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Perirenal adipose tissue and clear cell renal cell carcinoma: the triad cancer cell-macrophage-adipocyte

Rafaela Sofia Gonçalves Rodrigues

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Perirenal adipose tissue and clear cell renal cell carcinoma: the triad cancer cell-macrophage-adipocyte

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Orientador – Doutor Tânia Carina Bértolo da Cruz, PhD
Categoria – Junior Researcher
Afiliação – Instituto de Investigação e Inovação em Saúde (i3S)

Co-orientador – Professor Doutor Ricardo Jorge Teixeira Ribeiro
Categoria – Investigador, Professor e Médico
Afiliação – Instituto de Investigação e Inovação em Saúde da Universidade do Porto
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ABSTRACT

Renal Cell Carcinoma (RCC) is one of the most frequent malignancies of the urinary tract and the deadliest urological cancer. The most common RCC histologic subtype is clear cell RCC (ccRCC). This disease is characterized by important metabolic alterations that result in clear cell cytoplasm with lipid and glycogen accumulation. Furthermore, ccRCC is considered an immunogenic tumour, as it is characterized by the infiltration of immune cells, among which macrophages, reported to potentially have a relevant role in disease development and progression. Noteworthy, obesity is an established risk factor for ccRCC despite it has been demonstrated to contribute towards better prognosis, the so-called obesity paradox.

The role of adipose tissue in the renal cancer milieu remains unclear, yet an outstanding piece of data, obtained from murine xenograft models, shows that distant adipose tissue stem-like cells can be recruited to the tumour region. This striking observation highlights the existence of a crosstalk between the tumour compartment and outlying adipocytes, supporting the importance of further dissecting the mechanisms underlying the obesity paradox.

To start unveiling the complexity of tumour and adipose tissue crosstalk, we set out to disclose the molecules entangled in the interplay between the triad composed by cancer cells, macrophages, and adipocytes. Following this premise, we proposed to: i) investigate the influence of perirenal adipose tissue adipocytes on macrophage and on tumour cell profile in ccRCC context, ii) discover potential key molecules of this triad communication, and iii) investigate the triad localization and validate the uncovered players of the triad interplay in ccRCC patient samples. For that, a 2D cell culture model, in which cancer cells were challenged with macrophages and adipocytes, was established and the phenotype of each cell type was assessed. Additionally, ccRCC perirenal adipose tissue patient samples histology, from two different anatomical regions (juxta-tumoral and distant to the tumour), were characterized and used for further validation of candidate key players.

In vitro data suggested that the ccRCC cell line 786-O influence macrophages by triggering polarization into an anti-inflammatory phenotype. In addition, in a 2D-model, our data supports the existence of a molecular crosstalk established between cancer cells, macrophages and adipocytes. Immunohistochemical analyses in perirenal adipose tissue demonstrated that macrophages are present in perirenal adipose tissue of ccRCC patients, although no differences were identified between juxta-tumoral and distant to the tumour regions. Absence of differences in those areas was also found for adipocytes areas.

Findings from this study provided preliminary information on the triad crosstalk, supporting a role for adipocytes as a novel player in the tumour microenvironment. Data presented here will foster additional studies in larger samples to clarify the mechanistic oncogenic communication between the tumour itself and the surrounding cells.

Keywords: Clear cell renal cell carcinoma, perirenal adipose tissue, obesity paradox, macrophage, adipocyte, tumour microenvironment, cell-cell crosstalk.

RESUMO

O carcinoma de células renais (CCR) é um dos tumores malignos mais comuns e letais do trato urinário. O subtipo histológico mais frequente é o carcinoma de células renais de células claras (CCRcc). Esta doença é caracterizada por importantes alterações metabólicas que têm como resultado morfológico uma acumulação de lípidos e glicogénio no citoplasma das células renais, conferindo-lhes uma tonalidade mais clara. Além disso, o CCRcc é um tumor imunogénico, apresentando um infiltrado de células imunes, de entre as quais os macrófagos, descritos como tendo um papel relevante no desenvolvimento e progressão da doença. Um importante fator de risco associado ao CCRcc é a obesidade. Curiosamente, vários estudos epidemiológicos revelaram que esta condição pode contribuir para um melhor prognóstico em determinados contextos, um fenómeno designado de paradoxo da obesidade.

De facto, o papel do tecido adiposo no microambiente do carcinoma renal permanece ambíguo. Não obstante, estudos realizados em ratinhos comprovaram a existência de recrutamento de células estaminais de tecido adiposo para a região do tumor. Esta notável descoberta destaca a existência de uma comunicação entre o tumor *in situ* com os adipócitos periféricos, o que aponta, uma vez mais, para a importância da compreensão do papel dos adipócitos no microambiente tumoral.

Seguindo estas premissas, com o objetivo de desvendar a complexidade do ccRCC, estabelecemos um modelo de cultura 2D, de forma a revelar as moléculas envolvidas na interação entre a tríade composta por células tumorais, macrófagos e adipócitos. Além disso, avaliamos a histologia do tecido adiposo perirrenal de pacientes com ccRCC de duas regiões anatómicas diferentes (justa-tumoral e distante do tumor), para confrontar potenciais diferenças. Finalmente, também investigámos a presença de macrófagos nessas áreas.

Assim, neste projeto tivemos como principais objetivos: i) investigar a influência dos adipócitos, provenientes do tecido adiposo perirrenal, no perfil dos macrófagos e das células tumorais; ii) descobrir potenciais moléculas-chave envolvidas na comunicação estabelecida entre células tumorais, macrófagos e adipócitos; iii) investigar a localização da tríade, bem como validar os candidatos moleculares envolvidos na interação das células tumorais, macrófagos e adipócitos, em amostras de pacientes com CCRcc.

Os dados *in vitro* aqui apresentados comprovam que as células 786-O têm de facto influência sobre os macrófagos, estimulando a sua modulação para um perfil anti-inflamatório. Além disso, os resultados no modelo 2D aqui estabelecido, suportam a existência de comunicação entre os diferentes tipos celulares. Como resultado das análises imunohistoquímicas, verificou-se a presença de macrófagos nas amostras de tecido adiposo perirrenal, embora não tenham sido identificadas diferenças evidentes quanto à sua distribuição entre regiões, distante e justa-tumoral. Além disso, comparando amostras destas duas regiões anatómicas, no que diz respeito ao número de adipócitos e à sua área, também não foram observadas diferenças estatisticamente significativas.

Em conclusão, este trabalho reúne resultados promissores no contexto de CCRcc, uma vez que reporta a existência de uma interação entre as células tumorais, os macrófagos e os adipócitos. No futuro será importante esclarecer e dar robustez aos dados resultantes do trabalho aqui desenvolvido, por forma a descobrir os mecanismos-chave responsáveis pela comunicação oncogénica que se estabelece entre o próprio tumor e as células circundantes. Esta estratégia irá potencialmente contribuir para o esclarecimento do paradoxo da obesidade, e potenciar a identificação de novos alvos terapêuticos.

Palavras-chave: Carcinoma de células renais de células claras, tecido adiposo perirrenal, paradoxo da obesidade, macrófagos, adipócitos, microambiente tumoral, comunicação entre células.

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List of Abbreviations

3p - Short arm of chromosome 3

ACSL1 - Acyl-CoA Synthetase Long Chain Family Member 1

AdipoR - Adiponectin receptor

AKT - Protein kinase B

AMP - Adenosine monophosphate

AMPK - AMP-activated protein kinase

ASC - Adipose-derived stem cells

AT - Adipose tissue

ATP - Adenosine triphosphate

BAT - Brown adipose tissue

BMI - Body mass index

BSA - Bovine Serum Albumin solution

CAA - Cancer-associated adipocyte

CCL - Chemokine ligand of the CC family

ccRCC - Clear cell renal cell carcinoma

CD - Cluster of differentiation

cDNA - complementary DNA

CM - Conditioned medium

cfDNA - Circulating cell-free DNA

CT - Computed tomography

CXCL - Chemokine ligand of the CXC family

CYP19 - Aromatase cytochrome P450

DAB - 3,3'-Diaminobenzidine

DMEM/F12 - Dulbecco's modified Eagle medium/ Nutrient Mixture F-12

DNA - Deoxyribonucleic acid

dNTPs - Deoxynucleoside triphosphate

DTT - Dithiothreitol

ECM - Extracellular matrix

EDTA - Ethylenediaminetetraacetic acid

ELISA - Enzyme-Linked Immunosorbent Assay

EMT - Epithelial–mesenchymal transition

ERK - Extracellular signal–regulated kinases

FBS - Fetal bovine serum

FA - Fatty acid

FABP - Fatty-acid-binding proteins

FATP - Fatty Acid Transport Protein

FFA - Free fatty acid

GM3 - Membrane-bound glycosphingolipid

H&E - Haematoxylin and eosin

HBSS - Hanks' Balanced Salt Solution

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF - Hypoxia inducible factor

HLA - Human leukocyte antigen

HRE - HIF response element

HRP - Horseradish peroxidase

HSL - Hormone-sensitive lipase

IFN - Interferon

IHC - Immunohistochemistry

IL - Interleukin

IRE1α- *Inositol-requiring transmembrane kinase/endoribonuclease 1α*

JAK - Janus kinase

KRHB - Krebs-Ringer HEPES Buffer

LOH - Loss of heterozygosity

LPA - Lysophosphatidic acid

MAPK - Mitogen-activated protein kinase

M-CSF - Macrophage colony stimulating factor

MDSC - Myeloid-derived suppressor cells

MMP - Matrix metalloproteinase

mRNA - Messenger ribonucleic acid

mTOR - Mechanistic target of rapamycin kinase

mTORC - Mechanistic target of rapamycin complex

NETs - neutrophil extracellular traps

NF-κB - nuclear factor kappa B

NK - natural killer

OBR - Leptin receptor

PA - Phosphatidic acid

PBMCs - Peripheral blood mononuclear cells

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

PD-1 - Programmed cell death protein 1

PDGF - Platelet-derived growth factor

PD-L1 - Programmed death-ligand 1

PI3K - Phosphoinositide 3-kinase

p65 - Transcription factor p65 (human) NF-kappa-B

PMSF - phenylmethylsulfonyl fluoride

PPARγ/α - Peroxisome proliferator-activated receptor gamma/alpha

PUFAs - Polyunsaturated fatty acids

qRT-PCR - Quantitative real-time PCR

RCC - Renal cell carcinoma

RNA - Ribonucleic acid

ROS - Radical oxygen species

RPMI - Roswell Park Memorial Institute Medium

RT - Room Temperature

S1P - Sphingosine-1-phosphate

SD - Standard deviation

STAT1 - Signal transducer and activator of transcription 1

STAT3 - Signal transducer and activator of transcription 3

SWI/SNF - Switch/sucrose non-fermenting complex

TAM - Tumour-associated macrophage

TAG - Triacylglycerol

TBST20 - Tris-Buffered Saline with Tween 20

TGFβ - Transforming growth factor beta

TME - Tumour microenvironment

TNF - Tumour necrosis factor

Treg - Regulatory T cell

UCP1 - Uncoupled protein 1

VEGF - Vascular endothelial growth factor

VEGFR - VEGF receptor

VHL - von Hippel-Lindau tumour suppressor gene

WAT - White adipose tissue

I. INTRODUCTION

1 – RENAL CELL CARCINOMA, CLEAR CELL SUBTYPE

1.1. Epidemiology and risk factors

According to GLOBOCAN, kidney cancer is the fourteenth most incident cancer worldwide, higher in developed countries as shown in **Figure 1**^(1, 2). Its incidence is 2:1 ratio in men versus women and increases with age⁽²⁾. Since the 1990s, incidence rates have been rising, whereas mortality stabilized and gradually declined^(3, 4). This apparently conflicting epidemiological data might be due to either dietary and lifestyle changes, increasing obesity, or widespread use of imaging exams for other causes, resulting in enhanced incidentaloma detection rate. In favor of diet and lifestyle, comprehensive meta-analysis reported that fruit and vegetables intake protected from renal cancer development⁽⁵⁾. Moreover, physical activity has been associated with a decreased risk of this disease⁽⁶⁾.

Renal cell carcinoma (RCC), is the most common type of kidney cancer, corresponding to more than 90% of all cases⁽⁷⁾. Epidemiological studies pinpoint renal cell carcinoma (RCC) as one of the deadliest urological neoplasm, particularly, in developed world^(8, 9). RCC arises from renal tubular epithelial cells and according to its heterogenous histology and molecular characteristics, it can be classified into subtypes: clear cell renal cell carcinoma (ccRCC), accounting for 75%; papillary renal cell carcinoma types 1 and 2 (pRCC), corresponding to 12%; chromophobe renal cell carcinoma (chRCC), about 4% of the RCC cases; oncocytoma (4%); collecting duct (less incidence than 1%), and unclassified RCC variants (3-5%)⁽¹⁰⁻¹²⁾. ccRCC is the most frequent and aggressive and is associated with higher mortality rate⁽¹³⁾. Its incidence and mortality are influenced by numerous factors including hypertension, cigarette smoking, and obesity. In addition, RCC family history, race, age, gender, acquired renal cystic disease, diabetes, urinary tract infection, nutritional and dietary factors, as well as metabolic, immunologic, and genetic factors, have also been associated with this disease⁽¹¹⁾. Of note, when ccRCC is diagnosed before 46 years of age, the probability of having a mutation in both alleles of the tumour suppressor genes responsible for the disease development is lower, thus this is a reason to consider the hypothesis of a possible hereditary cancer⁽⁸⁾.

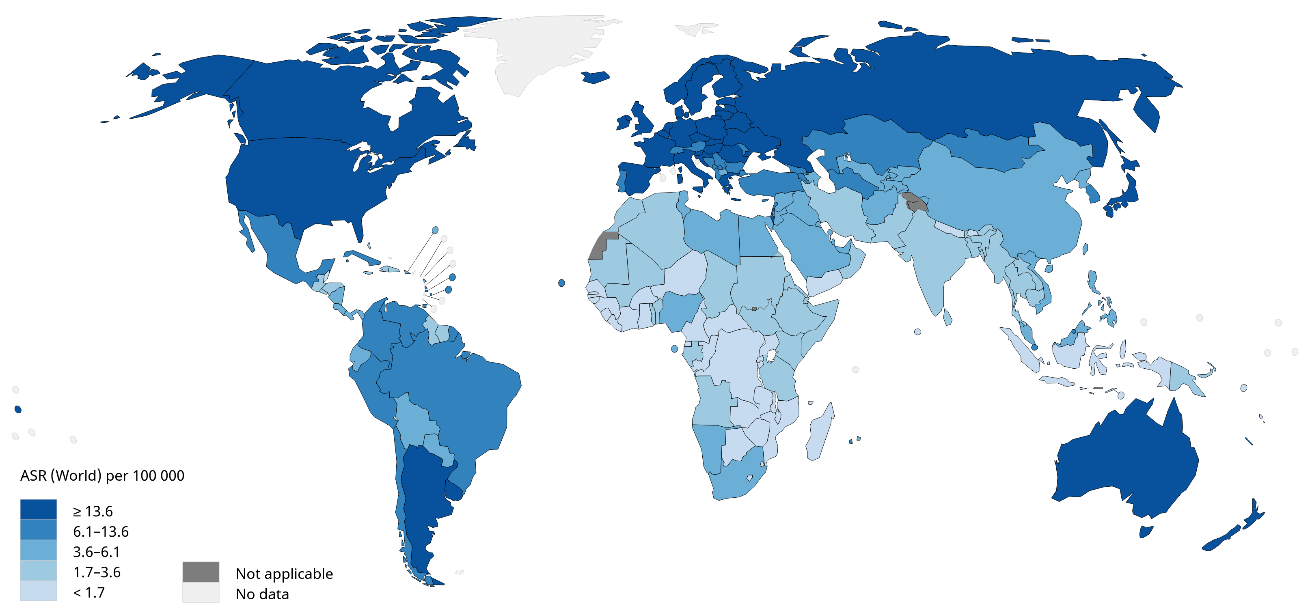


Figure 1 - Estimated incidence of kidney cancer (age standardized rates, ASR), for both sexes, worldwide.

Adapted from Global Cancer Observatory, 2021⁽¹⁴⁾.

1.1.1 An obesity paradox?

One of the greatest concerns worldwide is obesity, a metabolic disease characterized by an increase in the white adipose tissue (WAT), in which *body mass index (BMI) is $\geq 30\text{kg/m}^2$* (WHO, 2021). The obese phenotype is itself a risk factor for developing multiple, severe pathologies, including ccRCC⁽¹⁵⁾.

Overweight/obese patients have statistically significantly longer overall survival than normal-weight ccRCC-treated patients. This pattern is observed regardless of the treatment chosen and the stage/grade of ccRCC^(16, 17). In line with this, a recognized epidemiological phenomenon, designated obesity paradox, describes the double and paradoxical role of the adipose tissue as it increases the risk of ccRCC development but is also associated with improved overall ccRCC patient survival. The mechanisms that link obesity with risk of developing ccRCC are yet unknown, however, hypoxia, hormonal imbalances, and inflammation, are likely to contribute^(15, 18-20). The mechanisms by which adipose tissue accumulation may benefit the outcome of ccRCC-treated patients are not clear, yet it has been hypothesized that ccRCC tumours from obese patients present adipocyte hypertrophy leading to i) angiogenesis and better drug delivery (e.g. tyrosine kinase inhibitors or checkpoint

inhibitors), ii) inflammation at the tumour milieu with increased immunovigilance, and iii) hypoxia that further stimulates angiogenesis^(19, 21). Furthermore, the fact that obese patients could easily preserve an adequate nutritional status when compared to normal-weight patients may be an additional reason of having a better outcome after treatment, reducing the risk for cachexia as well as other complications^(16, 17). Additionally, some authors reported that peritumoral adipose tissue acts as an immune reservoir of activated immune cells that might influence tumour microenvironment toward a pro- or anti-tumoral behavior^(19, 22).

Despite epidemiological evidence of the RCC causally-associated obesity, most studies use BMI to classify obesity, although it is an imprecise estimate of adiposity. This raised the question of accuracy of the data from obese ccRCC patient and their relationship with overall survival. It is possible that reverse causality in regard to cancer cachexia (weight loss is a consequence of cancer), collider stratification bias, or other methodological explanations, formulate confounding results concerning the obesity paradox, making the elevated BMI an inaccurate factor^(18, 23). Some authors defend that other adiposity measures, such as waist circumference or CT-scan or MRI assessment of subcutaneous, visceral, intramuscular or intermuscular adipose tissue, are better to reliably evaluate obesity⁽²⁰⁾.

1.2. Molecular basis of ccRCC

The most relevant genetic hallmark of ccRCC, which occurs in over 90% of the patients, is the loss of the short arm of chromosome 3 (3p). This alteration leads to loss of important genes such as Von Hippel-Lindau (*VHL*) tumour suppressor gene, located on 3p25-26⁽²⁴⁻²⁶⁾. *VHL* is a gene related with Von Hippel-Lindau syndrome, an inherited disease associated with increased risk for the development of several cancer types⁽²⁴⁾. As a tumour suppressor gene, *VHL* is functional even with the allele of the remaining chromosome 3⁽²⁵⁾, however, along time there is often loss of heterozygosity (LOH) meaning that after a *first hit* (3p deletion) oncogenic driver mutation, deletion or epigenetic silencing event (e.g. promotor hypermethylation) on the other *VHL* allele (*second hit*) may follow. As a consequence, inactivation of *VHL* gene function occurs⁽²⁷⁾. These molecular alterations are reported as key early events in ccRCC development and progression, and they can occur at the somatic or, less commonly, at the germline landscape, when associated with inherited syndromes⁽²⁸⁾. *VHL* gene encodes for an oxygen sensing mediator protein (VHL), which is a recognition component that makes part of an E3 ubiquitin ligase complex involved in the regulation of the hydroxylated hypoxia-inducible-factor alpha (HIF- α), namely, its ubiquitination and degradation under normoxic conditions. HIF- α (oxygen-sensitive alpha-subunit) and HIF- β (*constitutively expressed beta-subunit*) are the subunits that constitute the transcription factor HIF and together they trigger several processes such as, cellular adaptation to hypoxia conditions and regulation of gene expression. As presented in **Figure 2**, HIF signaling is influenced by the microenvironment oxygen concentration, meaning that in the presence of oxygen molecules a process of hydroxylation on the HIF- α conserved proline residues occurs, resulting in a biochemical modification that allows HIF recognition and binding to its negative regulator, VHL. Such reaction triggers HIF ubiquitination and subsequent proteosomal degradation preventing HIF migration into the nucleus and subsequent transcription of its target genes. Due to the high level of cell proliferation and aberrant new blood vessels formation during cancer development, a scenario of hypoxia commonly occurs. This results in an inefficient tissue oxygenation, thereby there is lack of HIF- α hydroxylation, enabling its recognition and degradation through VHL regulator. Such regulation allows HIF translocation into the nucleus where HIF- α dimerizes with HIF- β to form transcriptionally active HIF molecules which will bind to hypoxia responsive elements (HRE) inducing the transcription of target genes involved in several processes namely, glucose uptake, angiogenesis, erythropoiesis, cell proliferation, apoptosis, cell-cell, and cell-matrix interactions genes^(8, 28). In ccRCC patients with *VHL* inactivation there is no regulation of HIF- α by *VHL* protein, resulting in the overexpression of HIF transcription factor, regardless of oxygen availability, driving the downstream constitutive activation of the hypoxia pathway, ultimately contributing to the tumour growth^(8, 28).

Apart from genetic factors, epigenetic modifications have been reported as important players in ccRCC development⁽²⁹⁾. Several inactivation mutations were identified, along with *VHL* loss, in genes such as *BAP-1* (encoding the histone deubiquitinase BRCA1 associated protein-1), *PBRM1* (related with SWI/SNF chromatin remodeling complex), *SETD2* (encoding histone methyltransferase), and other genes with epigenetic modifier function^(30, 31). Of note, *PBRM1* tumour suppressor gene encodes BAF180, a subunit of the SWI/SNF ATP-dependent chromatin remodeling complex responsible for regulating gene expression through remodeling DNA compactation for possible transcription sites (euchromatin sites, less compacted) or transcription-repressed sites (heterochromatin, less accessible sites). Several reports based in *in vitro* studies demonstrated that the *PBRM1* inactivation promotes tumorigenesis and tumour development through yet unknown mechanisms⁽³²⁻³⁴⁾.

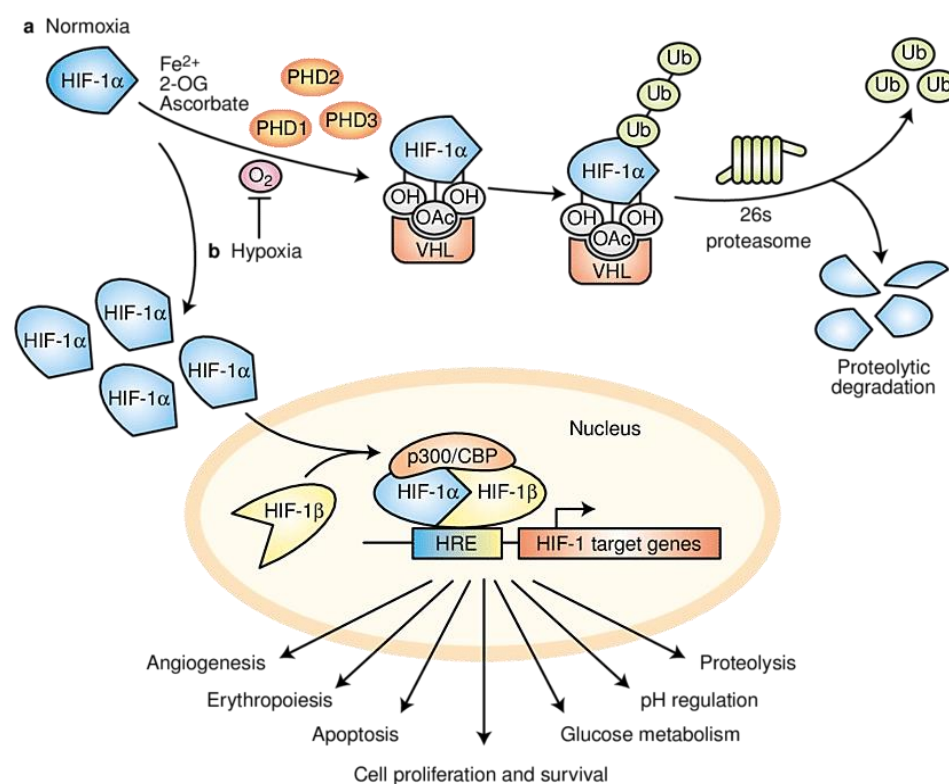


Figure 2 – Regulation of the transcription factor HIF under normoxia and hypoxia.

Under normoxic conditions (a), HIF- α subunits are hydroxylated on proline residues by prolyl hydroxylases (PHDs) and then ubiquitinated by an E3-ubiquitin ligase complex containing VHL, these modifications result in HIF- α proteolysis. In hypoxia (b), there is no hydroxylation of HIF- α thus this subunit is translocated to the cell nucleus where it heterodimerizes with HIF- β . Then, active HIF binds to the HRE of the DNA resulting in hyper-transcription of the target genes such as erythropoiesis, glycolysis, and angiogenesis genes, thus favoring tumour evolution. Adapted from Carroll and Ashcroft, 2005 ⁽³⁵⁾.

Nargund *et al.* proposed a theory that encompasses genetic and epigenetic events as a multistep process for ccRCC development (Figure 3). Using *in vivo* mouse models, authors found evidence that the loss of VHL followed by the inactivation of PBRM1, both involved in the HIF/STAT3 pathway as tumour suppressors as well as the hyperactivation of mTORC1 with oncogenic potential, are the pre-required drivers for the ccRCC tumorigenesis ^(34, 36).

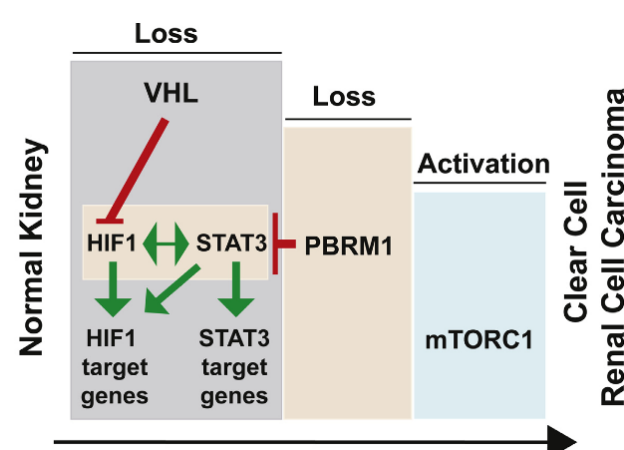


Figure 3 – Theory for ccRCC initiation.

In the normal kidney the most common oncogenic event that could occur is VHL gene mutation, thereby HIF become constitutively activated. The second most reported event is in epigenetic landscape, inactivation of PBRM1 tumour suppressor gene, activating overexpression of STAT3/HIF pathway. As the third and last event for this carcinogenesis process is the mTORC1 activation, involved in the activation of genes related with anabolism ⁽³⁴⁾. Altogether, it is proposed that such events result in ccRCC development.

During tumour development, cancer cells face different challenges such as hypoxia, immune defense, and nutrient limitation. To overcome such stress, malignant cells go through aberrant metabolic shifts, particularly, a decreased of the Krebs cycle activity and an increase of the pentose phosphate pathway, upregulated fatty acid synthesis activity, and aerobic glycolysis, the so-called Warburg Effect. These metabolic alterations occur in ccRCC and are related with a worse prognosis. Moreover, as a result of the lipid and glycogen accumulation within the tumour cells, there is the induction of a morphological hallmark of ccRCC, the typical clear cell cytoplasm ^(37, 38). Furthermore, as a consequence of HIF constitutive activation that targets VEGF expression, triggering angiogenesis, another relevant ccRCC feature occurs, the elevated tumour vascularization ⁽⁸⁾. The accumulation of HIF and the activation of oncogenes also results in the increase of erythropoietin, PDGF- β , TGF- α , glucose transporters, mitochondrial enzymes, and glycolytic enzymes levels ⁽³⁹⁾.

In ccRCC, with the VHL gene mutation and/or PI3K/AKT/mTOR enhanced activity, there is an accumulation of the transcription factor HIF,

driving to the pyruvate dehydrogenase kinase (PDK) overexpression, which is a key mitochondrial enzyme, that through phosphorylation, inhibits the pyruvate dehydrogenase complex (PDC) activity, responsible for the pyruvate conversion into acetyl-CoA, needed for Krebs Cycle initiation, thus resulting in a reduction of the mitochondria activity (oxidative phosphorylation), concomitantly with an increase in the glycolysis. The glycolysis end products and intermediate metabolites accumulate in the cells and are used for synthesizing nucleic acids, amino acids and lipids, processes essential for the tumour progression and maintenance. Pyruvate is the major energy source for the tumour cells, converted in lactate by fermentation. In the ccRCC development, glycolysis is a crucial pathway, causing a reduction in the oxygen need, also triggers new blood vessels formation (angiogenesis) for a major uptake of oxygen into the tumour tissue ^(37, 40-42).

2- Tumour microenvironment in ccRCC

Carcinogenesis is a multistep and complex process, involving several alterations in the cells and, consequently, in its microenvironment. The ccRCC tumour microenvironment (TME) is comprised of cellular and non-cellular components, as shown in **Figure 4**, among which macrophages, lymphocytes, fibroblasts, pericytes, adipocytes, extracellular matrix, growth factors, cytokines, and hormones. Altogether, these players have a key role on the neoplasia outcome, tipping the balance towards tumour progression or eradication ⁽⁴³⁻⁴⁵⁾.

Regarding ccRCC tumour-infiltrating immune cell profile it is yet not fully clarified, notwithstanding, it has been reported that ccRCC TME has an immunosuppressive infiltrate composed of a myriad of immune cells, leveraging the immunotherapy efficacy ⁽⁴⁶⁻⁴⁹⁾. In advanced ccRCC, generally, CD8⁺ T cells became terminally exhausted due to the constant stimulus, the expression of pro-inflammatory molecules is decreased, and M2-like anti-inflammatory macrophage profile is enhanced. Such settings trigger an aberrant immune cell function contributing to the immune escape, immunotolerance and drug resistance, fostering the tumour success ^(44, 45, 50).

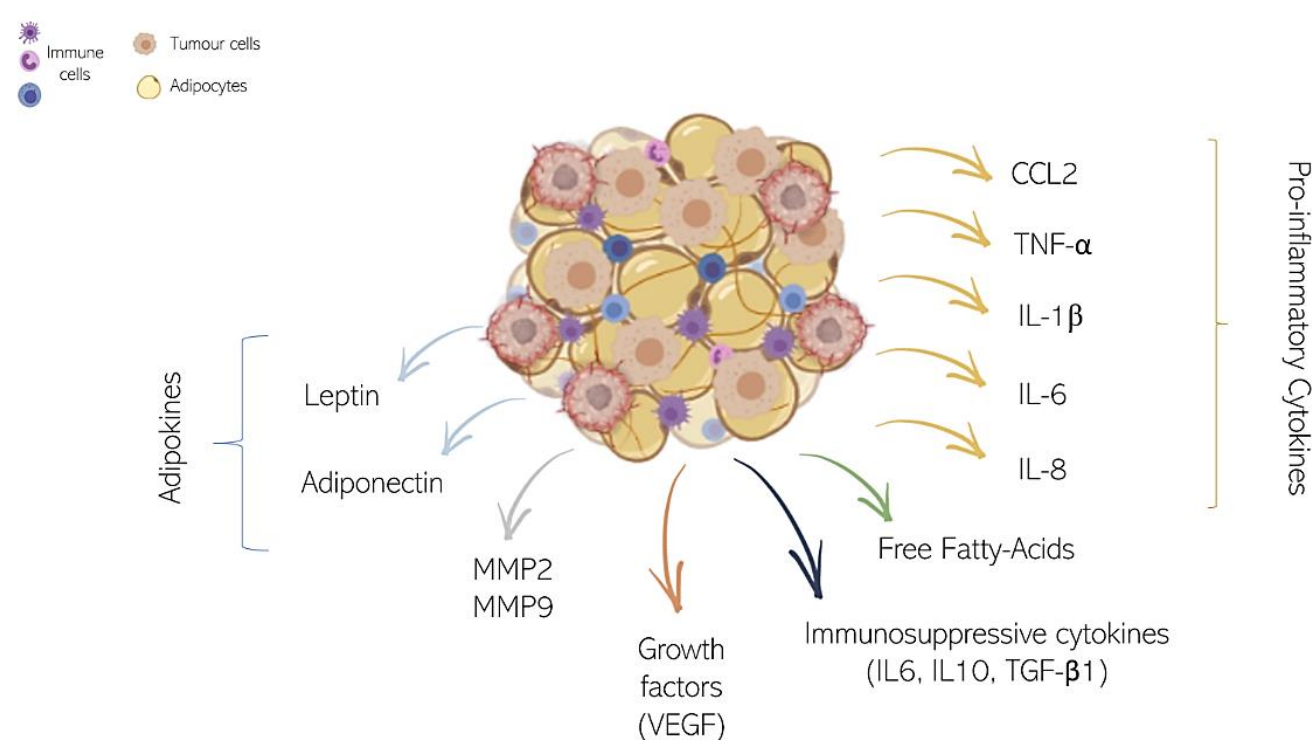


Figure 4 – ccRCC tumour microenvironment complexity.

Represented here are some of the cellular components (macrophages, leukocytes, neutrophils, tumour cells, and adipocytes) of ccRCC niche, as well as some of the potential key molecules involved in this disease (CCL2, TNF-α, IL-1β, IL-6, IL-8, IL-10, TGF-β1, free fatty-acids (FFA), VEGF, MMP2, MMP9, Leptin, Adiponectin).

2.1. Macrophages

Monocytes in the blood vessels, are recruited to distinct areas, according to the stimulus present in the microenvironment. In the tumour context, monocytes are recruited through CCL2/4/5, M-CSF, IL-6, VEGF, and PDGF, and differentiated into tumour associated macrophages (TAM), developing an important role in tumour progression and in metastatic niches formation ⁽⁵¹⁻⁵³⁾. These phagocytes are able to be modulated into different profiles due to their phenotype plasticity, playing distinct roles in tumour development or eradication (**Figure 5**) ⁽⁵⁴⁾. Interestingly, it has been reported that M1-like macrophages can give rise to the anti-inflammatory phenotype, and M2-like macrophages can be transdifferentiated toward the M1-like profile ⁽⁵⁵⁻⁵⁸⁾. In detail, macrophages may be polarized into classical activated macrophages corresponding to a pro-inflammatory phenotype, in the presence of LPS (lipopolysaccharide) and Th1 secreted cytokines (TNF-α and IFN-γ) ^(56, 59). These M1-like macrophages are key players in innate and acquired immunity, being involved in: anti-tumoral processes, phagocytosis,

and elimination of senescent or apoptotic cells, external foreign agents and some neoplastic cells; adaptive immune response triggering, and, adaptative response activation via secreted molecules or cell-cell contact (T and B cells), or antigen presentation ⁽⁶⁰⁾. Pro-inflammatory macrophages express at their cell surface molecules such as CD80, CD86, MHC-II, among others and secrete pro-inflammatory cytokines as $TNF-\alpha$, $IL-1-\beta$, $IL-6$, $IL-12$, and $IL-23$. The cytokine secretion by pro-inflammatory macrophages is regulated by several molecules, among which NF-kB/STAT1, STAT3, IRF4, and HIF1- α ^(56, 59). In addition, these phagocytes prefer glycolysis to obtain energy, contrariwise to the M2-like macrophages, which prefer fatty acids oxidation and mitochondrial respiration ^(56, 59, 61, 62). Alternative activated macrophages (M2-like) can be stratified in four different subtypes: M2a, M2b, M2c, and M2d. M2a, induced by IL-4 and IL-13, present anti-inflammatory/immunosuppressive profile, allowing these cells to counteract the inflammation process, resolving the immune response exacerbation and tissue remodeling ⁽⁶³⁾. These immune cells are described as expressing CD206, CD163, CD36, and producing, VEGF, EGF, PDGF, IL-8, TGF- β , IGF-1. M2b, are induced with the presence of TLR ligands or by IL-1R agonists, secreting pro- and anti-inflammatory cytokines. M2c cells are highly anti-inflammatory and are induced by the presence of glucocorticoids or IL-10, secreting IL-10 and TGF- β . *M2d (tumour associated macrophages)* are induced by the presence of TLR ligands and A2R agonists, or by IL-6. They are responsible for the secretion of a wide range of molecules such as, IL-10, TGF- β , and VEGF as well as low levels of $IL-12$, $TNF-\alpha$, and $IL-1\beta$. The latter presents an immunosuppressive activity, favoring tumour development through their involvement in angiogenesis, proliferation, stemness, matrix remodeling, invasiveness and survival processes ^(53, 56, 59, 61, 64, 65).

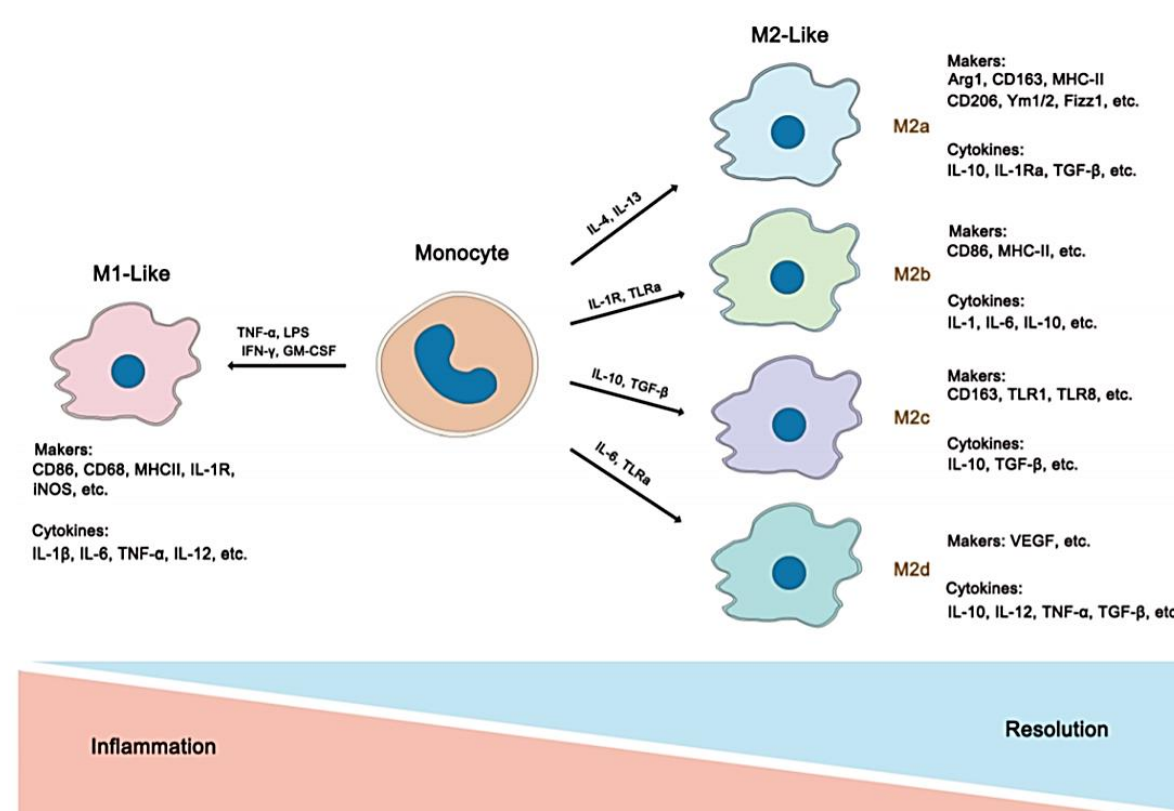


Figure 5 – Macrophage’s phenotypes characterization.

Depending on the stimulus, monocytes can be polarized into two subtypes, pro-inflammatory (M1-like) and anti-inflammatory (M2-like). The latter can be divided into four different phenotypes M2a, M2b, M2c, and M2d. These immune cells play distinct functions and are distinguished by their specific pattern of secreted molecules and receptors expression. Pro-inflammatory macrophages are related with Th1 response, on the other hand, M2-like macrophages are generally associated with immune tolerance. Specifically, (1) M2a is commonly associated with matrix remodeling and adaptative immune responses, (2) M2b is mostly related with immune system regulation, (3) M2c mainly functions in anti-inflammatory processes and phagocytosis, and (4) M2d, is associated to pro-tumoral processes, such as angiogenesis ⁽⁵⁹⁾.

2.2. Adipose tissue

Adipose tissue is an endocrine organ that secretes extracellular vesicles and hormones that will act in specific targets. It is localized in different anatomic regions namely, under the skin being designate subcutaneous adipose tissue, at the organs surrounding, also called visceral adipose tissue, and in the kidney where it has the designation of perirenal adipose tissue ^(66, 67). This organ is composed of adipocytes (varying in number and size, accordingly with the individual), preadipocytes, and the denominated stromal vascular fraction, constituted by pericytes, monocytes, macrophages, endothelial cells, fibroblasts, pluripotent adipose-derived stem cells and stromal cells. Adipose tissue functions in energy homeostasis maintenance, insulin sensitivity, immunity, angiogenesis, adipogenesis, body temperature regulation, and extracellular matrix restructuring ⁽⁶⁸⁾. On average, adipose tissue of normal-weight individuals comprises a quarter of the body mass, in contrast, in obese individuals, it can comprise up to seven-tenths of the body mass ^(69, 70). The accumulation of adipose tissue in obese individuals is not only due to an increase in the adipocytes number (hyperplasia) but also an increase in the adipocytes volume (hypertrophy), which contribute to the

inflammatory process associated with this disease ^(66, 67, 71, 72). Furthermore, the adipose tissue phenotype and microenvironment suffers alterations in the normal-weight condition vs obesity, particularly, under normal-weight conditions, the adipose tissue microenvironment is characterized as anti-inflammatory, with M2-like associated cells and the respective cytokines. Contrariwise, in the course of obesity, adipocytes accumulate lipids becoming hypertrophic, and ultimately, they die releasing their cellular substances to the extracellular milieu. The referred process functions as a signal to trigger innate immune responses, polarize macrophages towards a pro-inflammatory state (M1-like phenotype), recruitment of pro-inflammatory immune cells, inflammatory cytokines secretion, and all the features of an inflammatory milieu. As such, processes such as fibrosis and uncontrolled inflammation are recurrent in the obesity status, favoring cancer initiation, promotion, and progression ⁽⁷³⁾.

Regarding the adipose tissue phenotype, it can be classified as white (the most abundant type), brown, and beige, each one with a specific associated gene signature. White adipocytes, differentiated from preadipocytes, morphologically identified by a single lipid droplet, are responsible for energy homeostasis maintenance by the storage of triacylglycerol (TAG) and mobilization of lipids. In contrast, brown adipocytes contain several lipid droplets and mitochondria, and are described as existing only in human neonatal and early childhood periods, producing heat (thermogenesis) through the high levels of expression of uncoupling protein 1 (UCP1) by a process of fatty acids oxidation ⁽⁷⁴⁾. Finally, beige adipocytes are derived from white adipocytes, and phenotypically present characteristics of white and brown adipocytes, with low expression of UCP1. Peroxisome proliferator-activated receptor (PPAR- γ) *is a transcription factor that regulates this process of adipocytes differentiation through the regulation of the expression of hormone sensitive lipase (HSL), adiponectin, and fatty acid binding protein (such as FABP-4). These proteins also have a role in the tumour cells and adipocytes interplay. Interestingly, there is a process called “browning of white adipose tissue (WAT)”, a characteristic inherited to WAT, in which, their beige associated adipocytes, if stimulated, transdifferentiate into a brown fat-like phenotype through mechanisms involving PPAR γ , ultimately increasing the thermogenesis process* ^(67, 74).

2.3. Adipose tissue and cancer

A number of tumours develop closely to the adipose tissue depots, an example is ccRCC which localizes fairly close to the perirenal adipose tissue ⁽⁷⁵⁾. In such tumours, it is known that adipocytes integrate its microenvironment, being reprogrammed into cancer associated adipocytes (CAA). Also, some of these cells go through a process called delipidation in which the mature cells dedifferentiate into pre-adipocytes, releasing lipids and free-fatty acids that may be utilized by tumour cells for their maintenance ^(76, 77). Moreover, pre-adipocytes are able to differentiate into fibroblast-like cells which may replace mature adipocytes in tumours with an adipocyte-rich microenvironment thus reducing the numbers of adipocytes in the cancer milieu ⁽⁷⁶⁾. In addition, the free fatty acids released from adipocyte dedifferentiation are the building blocks of more complex lipid identities. These bioactive molecules act as second messengers, being responsible for the transduction of signals, regulating signaling pathways involved in tumorigenesis as well as influencing the lipidic composition of the cellular membranes where they alter lipid *layers’ organization, dynamics, and function* ^(64, 65). Concomitantly, cancer cells increase the expression of fatty acids importers and associated enzymes as CD36, FATP4, and ACSL1 to assist in lipid uptake as a means to sustain malignant cell proliferation ⁽⁷⁸⁻⁸¹⁾.

Apart from acting locally, adipocytes have the capability of acting systemically through the release of molecules named adipokines or adipocytokines. Leptin and adiponectin are the most studied adipokines and they play a relevant role in metabolic homeostasis. When leptin and adiponectin are deregulated, as it is the case of obesity condition it is possible that tumorigenesis and tumour progression are triggered (**Figure 6**). Indeed, there are reports regarding ovarian, prostate, breast, and renal cell tumours associating increased leptin levels with tumour progression ^(67, 82).

Leptin is a multirole protein, regulates appetite and the body weight, bone formation, survival, stemness, neovascularization, mitogenic cascades, immune suppression, EMT, and even reproductive functions. Generally, it is found in higher levels in obese individuals ⁽⁸³⁻⁸⁵⁾. In the molecular point of view, leptin is the ligand of Ob-Rb and the binding to this receptor triggers the PI3K/AKT and JAK/STAT3 signaling cascades. The activation of such pathways results in the induction of pro-inflammatory cytokines such as IL-6 and IL-8, ultimately promoting malignant cell invasiveness and proliferation, angiogenesis, and other pro-tumorigenic processes ⁽⁶⁷⁾. Adiponectin, which is decreased in obesity states, is a ligand of AdipoR1 and AdipoR2, and is reported to have an anti-tumoral role ⁽⁷²⁾. This is supported by several studies reporting that adiponectin receptors expression in tumoral tissues is decreased when compared to the non-tumour sites. AdipoR1 and AdipoR2 play a key role in the regulation of lipid and glucose metabolism, anti-apoptotic and anti-inflammatory processes. In detail, adiponectin binding to AdipoR1, activates AMPK pathway, triggering processes involved in fatty acids and glucose metabolism. Furthermore, AMPK pathway induces cell cycle *arrest and apoptosis in various tumour types. In its turn, AdipoR2 promotes PPAR α function and insulin sensitization. Thus, lower adiponectin levels suppress the PPAR α signaling pathway, downregulating CD36 and acetyl-CoA oxidase, and increasing the amount of insulin available.* Of note, both of adiponectin receptors promote destruction of intracellular ceramide, increase the level of sphingosine 1-phosphate, and have anti-inflammation, anti-atherosclerosis, anti-angiogenesis, and anti-diabetes roles ^(67, 86). Adiponectin inhibits leptin signaling and pro-tumoral phenotype ⁽⁶⁷⁾.

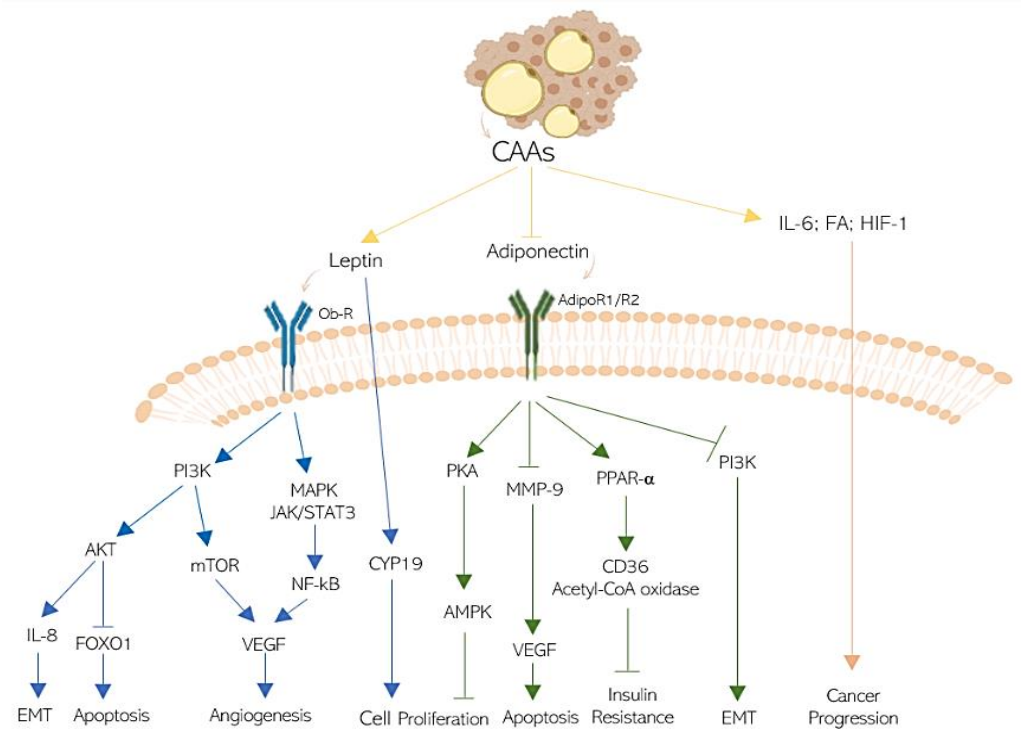


Figure 6 – CAAs signaling pathways that promote tumorigenesis.

CAAs secrete leptin which binds to its receptor OB-R activating PI3K and MAPK pathways to promote cancer EMT and angiogenesis, respectively. Moreover, this hormone directly activates aromatase (CYP19) to enhance cell proliferation. Notwithstanding, CAAs reduce the expression of adiponectin that has contrary functions to that of leptin, inhibiting EMT and stimulating cancer cell apoptosis in part through the downregulation of MMP-9 expression. Meanwhile, adiponectin triggers PPAR- α expression, stimulating CD36 and acetyl-CoA oxidase that ultimately could inhibit insulin resistance. Additionally, CAAs increase IL-6, FA, and HIF-1 α expression thus promoting cancer progression⁽⁶⁷⁾. Adapted from Schmid and Varner, 2010⁽⁵⁹⁾.

2.3.1. Hallmarks of cancer and lipid metabolism

At the metabolic level, the most relevant modification required for the success of the tumour is the rewiring of the cell's normal metabolism into an upregulated aerobic glycolysis, this is, the uptake of high glucose levels to obtain energy, the so-called, Warburg Effect^(87, 88). Such metabolic change allows cells to increase the amount of ATP faster than with the oxidative phosphorylation on mitochondria. Importantly, lipids molecules and lipid metabolism were reported to be associated in all of the cancer hallmarks described by Hanahan & Weinberg, supporting cancer initiation, progression, and metastization^(64, 89, 90), as described in Figure 7.

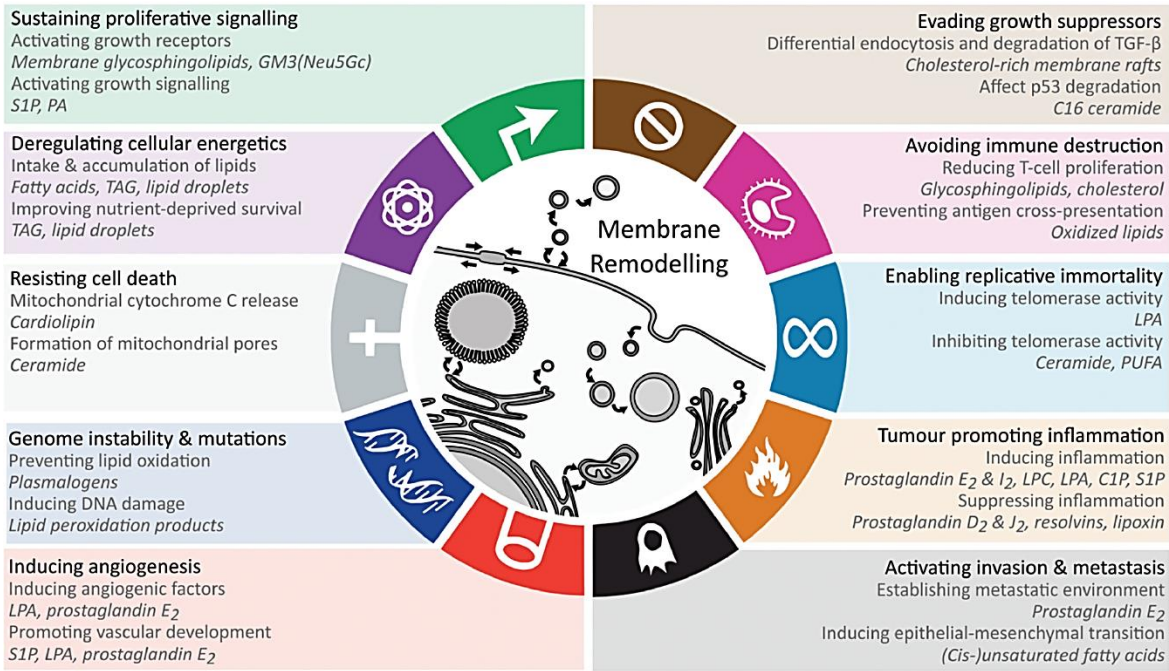


Figure 7 – Lipid metabolism pathways and cancer hallmarks.

Each of the hallmarks are related with the respective lipid associated mechanism and the key molecules involved in each pathway⁽⁹⁰⁾.

Specifically, membrane glycosphingolipids and ganglioside GM3, an evolution fixed neoantigen for cancer immunotherapy, were reported as having a key role in the activation of growth receptors, together with sphingosine-1-phosphate (S1P) and phosphatidic acid (PA), that activates growth signaling, these are key mechanisms involved in sustaining proliferative signaling. Concerning the dysregulation of cellular energy, the molecules reported as having an influence in such process are triacylglycerols (TAG), lipid droplets, and fatty acids. Resisting cell death is one of the most common features of malignant cells, and the main processes involved are the formation of mitochondrial pores and mitochondrial cytochrome C release. For the tumour to succeed, cancer cells must acquire the potential to induce angiogenesis, a feature in which S1P,

lysophosphatidic acid (LPA), and prostaglandin E2 are associated. In addition, C16 ceramide and cholesterol-rich membrane rafts are related with the tumour ability to evade growth suppressors. Also, glycosphingolipids, cholesterol, and oxidized lipids are related with the tumour capacity to perform immune destruction escape. Another hallmark is immortality, a key quality for tumour survival and replication, once again, lipids (LPA, polyunsaturated fatty acids - PUFAs, and Ceramide) enable this process. Additionally, it is well-known that inflammation may lead to cancer and vice-versa, and with no exception, lipid molecules are also involved in the regulation of this interplay. Finally, to surpass the primary localization, invasion, and metastasis are hallmarks triggered by (Cis)-unsaturated fatty acids and prostaglandin E2. Altogether, these evidences suggest that the various cancer hallmarks are influenced by specific lipid molecules ⁽⁹⁰⁾.

3. Tumour cells-macrophage-adipocyte crosstalk

In solid tumours, macrophages are chemoattracted by the secretion of CCL-2, -4, and -5 by cells present at the tumour microenvironment, such as cancer cells, resident macrophages, and adipocytes ^(91, 92). Within this niche, macrophages may differentiate into distinct profiles to display different roles, having either immunosuppressor or pro-tumoral functions ^(61, 91). As previously stated, ccRCC is widely recognized as an immune-rich tumour with macrophage infiltration ^(61, 93-95). Interestingly, increased tumor-associated macrophages (TAMs) infiltration in ccRCC microenvironment has been associated with poor prognosis ⁽⁹⁶⁾.

As mentioned above, ccRCC development occurs in the vicinity of the perirenal adipose tissue, as such it is likely that this organ might influence tumour initiation and development. This process is illustrated in **Figure 8**. In fact, adipose tissue produces anti-inflammatory cytokines, such as IL-10, IL-13, and TGF- β *that maintain the tumour and block the recruitment of anti-tumorigenic immune cells* ⁽⁹¹⁾. As for the pro-inflammatory cytokines secreted by adipocytes, such as IL-6 and TNF- α , *they display pro-tumorigenic functions through the activation of the JAK/STAT signaling pathway*. This culminates in a pre-metastatic niche formation ^(97, 98). In addition, leptin production triggers the downstream signaling pathway involving STAT3, which increases inflammation, promoting tumour invasion and progression ⁽⁹¹⁾. Importantly, at this permissive tumour niche, there is evidence that hypoxia could trigger the inflamed adipocytes to recruit M1-like macrophages, forming a crown-like structure *around the outside adipocyte's membrane* ⁽⁷²⁾. These macrophages destroy the adipocytes (in a process called lipolysis) releasing molecules that include FFA. Such mechanism, provides an energetic boost to the tumour, as well as triggering local inflammation that in turn attracts monocytes that are polarized into immunosuppressors cells through the microenvironmental selective pressure⁽⁹¹⁾. Importantly, some of these released lipids may act as signaling molecules mediating some pro-tumorigenic events ^(72, 91). Moreover, lipolysis also causes circulating cell-free DNA (cfDNA) release, which is recognized by macrophages, triggering the increased production of CCL-2 feeding the loop for monocyte recruitment that once polarized into an anti-inflammatory profile produce cytokines with pro-tumorigenic functions ⁽⁹¹⁾. Such M2-like macrophage polarization is assisted by neutrophils through IL-4 and TGF- β *secretion and maintains the immune tolerance for tumour growth* ^(68, 82).

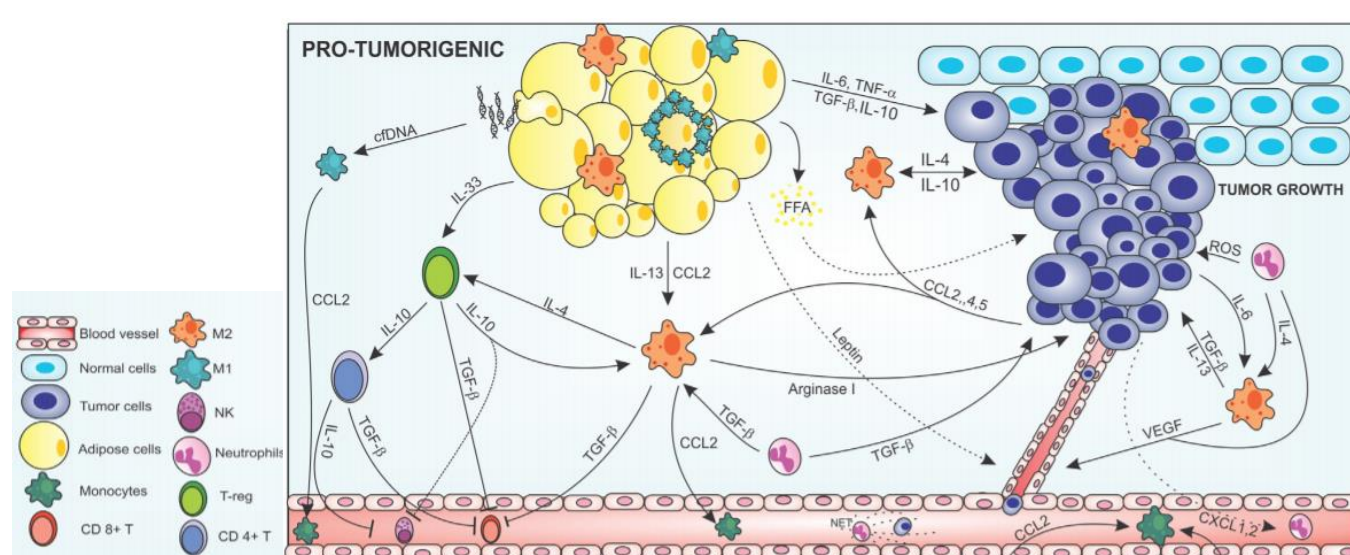


Figure 8 – The triad interplay: cancer cell, macrophage, and adipocyte at the tumour milieu.

In the tumour microenvironment macrophages are attracted due to the release of chemoattractant molecules by cancer cells, such as CCL-2, -4, and -5. The adipose tissue also produces anti- and pro-inflammatory mediators, the first are produced to maintain the tumour and block the recruitment of immune cells with anti-tumorigenic functions. The second, act as pro-tumorigenic through JAK/STAT pathway signaling. Leptin, a hormone secreted by adipocytes that leads to neovascularization, increases tumour invasion capacity. Inflamed adipocytes in the tumour context can attract M1-like macrophages, forming a crown-like structure around adipocytes in order to cause cell destruction, accompanied with the release of molecules that include FFA and give an energetic boost to malignant cells. Due to lipolysis the released cfDNA is recognized by macrophages, triggering the increased production of CCL2, that in turn, enhances monocyte recruitment. The latter will be further differentiated and polarized into an anti-inflammatory profile, producing cytokines with pro-tumorigenic functions. In addition, neutrophils, that are cells present at the tumour microenvironment through C-X-C Motif Chemokine Ligand 2 (CXCL2) recruitment, secrete IL-4 and TGF- β polarizing macrophages in an M2-like profile. Such anti-inflammatory macrophages produce VEGF, increasing angiogenesis at the tumour milieu. Subsequently, in blood vessels, neutrophils have the capability of neutrophil extracellular traps (NETs) formation, a structure that enhance cancer cell stability and invasion ^(72, 91).

II. OBJECTIVES

This project is based on understanding the crosstalk between cancer cells, macrophages, and adipocytes, using ccRCC as a model. The choice of ccRCC relies on five premises: i) it is the most frequent type of RCC, ii) its anatomical location at the vicinity of perirenal adipose tissue, iii) the lipid and glycogen accumulation in the cytoplasm of tumour cells, iv) epidemiological evidence reporting the obesity paradox exists in this disease and, v) it is considered an immunogenic tumour with macrophages at the malignant niche.

Herein, 786-O ccRCC cells, adipocytes, and macrophages interplay will be analyzed in a 2D model to have an insight on their molecular communication. Additionally, using perirenal adipose tissue samples from ccRCC patients, we will characterize the histology of two distinct anatomic regions, juxta-tumoral and distant to the tumour. The presence of macrophages on the referred samples will also be assessed. With this strategy we expect to contribute for *the clarification of the obesity paradox, a “hot topic”* on renal cancer research.

To accomplish the projects ‘aims, we defined the following specific objectives:

- 1) Investigate the influence of perirenal adipose tissue adipocytes on macrophage and tumour cell profile in ccRCC context;
- 2) Discover potential key molecules of the triad communication;
- 3) Characterize macrophages infiltration in perirenal adipose tissue from ccRCC patient samples and evaluate juxta-tumoral and distant to the tumour histological differences.

III - MATERIAL AND METHODS

3.1. CLINICAL SAMPLE HARVESTING

Perirenal adipose tissue sample harvesting was performed from patients with renal tumours submitted to surgery. Briefly, collected perirenal adipose tissue was washed with PBS and divided into small pieces for: i) adipocyte isolation [immediately placed in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Invitrogen) supplemented with 10% of fetal bovine serum (Biowest, cat. Nº S181BH-500)], ii) RNA extraction (RNAlater storage, Invitrogen), and iii) histology (formaldehyde storage). All sample collection procedure was performed in accordance with the recommendations of the Ethics Committee of Centro Hospitalar do Porto (CHUP) (103-DEFI/107-CE), after kidney tumour patients voluntarily agreed to sign the informed consent. This study followed the Principles of the Helsinki Declaration.

3.2. HISTOLOGY ANALYSIS OF PERIRENAL ADIPOSE TISSUE

Histology processing and evaluation was performed at the i3S Histology and Electron Microscopy (HEMS) unit, specifically, immunohistochemistry (IHC) and Hematoxylin and Eosin (H&E) staining's. Slides were observed thereafter, under a Light microscope Olympus with a DP 25 Camera in order to analyze tissue structure, morphology, specific staining, as well as immune cell population presence. The visualization, analysis, and comparison of the tissue sections was made in images acquired in 10x and 20x magnifications. From each slide 5-10 representative images were taken, and each condition was further analyzed with quantification tools using Fiji Software.

3.3. ADIPOCYTE SIZE MEASUREMENTS

Perirenal adipose tissue samples, from juxta-tumoral and distant to the tumour regions, stained with H&E, were observed and 5-10 random representative images with a 10x objective were taken. Micrographs were then analyzed to obtain the area of 100 adipocytes per condition. The adipocyte area measurements were carried out using Fiji software and plotted in GraphPad software.

3.4. IMMUNOHISTOCHEMISTRY

IHC was used for the detection of macrophages in the samples from perirenal adipose tissue, using the CD68 marker for monocytes (1:200 CD68 Antibody Polyclonal, PA5-32331, Thermo Fisher). Of note, the positive control used was a tonsil sample. Paraffin blocks were sectioned *into 5 µm slices and tissues samples were deparaffinized, hydrated. Antigen retrieval was performed by incubating the sections* in commercial buffer from the Thermo Fisher Scientific kit (UltraVision Quanto Detection System HRP – Ref. TL-060-QHL) for 40 minutes at 98°C. Then, slides rested at 37°C for 20 minutes and were washed in TBST20 (Tris-buffered saline with 0.1% Tween® 20 detergent), twice, 5 minutes each. After, endogenous peroxidase activity was blocked by incubating each tissue with 100 µL of Peroxidase Blocker Solution for 10 minutes at RT, followed by 2 washing steps in TBST20, 3 minutes each. Blocking of non-specific binding was performed by incubating the slides with 100 µL of Ultra V Block for 10 minutes at RT. Primary antibody dilution was achieved with a commercial antibody diluent, in a solution of 1:200 (CD68). Each slide was then incubated overnight at 4°C. After a 15 minutes washing step with TBST20, slides were incubated in the secondary antibody (TL-060-QPH) for 10 min, RT. Next, tissue samples were exposed to Ultravision Quanto-HRP ThermoScientific (composed of hydrogen peroxide blocking solution, Ultra V block, Secondary Antibody/Primary Antibody Amplifier, Polymer-HRP, and DAB/Substrate) for 10 minutes *at RT, washed, and incubated for 3 to 5 minutes in a DAB solution (prepared accordingly to the manufacturers' instructions)*. Reaction was stopped using distilled water and counterstain was assessed with Meyer's Haematoxylin. Samples were then dehydrated and cleared in xylol, prior to mounting in Entellan medium.

3.5. ccRCC CELL LINE MAINTENANCE

The ccRCC cell line used was 786-O, derived from a primary tumour, with VHL gene mutation. It was cultured in RPMI-1640 (Gibco, Invitrogen) media, supplemented with 1% penicillin-streptomycin (Pen/Strep) (Biowest, 100x, cat no. L0022-100) and 10 % fetal bovine serum (FBS) (Biowest, cat. Nº S181BH-500). The conditions for cell maintenance were 37°C in a humidified atmosphere of 5% CO₂ in the incubator. In order to detach the cells, an incubation with 0.05% trypsin (Sigma-Aldrich, T4799) for 5 minutes at 37°C was used, and next, an inactivation of the enzyme using RPMI-1640 medium containing 1% Pen/Strep (Biowest) and 10% FBS was performed. Ideally, the cells used for further analysis had over 80% confluence.

3.6. MONOCYTE ISOLATION AND MACROPHAGE DIFFERENTIATION

In order to perform human monocytes isolation, buffy coats from healthy blood donors were centrifuged at 1200 x g (speed 5, break 0) for 30 minutes at RT. *The “white ring” corresponding to peripheral blood mononuclear cells (PBMCs) was collected and further incubated, at RT, in a horizontal shaker roller mixer for 20 minutes, with 67 µL of RosetteSep-Human Monocyte Enrichment Cocktail (StemCell Technologies) per mL of PBMCs.* After, this mixture was carefully diluted (1:1) in phosphate buffered saline with 2% fetal bovine serum, added over Ficoll-Histopaque1077 (Sigma-Aldrich), and centrifuged 1200 x g for 30 minutes (speed 0, break 0) at RT. The white ring, composed of monocytes was collected, washed in PBS 1X, and centrifuged at 1300 rpm for 6 minutes (speed 9; brake 9) at RT. The supernatant discarded and the

pellet was washed twice before cells were resuspended in 10 mL of RPMI1640 (Invitrogen) supplemented with 10% FBS and 1% penicillin-streptomycin (Biowest). Cells were seeded in 6-well cell culture plates (1.2×10^6 cells/well), placed in an incubator for 7 days, at 37°C, 5% CO₂. To achieve macrophage differentiation 50 ng/mL of M-CSF (ImmunoTools) was added to the cell culture. After the incubation period, the medium was refreshed by adding 1,5mL of RPMI supplemented with 10% FBS and 1% P/S per well, and cells were further incubated for 3 days at 37°C, 5% CO₂ ⁽⁹⁹⁾. Then, at day 10, differentiated macrophages were used in mono, co- and tri-culture experiments. This procedure has Ethical Committee approval from Centro Hospitalar Universitário de São João (CHUSJ), protocol 90/19, and follows the Helsinki Declaration criteria.

3.7. MACROPHAGES:786-O CELLS INDIRECT CO-CULTURE

786-O (1.5×10^5 cells/well) cells were seeded within the inserts (Falcon®, no. 353104), on a corresponding volume of 800 µL of cell suspension per well of a 6-well plate (Falcon®, no. 353046) containing 1,2mL of RPMI-1640 supplemented with 10% FBS and 1% P/S (complete media). The monoculture was performed during 72 hours at 37°C, 5% CO₂. After the incubation period, the biological material was collected, as explained in detail below.

Macrophage monolayers had their culture medium renewed to a volume of 1,2mL per well. Next, 6 well cell culture inserts with a pore size of 1.0 µm (Falcon) were set above each well of the 6-well cell culture plate and 800 µL of the 786-O cell suspension (1.5×10^5 cells/well) were seeded onto each insert. Macrophage monocultures, maintained in 2mL of RPMI complete media, were used as control of the experience. Cells were further incubated for 3 days at 37°C in a 5% CO₂ atmosphere. After the incubation period biological material from monocultures and co-cultures, namely RNA, cell lysates, cells and conditioned medium were collected to further analysis.

3.8. ADIPOCYTES ISOLATION

Perirenal adipose tissue samples from juxta-tumoral and distant to the tumour regions, collected from kidney tumour patient surgical resections were sliced into small pieces (**Figure 9A**), and its weight was measured. The adipose tissue was further separated from the fibrous material and minced in a Petri Dish containing the medium DMEM/F12 prior to the digestion with collagenase buffer 1x (HBSS + CaCl₂ + MgCl₂, 2% BSA) for approximately 30 to 40 minutes at 37°C (**Figure 9B**). The digested material was passed through a 250µm strainer, and the material was washed 3 times with KRHB buffer (1x KRH, 25 mM HEPES, 2 mM glucose, 2% BSA), using a system to separate the pre-adipocytes from the floating mature adipocytes (**Figure 9C**). Next, the floating mature adipocytes were transferred into a 50ml falcon and centrifuged at 50 x g for 3 minutes to pack the cells and separate free lipids (**Figure 9D**). Then, using a syringe, with an 18-gauge needle, free lipids were removed ⁽¹⁰⁰⁾. Of note, all of the experiments involving adipocytes, were performed following the same scheme for adipocytes from juxta-tumoral and distant to the tumour regions.

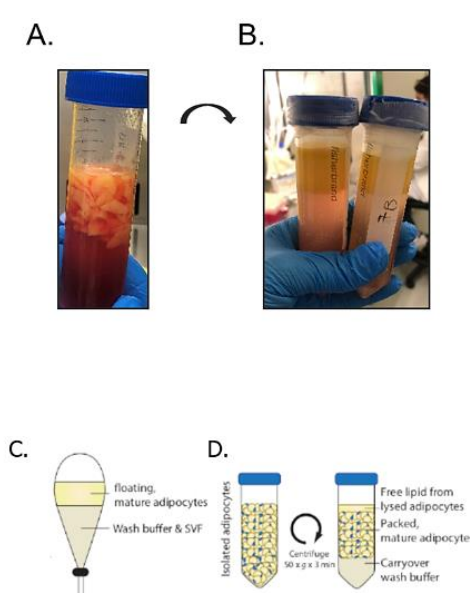


Figure 9 – Adipocyte's isolation approach.

A. Collected perirenal adipose tissue sample in DMEM/F12; **B.** Digested perirenal adipose tissue samples; **C.** Separation system for adipocytes isolation; **D.** Packing of mature adipocytes.

3.9. ADIPOCYTE CHALLENGING ASSAYS

3.9.1. Mature adipocytes seeding – Adipocytes monoculture

For adipocyte monoculture, 30 µL of the mature adipocytes were transferred onto each of the culture-specific inserts (24-well plate) carefully (**Figure 10A**). Then the membranes were inverted with the adipocytes on top, to make a surface-tension (**Figure 10B**). The plate with adipocytes was lowered into the wells containing 1 mL of RPMI medium and transferred into an incubator until further use in multiple culture procedures (**Figure 10C, D**) ⁽¹⁰⁰⁾. Note that in case of 6-well plate, we pipette 100 µL of the adipocytes cellular suspension.

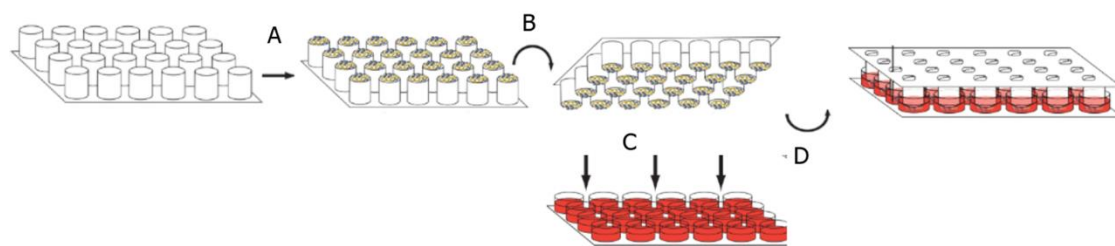


Figure 10 – Adipocyte culture method.

A. Pipette 30 μL of the mature adipocytes onto each of the inserts (24-well plate); **B.** Carefully, invert the membranes with the adipocytes on top, to make a surface-tension; **C.** Lower the plate with adipocytes into the wells containing 1 mL of RPMI medium; **D.** Transfer the plate into an incubator until further use (adapted from Alexandersson, Harms and Boucher 2020).

3.9.2. Macrophages direct co-culture with conditioned medium of adipocytes

After 10 days of macrophages and adipocytes monoculture, the medium of the macrophages was removed and 500 μL of the conditioned medium of adipocytes monoculture was added together with 500 μL of fresh medium DMEM/F12. This co-culture was established during 3 days and then the conditioned medium, the cells, and macrophage RNA were collected.

3.9.3. Macrophages direct co-culture with adipocytes

After one week of macrophages monoculture establishment, the medium of the macrophages was refreshed and 100 μL of the adipocytes suspension was plated according to the protocol above mentioned. This co-culture was established during 3 days and then, the conditioned medium, the cells, and RNA (from macrophages and adipocytes) was collected.

3.9.4. Adipocytes indirect co-culture with 786-O cells

After adipocyte isolation and plating into the specific inserts, 100 μL of 786-O cells (20 000 cells per well in 24-well plates) was added. This co-culture was established during 3 days and afterwards the conditioned medium, the cells and RNA of the both cell types, were collected for further analysis. Of note, when this procedure was performed in 6-well plates the volume of 786-O cell suspension per 100 μL of adipocytes suspension was adjusted.

3.9.5. Macrophages: 786-O: adipocytes tri-cultures

After adipocyte isolation, we used differentiated macrophages to establish the tri-culture. First, we refresh the macrophages media. In parallel, 100 μL of the mature adipocytes was plated onto each of the inserts of the 6-well plate, which were then placed above the macrophages. Finally, 1.5×10^5 cells per well of 786-O cells were plated within the insert containing the adipocytes. These tri-cultures were established during 3 days. Finally, the conditioned medium, the cells and RNA of each cell type were collected for further analysis.

3.10. CONDITIONED MEDIUM COLLECTION

From the plates used for cell culture, conditioned medium (CM) was collected, namely, from 786-O cells monoculture, macrophages monoculture, macrophage:786-O co-culture, adipocytes monoculture, adipocytes:786-O co-culture, macrophages with adipocytes CM, macrophages: adipocytes co-culture, and tri-culture. The collected CM were centrifuged at 1500 rpm for 5 minutes at 4°C and stored at -80°C until further use.

3.11. CELL LYSATES OF MACROPHAGES AND 786-O CELLS

Cells were washed with PBS 1x and incubated with 60 μL of RIPA buffer [50 mM Tris-HCl (pH=7.5), 150 mM NaCl, 2 mM EDTA and 1% NP-40] supplemented with a cocktail of proteases and phosphatases inhibitors [1mM phenylmethanesulfonylfluoride (PMSF), 3 mM sodium orthovanadate (Na_3VO_4), 20 mM sodium fluoride (NaF), 25 mM sodium pyrophosphate tetrabasic ($\text{Na}_4\text{P}_2\text{O}_7$), 10 mg/mL aprotinin, 10 mg/mL leupeptin], during 15 minutes on ice. Next, a cell scraper was used to detach the cells. After, lysates were centrifuged at 13000 rpm for 10 minutes at 4°C, the supernatant was collected and preserved at -80°C until further use. Protein concentration was determined using the DC™ Protein Assay Kit (Bio-Rad).

3.12. RNA EXTRACTION OF MACROPHAGES AND ccRCC CELLS

Total RNA was extracted from one well of each cell culture condition using EXTRAzol according to manufacturer’s instructions. Briefly, the medium was removed from the wells and stored for further use (conditioned medium, as above) while cells were disrupted by adding 1mL of EXTRAzol (EM30, Blirt). After 5 minutes incubation at RT, 190 µL of chloroform was added, following a vigorous shake. The cell suspension was centrifuged at 12000 x g for 10 minutes at 4°C and the aqueous phase was transferred to another tube. RNA precipitation was accomplished by adding 500 µL of cold isopropanol. After an incubation period of 10 minutes RT, a 10-minute centrifugation at 12000 x g at 4°C was performed. The supernatant was removed, and the pellet was washed once with 1mL of 75% ethanol. Finally, the pellet was centrifuged at 7500 x g for 5 minutes at 4°C, air-dried and dissolved in 25µL of Ultra-Pure Water. Nanodrop Spectrophotometer ND1000 (Thermo Scientific) was used to perform a quantitative and qualitative evaluation of the RNA. Samples were stored at -80°C for further analysis.

3.13. RNA EXTRACTION OF ADIPOCYTES

Total RNA was extracted from adipocytes using QIAzol, according to the manufacturer’s protocol. First, cells were centrifuged at 12000 rpm for 10 minutes at 4°C, the supernatant was discarded, and the adipocytes lysed by adding 1mL of QIAzol Lysis Reagent (QIAGEN no. 79306) and by passing the lysate several times through a 1ml syringe with a 0.8 mm needle. Then, cell homogenate was centrifuged as above and the lipid fraction at the top was removed. Next, 200 µL of chloroform was added and solution was vigorously mixed during 3 minutes. Finally, the upper aqueous phase containing the adipocyte RNA was transferred to a new tube and 600 µL of 70% ethanol was added to achieve RNA precipitation. Isolated RNA was kept at -80°C.

3.14. COMPLEMENTARY DNA FIRST-STRAND SYNTHESIS

Complementary first-strand DNA was synthesized according to Table 1. First, RNA samples and water were added into a nuclease-free microcentrifuge tube (Table 1). Then, a master mix containing random primers and dNTPs was performed, from which 2 µL were added to each microcentrifuge tube. This solution was heated to 65°C in a thermocycler (BIOMETRA T1 Thermoblock Thermal Cycler) for 5 minutes. A second master mix was prepared containing first-strand buffer, DTT, NZYRibonuclease Inhibitor, Nuclease Free-Water and NZY M-MuLV RT Enzyme, 8 µL of this solution were added to each previously prepared sample. cDNA synthesis was achieved following the cycles: 25 °C for 10 minutes, 37°C for 50 minutes and 70°C for 15 minutes. Finally, cDNA was stored at -80°C.

Table 1 – Component’s list used to the synthesis of cDNA.

REACTION COMPONENTS	VOLUME/TUBE
FIRST ADD TO THE TUBES:	
RNA SAMPLE	1000ng/conc. RNA sample
NUCLEASE FREE-WATER (INVITROGEN)	Up to 10 µL
MASTER MIX 1:	
RANDOM PRIMERS (250 NG/µL, INVITROGEN)	1 µL
10 MM DNTP MIX (BIORON)	1 µL
MASTER MIX 2:	
5X FIRST-STRAND BUFFER (INVITROGEN)	4 µL
0.1 M DTT (INVITROGEN)	2 µL
NZYRIBONUCLEASE INHIBITOR (20 UNITS/ µL, NZYTECH)	1 µL
NUCLEASE FREE-WATER (INVITROGEN)	0.5 µL
NZY M-MULV RT ENZYME (NZYTECH)	0.5 µL

3.15. QUANTITATIVE REAL-TIME PCR (qRT-PCR)

Using the cDNA previously synthesized, qRT-PCR was performed using a TaqMan Universal PCR Master Mix (KAPA PROBE FAST qPCR Master Mix, IKAPA Biosystems) and probes for IL-6, TGF-β1, IL-8 and, IL-10. As housekeeping gene for cell lines and macrophages, GADPH was used (Table 2). This reaction was carried out in a 7500 Real Time PCR system (Applied Biosystems). The program was 1 step of 95 °C

(20 seconds) followed by 40 cycles of 95°C (3 seconds) and 60 °C (30 seconds). Each reaction was accomplished in duplicated and relative mRNA expression of the *target genes* was *normalized to the levels of the housekeeping gene prior to using the comparative $\Delta\Delta CT$ method*.

Table 2 – Gene assays details for qRT-PCR.

Gene	Assay ID
<i>IL-6</i>	Hs.PT.58.40226675
<i>TGF-β1</i>	Hs.PT.58.39813975
<i>GAPDH</i>	Hs.PT.39a.22214836
<i>IL-8</i>	Hs.PT.58.39926886.g
<i>IL-10</i>	Hs.PT.58.2807216

3.16. ELISA ASSAY

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the secreted cytokine levels of *IL-1 β* , *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12p70*, *TNF- α* , *TGF- β* and, *IFN- γ* , in *CM* of *mono*, *co-* and *tri-cultures*, using the *LEGEND MAX™ Human ELISA Kits (BioLegend)*, according to *manufacturer’s instructions*. Briefly, *CM* was added to a 96-well pre-coated plate and incubated overnight at 4°C. Then using the commercial detection antibody and Avidin-HRP solutions, the proteins bounded to the capture antibody were detected. Of note, all of the incubation steps were intercalated with washes, using washing buffer solution. The ultimate step was the addition of TMB substrate solution, that might induce a colour change depending on the antigen capture. Absorbance was read at 450 nm and subtracted to the absorbance at 570 nm, in order to plot the values on a standard curve to determine the protein target levels.

3.17. WESTERN BLOTTING

Cell lysates were diluted in 4x Laemmli buffer (Bio-Rad), denatured at 94°C for 5 minutes, and loaded onto a 10% polyacrylamide gel, followed by electrophoresis at 25 mA per gel. Transference of proteins was performed at 100 V for 2h into Amersham™ Protran™ 0.45 NC nitrocellulose blotting membranes (Cytiva). Subsequently, each of the blotting membranes was blocked with Bovine Serum Albumin (BSA, Jackson Immuno Research) for 1h and incubated with primary monoclonal antibodies, namely, AKT (rabbit, 1:1000), p-AKT (mouse, 1:1000), STAT1 (rabbit, 1:1000), p-STAT1 (rabbit, 1:1000), STAT3 (mouse, 1:1000), p-STAT3 (rabbit, 1:1000), p65 (rabbit, 1:1000), p-p65 (rabbit, 1:1000), ERK (mouse, 1:1000), p-ERK (rabbit, 1:1000), SRC (rabbit, 1:1000), p-SRC (rabbit, 1:1000) at 4°C overnight. Of note, as loading control, membranes *were incubated with a primary monoclonal antibody for β -actin* (mouse, 1:500) detection, during 1h at RT. After, membranes were washed and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies, anti-mouse (1:3000) or anti-rabbit (1:5000), for 1 hour at RT. Finally, signal detection was assessed with the Amersham ECL Western Blot detection kit (Bio-Rad).

3.18. FLOW CYTOMETRY

After removing the media, cells were washed with PBS and harvested with Accutase®. Collected cells were then centrifuged for 5 minutes at 1500 rpm and stained for flow cytometry analysis. Antibodies solutions were performed as indicated in **Table 3**, according to the specific cell type. Cells were resuspended in the respective antibody solution, incubated for 40 minutes at 4°C and, washed three times in FACS buffer (PBS 1x; 2%FBS; 0,01% sodium azide). Finally, cells were resuspended in FACS buffer, filtered and, acquired on a FACS Canto flow cytometer (BD Biosciences) using FACS Diva software. Data analysis was performed with FlowJo software.

Table 3 – Antibody specifications used in flow cytometry approach.

	Antibody	Reference and specificities
Macrophages	CD14-APC	Cat 21450146 Immunotools Clone OFC14D Mouse IgG
	CD86-FITC	Cat 21480863 Immunotools Clone BU63 Mouse IgG1
	CD163-PE	Cat 556018 BD Pharmingen Clone GHI/61 Mouse BALB/c IgG1, κ
	HLA-DR-FITC	Cat 21278993 Immunotools Clone MEM-12 Mouse IgG1, κ
	CD206-PE	Cat 321106 Biolegend Clone 15-2 Mouse IgG1, κ
Cancer Cells	PDL1 (CD274)-FITC	Cat 558065 BD Pharmingen Clone MIH1 Mouse IgG1, κ
	PD1 (CD279)-PerCP-Cy 5.5	Cat 561273 BD Pharmingen Clone EH 12.1 Mouse IgG1, κ
	HLA-ABC-PE	Cat 21159034 Immunotools Clone W6/32 Mouse IgG2a
	CD47-FITC	Cat 21270473 Immunotools Clone MEM-122 Mouse IgM, κ
Adipocytes	DAPI	Sigma Aldrich D9542-5MG Molecular Probes 5 µg/mL
	HSC-LipidTox-Alexa Fluor® 647 dye or Cy®5 dye	Invitrogen Green Neutral Lipid Staining Molecular Probes

3.19. STATISTICAL ANALYSIS

The data obtained in the flow cytometry and ELISA assays was analyzed using the GraphPad Prism 9 software (GraphPad Software, San Diego, California USA). Data were tested for the normality of the distribution using Shapiro-Wilk normality tests and the appropriate test was applied accordingly. Briefly, in the case of two variables, if the distribution was normal, the parametric Student’s t-test was employed, otherwise the non-parametric Mann–Whitney test was used. In case of more than two variables, one-way ANOVA was chosen. The statistical significance was reached for $p \leq 0.05$.

IV – RESULTS

SECTION A - UNDERSTANDING THE COMMUNICATION ESTABLISHED BETWEEN 786-O ccRCC CELLS, MACROPHAGES, AND ADIPOCYTES

(manuscript in preparation)

1. Macrophage profile is altered upon culture with ccRCC cells

To understand if ccRCC cells have the ability to alter macrophage profile, we analyzed the expression of different polarization markers upon macrophage culture with 786-O cells. Specifically, we assessed the percentage of cells expressing M1-like phenotype molecules, CD86 and HLA-DR, and M2-like markers, CD163 and CD206. The expression of each referred molecule in each cell was also determined (MFI). Concerning macrophages from co-culture with 786-O cells, our data shows a significant decrease of the percentage of macrophages expressing CD86 marker, despite the number of molecules expressed (MFI) not being altered, as observed in **Figure 11**. Concomitantly, there is a trend towards an increase of the percentage of macrophages expressing CD206 marker and an increase of CD206 molecules expressed by each of these phagocytes upon exposure to 786-O cell line. In addition, our results indicate that the presence of ccRCC cells does not affect the macrophage HLA-DR (MHC-II) and CD163 expression. Altogether, these data indicate that, upon challenge with 786-O ccRCC cell line, macrophage phenotype is altered.

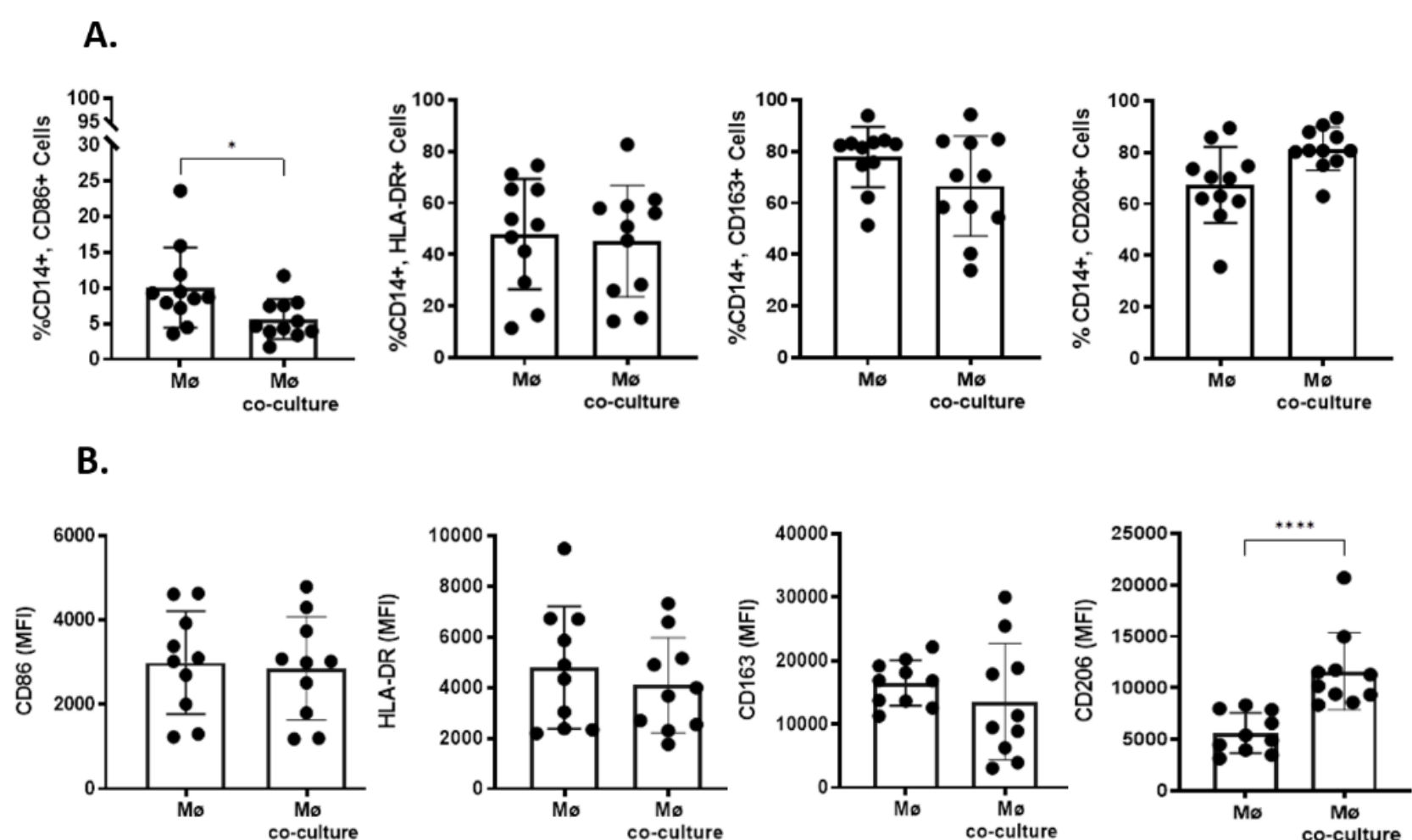


Figure 11 – Macrophage profile changes upon culture with 786-O cells.

A. Scatter-plot represents the percentage of CD14 positive cells expressing CD86, HLA-DR, CD206, and CD163 in macrophage monolayers (Mø) and in macrophages obtained from co-culture with 786-O cells (Mø co-culture); (n=11). **B.** Shown is the number of molecules (MFI, mean fluorescence intensity) expressed by macrophages from monoculture (Mø) and by macrophages in co-culture with 786-O cells (Mø co-culture). Each point indicates one different experiment that corresponds to a different monocyte donor (n=10). Statistical analysis was performed on GraphPad and the Mann-Whitney test was applied in CD163 and CD206 markers analysis while Student's t-test was used for HLA-DR and CD86. * $P \leq 0.05$, **** $P \leq 0.0001$.

2. ccRCC cells immunogenicity is not influenced by macrophages

Since our results suggest a communication between 786-O cells and macrophages, we analyzed whether ccRCC cells change their immunogenicity profile in the presence of macrophages. For that, the expression of HLA-ABC (antigen presentation molecule or MHC-I), CD47 (“do not eat me” signal), PDL-1 (negative regulator ligand of T Cells), and PD-1 (negative regulator molecule of T Cells) markers was assessed.

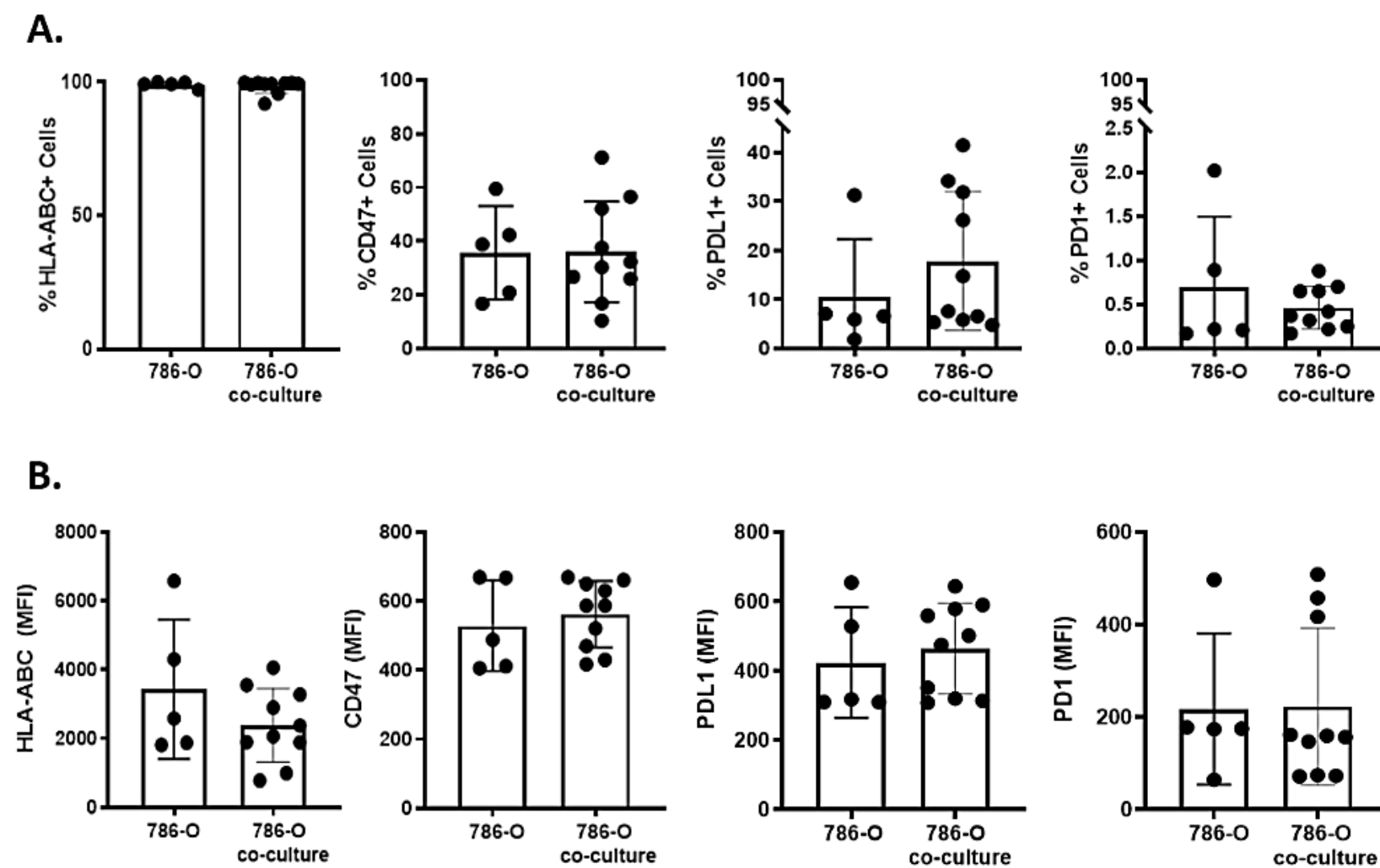


Figure 12 – The immunogenicity profile of 786-O cells does not change after culture with macrophages.

A. Scatter-plot represents the percentage of 786-O cells expressing markers of immune activation (HLA-ABC) and immune-escape (CD-47, PDL-1 and PD-1) in monoculture (786-O) and in co-culture with macrophages (786-O co-culture). **B.** Shown is the number of molecules (MFI, mean fluorescence intensity) expressed by tumour cells from monoculture (786-O) and by 786-O in co-culture with macrophages (786-O co-culture). Each point indicates one different experiment that corresponds to different monocytes donors (786-O monoculture, n=5; 786-O co-culture, n=10). Statistical analysis was performed on GraphPad and the Student's t-test (HLA-ABC and CD47), or Mann-Whitney test (PDL-1 and PD-1) were applied.

As shown in **Figure 12**, there are no differences in the expression of immunogenicity markers in 786-O cells from the co-culture condition when compared to the monoculture. As such, our results indicate that macrophages do not seem to exert an influence on the 786-O immunogenicity molecules analyzed herein. Moreover, flow cytometry results suggest that on one hand this ccRCC cell line does not express higher amount of immune-escape molecules, and on the other hand, HLA-ABC expression is likely a feature of 786-O cell line.

3. Macrophage and 786-O cell interplay modulates the secretion of pro- and anti-inflammatory cytokines

So far, our results indicate that macrophages alter the expression of CD86 and CD206 when cultured with 786-O cells, thus suggesting an interplay between such phagocytes and ccRCC cells. In order to understand if the secretome is also altered when macrophages and 786-O cells are communicating, we characterized the cytokine profile on the medium of macrophages and tumour cells co-cultures. For that, we performed ELISA assays for pro-inflammatory molecules (TNF- α , IL-8, IL-1 β , IL-12p70), anti-inflammatory cytokines (TGF- β 1, IL-10, IL-4), as well as those with pleiotropic function depending on the microenvironment context (IL-6, IFN- γ). *Of note, IFN- γ and IL-4 levels were not detected, thereby these data are not shown (n=13).* Analyzing the obtained data, it is verified that there are significant differences on TGF- β 1 secretion being enhanced in the co-culture when compared to the monoculture conditions (**Figure 13 B**). Moreover, IL-10 levels are decreased in the co-culture when compared to macrophage monocultures and it is not secreted by 786-O cells. On the other hand, IL-6 cytokine is not secreted by macrophages, but presents high secretion levels in 786-O cells alone and in the medium from the co-culture with macrophages. As for the pro-inflammatory cytokines, TNF- α and IL-1 β , our results indicate that they are either not secreted or have very low levels in the monocultures as well as in the co-cultures. Moreover, high IL-8 levels seem to exist in macrophage and 786-O monocultures as well as in the co-cultures between both cell types. Regarding IL-12(p70) secretion levels, there are no significant differences between the culture conditions, and a high variation in the secretion profile found between monocytes donors (**Figure 13 A**).

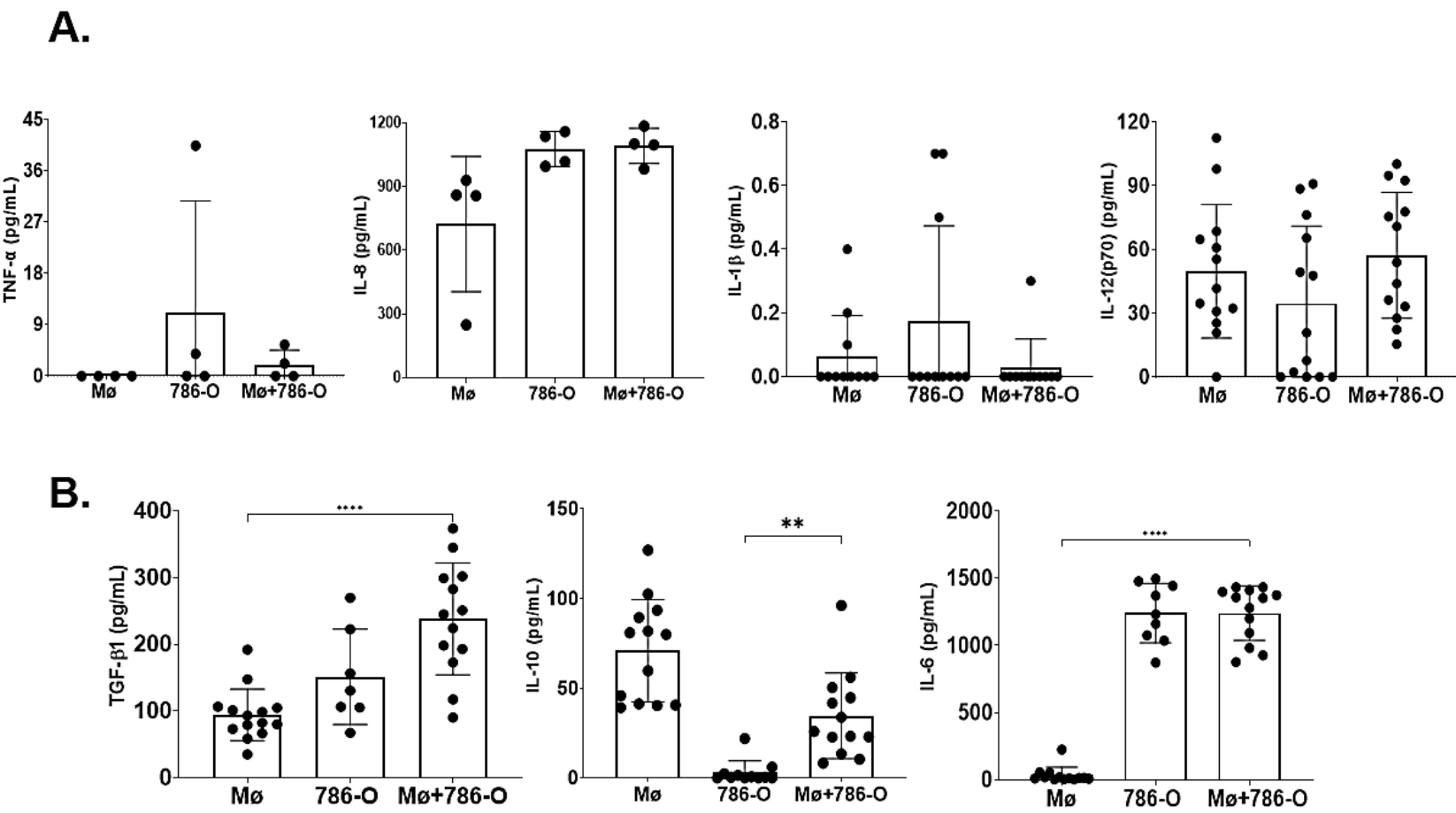


Figure 13 – Cytokine secretion is altered when ccRCC cells and macrophages communicate.

A. Secreted levels (pg/mL) of TNF- α ($n=4$), IL-8 ($n=4$), IL-1 β ($n=11$), and IL-12(p70) ($n=13$) on the conditioned medium of cell monocultures (Mø or 786-O cells) and of macrophages and 786-O cells co-cultures (Mø +786-O). Each point indicates one different experiment that corresponds to a different monocyte donor. **B.** Shown is the concentration of TGF- β 1 ($n=13$), IL-10 ($n=13$), and IL-6 ($n=14$) on cell monocultures (Mø or 786-O cells) and co-culture of macrophages with 786-O cells (Mø +786-O) collected media. Each point indicates one different experiment performed with a different monocyte donor. The statistical analysis was executed on GraphPad and the one-way ANOVA test was used as distributions follow the normality (** $P \leq 0.01$; **** $P \leq 0.0001$).

To further understand the previous data, we investigated if IL-8, TGF- β 1, IL-10, and IL-6 secretion, found in the co-cultures, was produced by macrophages, tumour cells or both cellular types. To achieve this, we analyzed the levels of the cytokine transcripts from macrophages and 786-O cells obtained from mono- and co-cultures. Of note, IL-10 mRNA levels in 786-O were not detected, meaning that in tumour cells, this gene is not transcriptionally active (data not shown).

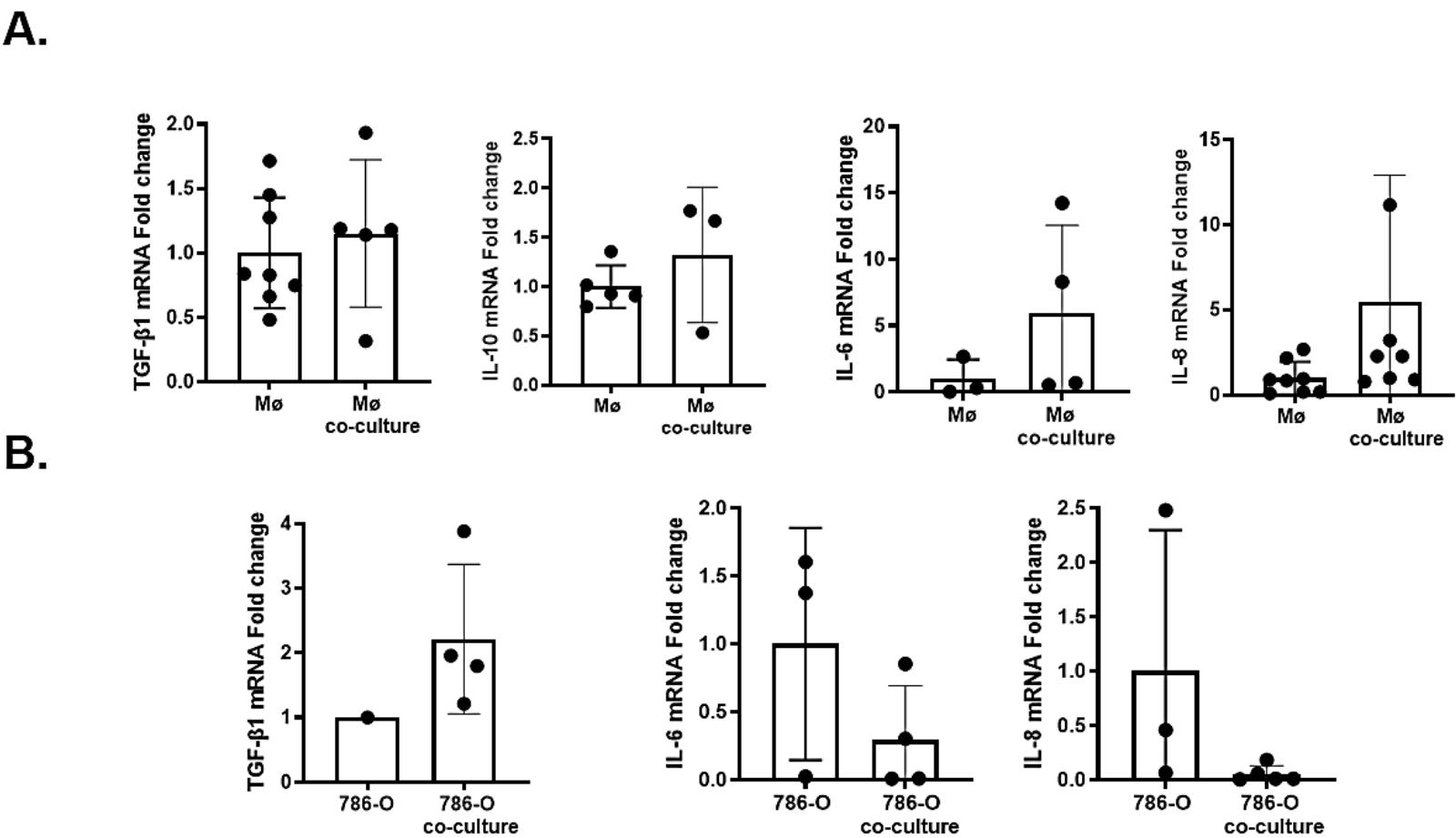


Figure 14 – The interplay between macrophages and ccRCC cells alters their cytokine profile.

A. Data here depicted is represented as fold-change comparing macrophages from co-culture with 786-O cells (Mø co-culture) with the macrophage monocultures (Mø). Values were normalized to GADPH (housekeeping gene) expression. Each point refers to a different monocyte donor. **B.** Depicted is IL-8, TGF- β 1, and IL-6 gene expression as fold-change comparing 786-O from co-culture (786-O co-culture)

with ccRCC cells from monocultures (786-O). Bars represent mean values $\pm$ standard deviation. No statistically significance was found.

As shown in **Figure 14**, *TGF- β 1 expression is enhanced in 786-O cells but not in macrophages collected from the co-cultures*. As such, the significant increase observed in *TGF- β 1 secreted levels (Figure 13 B)* is likely to be a modulation of ccRCC cells secretome upon crosstalk with macrophages. As for IL-10, no major changes were observed in macrophage gene expression and no transcript was detected in 786-O cells either in the monoculture or in the cells obtained from the co-culture. Thus, it is likely that the decrease of IL-10 in co-cultures, uncovered in ELISA assays, may be due to protein regulation and not alterations at the mRNA levels.

High levels of IL-6 secretion were found in 786-O monocultures and co-cultures conditions (**Figure 13 A**). However, a decrease in *IL-6* gene expression in ccRCC cells obtained from co-culture was found. Thus it is possible that macrophages, which present enhanced IL-6 mRNA levels when in co-culture with 786-O cells, tip the final balance of IL-6 in the co-culture medium (**Figure 14**). This clearly suggests a crosstalk between these cells mediated most likely by secreted factors. The expression levels of IL-8 are yet difficult to integrate, still it is possible that macrophages, upon culture with 786-O, up-regulate *IL-8* expression while malignant cells negatively regulate this cytokine mRNA expression. Note that, some of the results analyzed herein need to be completed with a higher number of experimental replicates before taking reliable conclusions. Nevertheless, these data point to an effective communication between macrophages and ccRCC cells.

4. STAT1 and STAT3 activation is significantly increased in macrophages cultured with 786-O cells

So far, the previous results indicate that there are significative differences in IL-10, IL-6, and *TGF- β 1 secreted cytokines levels*. Thus, we wanted to understand which signaling pathway is triggered as a means to induce such changes. To achieve this, we analyzed the expression of STAT1, STAT3, AKT, Src, p65, and ERK, proteins associated to the regulation of the above-mentioned cytokines. First, the total protein levels were assessed to understand whether there were alterations in the expression of such proteins in macrophages collected from mono- and co-culture with 786-O cells. Yet, as shown in **Figure 15**, no major changes were observed.

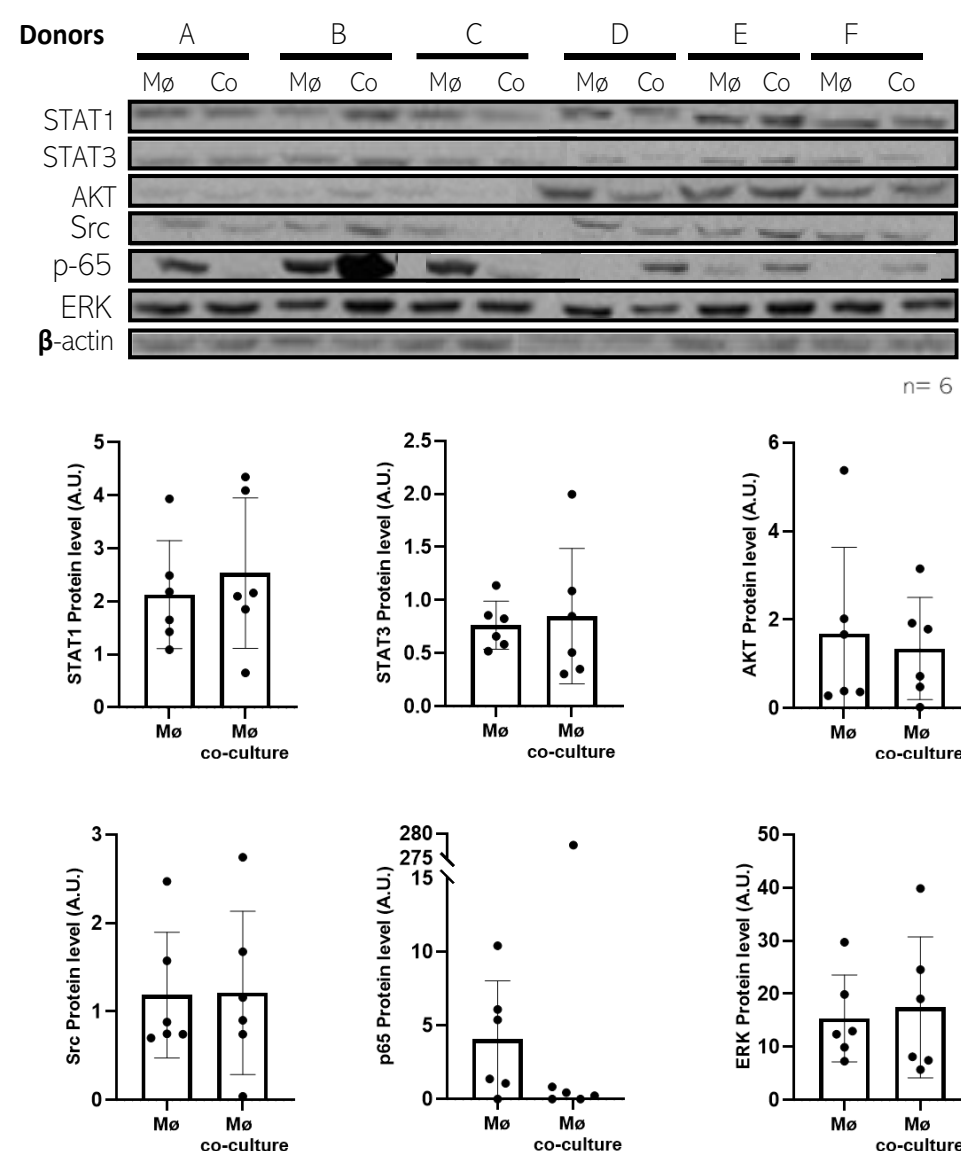


Figure 15 – No major differences in the expression of macrophage proteins associated to cytokine regulation.

Expression of STAT1, STAT3, AKT, Src, p65, ERK in macrophages from mono- (Mø) and co-cultures with 786-O cells (Mø co-culture). Western blots depicted herein indicate the protein expression of macrophages' donor A to F. At the bottom, protein quantifications by densitometry are plotted (n=6). Statistical analysis was performed on GraphPad and the Student's t-test, or Mann-Whitney test were applied.

Secondly, we tested if the activation of these proteins, via the expression of the phosphorylated form, was changed. The results indicate that there is a significant increase in the p-STAT1 and p-STAT3 protein levels in macrophages from co-culture with 786-O, when compared to macrophages from monoculture (**Figure 16**). Moreover, p-AKT and p-ERK levels seem to be decreased in macrophage lysates obtained from

co-cultured with 786-O cells. As for p-Src and p-p65 the results are unclear and require additional experimental replicates.

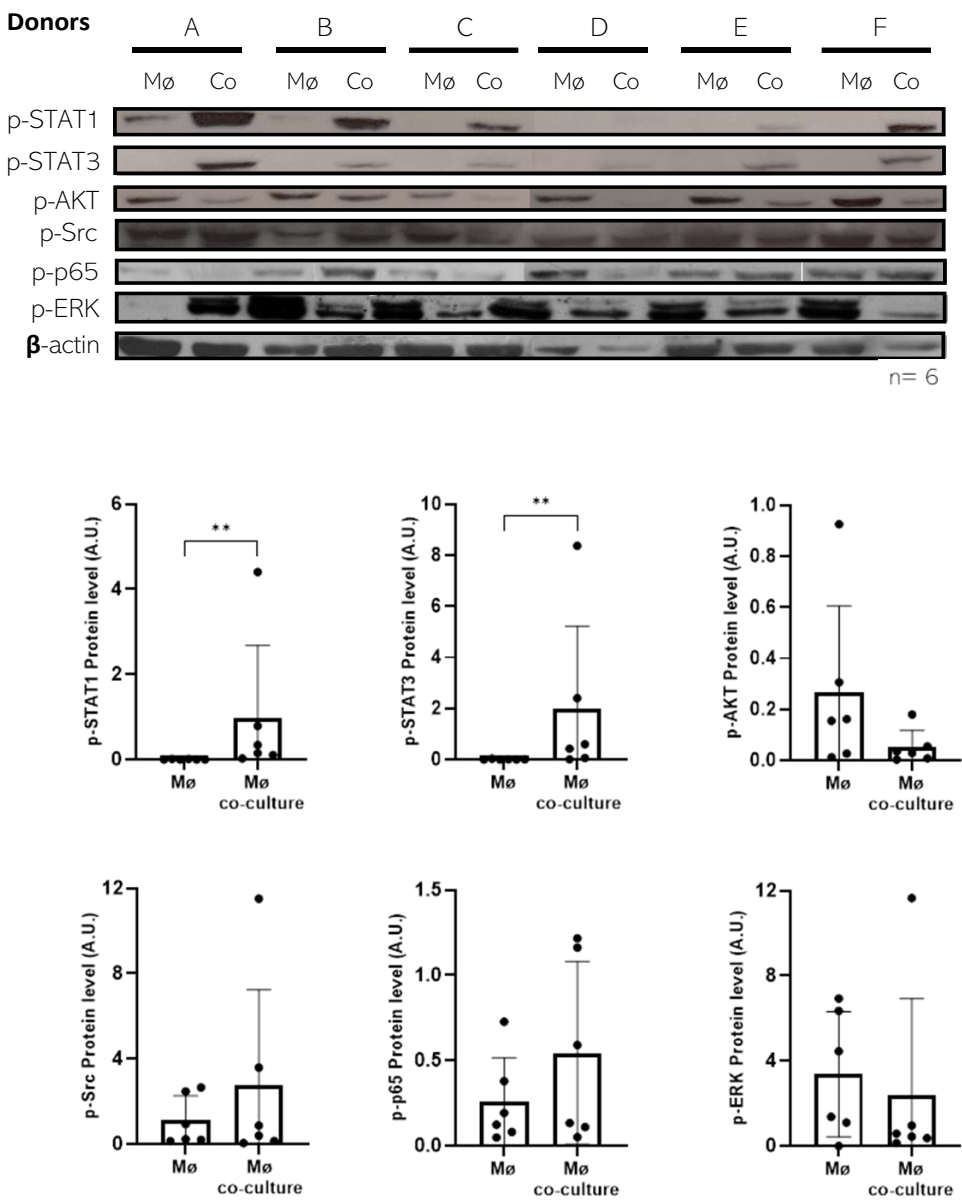


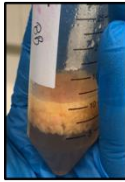
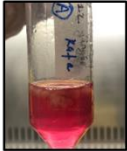
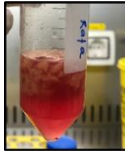
Figure 16 – Levels of protein activation in macrophages lysates.

Macrophage p-STAT1, p-STAT3, p-AKT, p-Src, p-p65 levels in mono- (Mø) and co-cultures with 786-O cells (Mø co-culture). Western blotting analyzes of macrophage lysates obtained from donor A to F. The bottom panel depicts protein quantifications by densitometry (n=6). Statistical analysis was performed on GraphPad and Student's *t*-test or Mann-Whitney test were applied.

5. Preliminary data suggests a close crosstalk between 786-O cells, macrophages, and adipocytes

To understand the impact of the triad interplay on macrophage polarization and on 786-O immunogenicity profile, we performed tri-cultures with 786-O cells, macrophages, and adipocytes from juxta-tumoral and distant to the tumour perirenal adipose tissue. To achieve this, we performed three different experiments with isolated adipocytes from juxta-tumoral and from distant to the tumour perirenal adipose tissue from *three different kidney tumour's patients, who's relevant clinicopathological data is represented in Table 4*. Of note, these are very preliminary results since the cohort is still very small, not fully restricted to ccRCC samples, and is still ongoing. As such, the experiments performed here with adipocytes isolated from fresh perirenal adipose tissue samples were obtained not only from ccRCC patients, but also from other kidney tumours subtypes. In addition, a number of controls are yet to be performed, specifically, it is important to highlight that it was not possible to obtain data of macrophages co-cultured with adipocytes from juxta-tumoral perirenal adipose tissue (Mø + Adip. Just.) (Figure 17). Regarding macrophage profile it is possible to observe that there is heterogeneity between patients, reflected by the differences in the expression of the macrophage profile-associated markers. Importantly, one must have in mind that replicate differences may occur due to the distinct kidney tumour histological types (Table 4) from which adipocytes were collected from. In detail, concerning patient 2, macrophage phenotype when in culture with adipocytes, presents a trend towards an increase of the phagocyte percentage expressing M2-like markers, despite the number of molecules expressed (MFI) per cell not being altered. As for patient 3, a mixed macrophage phenotype is clearly stimulated in the tri-culture with adipocytes and 786-O cells. Additionally, tri-cultures with 786-O cells and adipocytes from the juxta-tumoral perirenal adipose tissue or with adipocytes from distant to the tumour perirenal adipose tissue seem to present a different molecular signature (Figure 17).

Table 4 – Clinicopathological data from patients with kidney tumours whose adipocytes were collected for in vitro cultures experiments.

PATIENT CODE	PATIENT CHARACTERISTICS	PERIRENAL ADIPOSE TISSUE SAMPLE	
		JUXTA-TUMORAL	DISTANT TO THE TUMOUR
Patient 1 (▲)	Histology: ccRCC Gender: Female Comorbidities: Obesity		
Patient 2 (●)	Histology: renal oncocytoma Gender: Female Comorbidities: none		
Patient 3 (■)	Histology: RCC papillary type I Gender: Female Comorbidities: none		

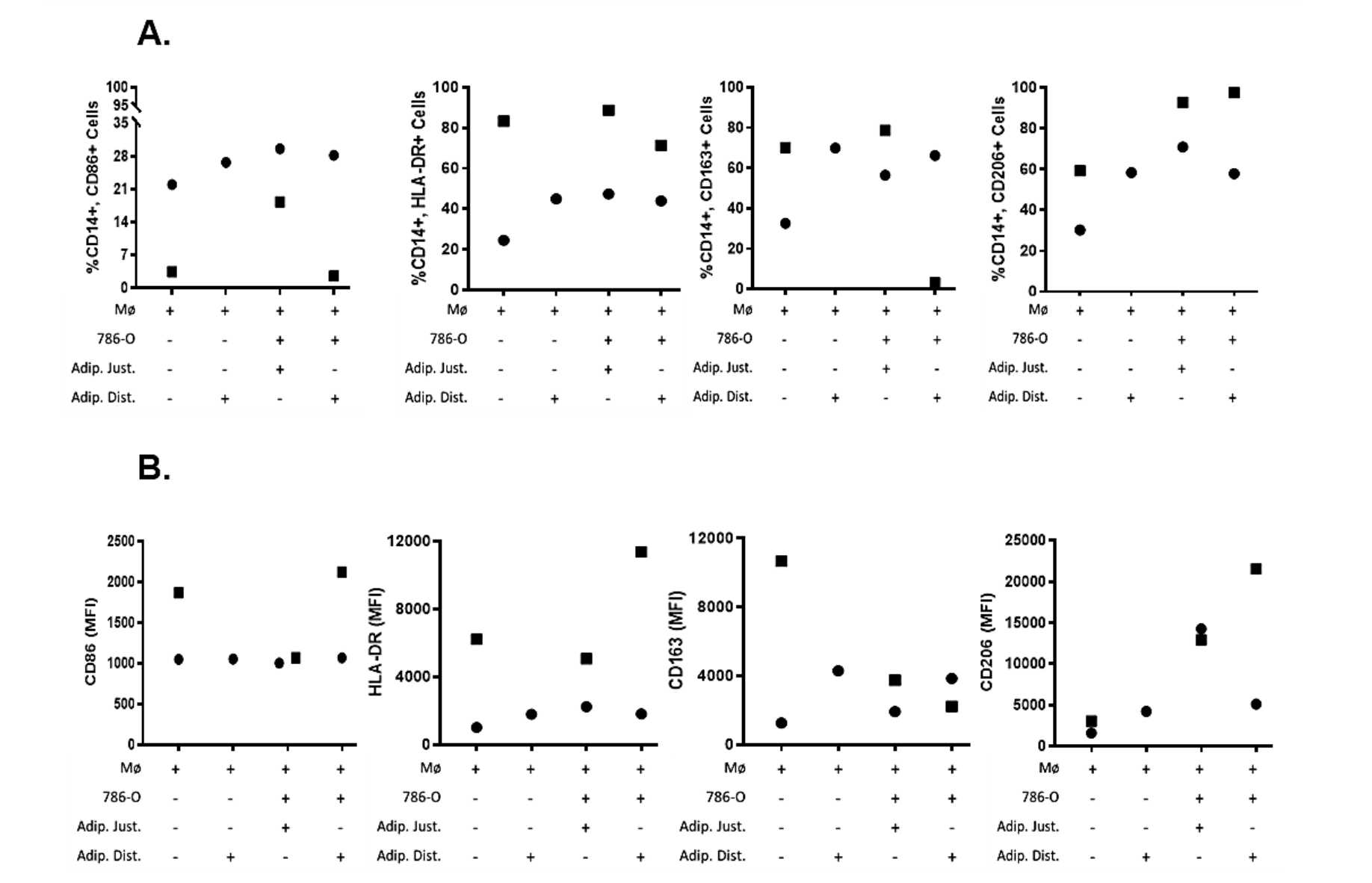


Figure 17 – Macrophage phenotype is altered upon culture with ccRCC cells and adipocytes.

A. Scatter-plot represents the percentage of CD14 positive cells expressing CD86, HLA-DR, CD206, and CD163 in macrophage monolayers (Mø), macrophages co-cultured with adipocytes from distant to the tumour perirenal adipose tissue (Mø + Adip. Dist.), macrophages from tri-culture, with 786-O and adipocytes from juxta-tumoral perirenal adipose tissue (Mø + 786-O + Adip. Just.) or from distant to the tumour perirenal adipose tissue (Mø + 786-O + Adip. Dist.). **B.** Illustrated is the number of molecules (MFI, mean fluorescence intensity) expressed by macrophages from monoculture (Mø), macrophages co-cultured with adipocytes from distant to the tumour perirenal adipose tissue (Mø + Adip. Dist.), macrophages from tri-culture, with 786-O and adipocytes from juxta-tumoral perirenal adipose tissue (Mø + 786-O + Adip. Just.) or from distant to the tumour perirenal adipose tissue (Mø + 786-O + Adip. Dist.). Each different symbol indicates one different experiment that corresponds to a different adipocytes and macrophages donor (n=2).

With regard to the tumour cells, macrophages, and adipocytes crosstalk, and their influence on the ccRCC cell immunogenicity markers, we detected no differences in all of the markers analyzed for patient 2. Yet, in patient 1 a decrease in the percentage of 786-O cells that express

HLA-ABC marker when in co-culture with adipocytes was observed. Finally, in patient 3 there are no alterations in HLA-ABC, and PD-1 expression comparing 786-O monoculture with tri-culture using adipocytes from juxta-tumoral perirenal adipose tissue. However, when looking to the tri-culture with adipocytes isolated from distant to the tumour perirenal adipose tissue we observed modulations in the percentage of 786-O cells expressing HLA-ABC, PD-1. Regarding PDL-1 marker, data points to an increase in 786-O cultured with macrophages and adipocytes (**Figure 18**).

Altogether, these piece of very preliminary data suggests that both macrophages and ccRCC cells crosstalk with adipocytes from the perirenal adipose tissue, independently of the renal tumour subtype. Moreover, results gathered herein point to differences in the expression of specific macrophage markers and immunogenicity molecules, when cells are challenged with adipocytes from distinct anatomic regions.

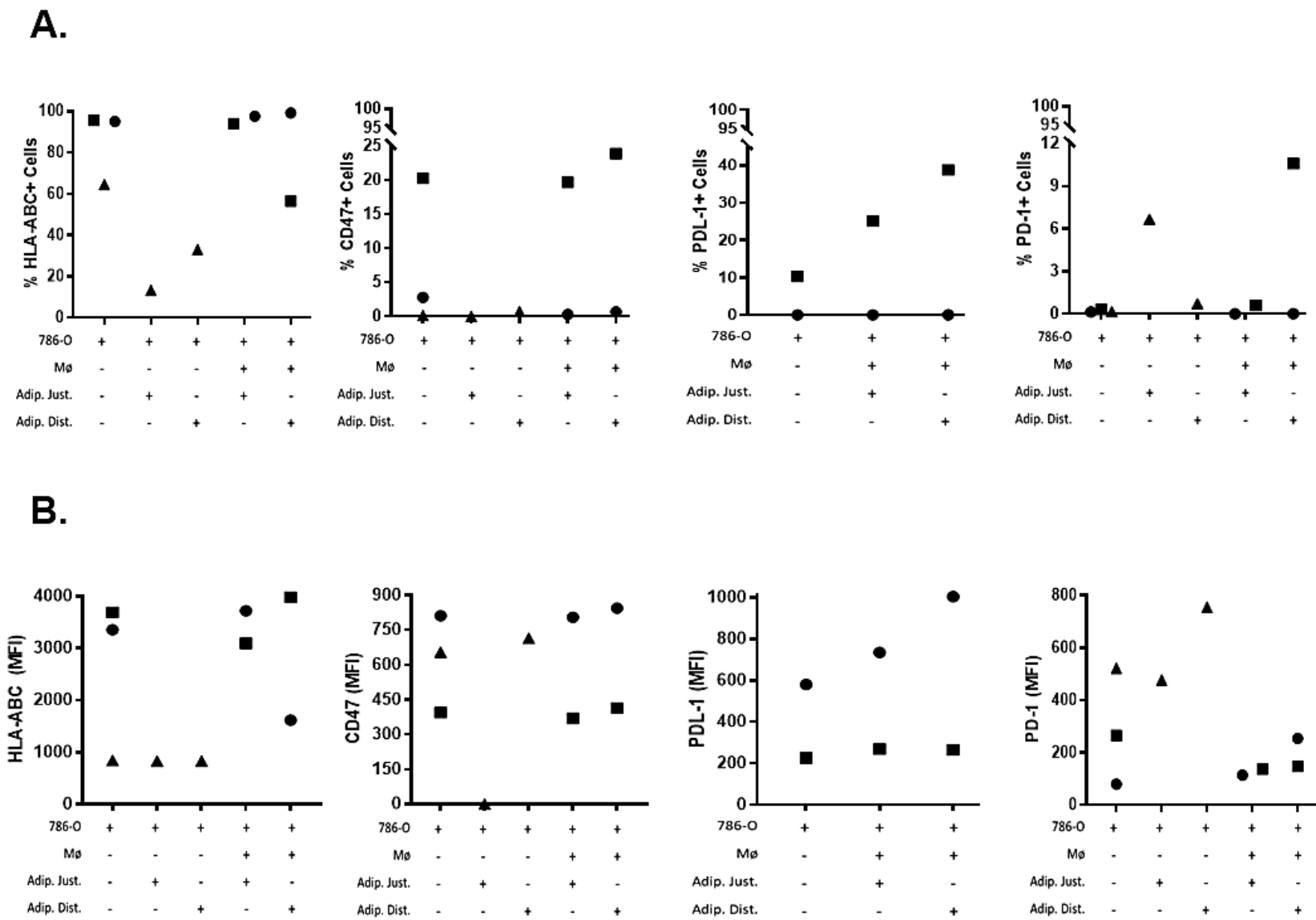


Figure 18 – Immunogenicity profiling of 786-O cells collected from tri-cultures suggest a crosstalk between the triad.

A. Scatter-plot represents the percentage of 786-O cells expressing markers of immune activation (HLA-ABC) and immune-escape (CD-47, PDL-1 and PD-1) in monoculture (786-O), 786-O in co-culture with adipocytes from juxta-tumoral perirenal adipose tissue (786-O + Adip. Just.), and from distant to the tumour perirenal adipose tissue (786-O + Adip. Dist.), also is represented 786-O in tri-culture with macrophages and adipocytes from juxta-tumoral perirenal adipose tissue (786-O + Mø + Adip. Just.), and from distant to the tumour perirenal adipose tissue (786-O + Mø + Adip. Dist.). **B.** Shown is the number of molecules (MFI, mean fluorescence intensity) expressed by tumour cells from monoculture (786-O), 786-O in co-culture with adipocytes from juxta-tumoral perirenal adipose tissue (786-O + Adip. Just.), and from distant to the tumour perirenal adipose tissue (786-O + Adip. Dist.), also is represented 786-O in tri-culture with macrophages and adipocytes from juxta-tumoral perirenal adipose tissue (786-O + Mø + Adip. Just.), and from distant to the tumour perirenal adipose tissue (786-O + Mø + Adip. Dist.). Each different symbol indicates one different experiment that corresponds to different adipocytes and monocytes donors (n=3). Moreover, must be underline the fact that for PDL-1 marker, it was not possible to analyze 786-O from the co-culture conditions with adipocytes.

6. Adipocytes and macrophages communication does not seem to require cell-cell contact

To further investigate if the adipocyte's secreted factors are indeed sufficient to promote alterations in the macrophage profile, we used conditioned medium from ccRCC adipocytes to stimulate macrophages and analyzed the phenotype markers CD86 and HLA-DR (M1-like) as well as CD163 and CD206 (M2-like) (**Figure 19**). Analysis of this trial experiment revealed that there are differences in the macrophages profile when these are stimulated with adipocytes conditioned medium. In fact, CD86, HLA-DR, and CD206 are modulated and point into a M2-like phenotype subversion of macrophages, suggesting that adipocytes may contribute to an anti-inflammatory milieu. As stated before, the same pattern was observed in macrophages under 786-O influence, thus both adipocytes and ccRCC cells appear to modulate macrophage profile into an immunosuppressive phenotype, contributing for the establishment of a tolerogenic microenvironment in ccRCC context. Nevertheless, these data must be further confirmed as, so far, only one experiment was performed.

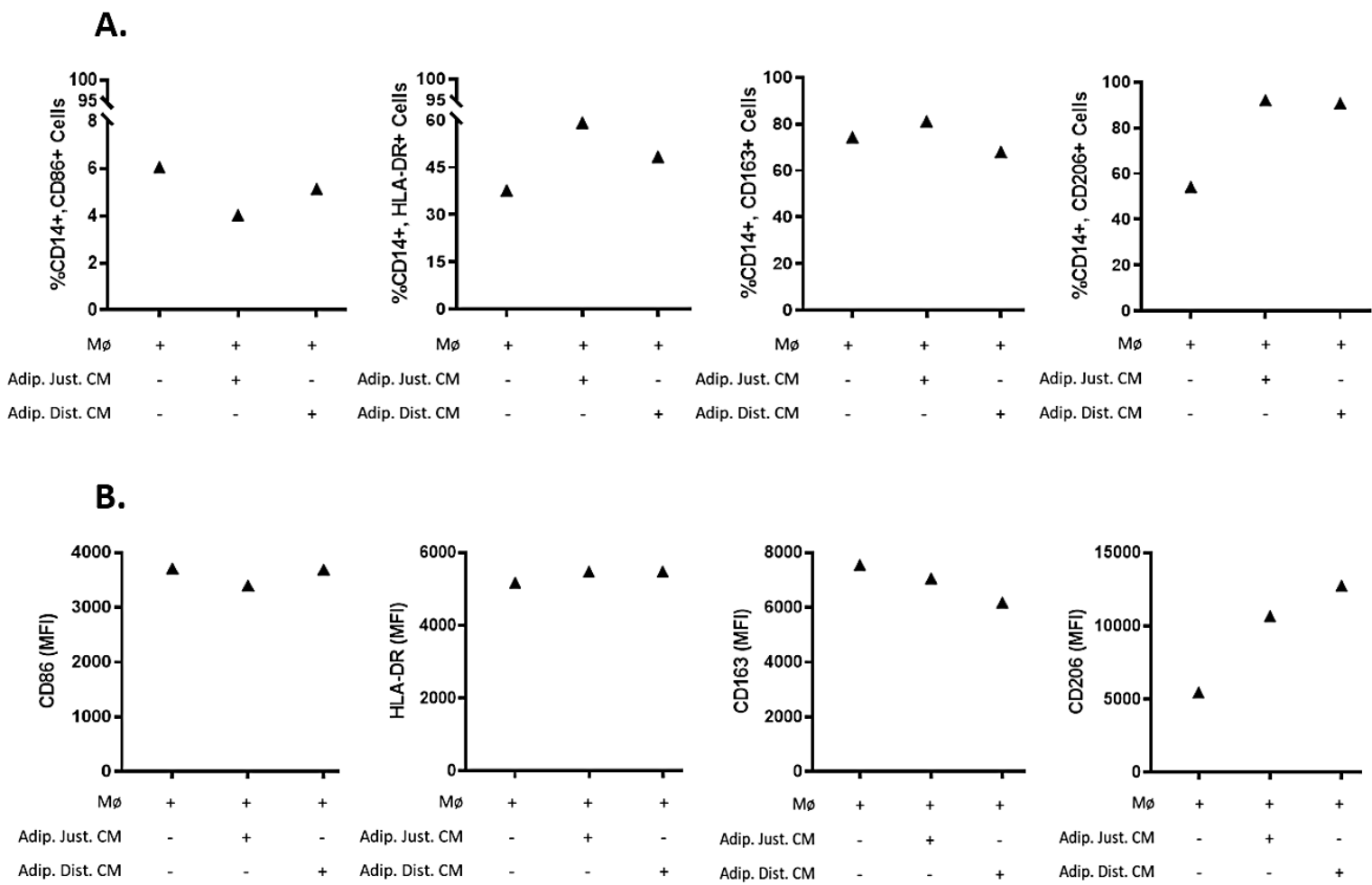


Figure 19 – Macrophage and adipocyte communication is likely to be dependent of soluble factors.

A. Scatter-plot represents the percentage of CD14 positive cells expressing CD86, HLA-DR, CD206, and CD163 in macrophage monolayers (Mø) and in macrophages obtained from culture stimulated with adipocytes conditioned medium from juxta-tumoral (Mø + Adip. Just. CM) or with distant to the tumour perirenal adipose tissue (Mø + Adip. Dist. CM). **B.** Shown is the number of molecules (MFI, mean fluorescence intensity) expressed by macrophages from monoculture (Mø) and by macrophages in culture with adipocytes conditioned medium from juxta-tumoral (Mø + Adip. Just. CM) or with distant to the tumour perirenal adipose tissue (Mø + Adip. Dist. CM), (n=1).

7. Differences in tri-cultures cytokine profile suggests a communication between macrophages, ccRCC cells and adipocytes

Our data, indicates that an interplay between ccRCC cells, macrophages, and adipocytes is likely to exist. In order to understand the influence of the triad crosstalk in cytokine expression, we evaluated the conditioned media of monocultures and tri-cultures, using adipocytes obtained from oncocytoma patient samples (Patient 2). As shown in Figure 20, there is a decrease in the IL-1 β secretion in the tri-culture condition when compared with the controls (adipocytes monoculture). The contrary is observed for IL-10 and TGF- β secretion. Regarding IL-6 secretion, it is observed a decrease in adipocytes from juxta-tumoral perirenal adipose tissue when in tri-culture, and the opposite effect occurs in case of the distant to the tumour perirenal adipose tissue. This suggests, once again, a difference associated to the anatomical localization of the adipose tissue. Importantly, our data indicates that the perirenal adipose tissue most likely has distinct cues when in close vicinity of the tumour.

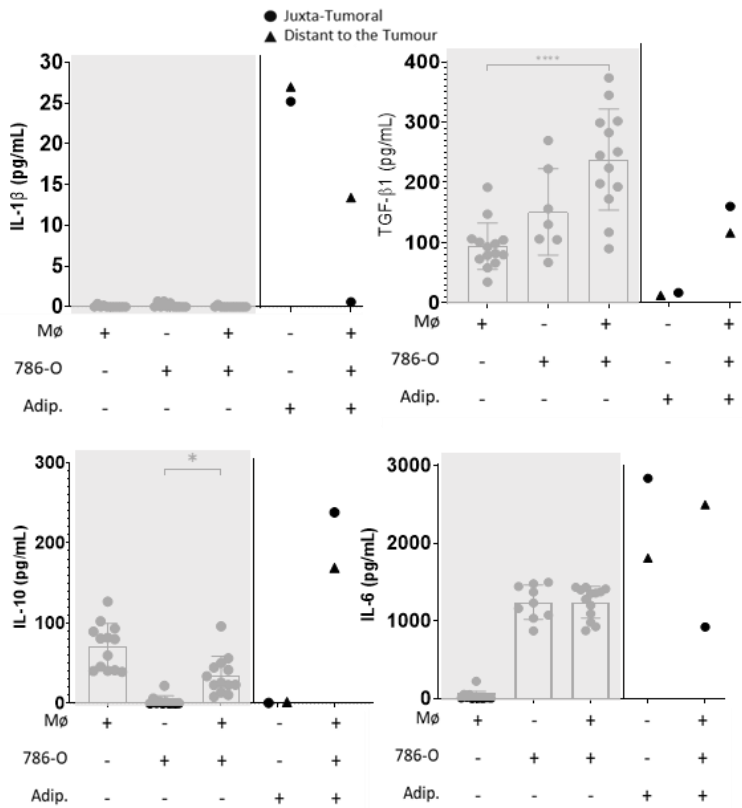


Figure 20 – Cytokine expression is modulated by adipocytes.

Represented are the concentrations (pg/mL) of IL-1 β , TGF- β 1, and IL-10 on the conditioned medium of adipocytes monoculture (from juxta-tumoral or distant to the tumour perirenal adipose tissue) and tri-culture of 786-O cells, macrophages, and adipocytes from the different anatomical locations (n=1). Each point indicates one experiment corresponding to the juxta-tumoral perirenal adipose tissue (●), and to distant to the tumour perirenal adipose tissue (▲). Of note, in grey are the cytokine profile of the conditions previously evaluated in this thesis, in order to facilitate the comparison.

Section B - Characterization of perirenal adipose tissue of the ccRCC patient cohort

8. Histology of perirenal adipose tissue from ccRCC patients

A biobank of samples from ccRCC patient was started, with collection of fresh samples for the projects’ experiments as well as a paraffin-embedded juxta-tumoral and distant to the tumour adipose tissue biorepository with associated clinicopathological information. So far, we have a cohort of 6 patients with ccRCC, and 6 patients of other kidney neoplasms. Of note, only one obese ccRCC patient is included in the ongoing cohort.

To investigate histological differences that could exist between obese and lean perirenal adipose tissue from ccRCC patients, the morphological appearance of juxta-tumoral and distant to the tumour (control) perirenal adipose tissue of different patients was compared (Figure 21). Hematoxylin and eosin (H&E)-stained paraffin-embedded sections of both adipose areas were analyzed regarding its morphological aspect in two representative ccRCC patients.

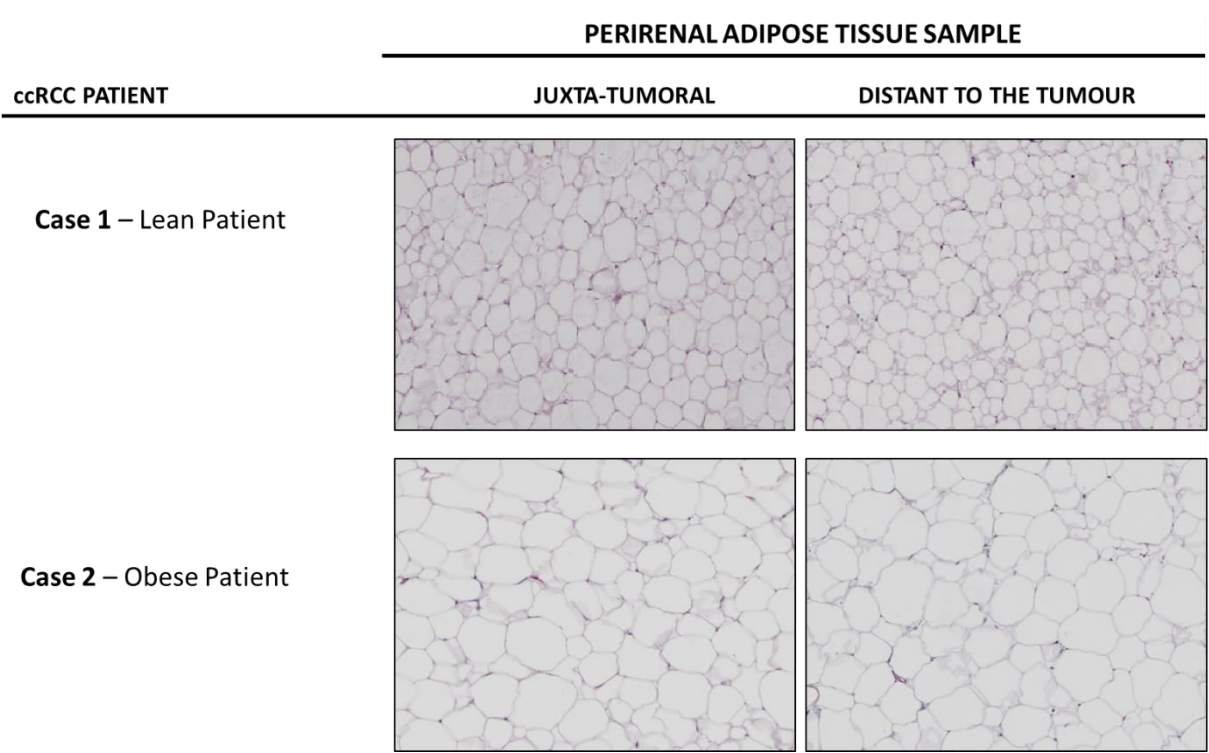


Figure 21 – Morphological comparison between two distinct areas of perirenal adipose tissue of a lean and an obese ccRCC patient.

Representative images of H&E cross-sectional sections of both perirenal adipose tissue from juxta-tumoral and distant to the tumour areas, from one lean ccRCC patient and one obese (n=6).

So far, by comparing these results and due to the scarcity of samples from obese patients, we were not able to take solid conclusions regarding the differences observed between juxta-tumoral perirenal adipose tissue and the control. However, it is possible to observe that when analyzing the perirenal adipose tissue samples of obese and lean ccRCC patients, one identifies connective tissue and adipocytes, fat-storing cells, within adipose tissue. Also, it was verified that in both conditions a unique large unilocular lipid droplet occupy most of the cell cytoplasm (**Figure 21**).

9. Quantitative analysis of adipocytes from ccRCC juxta-tumoral perirenal adipose tissue samples does not present differences when compared to control

To understand if the distance of the tumour has influence on the adipocyte size, a quantitative analysis of the lipid droplets and the adipocyte area was performed in juxta-tumoral and distant to the tumour perirenal adipose tissue samples.

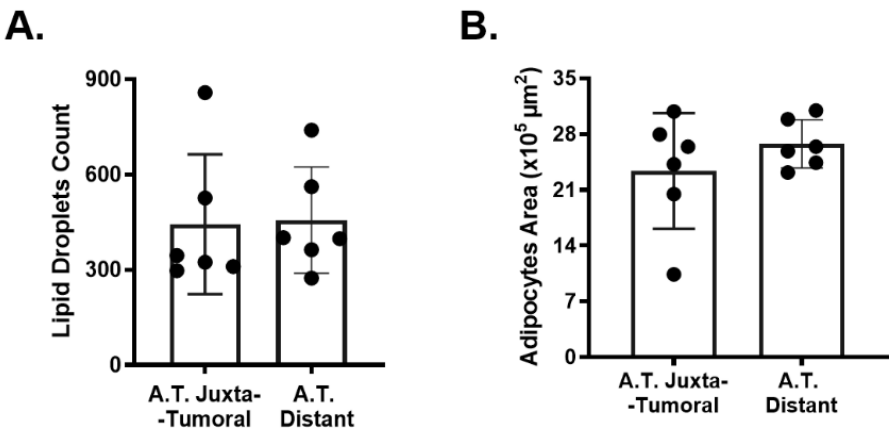


Figure 22 – Quantitative analysis of ccRCC perirenal adipose tissue.

A. Microscopic counting of the adipocytes within perirenal adipose tissue samples from juxta-tumoral and distant to the tumour regions. **B.** Quantification of individual adipocytes area in perirenal adipose tissue samples from juxta-tumoral and distant to the tumour regions of the aforementioned H&E-stained sections (n=6). Histological images were analyzed to calculate the average size of adipocytes using ImageJ Software. For the analysis, 10 random images were captured for every sample.

In fact, microscopic determination of the cross-sectional area of individual adipocytes from the two different tissues revealed no differences between the samples of perirenal adipose tissue obtained from the distinct anatomical areas (**Figure 22**). These measurements also demonstrated no apparent influence of the tumour cues in the adipocyte area. Stratification of lean vs obese individuals was not performed due to lack of obese ccRCC patient samples (n=1).

10. Macrophages localization within the ccRCC patient samples of perirenal adipose tissue

To assess if there are differences in macrophage localization in ccRCC context, an immune-staining with anti-CD68 (macrophages marker) was performed in paraffin-embedded sections of juxta-tumoral and distant to the tumour perirenal adipose tissue from ccRCC patients (**Figure 23**).

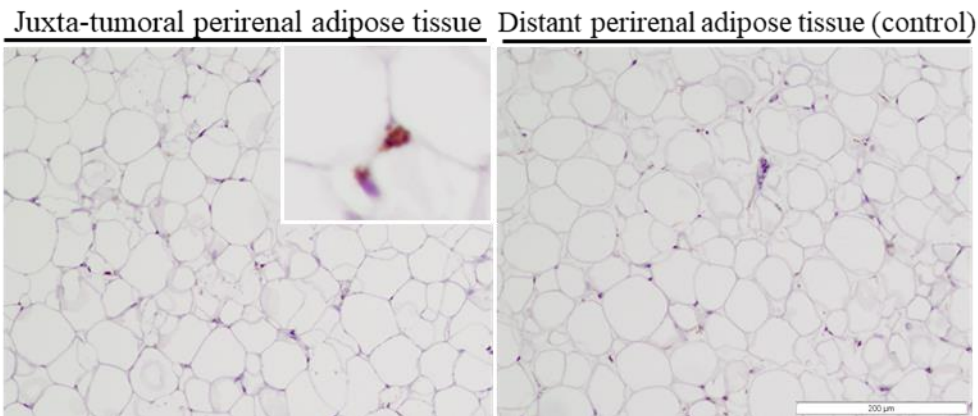


Figure 23 – Immunohistochemical staining of Perirenal Adipose Tissue from ccRCC patient samples with anti-CD68.

Illustrated here are micrographs of patient adipose tissue, FFPE processed and stained with a macrophage marker CD68 as depicted in the inset (brown). Shown are representative images of juxta-tumoral and distant to the tumour (control) perirenal adipose tissue from a cohort of n=6 ccRCC patients.

Analyzing the data obtained herein, ccRCC juxta-tumoral perirenal adipose tissue does not present evident difference regarding CD68+ staining when compared to the control (**Figure 23**). Specifically, it is possible to observe macrophages (brown staining) in the middle of adipocytes, that could in theory be recruited to the tumour milieu.

V – DISCUSSION

Clear Cell RCC is the most common subtype of RCC, recognized by glucose and fatty acids metabolism alterations, and by lipid and glycogen accumulation, which result in morphological alterations, such as cell clear cytoplasm in malignant cells^(101, 102). This carcinoma is known to have infiltration of immune cells such as macrophages, reported to play a relevant role in cancer development and progression^(61, 93-96). Importantly obesity seems to play a dual role in ccRCC, with adipose tissue accumulation being a risk factor for the disease development, but also contributing for better disease outcome in treated patients^(15, 16, 19, 22). This phenomenon, called obesity paradox, emphasizes the relevance of studying the role of adipose tissue in the context of ccRCC⁽²⁰⁾. As a means to contribute for the disclosure of this paradox, we focused on understanding the interplay between ccRCC cells, macrophages, and adipocytes, by investigating how this triad communicates. For that, we established co-culture models and started to assess if ccRCC cells have an impact on macrophages profile. The obtained data clearly demonstrates that, in the presence of 786-O cells, macrophages, which are a quite heterogeneous population, exhibit a significant decrease on the pro-inflammatory co-stimulatory receptor CD86, and an apparent increase on the anti-inflammatory mannose receptor CD206 expression. Moreover, the number of CD206 molecules expressed by each macrophage was clearly enhanced. Such results suggest that these phagocytes are modulated into an anti-inflammatory-like phenotype by this ccRCC cell line. Interestingly, there is evidence that monocytes stimulated by 786-O conditioned media differentiate into a monocytic myeloid-derived suppressor cell (MDSC)-like phenotype, characterized by low cellular expression of the HLA-DR and TAM-like suppressive functions⁽¹⁰³⁾. Herein, we did not detect changes in HLA-DR expression or molecule numbers per cell, what could be explained by the different experimental settings. In our study, we used already differentiated macrophages in indirect co-cultures with cancer cells, as such it is plausible that cell-cell contact between 786-O cells and macrophages may occur through pseudopodia, resulting in different molecular regulations^(104, 105). Still, our results support that 786-O cells may lead to the modulation of macrophages into an immunosuppressive and pro-tumoral phenotype.

We also evaluated whether cancer cells, upon challenge with macrophages, changed the expression of various immunogenicity markers namely, HLA-ABC, CD-47, PDL-1, and PD-1. Our results indicated however, that when comparing 786-O mono-culture and co-cultures with macrophages, there were no changes in the percentage of cells expressing such markers, or in the number of the referred immunogenicity molecules per malignant cell. Not surprisingly, this cancer cell line was characterized here as highly positive for HLA-ABC, as reported by Trotta and colleagues⁽¹⁰⁶⁾. Of note, in RCC patient samples, a downregulation of HLA-ABC in tumour cells was reported^(107, 108). Such finding does not fit our data, most likely because our experimental settings do not include all the TME components, particularly T lymphocytes. This may justify the absence of a selective pressure for 786-O to downregulate MHC-I expression. Taking in consideration all the immunogenicity markers analyzed, it is possible that the 786-O cell line does not present the main classical mechanisms of immune system evasion. To further confirm the alterations observed and understand whether ccRCC cells hold mechanisms to induce a tolerogenic tumour microenvironment, we assessed the cytokine profile of the conditioned media obtained from 786-O and macrophages mono- and co-cultures. Indeed, an anti-inflammatory milieu characterized by high *TGF- β 1 levels*, *presence of IL-10*, and low pro-inflammatory cytokines secretion was detected when 786-O were confronted with macrophages. These results further suggest that ccRCC cells may have strategies to subvert macrophages into a more permissive phenotype, guaranteeing a modulation of the TME into a pro-tumoral milieu⁽¹⁰⁹⁾. Interestingly, IL-6 and IL-8 were also found to be highly secreted by 786-O cells, and abundant in the media collected from ccRCC cells and macrophages co-cultures. These cytokines alterations were already reported and associated with poor prognosis in RCC patients⁽¹¹⁰⁾.

The existence of an interplay between ccRCC cells and macrophages was once again highlighted, when evaluating the players that secrete each cytokine. Interestingly, we observed that 786-O cells express high *TGF- β 1 mRNA* levels, when in co-culture with macrophages, and that low IL-6 expression by ccRCC cells in co-culture, seemed to be compensated by macrophage IL-6 mRNA levels. Regarding IL-10, we have hypothesized that its regulation does not occur at the mRNA but at the protein level, yet this must be further confirmed. Nevertheless, all evidences point, once again, to the existence of a crosstalk between malignant cells and macrophages.

To start scrutinizing the mechanisms that lead to the referred cytokine modulations, total and phosphorylated levels of AKT, ERK, STAT1, STAT3, Src, and p65 were assessed. The activated forms of STAT1 and STAT3 were found increased in macrophages co-cultured with ccRCC cells, despite the absence of alterations in their total form. In addition, p-AKT and p-ERK levels appeared to be decreased in macrophages obtained from co-culture with 786-O. Interestingly, other studies had also described an increase on the expression and activation of STAT1 and STAT3 in TAMs⁽¹¹¹⁾. Notwithstanding, these proteins are known to have opposite roles, since STAT3 may function as a suppressor of STAT1 activity. Moreover, IL-6 was already reported to induce STAT3 cascade triggering pro-tumoral events⁽¹¹²⁾. As such, it is possible that the increased IL-6 secretion by 786-O cells *result in the activation of STAT3 signaling pathway in macrophages, triggering IRE1 α* , and subsequently leading to extracellular matrix degradation, cancer cell invasion and, tumour progression⁽¹¹³⁾.

To fully understand ccRCC pathogenesis one must include adipocytes particularly in renal carcinomas where an anatomical proximity to perirenal adipose tissue exists. Moreover, adipocytes are known to make part of the tumour landscape and, in solid carcinomas, these fat depots were reported to display a pro-tumoral role, contributing to the establishment of a permissive niche for tumour development⁽¹¹⁴⁾. Our very preliminary data supports that adipocytes are indeed novel and important players in the tumour milieu. Given time constrictions, only three experiments with adipocytes, isolated from different histological subtypes of kidney tumours, and co-cultured with ccRCC cells and macrophages were performed. Nevertheless, our results evidenced that, in the presence perirenal adipose tissue-derived adipocytes,

macrophage profile was modulated, 786-O immunogenicity molecules were changed, and the cytokine profile of the microenvironment was also altered. Additionally, the trial experiments comparing adipocytes isolated from juxta-tumoral and distant to the tumour perirenal adipose tissue, indicate that the anatomic proximity to the tumour may provide this fat-containing cells with distinct regulatory cues. Nevertheless, due to the novelty of this study, the limited access to ccRCC perirenal adipose tissue samples, and the kidney tumour patient histological differences, one must carefully analyze the results and keep in mind that data presented here may be masked by adipocyte heterogeneity. Still, collected data must be considered as an outstanding starting point to clarify the molecular and mechanistic oncogenic crosstalk between cells that compose the ccRCC tumour microenvironment and, a small piece of the puzzle to start unveiling the obesity paradox.

This project also contemplates the gathering of the building blocks required to investigate the adipose tissue role in ccRCC through the assembly of a paraffin-embedded ccRCC sample biobank. Such strategy allowed the preliminary characterization of ccRCC perirenal adipose tissue from lean and obese patients that may assist in the clarification of the obesity paradox. Yet, so far, due to the scarcity of samples from obese patients we were not able to take conclusions. Also, the histological analysis of perirenal adipose tissue, collected from juxta- or distant to the tumor regions from 6 ccRCC patients (ongoing cohort), revealed that the proximity to the tumour had no significant influence on the adipocyte size or area. We took advantage of having such a valuable biological material and investigated macrophage presence in perirenal adipose tissue from juxta- and distant to the tumor regions, yet no apparent differences were detected regardless of its proximity to the tumour. However, it is essential to notice that sample collection difficulties and time constraints (*i.e.* COVID-19 pandemic) may have influenced the results obtained herein. As such, it is essential to continue pursuing the replicates and controls required to complete each experimental setup. Notwithstanding, to the best of our knowledge, this is the first study establishing a crosstalk between ccRCC cells, macrophages, and adipocytes. Importantly, the biological material and data gathered in this thesis contribute with an insight on the mechanisms underlying ccRCC.

VI - CONCLUSION AND FUTURE PRESPECTIVES

ccRCC is considered a metabolic disease, chemo- and radioresistant, highly vascularized, and is associated with elevated mortality. It is a “*hot tumour*” meaning that immune cells are infiltrated in the tumour milieu, for example macrophages, which are described to have a relevant role in ccRCC development and progression. One of the reported ccRCC risk factors is obesity, a major societal burden of the twenty-first century. Nevertheless, a number of epidemiological studies show that obesity can also contribute to better disease outcome in certain scenarios, the so called, obesity paradox. Additionally, the tumours in the kidney are in close contact with adipocytes that compose the perirenal adipose tissue, which may contribute to the tumour fate. Altogether, these facts support the importance of further dissecting the mechanisms underlying the interplay between ccRCC cells, macrophages, and adipocytes.

The present work aimed at characterizing the interplay between the above-mentioned cellular triad. For that, we employed a 2D cell co-culture model as a means to confront the different cell populations and investigate which molecules are involved in ccRCC cells, macrophages, and adipocytes communication. Our results support the existence of a crosstalk between 786-O cells, macrophages, and adipocytes. This was shown in the co-culture assays between tumour cells and macrophages where it was observed that ccRCC cells modulate macrophage profile into an anti-inflammatory-like phenotype. Additionally, alterations in the macrophage STAT1 and STAT3 activation, probably triggered by IL-6 and IL-10 secretion, observed in the co-cultures, also highlight an interplay between malignant cells and macrophages. Altogether these data point to a possible subversion mechanism owned by cancer cells as a means to survive. The very preliminary tri-cultures results also reinforce the existence of 786-O and macrophage communication and, importantly they pointed to a role of adipocytes in the triad interplay. Taken together, our provocative input proposes that the triad communication might result in the microenvironment modulation to an anti-inflammatory and permissive milieu, contributing to cancer development. The challenge of characterizing ccRCC perirenal adipose tissue from lean and obese patients, still ongoing, thus due to the insufficient number of samples from obese patients we were not able to take conclusions, while perirenal adipose tissue CD68 staining showed macrophage presence regardless of the samples distance to the tumour.

In conclusion, this study novelty highlights the current perspective that the tumour microenvironment must be investigated as a multidisciplinary landscape of cellular and non-cellular components that are inter-related, driving biological modifications in their milieu, triggering the outcome of tumour growth. In line with this, there is an urgent need to scrutinize the molecular mechanisms underlying ccRCC cells, macrophages, and adipocytes interaction as this is a fairly new research field. Thus, in the future, it is essential to further confirm whether and how the triad interaction occurs. For that we propose a number of experiments namely, i) the increase of tri-culture replicates to further characterize tumour cells, macrophage and adipocyte molecular alterations, ii) analyze the molecules involved in the oncogenic communication processes as cancer cell invasion, tumor progression, cell survival, stemness, and immune escape markers, and iii) validate the candidate key players uncovered, in human ccRCC surgical resections (already available in the ccRCC Biobank that resulted from this project). After clarifying the communication between 786-O cells, macrophages, and adipocytes, it would also be important to compare the obtained results with other ccRCC cell lines, for example Caki-1, to ascertain if differences in the tumour stage could influence the triad interplay. With these strategies we expect to contribute for the understanding of the obesity paradox and power the discovery of new candidates that once targeted may tip the balance towards patient survival.

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