Histological classification of tumor

1.3 Gastrointestinal tumour

Gastrointestinal cancer represents on organs of digestion including: esophagus, stomach, biliary system, pancreas, small intestine, large intestine, rectum and anus (Figure 3, 4), frequently the diagnosis is based on endoscopy, followed by biopsy of ambiguous tissue. The treatment depends on the location of the tumor, as well as the type of cancer cell and whether it has invaded other tissues or spread elsewhere. These factors also determine the prognosis.

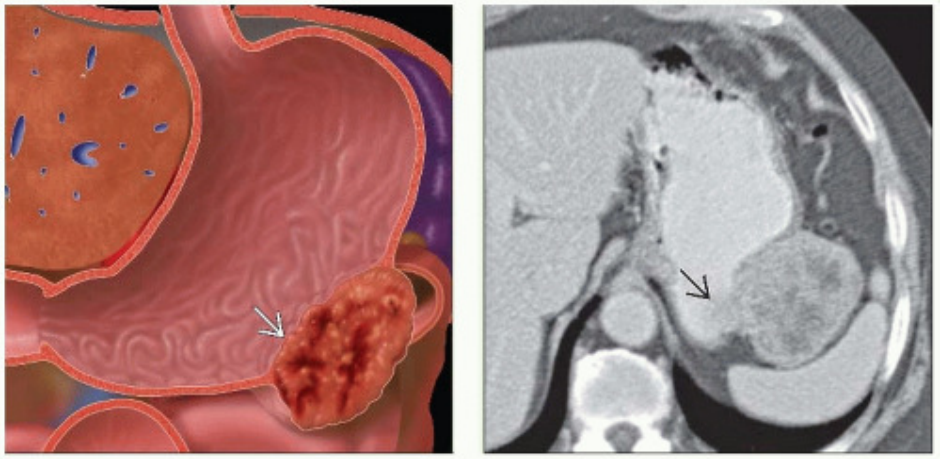


Figure 3: Anatomic representation of a gastric stromal tumor shows and grow submucosal mass image with internal necrosis. (Right) Axial contrast-enhanced computed tomography (CECT shows a soft tissue density left upper quadrant mass. The origin of the mass may not be evident, except for a small projection image into the gastric lumen (Jeffrey, 2016).

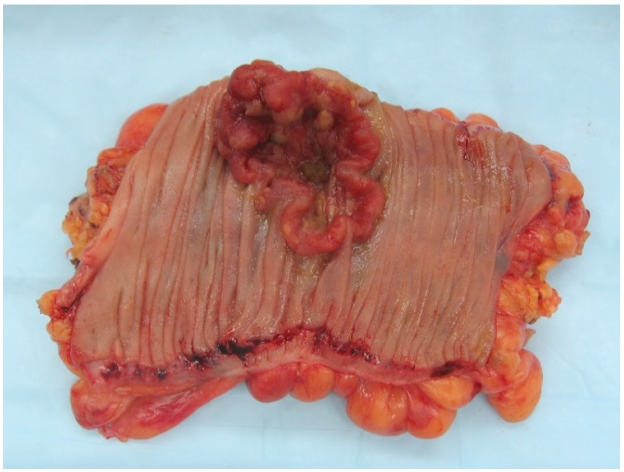


Figure 4: colectomy specimen. ("II Neoplasms". World Health Organization 2014).

In general, gastrointestinal and the accessory organs of digestion (pancreas, liver, and gall bladder) are responsible for more cancers and more deaths from cancer than any other system in the body (Bjelakovic et al, 2008; Yamada et al, 2009). There is significant geographic variation in the rates of different gastrointestinal cancers.

1.3.1 Gastric cancer

Gastric cancer is the cancer of the stomach mucosa. The main symptoms are heartburn, upper abdominal pain, nausea and loss of appetite (Raymond, 2007); later symptoms are weight loss, yellowing of the skin and whites of the eyes, vomiting, difficulty swallowing, and blood in the stool among others. This cancer can propagate from the stomach to other parts of the body.

Generally, the common cause is infection by the bacterium *Helicobacter pylori*, which represents more than 60% of cases (Chang, 2010; Amadei et al, 2016; Chmiela et al, 2017). Other common causes include eating acid aliments and smoking. Genetically speaking, 10% of cases are due to inherited genetic factors.

Gastric cancer is relatively common and is representing the second leading cause of cancer mortality in the world. Annually, more than 700,000 deaths of patients with gastric cancer, and almost 1,000,000 new gastric cancer cases, present in the entire world (Ferlay et al, 2013). But, a few of patients with gastric cancer are suitable for surgical treatment because of the high proportion of advanced tumors at the time of presentation (Carneiro, 2014). Anyway, this intervention remains the most important therapeutic means for patients with gastric cancer to achieve long-term survival.

The chemotherapy is another way to treat stomach cancer but has no firmly established standard of care, because stomach cancer has not been particularly sensitive to drugs. If used, has usually served to reduce the size of the tumor, relieve symptoms of the disease and increase survival time (Scartozzi, 2007). Clinical researchers have explored the benefits of giving chemotherapy before

surgery to shrink the tumor, or as adjuvant therapy after surgery to destroy remaining cancer cells (Galizia, 2014).

1.3.2 Colorectal cancer

Colorectal cancer is the growth of cancer from the colon or rectum (Bibbins et al, 2016). Blood in the stool, a change in bowel movements, weight loss, and feeling tired all the time are the main symptoms of this cancer.

Globally, colorectal cancer represents the third prevalent type of cancer, 10% are the newly diagnosed cancer cases and 10% are the cancer deaths (Siegal et al, 2012). It is more common in developed countries, where more than 65% of cases are found and is less common in women than men (National cancer institute. 2012) (Figure 5).

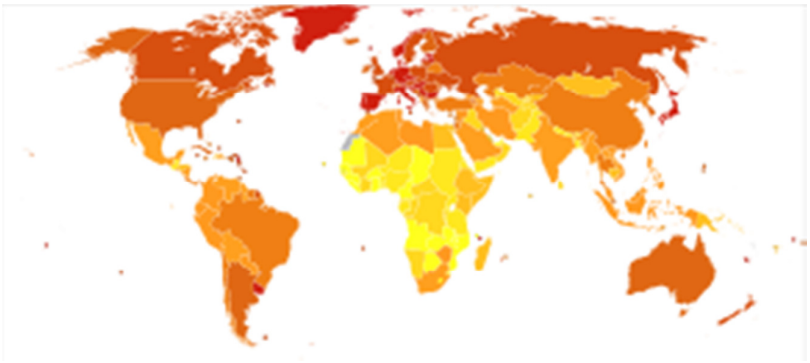


Figure 5: Colon and rectum cancer deaths per million persons in 2012

The causes of the colorectal cancers are often old age and lifestyle factors, with only a small number of cases due to underlying genetic disorders.

Furthermore some risk factors like diet, obesity, smoking, and lack of physical activity. Another risk factor is inflammatory bowel disease, which includes Crohn's disease and ulcerative colitis (World cancer. 2014). Some of the inherited genetic disorders that can cause colorectal cancer include familial adenomatous polyposis and hereditary non-polyposis colon cancer. However, these represent less than 5% of cases. It typically starts as a benign tumor, which over time becomes cancerous.

For colorectal cancer, the Treatments can include some combination of surgery, radiation therapy, chemotherapy and targeted therapy.

1.4 Tumor microenvironment:

Decades of studies have established that complex interactions with the anatomical microenvironment where tumor initiates may be crucial to drive cancer progression and metastasization. Tumor microenvironment (TME) comprises multiple cellular and biochemical components that may act promoting or mitigating cancer progression (Mbeunkui et al, 2009: Hanahan et al, 2011; Spaw et al, 2016) (Figure 6).

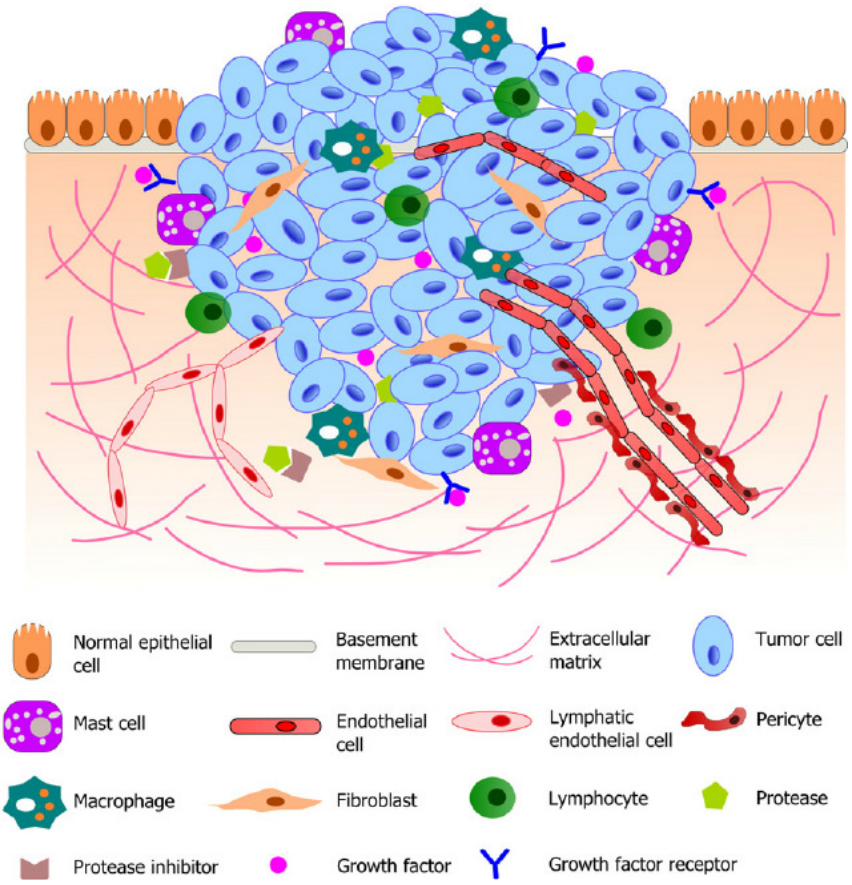


Figure 6: Schematic illustration of a typical tumor microenvironment. Cancer cells reside in a complex microenvironment containing various supporting cells, extracellular matrix (ECM) and a suite of signaling molecules. These environmental components collectively contribute to the tumor-stromal interaction and tumor progression.

These components include:

- The cellular components: consist on the microorganisms and leukocytes, plus fibroblasts, pericytes, endothelial cells, neural cells and adipocytes. The interact with one another as well as with tumor cells in different ways. These components contribute to the development of an abnormal extracellular matrix, and then promote to tumor growth, angiogenesis, invasion and metastasis.
- The non-cellular components (extracellular matrix): consist in one hand by the interstitial matrix formed by collagens, proteoglycans and glycoprotein, in the other hand the basement membrane formed by fibronectin, laminins, type IV collagen and linkage proteins.

1.4.1 Adipocytes

Among cellular components of TME, adipocytes are emerging as crucial players in tumor-stroma interaction. In some tumors, cancer cell growth and/
Or metastasis predominantly happened in adipocyte-rich microenvironment (D'Esposito et al, 2016). In fact, adipocytes represent the most abundant cell types surrounding cancer cells.

Adipocytes (Figure 7) derive from stromal progenitors residing within connective tissues and bone marrow (Majka et al, 2011). The interaction with tumor cells and the inflammatory signals acting within TME induces the conversion of stromal adipocytes into cancer-associated adipocytes, which display varied physiological alterations, including an increased expression of pro-inflammatory cytokines, growth factors, and adipokines (Dirat et al, 2011; Muller et al, 2013).

Cancer-associated adipocytes may even regress to acquire fibroblast-like phenotype, creating a desmoplastic appearance in the stroma. Furthermore, the lipid-rich adipocytes in the tumor microenvironment provide an abundant energy supply for transformed cancer cells. In cancers growing in adipose-rich tissues, fat stored in adipocytes may be utilized to fuel cell signalling, tumor growth and progression (Hardaway et al, 2014).

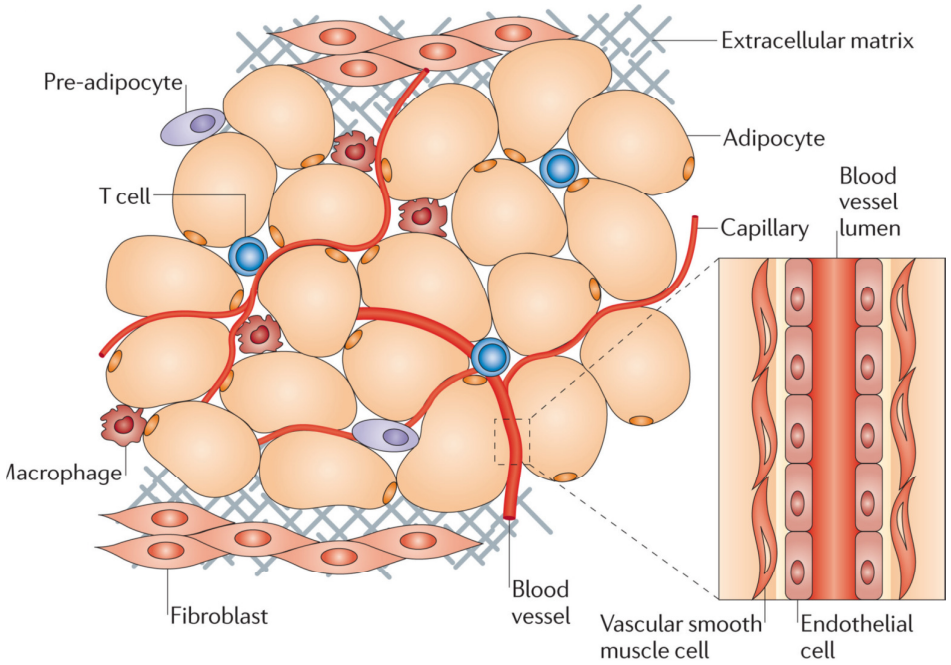


Figure 7: Components of adipose tissue (Ouchi et al, 2011)

Adipocytes are the main cellular component of adipose tissue, and they are crucial for both energy storage and endocrine activity. The other cell types that are present are precursor cells (including pre-adipocytes); fibroblasts, vascular cells and immune cells, and these cells constitute the stromal vascular fraction of adipose tissue. Vascular cells include both endothelial cells and vascular smooth muscle cells, which are associated with the major blood vessels. The blood vessels in adipose tissue are required for the proper flow of nutrients and oxygen to adipocytes, and they are the conduits that allow for the distribution of adipokines. Vascular cells also secrete, and are responsive to, adipose tissue-secreted proteins. Other active adipose tissue components include macrophages and T cells, which have major roles in determining the immune status of adipose tissue. The fibroblast-derived extracellular matrix functions to provide mechanical support and excess matrix can lead to adipose tissue dysfunction. Factors that are secreted by these different cellular components are critical for maintaining homeostasis in adipose tissue and throughout the body.

Adipocytes can be classified in two major subtypes morphologically and functionally different. Those are White and Brown adipocytes (Figure 8).

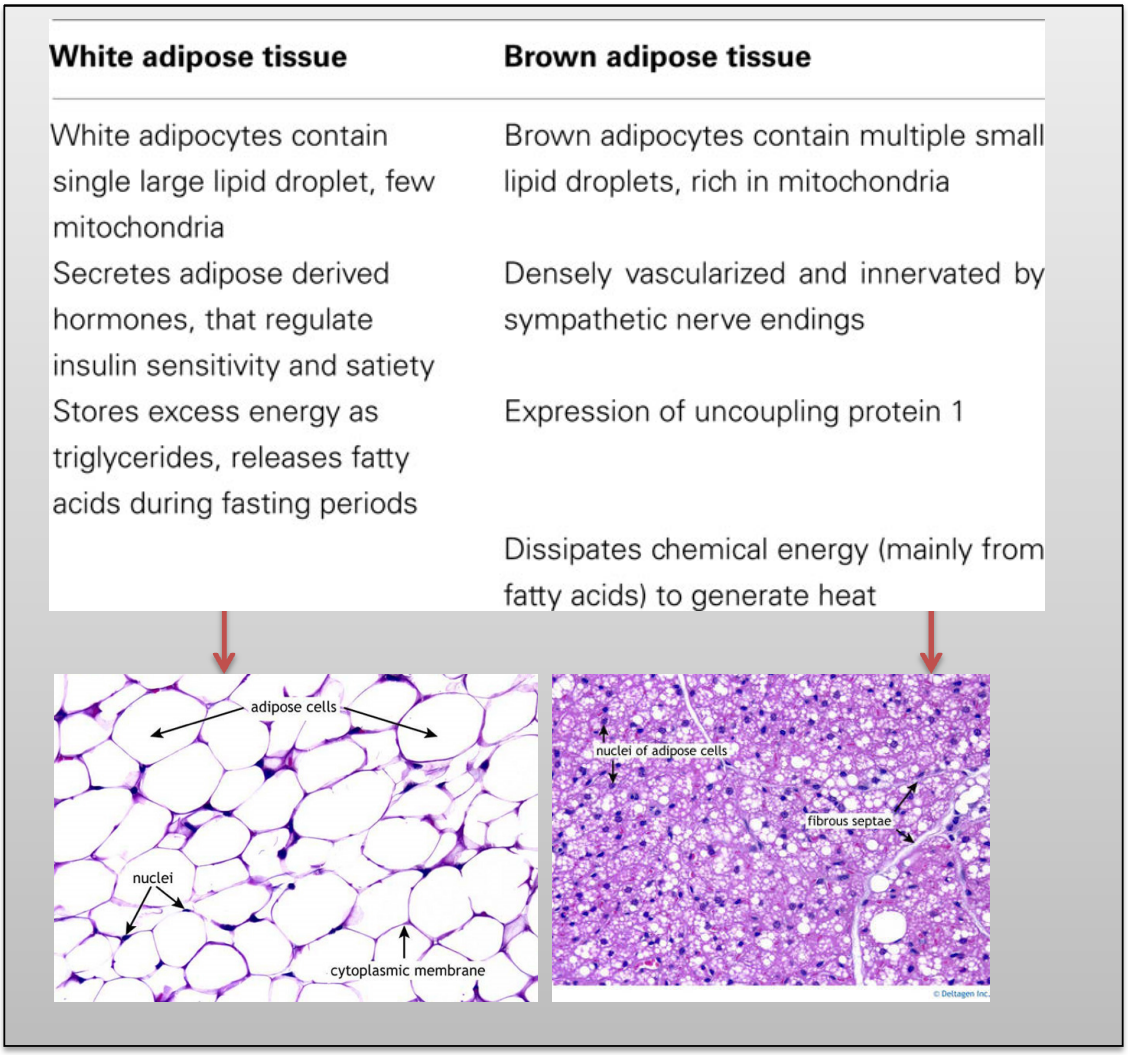


Figure 8: White and Brown adipose tissue.

White adipocytes are enormous cells (up to 200 µm of diameter) containing a single, large lipid droplet covering almost the entire cytoplasm area. They are specialized in the storage of lipids, primarily in the form of triacylglycerol, which are released as free fatty acids. In adult humans, are the dominant subtypes. The main depots are found in subcutaneous and visceral sites (Figure 9).

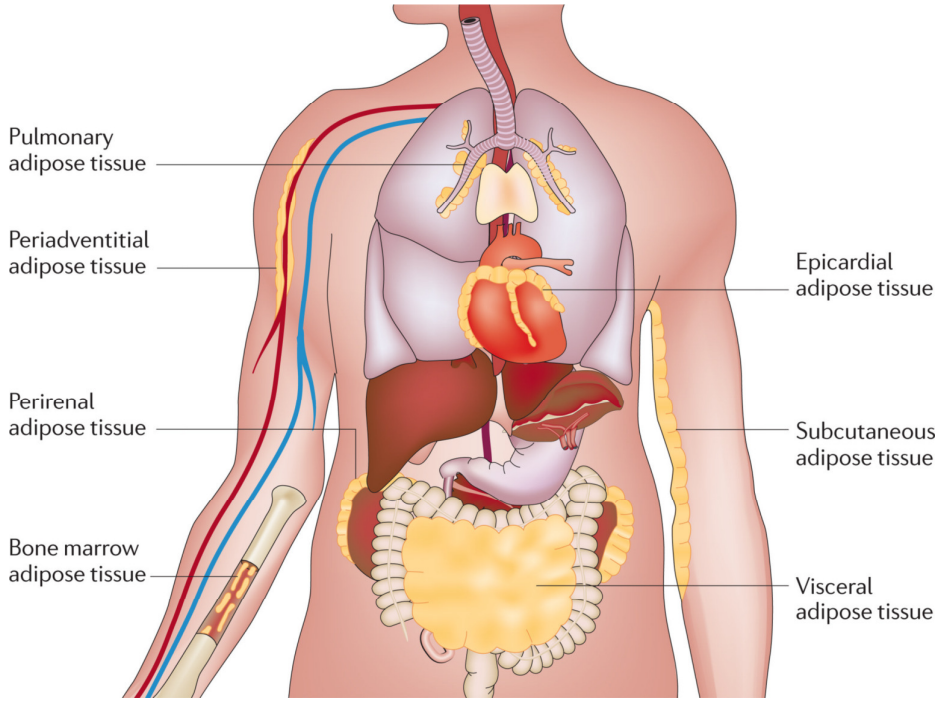


Figure 9: Adipose tissue depots. Adipose tissue is mainly found in subcutaneous and visceral depots (Nat Rev Immunol, 2010).

Visceral adipocytes are situated into the abdominal cavity between the organs (stomach, liver, intestines, kidneys, etc.). Visceral adipose tissue is including mesenteric, epididymal and perirenal white adipose tissue (Nagai et al, 2010). Unlike the subcutaneous adipocytes (Porter et al, 2009) visceral fat is strongly associated to obesity -related pathologies, like heart disease, stroke, and cancer (Landgraf et al, 2017).

Brown adipocytes are morphologically distinguishable from white adipocytes, as they are much smaller and display multiple small lipid droplets within their cytoplasm (Figure 8). Brown adipocyte is frequently found in small mammals like rodents. In humans is abundant in new-borns and infants, but is drastically reduced in adults (Van Marken Lichtenbelt et al, 2009).

Brown adipocytes exert a thermogenic function (Cannon et al, 2004).

Recently, it has been reported the existence of a third subtype of adipose tissue, consisting of brown-like adipocytes residing within white adipose

tissue. These adipocytes have been named “beige” or “brite” (brown in white) adipocytes (Vitali, 2012).

Adipose tissue functions as an endocrine organ (Kershaw et al, 2004), which plays a central role in regulating metabolic homeostasis. Dysfunctional endocrine properties of adipose tissue may result in disease-related alterations, including elevated release if inflammatory cytokines and increased infiltration of lymphocytes, macrophages, and stromal cells. The massive infiltration of macrophages into adipose tissue leads to chronic inflammation that not only modifies local metabolism, but also influences systemic energy homeostasis (Maury et al, 2007).

1.4.2 Adipose tissue and cancer

A large body of evidences have contributed to establish a strict association between adipose tissue and cancer in the contest of metabolic disorders such as obesity. Epidemiological studies have shown that obese patients have a drastically higher risk to develop cancer (Makki et al, 2013; Trevellin et al, 2015; Divella et al, 2016). In addition, higher concentration of pro-inflammatory cytokines has been found in the plasma of the obese-colorectal cancer patients compared to lean-colorectal cancer (Amor et al, 2016). These cytokines are likely to be released by adipose tissues located close to tumor cells (peritumoral adipose tissue) suggesting that obesity may exacerbate the tumor-promoting interaction between adipocytes and tumour cells (Conroy et al, 2016).

However, it still remains to be established whether a relevant pro-inflammatory effect might be exerted by peritumoral adipose tissue in non-obese patients with colorectal cancer.

In mice, it was found that adipocytes closely associated to a xenograft model of melanoma, exhibit an evident up-regulation of mouse cytokine including IL6, CXCL1, MCP1, MIP2 AND TIMP1 compared to distant non-tumoral adipose tissue (Wagner et al., 2012). In addition, peritumoral adipose tissue showed a reduced adipocyte size, vast fibrosis, increased angiogenesis and a dense macrophage infiltrate. Also, the CD11b+ macrophages were abundant

in peritumoral adipose tissue and resulted to overexpress Arg1, Nos2, Cd301, Cd163, Mcp1 and Vegf.

These results drive to hypothesize that the adipose tissue adjacent to tumor is part of a dynamic tumoral network that involves the cancer cells and their neighbouring cells (Amor et al, 2016) (Figure 10).

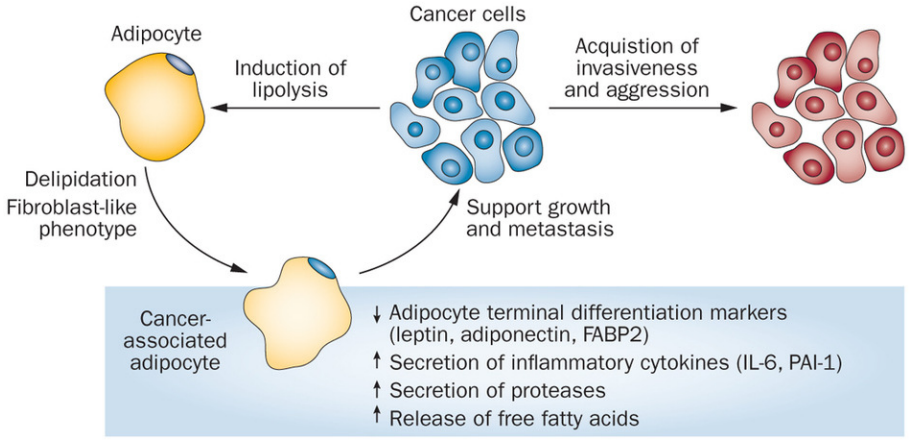


Figure 10. Reciprocal signalling between cancer-associated adipocytes and cancer cells.

According to the model depicted in the figure 10, cancer cells induce peritumoral adipocytes toward a partial de-differentiation with reduced lipid content and increased secretion of free fatty acid and pro-inflammatory cytokines, which, in turn, promote cancer cell aggressiveness. Macrophage recruited into the inflammatory foci at the tumor-adipocyte interface may further enhance this circuit, by producing pro-inflammatory cytokines themselves. Peritumoral adipose tissue lose the lipogenic ability due to a significant reduction in both lipoprotein lipase and fatty acid synthase gene expression and activity.

1.5 Aim of the work

Many studies have contributed to reveal the strong relationship between cancer and systemic disorders of adipose tissue, such as obesity and diabetes,

but almost nothing is known about the local interaction between cancer cells and peritumoral adipocytes in non-obese/non-diabetic patients.

In order to start bridge this gap we assess whether adipose tissue surrounding human colorectal tumors might be altered in terms of adipocyte morphology and inflammatory infiltration. Specifically we compare peritumoral and non-peritumoral adipose tissue (visceral fat distant from tumor lesion) for the adipocyte size and morphology, cell count of lymphocytes (CD3 positive cells, CD3+) and macrophages (CD68 positive cells, CD68+).

Chapter 2

Materials and methods

2.1. Patients

During my doctoral program until now, we have collaborated with Professor Giovanni De Manzoni surgeon and director of general surgery of oesophagus and stomach unit, at integrated university hospital of Verona.

Specimens, including peritumoral and non-peritumoral adipose tissue, were extracted from colon and stomach. Samples were taken off from patient with a gastrointestinal cancer that had not received chemotherapy, but had undergone surgical intervention such as subtotal/total gastrectomy or resection of the rectum. At the day of a surgical intervention, peritumoral/non peritumoral adipose tissue was carefully micro-dissected away from the surrounding tumor mass and from the distant fat of the same patient.

The peritumoral adipose tissue samples were collected from the fat very close to the tumor. Contrariwise, non-pertiumoral samples were taken from visceral fat surrounding the same organ of the tumor (either stomach or colon) but located distal from the tumor lesion. The patients had undergone subtotal or total gastrectomy, low anterior resection of the rectum, blind resection. All samples were conserved in formalin for at least one week, and then fixed with paraffin.

In the following part, we have the clinic card of patient, and we hide their names and give them a code for their privacy.

2.1.1. Patients with colorectal tumor

P1♂ (29/03/1945):

- Tumor in the left flexure of the colon,
- Intervention 29/10/2015: resection of transverse/ flexure left with packaging descending-transverse end-to-side anastomosis,
- Histological examination: less than 50% mucinous aspects of the large intestine with adenocarcinoma poorly differentiated G3,
- Maximum diameter: 6.5 cm,
- Infiltrating: the visceral wall-thickness and perivisceral fat (pT3),
- Progress: neoplastic expansive type,

- Vascular and lymphatic invasion: not evident,
- Perineural invasion: not evident,
- Tumor budding: absent,
- 24 pervisceral lymph-nodes free of metastases (0/24),
- Last Follow-up 09/09/2016: patient still alive, no disease recurrence.

P2 ♂ (25/12/1936):

- Adenocarcinoma ileocecal,
- Intervention 25/11/2015: blind resection with anastomosis packaging ileum-ascending lateral chest,
- Histological examination: mucinous aspects of the large intestine with adenocarcinoma poorly differentiated G3,
- Maximum diameter: 3.5 cm,
- Infiltrating: the visceral wall-thickness, the perivisceral fat and serous (pT4),
- Progress: neoplastic expansive type,
- Vascular and lymphatic invasion: not evident,
- Perineural invasion: not evident,
- Tumor budding: present,
- Last Follow-up: the patient died of postoperative complications.

P3 ♂ (30/09/1923):

- Adenocarcinoma of the rectum,
- Intervention 03/09/2016: low anterior resection with colorectal anastomosis termino-lateral and cholecystectomy,
- Histological examination: Cholecystitis free from neoplastic infiltration with chronic framework and adenomyosis foci,
- Moderately differentiated adenocarcinoma of the large intestine, with <50% of aspects mucinous,
- Infiltrating tumor: the bowel full thickness , the perivisceral fat,
- 17 lymph nodes free of metastases + 6 perivisceral lymph nodes free of metastases (N tot 0/23),
- Last Follow-up in November 2016: the patient still alive does not relapse

2.1.2 Patients with gastric tumor

P4 ♀ (10/08/1941):

- Adenocarcinoma gastric antrum,
- Intervention 11/10/2015: subtotal gastrectomy with lymphadenectomy D1, and packaging of gastro-jejunal anastomosis, cholecystectomy, resection of liver metastases IV segment,
- Histological examination: Gastric adenocarcinoma, tubular section moderately differentiated,
- The maximum diameter: 5 cm,
- Infiltrating tumor: the visceral wall-thickness with extension to serous tunic and packet nodal epigastric (pT4),
- Carcinomatosis vascular-lymphatic: present,
- Carcinomatosis perineural: present,
- Chronic cholecystitis, cholelithiasis, free of neoplastic involvement,
- Metastases in seven out of thirteen lymph nodes of the small curve (7/13), in a lymph node right gastroepiploic artery (1/1), in two out of five gastric artery lymph nodes left (2/5), an artery lymph node gastroepiploic left free of metastases (0/1) (N tot 10/20),
- Hepatic metastases of adenocarcinoma,
- Last Follow-up April 2016: hepatic recurrence.

P5 ♀(08/08/1941):

- Adenocarcinoma gastric antrum,
- Intervention 20/11/2015:subtotal gastrectomy with lymphadenectomy D1, and packaging of gastro-jejunal anastomosis, removal of three diaphragmatic nodules and macroscopic residual of transverse mesocolon,
- Histological examination: Gastric adenocarcinoma type mucinous, poorly differentiated,
- The maximum diameter of 6.5 cm,

- Infiltrating tumor: the visceral wall-thickness, the perivisceral fat and serous (pT4),
- Carcinomatosis vascular-lymphatic: present,
- Carcinomatosis perineural: present,
- Metastases in two out of four perivisceral lymph nodes (2/4),
- Lymph node metastasis and perilinfonodali in one in eight lymph nodes of the small curve (2/8), in six gastroepiploic left artery lymph nodes (6/6), in three gastroepiploic left artery lymph nodes (3/3) in two lymph nodes (2/2), in three out of four lymph nodes (3/4), in seven out of ten left gastric artery lymph nodes (7/10) (N tot 25/37),
- Fragments fibromuscolar with infiltrating adenocarcinoma,
- Omentum free of neoplastic involvement,
- Last Follow-up in August 2016: living patient

P6♂ (30/05/1946):

- Adenocarcinoma of the lesser curvature of the stomach
- Intervention 17/02/2016: total gastrectomy and D2 lymphadenectomy abdominal extended to para-aortic,
- Histological examination: Gastric adenocarcinoma, poorly differentiated,
- The maximum diameter: 6 cm,
- Infiltrating tumor: the visceral wall-thickness with extension to serous tunic (pT4),
- Carcinomatosis vascular-lymphatic: present,
- Carcinomatosis perineural: present,
- Perivisceral lymph node metastases in three (3/3),
- Omentum free of neoplastic involvement,
- Last Follow-up in February 2017: the patient still alive does not relapse.

P7♂(23/10/1948):

- Adenocarcinoma gastric antrum,
- Intervention 02/11/2016: subtotal gastrectomy with lymphadenectomy D1, and packaging of gastro-jejunal anastomosis, cholecystectomy,

- Histological examination: Gastric adenocarcinoma, mixed (tubular and mucinous), moderately differentiated,
- The maximum diameter: 3 cm,
- Infiltrating tumor: the visceral wall-thickness with extension to serous tunic (pT4),
- Carcinomatosis vascular-lymphatic: present
- Carcinomatosis perineural: not evident
- A lymph node metastases with adenocarcinoma (1/2), two localization of lymph nodes with adenocarcinoma (2/2), two gastroepiploic artery lymph nodes free of metastases (0/2) (N tot 3/6),
- Omentum free of tumor localization,
- Chronic cholecystitis,
- Last Follow-up in November 2016: the patient still alive, followed by 3 cycles adjuvant radiotherapy (45Gy) with radio sensitizing chemotherapy with capecitabine interrupted for severe thrombocytopenia,

P8 ♂ (04/08/1939):

- Gastric Adenocarcinoma of the small curve,
- Intervention 22/03/2016: total gastrectomy with D2 lymphadenectomy and packaging of gastro-jejunal anastomosis,
- Histological examination: Gastric adenocarcinoma, Tubular and poorly cohesive, moderately-poorly differentiated, Mixed (intestinal or diffuse),
- The maximum diameter: 4 cm,
- Infiltrating the visceral wall-thickness and adipose tissue perivisceral coming throughout the vicinity, apparently without infiltrate the tunica serosa (pT3),
- Carcinomatosis vascular-lymphatic: present,
- Carcinomatosis perineural: present,
- Four perivisceral lymph nodes (small curve) and 3 lymph nodes perivisceral (big curve), free of metastases (0/7), five right pericardial lymph nodes (0/5), nine claims pericardial lymph nodes (0/9), seventeen lymph nodes of the small curve (0/17) ten lymph nodes of the short gastric vessels (0/10), fourteen gastroepiploic left artery lymph nodes (0/14), twenty-two artery

gastroepiploic right lymph nodes (0/22), thirteen lymph nodes (0 / 13), eleven left gastric artery lymph nodes (0/11), seventeen common hepatic artery lymph nodes (0/17), four nodes of the celiac trunk (0/4), eight of the splenic artery proximal lymph nodes (0 / 8), a splenic artery distal lymph node (0/1), a hepatic artery lymph own (0/1) and four lymph nodes (0/4) free of metastases.

- Flap of momentum free of neoplastic involvement and lymph node metastasis-free (0/1) (N tot 0/144).
- Last Follow-up in July 2016: the patient still alive does not relapse.

All samples were conserved and fixed in 10% of formalin solution for at least one week then they were included in paraffin, once the tissue is embedded in paraffin, it is stable for many years.

2.2 Paraffin inclusion:

In this procedure, the tissue was dehydrated through a series of graded ethanol baths to displace the water, and then was infiltrated with paraffin, the technique is as follows:

- wash the tissue with the phosphate buffered saline solution (PBS): to remove excess of formalin,
- dehydrate with different concentrations of ethyl alcohol in ascending scale 70°, 80°, 90°, and overnight at 100°: to remove the aqueous component, which does not allow the entry of the paraffin in the tissue,
- transfer the tissue in xylene: to permit absolute alcohol replacement,
- Infiltration with paraffin at 58- 60°: to replace the xylene inside the histological piece with the paraffin, his hardness of the solid paraffin allows us to realize the histological sections.

The inclusion in paraffin was carried out at room temperature. After that, the cooled block of paraffin was ready for microtome cut, and to slice the piece at 7 mm of thickness. Then we leave slide for a while to dry in the room.

2.3 Hematoxylin and eosin

Hematoxylin and eosin stain are still essential for recognizing various tissue types and the morphologic changes that form the basis of contemporary cancer diagnosis. The stain has been unchanged for many years because it works well with a variety of fixatives and displays a broad range of cytoplasmic, nuclear, and extracellular matrix features.

Hematoxylin has a deep blue-purple color and stains nucleic acids by a complex, incompletely understood reaction. Eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining (Fischer et al, 2008).

Well-fixed cells show considerable intranuclear detail. Nuclei show varying cell-type- and cancer-type-specific patterns of condensation of heterochromatin (hematoxylin staining) that are diagnostically very important. Nucleoli stain with eosin. If abundant polyribosomes are present, the cytoplasm will have a distinct blue cast. The Golgi zone can be tentatively identified by the absence of staining in a region next to the nucleus. Thus, the stain discloses abundant structural information, with specific functional implications. Hematoxylin, generally without eosin, is useful as a counterstain for many immunohistochemically procedures.

This protocol describes hematoxylin and eosin staining of tissue.

Eosin Y (1% aqueous solution; Sigma), Hematoxylin, Mayer's (Sigma), Ethanol (95%, 100%), Mounting medium (Entellan, Sigma), Xylene, Coverslips.

To hydrate the tissue, the sections of paraffin embedded tissues are fixed in xylene, then in alcohol and finally in distilled water.

We Dip the slide into a Mayer's hematoxylin solution and agitate for 30-40sec.

We Rinse the slide in H2O for 1 min and we estimate the staining intensity at this point.

We stain the slide with 1% eosin Y solution for 10-30 sec with agitation.

We dehydrate the sections with one of change of 80% alcohol, one change of 95%, and two changes of 100% alcohol for 2min each. Some colorimetric substrates dissolve in alcohol.

We extract the slides from the alcohol with two changes of xylene for 5min.

We add one or two drops of mounting medium and we cover it with a coverslip.

2.4 Immunohistochemistry

The immunohistochemistry technique is able to identify specific molecules or structures of the intra and extra cellular compartment, is based on the principle of antigen-antibody conjugation, then in addition with detection systems (enzymatic) that the reaction took place to make visible at the microscope. In our case the reaction between antigen-antibody is detected with immunoperoxidase method and the sections will be observed under the microscope Olympus BX51, equipped with KY-F58 CCD camera (Merigo et al, 2008).

General Immunostaining protocol for paraffin peritumoral adipose tissue section is the follow:

- Deparaffinize the tissues as outlined below:
  - xylene : 2 changes, 5 min each
  - 100% alcohol: 2 changes, 2 min each
  - 95% alcohol: 2 washes, 2 min each
  - 70% alcohol: 2 changes, 2 min each
  - distilled H20: 1 change, 2 min
- Antigen retrieval: antigens can become masked during the preparation of the tissue. In our case, we use the sodium citrate buffer method (pH 6.0) in microwave for a total of 15 min. Slides must be cooled for 20-30 min before proceeding to immunostaining. Wash sections in PBS, 3 changes, 5 min each.
- Block endogenous peroxidase activity with 3% hydrogen peroxide (H_2O_2) in PBS for 10 min. Presence of endogenous peroxidase enzymes in the tissue can

severely limit the interpretation of immunoperoxidase staining. Wash slides in double distilled water for 5 min once.

- Rinse in PBS for 5 min with two changes.
- Block nonspecific binding with 5% normal goat or rabbit serum for 20 min at room temperature.
- Incubate with specific primary and secondary antibodies and precede for the detection with 3, 3'-diaminobenzidine (DAB) solution (Menon et al, 2015).

Antibody used in this study

CD68 (anti-macrophage): is a macrophage marker frequently used in many studies. The sections are incubated overnight with monoclonal mouse anti-human CD68 (1: 150, Thermo scientific).

Secondary Antibody: Incubate sections in Goat anti-mouse antibody (1: 200) diluted in blocking solution for 30 min.

Counter stain with hematoxylin and dehydrate sections as follows:

- Hematoxylin: 30-40 sec
- PBS: 2 changes, 3 min
- 70% Ethanol: 1 change, 2 min.
- 95% Ethanol: 1 change, 2 min.
- 100% Ethanol: 1 change, 2 min.
- Xylene, 3 changes, 2 min.

Mount section with Entellan and coverslip.

CD3 (anti-lymphocytes): is a polyclonal rabbit anti-human (ready to use, Thermo scientific), the sections are incubated overnight.

Secondary Antibody: Incubate sections in Goat anti-rabbit antibody (1: 200) diluted in blocking solution for 30 min.

Counter stain with hematoxylin and dehydrate sections as follows:

- Hematoxylin: 30-40 sec
- PBS: 2 changes, 3 min
- 70% Ethanol: 1 change, 2 min.
- 95% Ethanol: 1 change, 2 min.

- 100% Ethanol: 1 change, 2 min.
- Xylene, 3 changes, 2 min.

Mount section with Entellan and coverslip.

2.5 Microscope:

Immunoreactivity was studied with light microscopy under bright-field illumination. Quantitative evaluation was based on counts of CD68-CD3 and cells, as well as densitometric analysis of the immunosignal intensity.

For our analysis we have using a JVC CCD KY-F58 digital camera connected to the microscope and the image analysis software Image Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD).

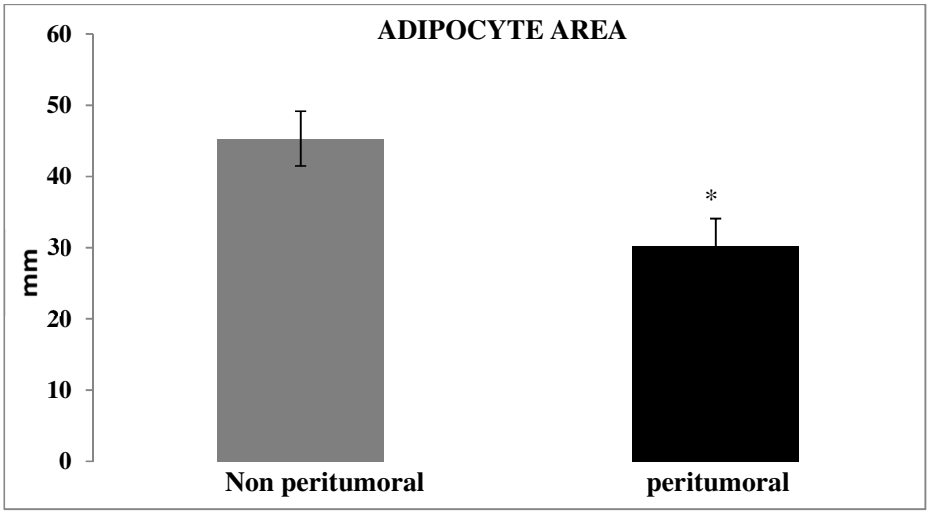
For each adipocyte, the area was measured. The Ferret's diameter (or Ferret Diameter FD) is a measure of an object size along a specified direction.

Chapter 3

Results

3.1 Adipocyte size and morphology

A



B

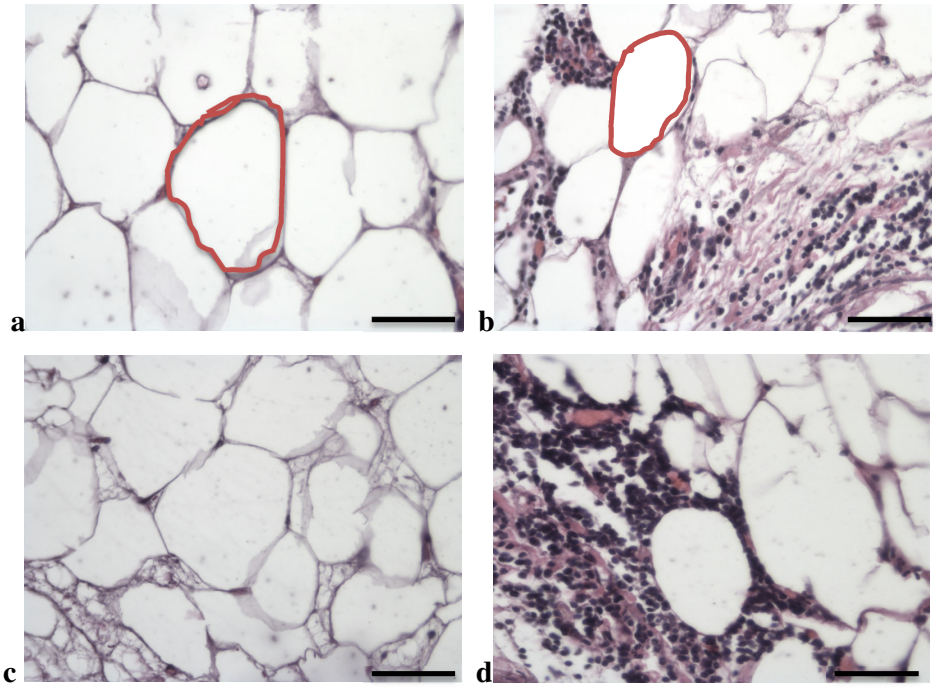


Figure 11: A: The histogram shows mean values ± SE of adipocyte size in peritumoral/non peritumoral adipose tissue, * p<0,05. B: The section of non-peritumoral (a and c) and peritumoral (b and d) adipose tissue stained with Hematoxylin Eosin. Red lines are indicative of the method used to measure

adipocyte size. On each slide, the contour of every adipocyte was manually lined and the inner area was measured using ImageJ software. Scale bar: 25um; all the other images are at the same magnification (20X).

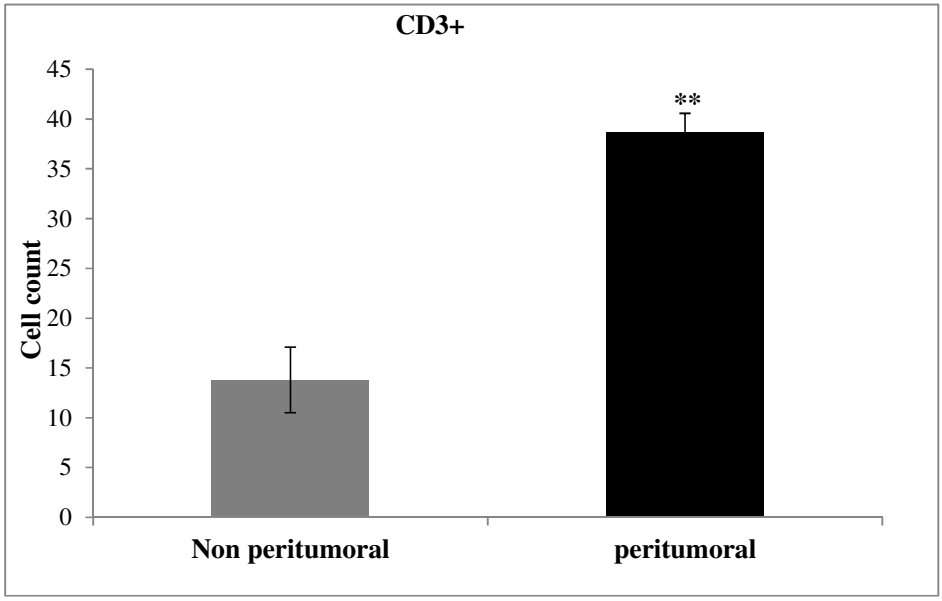
The mean size was 45±4 mm for non peritumoral adipocytes and 30±1,9 mm for peritumoral adipocytes (p=0.04, Student t-test) (Figure 11 A). Peritumoral adipocytes were significantly smaller than their normal (non peritumoral) counterparts (Figure11 ,B).

The adipocyte in non peritumoral fat (a,c) was characterized by a polyhedral and homogenous aspect while peritumoral adipocytes (b,d) were morphologically heterogenic.

There was no difference in the total number of adipocytes between non-peritumoral and peritumoral adipose tissue groups (data not shown).

3.2 Lymphocyte T cells /CD3+

A



B

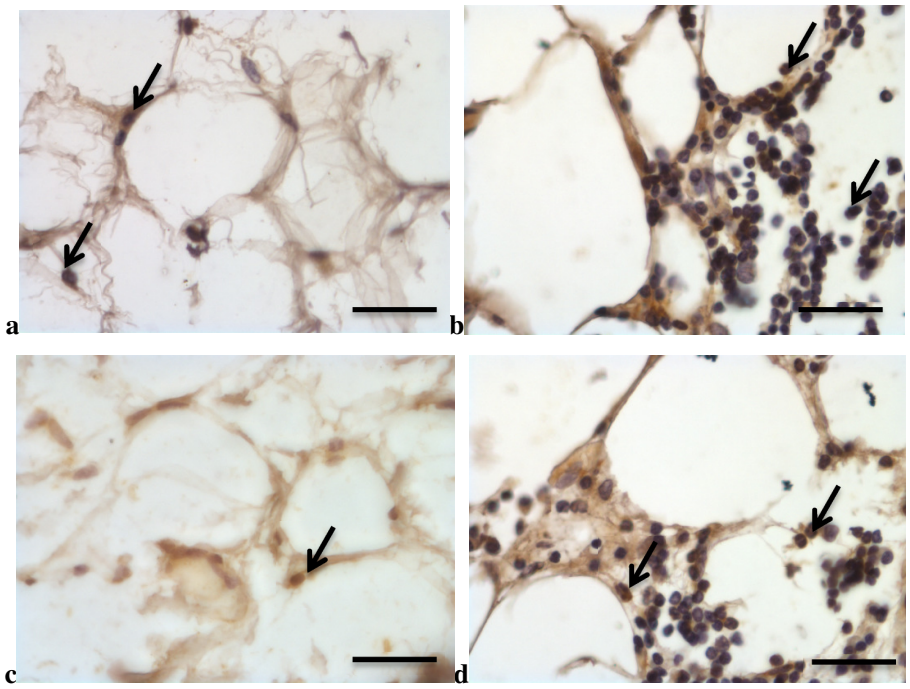


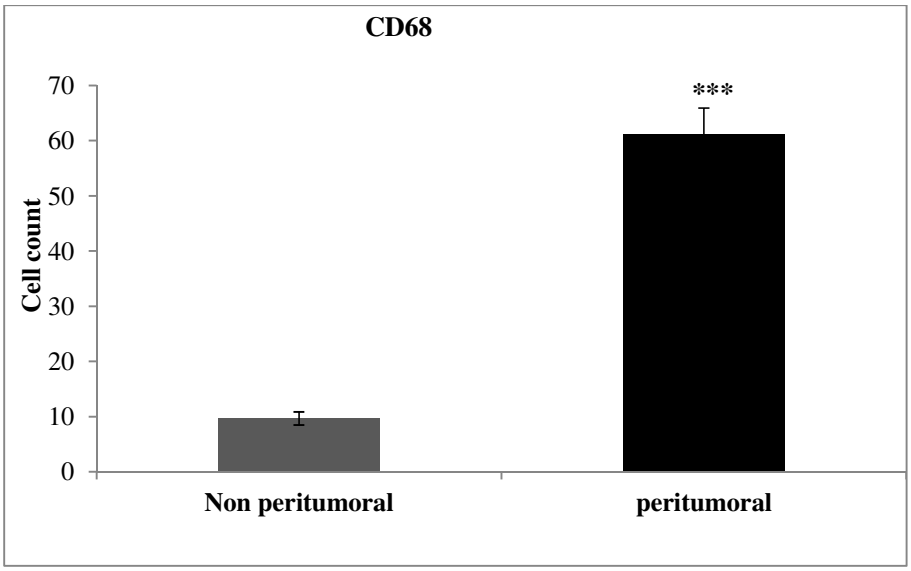
Figure 12: A: The histogram show mean values ± SE of lymphocyte cells T activated in peritumoral/non peritumoral adipose tissue, **p<0,01. B: sections of adipose tissue non peritumoral (a,c) and peritumoral (b,d) with CD3+immunohistochemistry. Black arrows indicate labeled lymphocyte cells. Scale bar: 25um; all the other images are at the same magnification (40X).

The number of activated CD3+ T cells lymphocytes (Figure 12) were much higher in peritumoral tissue (38,8±1,8 cells count) than in non peritumoral adipose tissue (13,8±3,3; P=0.009 Student's t test).

The lymphocytes infiltrated in peritumoral fat (Figure 12B, b and d) were mainly grouped in clusters of highly dense cellularity.

3.3 Macrophage /CD68+

A



B

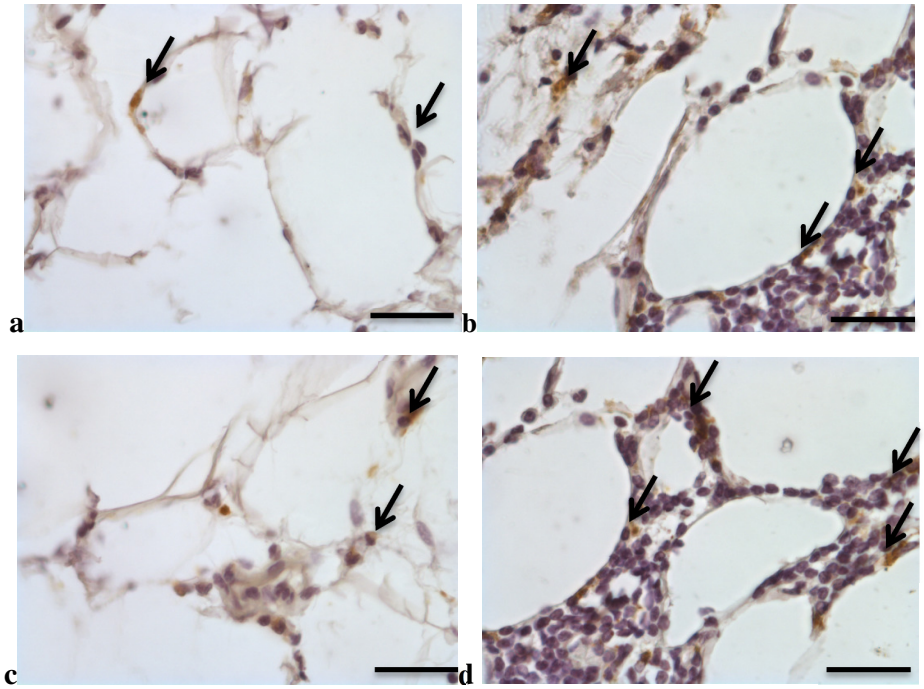


Figure 13: A: The histogram shows mean values ± SE of macrophage cells activated in peritumoral/non peritumoral adipose tissue, ***p<0,001. B: sections of adipose

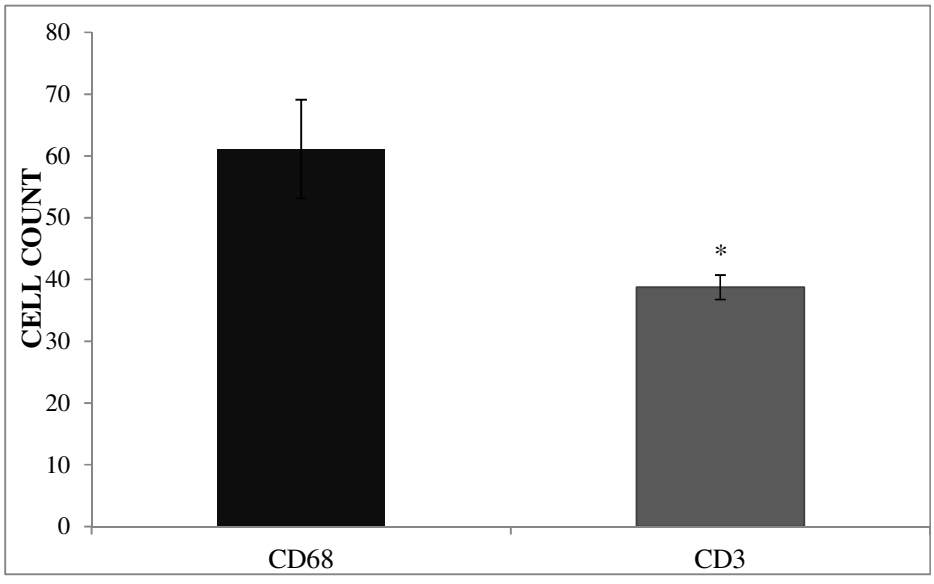
tissue peritumoral and non peritumoral with CD68+immunohistochemistry. Black arrow indicates labelling macrophage cells. B: sections of adipose tissue non peritumoral (a,c) and peritumoral (b,d) with CD68+immunohistochemistry. Black arrow indicates labelling macrophage cells. Scale bar: 25um; all the other images are at the same magnification (40X).

In peritumoral fat, a higher number of CD68+ macrophages were found (61,1±4,8 cells count activated) compared to non peritumoral adipose tissue (9,7±1,2 cells count activated; P=0,00026 Student's t test).

As for activated lymphocytes, CD68+ macrophages were organized in dense clusters surrounding adipocytes, while in the non peritumoral adipose tissue (b.d) the presence of these cells were extremely scarce and scattered around the adipocytes.

3.4 Macrophage Vs Lymphocytes in peritumoral adipose tissue

A



B

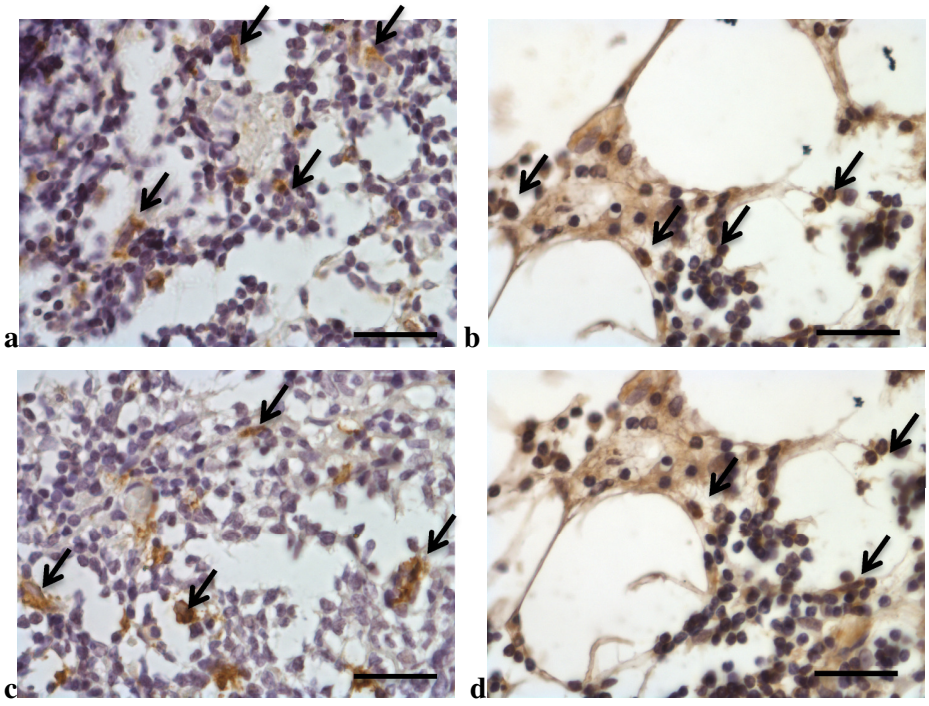


Figure 14: A: The histogram show mean values ± SE of macrophage and lymphocytes cells activated in peritumoral adipose tissue, *p<0,05. B: sections of adipose tissue peritumoral with CD68+ and Cd3+ immunohistochemistry. Black arrows indicate labelling macrophage and lymphocyte cells. B: sections of adipose tissue peritumoral with macrophage activation (a,c), (b,d) sections of adipose tissue peritumoral with lymphocytes. Scale bar: 25um; all the other images are at the same magnification (40X).

In literature, in the tumor microenvironment, the macrophages comprise the dominant portion of the leukocyte population (Wagner et al, 2012). We found that in peritumoral adipose tissue, the number of activated macrophages (Cd68+) was almost double the number of lymphocytes (Cd3+). Specifically, macrophages were 61,11±4,8/field, while lymphocytes were 38,8±1,8 (p=0,04).

Chapter 4

Discussion

Cancer associated adipocytes (CAAs) may contribute in tumor progression by multiple ways. First, peritumoral adipocytes have been shown to provide a metabolic support to the aberrant bioenergetics demands of cancer cells. The adipocyte-rich tissue surrounding tumor cells offers an easily accessible reservoir of lipids. *In vitro* experiments demonstrate that secreted paracrine signals from cancer cells induce lipolysis in adipocytes, causing them to release free fatty acids (Dirat B. 2011). In human and animal models of cancer, body composition evaluation and morphological analysis reveals adipose atrophy and presence of smaller adipocytes. This fat loss occurs in both visceral and subcutaneous depots. In addition, increased lipolysis and fat oxidation, decreased lipogenesis, impaired lipid deposition and adipogenesis, as well as browning of white adipose tissue may underlie adipose atrophy in cancer (Ebadi et al, 2014). In animal model of melanoma, it has been reported that peritumoral adipocytes have reduced size and extensive fibrosis (Wagner et al, 2013).

We found analogous results in human gastrointestinal cancer. Adipocytes surrounding either gastric or colorectal primary tumors display a significantly reduced size compared to adipocytes located distal from tumor lesion, which is likely the result of an increased lipolysis. Nevertheless, all adipocytes surrounding tumors still maintained a white adipocytes-like morphological phenotype, with a single, large lipid droplet covering almost the entire cytoplasm area.

On the other hand, peritumoral adipose tissue may contribute to tumor growth by biochemical interactions at the interface with cancer cells. Tumor invades stromal compartments that are rich in adipose tissue, and adipocytes function as endocrine cells to critically shape the tumor microenvironment. Multiple studies in the last decades have investigated the epidemiological association between cancer and metabolic and adipose tissue-related pathologies, such as obesity and diabetes (Park et al, 2014). These studies converged to establish the notion that metabolic disorders of adipose tissue, especially obesity, strongly contribute to tumor initiation and progression. However, the underlying mechanisms linking obesity to neoplastic diseases remain poorly

understood. Adipose tissue has long been considered a mechanically supportive storage of energy in the form of triglycerides. Nevertheless, it is currently recognized as one of the most important endocrine organs, which play a leading role in regulating interactions between metabolic and immune systems (Stern et al, 2016). Several lines of evidences suggest that the tumor promoting role of adipose tissue relies, at least in part, on systemic and paracrine release of cytokines by fat cells (Park et al, 2011), which cooperate to boost cancer cell proliferation and metastatic dissemination. The endocrine function of adipose tissue is alternated in obesity and the aberrant systemic release of cytokines by hypertrophic adipocytes has been extensively investigated as one of the major causes of the increased tumor burden in obese patients. The paracrine release of cytokines by peritumoral adipocyte have also been occasionally described and suggested as a potential modular of tumor biology. But, again, these few studies are limited to obese patients (Gnerlich et al, 2013), and are mainly aimed at identify microregional differences in tumor-promoting cytokine production between adipose sites nearby and distal from primary tumors in the context of obesity.

Our major aim here was to start assessing whether also in non-obese patients, peritumoral adipocytes may show any signs of increased inflammation, likely indicative of an increased release of pro-inflammatory cytokines at tumor-adipose tissue interface. We focused on gastrointestinal cancer since it is one of the most lethal neoplasms and assessed the frequency of activated lymphocytes and macrophages in comparison with non-peritumoral fat, based on the immunohistochemically staining with two respective highly specific markers that are CD3 for lymphocytes and CD68 for macrophages. Cd3 is known to be linked to the membranes of all mature T-cells, although it does appear to be present in small amounts in Purkinje cells (Amicarella et al, 2015). This specificity combined with the presence of CD3 at all stages of T-cell development, makes it a useful immunohistochemical marker for T-cells in tissue sections, and may hold an important key to unlocking effective cancer immunotherapy. Our results demonstrate that the level of lymphocytes

infiltrates in peritumoral fat is much higher than in non-peritumoral fat in gastrointestinal cancer.

The Accumulation of T cells in adipose tissue precedes macrophage infiltration causing a chronic low-grade inflammation (Quattromoni 2012). CD68+ cells were highly enriched in peritumoral adipose tissue. Generally, the macrophages are the dominant leukocyte population found in the tumor microenvironment. Accumulating evidence suggests that this tumor-associated macrophage actively promote all aspects of tumor initiation, growth, and development.

In conclusion, our results provide clinical evidences in support of the emerging notion that adipocytes at the tumor-stroma interface participate in a highly complex vicious cycle organized by cancer cells to promote tumor progression. Specifically our results suggest that peritumoral adipocytes might play a significant contribution to enhance tumor burden by fueling cancer cell's metabolic demands and providing mitogenic signals throughout the paracrine release of pro-inflammatory cytokines.

.

Chapter 4

References

References

Abrams A, Siegfried N. The Pan African Clinical Trials Registry: year one data analysis of the only African member of the World Health Organization Network of Primary Registries. 2010. J Evid Based ed.3(4):195-200.

Amedeo A, Bella C, Silvestri E, Prisco D, and D'Elios M. T Cells in Gastric Cancer. Clinical and Developmental Immunology. 2012. 10 pages.

Amor S, Iglesias-de la Cruz MC, Ferrero E, García-Villar O, Barrios V, Fernandez N, Monge L, García-Villalón AL, Granado M. Peritumoral adipose tissue as a source of inflammatory and angiogenic factors in colorectal cancer. 2016. Int J Colorectal Dis. 31:365–375.

Amicarella F, Muraro MG, Hirt C, Cremonesi E, Padovan E, Mele V, Governa V, Han J, Huber X, Droeser RA, Zuber M, Adamina M, Bolli M, Rosso R, Lugli A, Zlobec I, Terracciano L, Tornillo L, Zajac P, Eppenberger-Castori S, Trapani F, Oertli D, Iezzi G. Dual role of tumour-infiltrating T helper 17 cells in human colorectal cancer. 2015. GI Cancer. 0:1–13.

Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. 2005. Cancer Cell. Mar;7(3):211-7.

Bell D, Bell A, Roberts D, Weber RS, El-Naggar AK. Developmental transcription factor EN1--a novel biomarker in human salivary gland adenoid cystic carcinoma. 2012. Cancer. 1;118(5):1288-92.

Bibbins-Domingo K, Grossman DC, Curry S J, Davidson KW, Epling JW, García F AR, Gillman MW, Harper DM, Kemper AR, Krist AH, Kurth AE, Landefeld CS, Mangione CM, Owens DK., Phillips WR, Phipps MG, Pignone MP, Siu AL. Screening for Colorectal Cancer. 2016. JAMA. 315 (23): 2564–75.

Birbrair A, Zhang T, Wang ZM, Messi ML, Olson JD, Mintz A, Delbono O. Type-2 pericytes participate in normal and tumoral angiogenesis. 2014. American Journal of Physiology. Cell Physiology. 307 (1): C25–38.

Bjelakovic G, Nikolova D, Simonetti RG, Gluud C. Systematic review: primary and secondary prevention of gastrointestinal cancers with antioxidant supplements. 2008. Aliment Pharmacol Ther. 28(6):689-703.

Carneiro ZA, Biazzotto JC, Alexiou AD, Nikolaou S. Nitric oxide photorelease from a trinuclear ruthenium nitrosyl complex and it's in vitro cytotoxicity against melanoma cells. 2014. J Inorg Biochem. 134:36-8.

Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. 2004. Physiol RevJan;84(1):277-359.

Chang AH, Parsonnet J. Role of Bacteria in Oncogenesis. 2010. Clinical Microbiology Reviews. 23 (4): 837–857.

Catalán V, JavierGómez-A, Amaia R and Gema F. Adipose tissue immunity and cancer. 2013. Front Physiol. 4:275.

Chmiela M, Karwowska Z, Gonciarz W, Allushi B, Stączek P. Host pathogen interactions in Helicobacter pylori related gastric cancer. 2017. World J Gastroenterol. Mar 7;23(9):1521-1540.

Conroy MJ, Margaret RD, Donohoe CL, and Reynolds JV. Obesity-associated cancer: an immunological perspective. 2016. Proceedings of the Nutrition Society. 75, 125–138 .

D'Esposito V, Liguoro D, Ambrosio MR, Collina F, Cantile M, Spinelli R, Raciti GA, Miele C, Valentino R, Campiglia P, De Laurentiis M, Di Bonito M, Botti G, Franco R, Beguinot F, Formisano P. Adipose microenvironment promotes triple negative breast. 2016. Cancer cell invasiveness and dissemination. Oncotarget, Vol. 7, No. 17.

Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, Wang YY, Meulle A, Salles B, Le Gonidec S, Garrido I, Escourrou G, Valet P, Muller C. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. . 2011. Cancer Res. 71(7):2455-65.

Divella R, De Luca R, Abbate I, Naglieri E, Daniele A. Obesity and cancer: the role of adipose tissue and adipo-cytokines-induced chronic inflammation. 2016. Journal of Cancer .7(15): 2346-2359.

Ebadi M and Vera C. Mazurak. Evidence and Mechanisms of Fat Depletion in Cancer. 2014. Nutrients. 6, 5280-5297.

Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and eosin staining of tissue and cell sections. 2008. pdb.prot4986. doi: 10.1101/pdb.prot4986.

Galizia G, Sforza V, Gambardella V, Fabozzi A, Maddalena Laterza M, Andreozzi F, Ventriglia J, Savastano B, Mabilia A, Lieto E, Ciardiello F, and De Vita F. Treatment of gastric cancer. 2014. World J Gastroenterol. 20(7): 1635–1649

Gelderblom H, Hogendoorn PC, Dijkstra SD, Van Rijswijk CS, Krol AD, Taminiau AH, Bovée JV. The clinical approach towards chondrosarcoma. 2008. Oncologist. 13(3):320-9.

Gnerlich JL, Katharine A, Yao PS, Fitchev RA. Goldschmidt M, Bond C, Mona C, and Susan E. Crawford. Peritumoral Expression of Adipokines and Fatty Acids in Breast. 2013. Cancer. Ann Surg Oncol. 20:S731–S738.

Gospodarowicz MK, Miller D, Patti A, Groome M, Frederick L, Greene PA, Logan M.P.H, Sobin LH, The Process for Continuous Improvement of the TNM-Classification. The UICC TNM Project. 2004. Cancer. 1, / Volume 100 / Number 1.

Haggstrom. Nurses' opinions about a web-based distance course in a specialist education programme for the care of older people: a questionnaire study. 2009. Journal compilation, 177-184.

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. 2011. Cell.144(5):646-74.

Hardaway AL, Herroon MK, Rajagurubandara E, Podgorski I. Bone marrow fat: linking adipocyte-induced inflammation with skeletal metastases. 2014. Cancer Metastasis Rev. 33(2-3):527-43.

Hinrichs CS, Rosenberg SA. Exploiting the curative potential of adoptive T-cell therapy for cancer. 2014. Immunol Rev. 257(1):56-71.

Jeffrey S , Hochhauser D. Cancer and its Management. 2013. p. 43, 2013 (6th edn).

Jeffrey BR, Chemo-radiotherapy for localized pancreatic cancer: increased dose intensity and reduced acute toxicity with concomitant radiotherapy and protracted venous infusion 5-fluorouracil. 2016. Int J Radiat . Oncol Biol Phys. 40(1):93-9.

Jochems C, Schlom J. Tumor-infiltrating immune cells and prognosis: the potential link between conventional cancer therapy and immunity. 2011. Exp Biol Med. 236(5):567-79

Kershaw E, and Jeffrey S. Adipose Tissue as an Endocrine Organ. 2004. The Journal of Clinical Endocrinology & Metabolism. 89(6):2548–2556.

Landgraf K, Schuster S, Meusel A, Garten A, Riemer T, Schleinitz D, Kiess W, Körner A. Short-term overfeeding of zebrafish with normal or high-fat diet as a model for the development of metabolically healthy versus unhealthy obesity. 2017. BMC Physiol. 17(1):4.

Lemoine FJ, Marriott SJ. Accelerated G(1) phase progression induced by the human T cell leukemia virus type I (HTLV-I) Tax oncoprotein. 2001. J Biol Chem. 276(34):31851-7.

Makki K, Froguel P, and Wolowczuk I. Adipose Tissue in Obesity-Related Inflammation and Insulin Resistance: Cells, Cytokines, and Chemokines. 2013. ISRN Inflammation., Article ID 139239, 12 page.

Majka SM, Barak Y, Klemm DJ. Concise review: Adipocyte origins: weighing the possibilities.

Maury E, Ehala-Aleksejev K, Guiot Y, Detry R, Vandenhooft A, Brichard SM. Adipokines oversecreted by omental adipose tissue in human obesity. 2007. Am J Physiol Endocrinol Metab. 293(3):E656-65.

Mbeunkui F and Donald J. J Jr. Cancer and the tumor microenvironment. 2009.63(4): 571–582.

Menon U, Griffin M, Gentry-Maharaj A. Ovarian cancer screening--current status, future directions. 2015. Gynecol Oncol. 132(2):490-5.

Merigo F, Benati D, Galiè M, Crescimanno C, Osculati F, Sbarbati A. Immunohistochemical localization of cystic fibrosis transmembrane regulator and clara cell secretory protein in taste receptor cells of rat circumvallate papillae. 2008. Chem Senses. 33(3):231-41.

Müller J, Ehlers A, Burkhardt L, Sirma H, Steuber T, Graefen M, Sauter G, Minner S, Simon R, Schlomm T, Michl U. Loss of pSer2448-mTOR expression is linked to adverse prognosis and tumor progression in ERG-fusion-positive cancers. 2013. Int J Cancer. 132(6):1333-011. Stem Cells. 29(7):1034-40.

Nagai H, Matsui T, Kanayama M, Momiyama K, Shizawa K, Wakui N, Shinohara M, Watanabe M, Iida K, Ishii K, Igarashi Y, Sumino Y. Hepatotoxicity of intra-arterial combination chemotherapy in patients with liver cirrhosis and advanced hepatocellular carcinoma. 2010. Cancer Chemother Pharmacol66(6):1123-9

Ouchi N, Parker JL, Lugus JJ. and Walsh K. Adipokines in inflammation and metabolic disease. 2011. Nat Rev Immunol. 11(2): 85–97.

Porter SA, Massaro JM, Hoffmann U, Vasan RS, O'Donnel CJ, Fox CS. Abdominal subcutaneous adipose tissue: a protective fat depot? 2009. Diabetes Care. 32(6):1068-75.

Quatromoni J G, Evgeniy E. Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. 2012. Am J Transl Res;4(4):376-389.

Ruddon, Raymond W. Cancer biology. 2007. (4th ed.). Oxford: Oxford University Press. p. 223.

Scartozzi M1, Bearzi I, Pierantoni C, Mandolesi A, Loupakis F, Zaniboni A, Catalano V, Quadri A, Zorzi F, Berardi R, Biscotti T, Labianca R, Falcone A, Cascinu S. Nuclear factor-kB tumor expression predicts response and survival in irinotecan-refractory metastatic colorectal cancer treated with cetuximab-irinotecan therapy. 2007. J Clin Oncol. 25(25):3930-5.

Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012. 62(1):10-29.

Spaw M, Shrikant A and Sufi MT. Stromal Contributions to the Carcinogenic Process. 2016, Molecular carcinogenesis. Volume 56, Issue 4. Pages 1199–121.

Trevellin E, Scarpa M, Carraro A, Lunardi F, Kotsafti,A, Porzionato A, Saadeh L, Cagol M, Alfieri R, Tedeschi U, CalabreseF, Castoro C, and Vettor R. Esophageal adenocarcinoma and obesity: peritumoral adipose tissue plays a role in lymph node invasion. 2015. Oncotarget, Vol. 6, No. 13.

Van Marken L, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, Schrauwen P, Teule GJ. Cold-activated brown adipose tissue in healthy men. 2009. N Engl J Med. 360(15):1500-8.

Vitali A, Murano I, Zingaretti MC, Frontini A, Ricquier D, Cinti S. The adipose organ of obesity-prone C57BL/6J mice is composed of mixed white and brown adipocytes. 2012. J Lipid Res. 53(4):619-29.

Wagner M, Bjerkvig R, Wiig H, Melero-Martin J M, Lin RZ, Klagsbrun M, Dudley A. Inflamed tumor-associated adipose tissue is a depot for macrophages that stimulate tumor growth and angiogenesis. 2012. NIH Public Access. 15(3): 481–495.

Yamada Y, Itoh Y, Aoki S, Nakamura K, Taki T, Naruse K, Tobiume M, Zennami K, Katsuda R, Kato Y, Watanabe M, Nishikawa G, Minami M, Nakahira M, Ukai S, Sawada M, Kitamura A, Honda N. Preliminary results of M-VAC chemotherapy combined with mild hyperthermia, a new therapeutic strategy for advanced or metastatic transitional cell carcinoma of the urothelium. 2009. Cancer Chemother Pharmacol. 64(6):1079-83.