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Extracellular vesicles-derived *LAT1* and *ASCT2* mRNAs and colorectal cancer development

Cristina Sofia de Almeida

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“The scientist is motivated primarily by curiosity and a desire for truth.”

Irving Langmuir

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Abbreviations

A

APC- Adenomatous Polyposis Gene

AA- Amino Acids

AAT- Amino Acids Transporters

ATCC- American Type Culture Collection

ASCT2 - Alanine-Serine- Cysteine Transporter 2

B

BRCA1- Breast Cancer gene 1

BRCA2- Breast Cancer gene 2

BBB- Blood Brain Barrier

B2M - Beta-2- Macroglobulin

BSA- Albumin Bovine Fraction V

BTC- Biliary Tract Cancer

C

CRC - Colorectal Cancer

CCR- Cancro Colorretal

CIN- Chromosomal Instability

CIMP- Epigenomic CpG Island Methylator Phenotype

cDNA - complementary Deoxyribonucleic Acid

C_T- Cycle Threshold

CFSE - Carboxyfluorescein Succinimidyl Ester

CXCR4 - C-X-C motif Chemokine Receptor 4

CPNE3- Copine III

D

DEPTOR- DEP domain-containing mTOR-interacting protein

DMEM - Dubecco´s Modified Eagle´s Medium

DAPI- 4',6'-Diamino-2-Fenil-Indol

DTT- Dithiothreitol

E

ESMO- European Society of Medical Oncology

EGFR - Epidermal Growth Factor Receptor

EAA- Essential Amino Acids

4EBP1- Eukaryotic Initiation Factor 4E-Binding Protein

EV's - Extracellular Vesicles

EGF- Epidermal Growth Factor

EMT- Epithelial-Mesenchymal Transition

F

FBS - Fetal Bovine Serum

G

GbL/mLST8 - Mammalian Lethal with Sec13 Protein 8

GRP78- Endoplasmic Reticulum

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

H

HCT 116-VE's- Vesículas Extracelulares derivadas da linha celular HCT 116

HCT 116-EV's - Extracellular Vesicles derived from HCT 116 cell line

HCC- Hepatocellular Cancer

HIF-1α – Hypoxia-Inducible Factor 1

HSP60- Heat Shock Protein 60

I

IARC- International Agency for Cancer Research

ITS- Insulin – Transferrine – Selenium

K

KRAS- Kristen Rat Sarcoma Viral Oncogene Homolog

KRTAP5-4- Keratin Associated Protein 5-4

L

LAT1 - L-type Amino Acid Transporter 1

M

MSI- Microsatellite Instability

mRNA - messenger Ribonucleic Acid

mTOR – Mammalian Target of Rapamycin Complex

mTORC1- mTOR Complex 1

mTORC2- mTOR Complex 2

MAPK- Mitogen-Activated Protein Kinase

MVBs- Multivesicular Bodies

MAGEA3- Melanoma Antigen Family A3

O

OCDE- Organization for Economic Co-operation and Development

OSCC- Oral Squamous Cell Carcinoma

P

PTEN- Phosphatase and Tensin Homolog

p70-S6K- Ribosomal Protein 6 Kinase

PCR - Polymerase Chain Reaction

PI3K - Phosphoinositide 3-Kinase

PBS- Phosphate-Buffered saline

PFA- Paraformaldehyde

PVDF- Polyvinylidene Difluo-ride Membrane

R

RNA - Ribonucleic Acid

Raptor - Regulatory- Associated Protein of mTOR

RPMI- Roswell Park Memorial Institute Medium

RIPA- Radioimmunoprecipitation Assay Buffer

S

SLC - Solute Carrier Transporter

SDS-PAGE- Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

SSC- Side Scatter

T

TAA- Transportadores de Amino Ácidos

TEM - Transmission Electron Microscopy

TBS-T- Tris-buffered Saline (10X) with Tween20

V

VEGFA - Vascular Endothelial Growth Factor A

VE's- Vesículas Extracelulares

W

WHO - World Health Organization

WST-1- Water Soluble Tetrazolium Salt-1

Resumo

O cancro colorretal (CCR) é uma das principais causas de morte por cancro na população ocidental, sendo a nível mundial o terceiro cancro mais frequente. Estudos demonstram que aproximadamente 50% dos doentes desenvolvem doença metastática hepática no decurso da doença, o que indica a necessidade de identificação de novas moléculas envolvidas na oncobiologia do CCR, com potencial de serem usadas como biomarcadores de prognóstico e até futuros alvos terapêuticos. De acordo com a literatura admite-se que transportadores de aminoácidos (TAA) são capazes de regular a via de sinalização PI3K/Akt/mTORC1, via que é central na regulação da proliferação e metabolismo celular do CCR. Paralelamente, o aumento da expressão dos TAA, LAT1 e ASCT2, tem sido observado em diferentes modelos tumorais, associado ao crescimento, proliferação celular, angiogénese e a pior prognóstico. Atualmente, admite-se que as vesículas extracelulares (VE's) medeiam a comunicação celular e exercem diferente atividade biológica através da transferência de informação molecular (ácidos nucleicos, proteínas...) a células recetoras, podendo modificar fenótipos celulares e condicionar a progressão tumoral. Assim, no presente estudo analisamos o conteúdo de mRNAs derivados de VE's de uma linha celular de CCR e o seu potencial na modificação do fenótipo celular, usando uma linha celular de hepatocarcinoma (SK-HEP-1) e uma linha celular epitelial tubular proximal renal normal (HKC-8) como células recetoras.

Numa primeira fase, isolamos as VE's da linha celular de CCR HCT 116 (HCT 116-VE's), e estas foram caracterizadas por microscopia eletrónica de transmissão e por citometria de fluxo. Adicionalmente, quantificamos os níveis de mRNA de *LAT1, ASCT2, EGFR, HIF-1α, CXCR4* e *VEGFA* nas HCT 116-VE's. De modo a compreender o efeito da internalização das VE's nas células recetoras, fomos avaliar os níveis de mRNA e de proteína de LAT1 e ASCT2, assim como o seu impacto fenotípico nas células recetoras.

No presente estudo, validamos a presença dos mRNAs *LAT1, ASCT2, EGFR, HIF-1α, CXCR4* e *VEGFA* nas HCT 116-VE's. Os ensaios de internalização revelaram que as HCT 116-VE's foram capazes de induzir um aumento dos níveis do mRNA *LAT1*, com um consequente aumento de proteína, e um decréscimo do nível do mRNA *VEGFA* na linha recetora SK-HEP-1. Por outro lado, a internalização das HCT 116-VE's também teve a capacidade de estimular a migração e a proliferação celular na linha celular recetora SK-HEP-1. Na linha celular HKC-8, a internalização das HCT 116-VE's conduziu a um aumento dos níveis de mRNA de *LAT1*, com um consequente aumento de proteína, e um aumento dos níveis de mRNA de *HIF-1α*. Similarmente, a internalização das HCT 116-VE's também potenciou a capacidade de migração e de proliferação celular na linha celular recetora HKC-8.

Neste estudo, elucidamos o papel das VE's na modificação do fenótipo celular de células recetoras através da transferência dos mRNAs de TAA, nomeadamente o mRNA *LAT1.* Estudos futuros são imperativos de forma a validar o potencial do uso de TAA derivados de VE's como biomarcadores de prognóstico e o seu potencial como alvos terapêuticos no CCR.

Abstract

Colorectal cancer (CRC) is one of the main causes of cancer death in the western population, being the third most common cancer worldwide. Approximately 50% of the patients develop metastatic liver disease, showing the need for new molecular targets associated with CRC oncobiology, which can be potentially used as prognostic biomarkers. Recently, the amino acid transporters (AAT) have been showed to regulate the PI3K/Akt/mTORC1 signaling pathway which plays a crucial role in the regulation of CRC proliferation and metabolism. Additionally, increased expression of AAT, namely LAT1 and ASCT2, has been observed in different tumor models, including CRC, having been associated with growth, cell proliferation and angiogenesis. Currently, it is admitted that extracellular vesicles (EV's) mediate cellular communication and exert biological activity by transferring molecular information (DNA, mRNA, microRNAs, proteins…) to recipient cells, which can modify cellular phenotypes and condition tumor progression. Thus, in the present study, we analyzed the mRNAs' content of EV's from a CRC cell line. Moreover, using a hepatocellular carcinoma cell line and a normal renal proximal tubule epithelial cell line as recipients' cells, we analyzed these EV's ability to modify the cells' phenotypes.

Firstly, we isolated HCT 116-EV's (CRC-derived EV's) and we characterized them using Transmission Electron Microscopy and flow cytometry. Additionally, we quantified the mRNAs' levels of *LAT1, ASCT2, EGFR, HIF-1α*, *CXCR4* and *VEGFA* in the HCT 116-EV's. To understand the effect of their uptake in the recipient cells (SK-HEP-1 and HKC-8 cell lines), we evaluated the mRNA and protein levels of the LAT1 and ASCT2, as well as their phenotypic effect.

In this study, we validated the presence of *LAT1, ASCT2, EGFR, HIF-1α, CXCR4* and *VEGFA*'s mRNAs in HCT 116-EV's. The uptake studies showed that the HCT 116-EV's were able to induce an increase of *LAT1* mRNA levels, with consequent protein increase, and a decrease of *VEGFA* mRNA levels in SK-HEP-1 recipient cells. Furthermore, it also had an impact in the migration and proliferation capacity of these cells. On the other hand, the uptake of HCT 116-EV's led to an increase of *LAT1* mRNA levels, with consequent protein increase, and an increase of *HIF-1α* mRNA levels in HKC-8 recipient cells. Similarly, their uptake also had an impact in migration and proliferation capacity in HKC-8 these cells.

In this study, we clarified the role of EV's in the induction of phenotypic modifications in recipient cells, through ATT mRNA's transference, namely *LAT1*. Nevertheless, future studies are needed to validate the potential of ATT derived from EVs as prognosis biomarkers and future therapeutic targets in CRC.

1.Introduction

1.1. Cancer: General Concepts

Globally, cancer represents an important present and future disease, being the second leading cause of death after cardiovascular diseases (1, 2). According to the World Health Organization (WHO), in 2018, cancer was responsible for approximately 9.6 million of deaths and it is expected that the number of new cases will increase to 29.5 million in 2040 (3). In Portugal, the International Agency for Research of Cancer (IARC) estimates an increase of cancer cases of 19.5% by 2040 when compared to 2018 (figure 1).

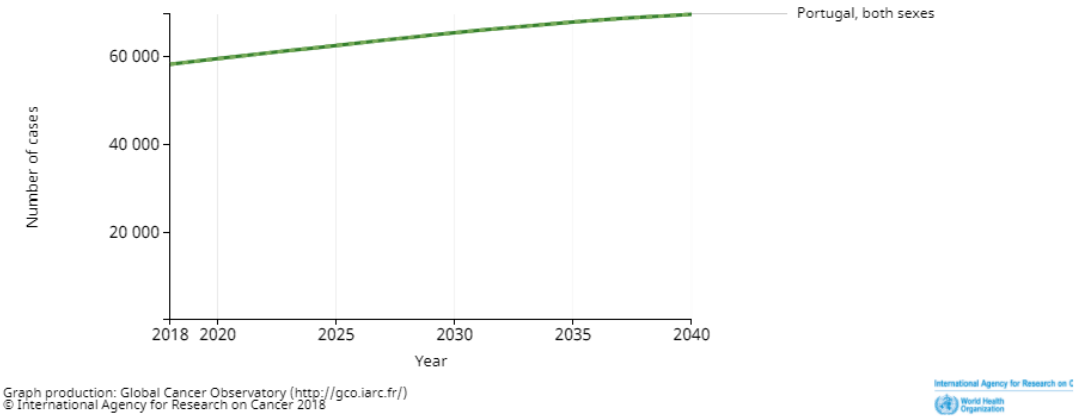

Figure 1. Estimated number of incidence cases, in Portugal, between 2018 to 2040, all cancers, in both sexes, all ages**.** GLOBOCAN 2018, IARC

Carcinogenesis is a multi-step process in which cells undergo metabolic and phenotypic changes, namely an increased proliferation rate, the capacity to evade to the immune system and the ability to invade distant tissues (4, 5). This process is the result of DNA alterations, that could be caused by endogenous or exogenous agents (6). Carcinogenesis can be conceptually divided in three main phases: initiation, promotion, and progression (7). The tumor initiation consists in the cell exposition to a carcinogenic agent (chemical, physical or biologic) which causes a genetic damage in the DNA molecule (8). During the promotion phase, promoter agents increase the capacity of initiated cells to proliferate (6, 7). In the progression phase, occurs the transformation of a preneoplastic cell into one that express a malignant phenotype. These malignant cells tend to acquire more aggressive characteristics over time, gaining the ability to invade beyond the immediate primary tumor location (6, 9).

During the carcinogenesis process, two key classes of genes suffer alterations: proto-oncogenes are activated and tumor suppressor loose function (8, 10). Oncogenes, such as *KRAS*, are like fuels for carcinogenesis and confer a selective advantage to the cell, since it is only necessary one mutated allele or their inappropriate expression for cancer development (10). On the other hand, tumor suppressor genes, such as Phosphatase and Tensin Homolog (*PTEN)* and adenomatous polyposis gene (*APC*), which are negative regulators of cell cycle, must suffer alteration in both gene copies in order to be associated with tumor development (10, 11). Tumor suppressor genes can be divided in two categories: caretakers, such as *BRCA*1 and *BRCA2* genes, which are responsible for genomic stability maintenance protecting the integrity of genome; and gatekeepers, such as *APC* gene, that are responsible for controlling or inhibiting directly cell growth (11). Overall, the activation of oncogenes or inactivation of tumor suppressor genes promotes the dysregulation of several signaling pathways such as RAS-MAPK and PI3K-AKT, which can lead to the activation of cell cycle and, consequently, cell proliferation (12).

In 2000, Hanahan and Weinberg suggested six essential characteristics that confer selective advantage to cancer cells compared to normal ones, supporting tumor growth and progression: apoptosis evasion, self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis limitless replicate potential and tissue evasion/metastasis (13). These characteristics are defined as the hallmarks of cancer (14, 15). A decade later, Hanahan and Weinberg introduced additional hallmarks, namely: reprogramming energy metabolism, evading immune response, genome instability and mutation and tumor-promoting inflammation (figure 2) (13, 16).

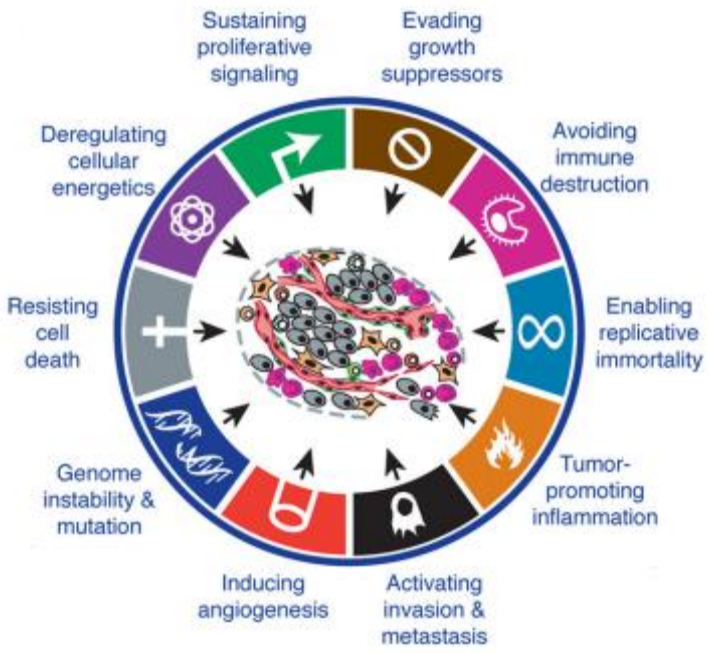

Figure 2. The hallmarks of cancer proposed in 2010, by Hanahan and Weinberg (Adapted from Hanahan D *et al.* (13)).

One important emerging hallmark of cancer is the dysregulation of metabolism in cancer cells. To sustain their relentless cell division, these cells undergo a complex metabolic rearrangement characterized by changes in metabolic pathways that are involved in biosynthetic processes and energy production (17, 18). One feature of tumor cells' metabolism is the capacity to acquire the needed nutrients, from a nutrient poor environment and use them to build new biomass and maintain viability (19). Thus, in order to fulfill the biosynthetic demands associated with proliferation of tumor cells, the cells must increase the import of nutrients, being one of the metabolic changes associated with cancer, the deregulation of glucose and amino acids uptake (19). In fact, emerging evidences show a relationship between metabolism and cancer, as part of a large effort to identify new therapeutic approaches (18, 20, 21).

1.2. Colorectal Cancer

Colorectal cancer (CRC) is one of the most common cancers worldwide, with 1 849 518 new cases in 2018, being the third most common neoplasia (22). CRC accounts for approximately 10% of all diagnosed cancers and it is the world´s second most deadly cancer (23). CRC is the second most common cancer diagnosed in women, and the third in men, being the incidence and mortality approximately 25% lower in woman (23, 24). Overall, CRC incidence is highly variable by region and 50% of all cases are diagnosed in high-HDI (high Human Development Index) countries (figure 3). However, in lower-HDI countries, there is an expected increase of 2.5 million of CRC new cases in 2035 (23, 25). Contrary, in high-HDI countries, the stabilization or even a decrease of the incidence rates is predicted, which could be consequence of the implementation of screening programmes (23).

According to the Organization for Economic Co-operation and Development (OCDE), in Portugal, CRC is the second most frequent cancer type in man and women (26). In 2018, 10 270 new cases were diagnosed which corresponds to 17.6% of the total number of new cancer cases (2, 26). Additionally, according to IARC, an increase of 26.2% in CRC cases in both sexes will be expected, in 2040.

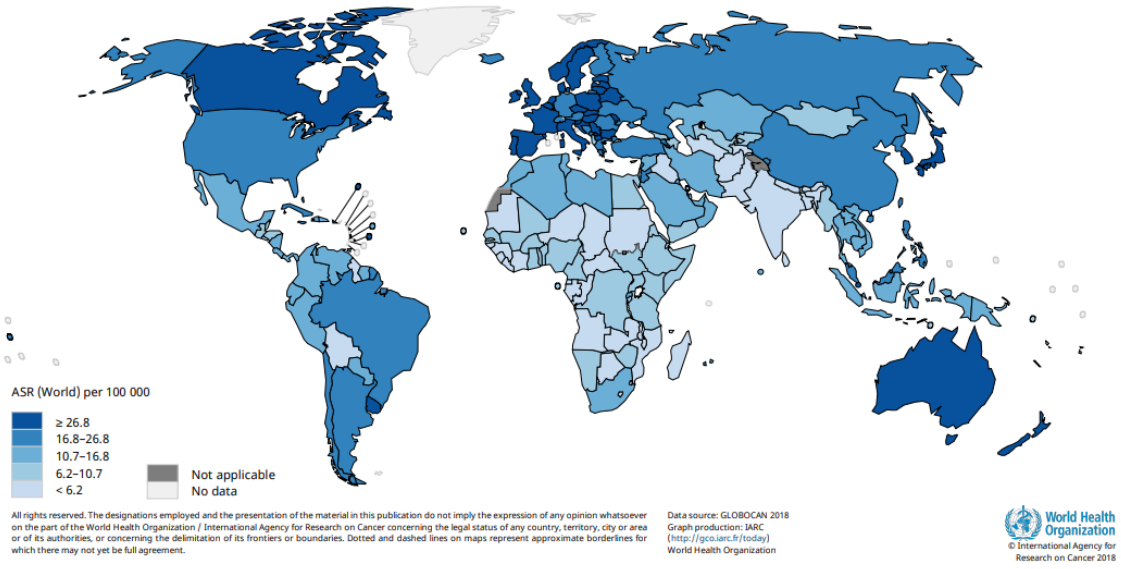

Figure 3. Estimated age-standardized incidence rates, in the world, in 2018, in colorectal cancer, both sexes, all ages. (GLOBOCAN 2018, IARC).

The development of CRC can be modulated by several factors, being the high alcohol consumption, overweigh, physical inactivity, tobacco smoking, diabetes mellitus, age, sex, personal or family history of CRC well established risk factors (27–29).

CRC comprises a heterogeneous group of diseases that arise from the accumulation of multiple genetic alterations. It's molecular progression can be consequence of alterations in multiple signaling pathways, being multiple genetic events required for tumor progression (25, 30). The CRC pathogenesis begins with the transformation of a normal colonic epithelium into a benign adenomatous polyp, which grows and develops into an advanced adenoma with high-grade of dysplasia, and later progresses to an invasive cancer. This sequence of events takes approximately 10 years and is known as "adenoma-carcinoma sequence" (31, 32) (33, 34). The CRC slow progression offers several opportunities to apply preventive strategies that also can be helpful for an early detection. In fact, screening programs do not only present an important role in early detection, but also contribute to morbidity and mortality reduction (23). Actually, several potential screening tests are used such as colonoscopy, flexible sigmoidoscopies, guaiac-based fecal occult blood tests and fecal immunochemical tests (27, 35, 36). In fact, American Cancer Society reports that 4 in 10 cases of CRC are found in early stages, being the 5-year survival rate in these cases near 90% (37). However, these screening tests have several limitations, including its invasive nature, the costs as well as their low sensitivity and specificity (27, 38).

Similarly to other cancers, the treatment options for CRC largely depend on the tumor stage, being the clinicopathological stage one of the most important prognostic factors (28, 39). According to the European Society of Medical Oncology (ESMO) guidelines, stage I and II patients' are frequently submitted to surgical tumor and local lymph nodes resection (28). Nevertheless, stage II patients can also be submitted to adjuvant chemotherapy, in the cases of poorly differentiated cancer, lymph vascular invasion, perineal invasion, <12 lymph nodes, bowel obstruction, localized perforation and positive margins (28). In CRC stage III, patients are submitted to surgical tumor removal, followed by adjuvant chemotherapy and radiotherapy (28). Lastly, in stage IV, the cancer spreads through the blood and lymph nodes to distant organs, in most cases to the liver, lungs, the abdominal wall or ovaries. Thus, the treatment can include a combination of neoadjuvant chemotherapy, surgery, radiotherapy and targeted therapy (28, 39).

Although the mortality rates have declined due to the improvement in diagnosis and treatment, CRC still represents one of the most lethal cancers (27). Metastasis are found in, approximately, 15-25% of CRC cases at the diagnosis, and increase to 50% during the course of the disease (40–42). Additionally, studies report that the liver is recognized as the most common initial organ of CRC metastasis establishment (43). In

fact, approximately 50% of CRC cases will develop liver metastasis, which are responsible for the death of two thirds of the patients (41, 43). Thus, future studies are imperative in order to clarify the exact mechanisms involved in disease progression and metastatic establishment, in order to improve the patients' follow up and new therapeutic opportunities identification.

1.2.1. Molecular biology of colorectal cancer

CRC can be defined as the result of a series of well-characterized histopathological modifications derived from specific genetic "hits" of several oncogenes and tumor suppressor genes (44). The pathogenic mechanisms associated with CRC development include three major pathways: chromosomal instability (CIN), microsatellite instability (MSI) and epigenomic CpG island methylator phenotype (CIMP) (45). CIN is characterized by imbalances in chromosomal number and loss of heterozygosity, and it is found in 60-75% of sporadic CRC (34). MSI is characterized by disfunction of mismatch repair genes (MMR), DNA damage repair system, and occurs mostly during replication which can lead to errors in base pairing, being found in 15% of CRC cases (46, 47). Moreover, according to the literature, genomic instability has a prognostic value, since MSI is associated with a favorable prognosis compared to CIN (48). Lastly, CIMP is estimated to occur in 20-30% of CRC cases, and it is described as an epigenomic instability characterized by multiple CpG islands hypermethylation, which can originate aberrant DNA methylation patterns in the tumors (46, 47, 49).

The majority of CRC cases (70-80%) occur sporadically (50). Sporadic CRC arises from the accumulation of a series of abnormal alterations in oncogenes and tumor suppressor genes, and it commonly occurs in patients with a median age of 70-75 years. On the other hand, cases in young people are mostly associated with a family positive history of CRC (50, 51).

In 1990, Fearon and Vogelstein proposed a CRC multistep carcinogenesis sequence through the association of mutations in *APC*, *K-RAS* and *p53* genes with CRC pathogenesis, being these alterations drivers and fuels for the disease progression (figure 4) (15, 34). This canonical sequence has been proposed to describe the mechanisms by which mutations in driver genes accumulate over time, leading to the progression of adenomas to invasive CRC (52, 53).

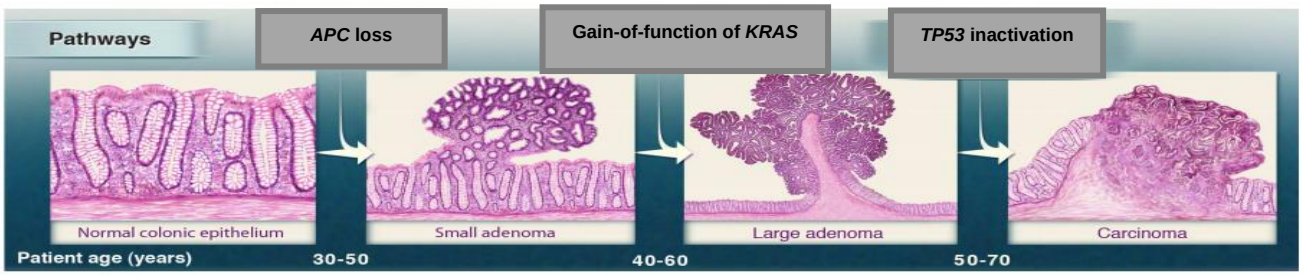

Figure 4. One of pathway implicated in CRC is chromosomal instability pathway (CIN). These is characterized by the mutation in tumor suppressor gene, adenomatous polyposis gene (APC). Subsequently, APC mutations are followed by activation of mutations in KRAS (proto-oncogene), which are deleted in CRC, and p53 genes (tumor suppressor gene), results confer growth advantages and leads to invasive cancers (Adapted from Mundade *et al.* (53)).

In the adenoma-carcinoma sequence, it is well documented that the loss of *APC* serves as an initiating event, followed by the accumulation of multiple mutations in genes such as *KRAS, TP53,* resulting in a progression of adenoma to cancer (54). *KRAS* is a member of the mitogen-activated protein kinase (MAPK) pathway and it is responsible for signal transduction from ligand-bound epidermal growth factor receptor (*EGFR*) to the nucleus (55). Furthermore, studies reported that high levels of EGFR have been related with aggressive disease, poor prognosis, decreased survival and poor response to therapy (55). In CRC, it is described that around 50% of colorectal cases present *EGFR* gene amplification (56).

The EGFR activation leads to the activation of multiple downstream signal transduction pathways, such as RAS-RAF-MAPK pathway and PI3K/AKT/mTOR pathway, involved in cell proliferation, metabolism and survival (22, 55, 57, 58).

mTOR is a serine/threonine protein kinase that belongs to two distinct multi-protein complexes commonly known as mTORC1 (mTOR Complex 1) and mTORC2 (mTOR Complex 2), which undergo a complex cross-talk that is important for physiological and tumoral growth (59–61). mTORC1 is involved in cell growth and contains Raptor (regulatory protein associated with TOR), GbL/mLST8 (mammalian lethal with Sec13 protein 8), and negative regulatory subnits PRAS40 and DEP domain-containing mTOR-interacting protein (DEPTOR). On the other hand, mTORC2 is associated with cell proliferation and survival and is composed by Rictor, mSin1, and Protor, GbL/mLST8 and DEPTOR (56, 62). Furthermore, mTORC1 can be indirectly activated by PI3K through AKT (63). AKT phosphorylates tuberous sclerosis complex 2 (TSC2), thereby dissociating the GTPase activity of the TSC1/TSC2 complex (64). The TSC1/TSC2 complex functions

as a GTPase- activating protein (GAS), and negatively regulates the activity of mTORC1, converting Rheb into its inactive form (63, 65).

The complex mTORC1 is located at the lysosomal surface and mediates the phosphorylation of 4EBP1 (eukaryotic initiation factor 4E-binding protein) and p70-S6K (ribosomal protein 6 kinase), which are essential regulators of protein translation (59, 66–68). Thus, one of the cellular processes associated with mTORC1 activity is cell growth, a process that is affected by nutrients bioavailability (69). In an amino acid (AA) high bioavailability microenvironment, mTOR is activated and translocates to the lysosomal surface where it interacts with the small GTPase Rheb, a direct activator of mTORC1, consequently regulating protein translation and other cellular processes (70). On the other hand, when there is an AA microenvironment deprivation, mTORC1 is inactive, being spread throughout the cytoplasm, and consequently increasing the breakdown of cellular proteins reducing protein biosynthesis (70, 71). In fact, Remaileh and co-workers reported that an AA starvation results in an inhibition of essential amino acids (EAA) exit of the lysosomal and consequent inhibition of mTORC1 (72). Thus, the availability of AA functions as a regulator of the mTORC1 pathway (73).

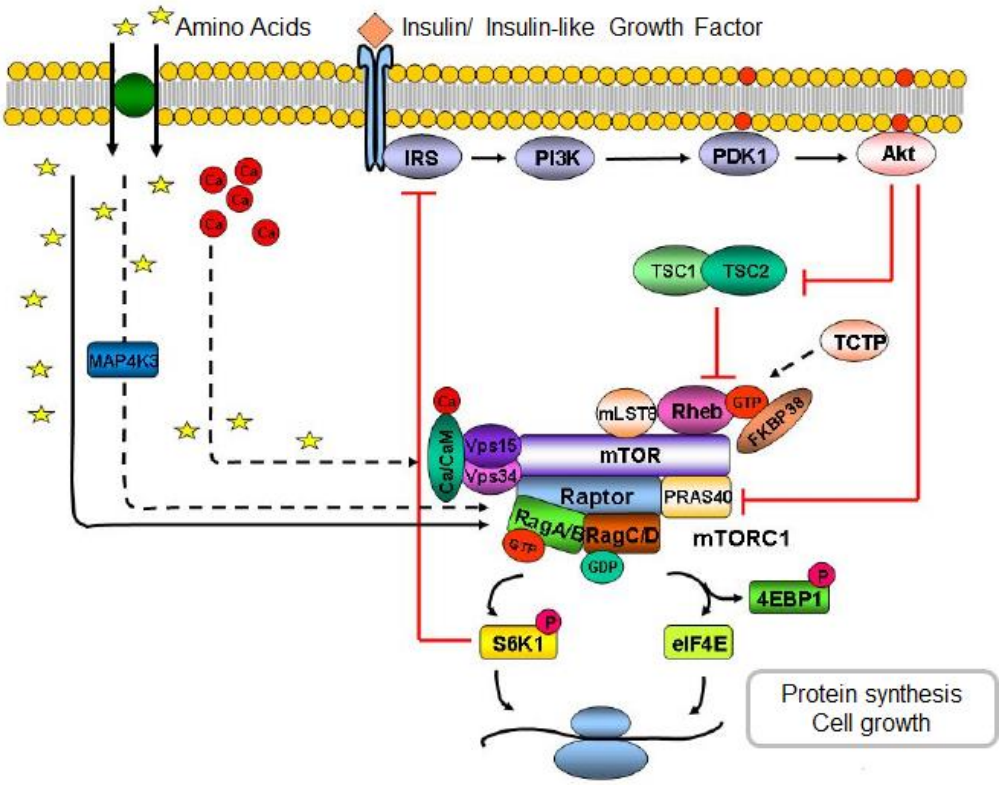

Figure 5. The mechanism of regulation of mTORC1. mTORC1 is a multiprotein complex constituted by Raptor, mLST8, PRAS40, which regulate the protein synthesis and cell growth by phosphorylation of S6K1 and 4EBP1. Once Rheb binding to mTOR, this complex is activated. Amino acids regulate mTORC1 activity by modulating the interaction between Raptor-mTOR. A amino acids starvation leads to S6K1 and 4EBP1 dephosphorylates, whereas, amino acid stimulation increase the S6K1 and 4EBP1 phosphorylation (Adapted from Kim *et al*. (60)).

1.3. Metabolism reprogramming and amino acids transporters

In order to sustain increased growth and proliferation, tumor cells undergo metabolic adaptations (61). Metabolic reprogramming is a hallmark of malignancy and an area of interest in cancer research since tumor cells grow rapidly by changing their own energy metabolism, specially under unfavorable conditions, such as pH alterations, hypoxia and nutrients deprivation (glucose, AA, micronutrients) (74–76).

It has been nearly a century since the discovery that normal and tumor cells' energy metabolism is different. In fact, tumor cells present a higher need of nutrients, being the AA bioavailability crucial to support cell proliferation and growth (17, 76). AA are molecules required for protein synthesis, with different functions. It is well documented that glutamine is an abundant nutrient that participates in energy formation, redox homeostasis, macromolecular synthesis and signaling in cancer cells (77). Glutamine and

glucose are the major plasma nutrients and sources of carbon metabolism, being estimated that the tumor cells consumes 10 times more glucose than non-proliferating normal cells (78). In fact, it has been described that, even under oxygen-rich conditions, most cancer cells undergo aerobic glycolysis, consuming higher amounts of glucose and producing high amounts of lactate, being this process known as the *Warburg* effect (13, 74, 79, 80).

In aerobic conditions, normal cells produce energy from mitochondrial oxidative phosphorylation, using pyruvate derived from glucose through the glycolytic pathway. On the other hand, under anaerobic conditions, energy is provided by glycolysis, and the pyruvate is mostly converted in lactate, that is preferentially exported from the cells (figure 6) (17, 81).

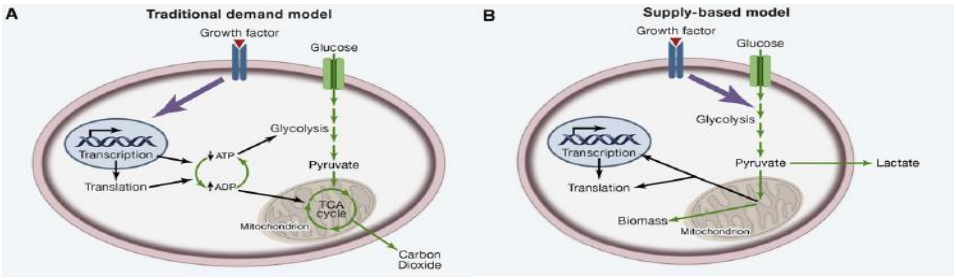

Figure 6. Schematic representation of Warburg effect. (A) In the traditional demand model, most of the flux from glycolysis is channeled toward Krebs cycle (TCA cycle). (B) In supply-based model, the most of the flux is channeled toward lactate synthesis (Adapted from Ward *et al*. (81)).

In cancer, the importance of glutamine as a nutrient results from its ability to donate its nitrogen and carbon into an array of growth-promoting pathways (77). Recently, Varshavi and colleagues described a molecular association between CRC that present oncogenic *KRAS* mutation and glutamine metabolism, since these cells exhibit special metabolic phenotypes, including differences in glycolysis, glutamine utilization and AA metabolism (82). Furthermore, glutamine is described as a signaling factor in the uptake of AA for the activation of *mTOR* (80). Thus, it has been shown that uptake of glutamine by cells regulates mTORC1 activity leading to the regulation of several mechanisms, including protein, lipid and nucleotide biosynthesis, and consequently, cellular growth and proliferation (66). Moreover, Nicklin and colleagues reported that EAA such as leucine, tryptophan, phenylalanine and arginine are the most potent stimuli for mTORC1 activation pathway (83–86).

In order to answer to the increase of anabolism and hypoxic tumor microenvironments, the fast metabolism of tumor cells will require an enhanced uptake of EAA, being the amino acids transporters (AAT) essential in the mediation og the traffic of amino acids between the outside and inside of cells (87, 88). In fact, several studies report that the Solute Carrier Transports (SLC) involved in cells' metabolism show important roles in response to pathological states, including the initiation and progression of tumors (89, 90).

1.3.1. Solute Carrier Transporters: LAT1/SLC7A5 and ASCT2/SLC1A5 transporters

SLC are a large family of transmembrane carriers that are involved in nutrient uptake and ion influx/efflux, presenting a ubiquitously distribution in different cells types (75, 89, 91). Over the years, more than 400 SLC transporters have been identified and grouped in 52 subfamilies (92, 93). They transport diverse solutes including AA, lipids, inorganic solutes, neurotransmitters and drugs across biological membranes (89). In normal tissues, SLC transporters are expressed in groups and patterns, mediating normal tissues functions, and they are categorized in different groups (´Systems`), depending of the basis of their substrate and sodium dependency (92, 94). In human diseases, including cancer, the expression patterns of these transporters can change. In fact, according to the literature, there are some SLC dysfunctions associated with CRC, such as the involvement of the L-type amino acid transporters (LATs) and SLC1 family in disease progression (90, 91, 94).

LAT1 (large amino acid transporter 1), known as SLC7A5, was identified in 1998 and belongs to SLC7 family (95). LAT1 is a transmembrane transporter involved in the EAA delivery in crucial body localizations, such as placenta and blood brain barrier (BBB) (94, 96). Furthermore, this transporter is a sodium (Na^+) and pH independent transporter and does not require ATPase, which facilitates diffusive transport (94). LAT1 forms a heterodimeric complex with the glycoprotein CD98 (4F2hc/SLC3A2), to import large and neutral AA (97). CD98 is a type II glycoprotein that functions as an integral subunit of LAT1, promoting stability and mediating its translocation to the plasma membrane (87). It preferentially transports large branched- chain and aromatic neutral EAA, including phenylalanine, tryptophan, leucine, isoleucine, methionine, histidine, tyrosine and valine into the cells, in exchange of the efflux of intracellular substrates (EAA or glutamine). Tryptophan, leucine, and histidine are recognized with high-affinity, and glutamine recognized much less efficiently (96, 98, 99).

LAT1 mRNA expression was detected in twenty seven tissue types, being the highest levels observed in brain, spleen, placenta, testis and low expression in colon (98, 100, 101). Furthermore, LAT1's expression is highly upregulated in multiple human cancers, including gastrointestinal, liver and kidney cancers (96, 102). In CRC tissues, Hayase and colleagues found a high expression of LAT1 in 72.4% of cases, and concluded that LAT1 could be a marker for malignant lesions, associated with poor patients' outcomes and survival (98, 102, 103). In cancer cells, it was demonstrated that the knockdown of *LAT1* can reduce leucine uptake and cell proliferation, indicating that LAT1 is a predominant transporter, essential for cancer growth (96). Moreover, studies that evaluate LAT1 levels in clinical settings as a potential drug target have been developed and were successful (94, 104). In 2010, Endo and co-workers developed a compound, named JPH203, that had the ability to inhibit LAT1, with a potent suppressive effect on cancer growth in animal models (94). Recently, Okano and co-workers, in a phase I study, observed that the JPH203 treatment was well-tolerated by patients' with CRC and biliary tract cancer (BTC). In fact, disease control was observed in two of the six CRC patients and in three of the five BTC patients (104).

ASCT2 (alanine, serine, cysteine transporter 2), also known as SLC1A5, belongs to SLC1 family and is predominantly localized in the cell membrane (74, 105). ASCT2 is an Na^+ and pH dependent transporter responsible for the transport of glutamine to the inside of the cell and induction of asparagine, serine, threonine efflux. This exchange of AA substrates is crucial for the uptake of glutamine by rapidly growing tumor cells (74, 88, 106). In 2012, Mootha and colleagues reported that tumor cells have a high necessity of glutamine uptake compared to other AA and, consequently, a glutamine starvation can interfere with tumor metabolism inhibiting tumor proliferation and progression (74).

ASCT2 is expressed in most human normal tissues including lung, large intestine, kidney, among others (74, 100, 105–107). According to Toda and co-workers, mutated *KRAS* can induce metabolic alterations that can modulate the AAT expression (108). The authors used two *KRAS*-mutated CRC cell lines (HCT 116 and DLD-1) and demonstrated a significant association between ASCT2 expression and *KRAS* mutation. When they introduced siRNAs targeting *KRAS,* a significantly reduction of ASCT2 was observed (108). In order to investigate which pathway was involved in this process, authors used specific inhibitors of Raf/MEK/ERK, and PI3K/Akt/mTOR pathways, and observed that both inhibitors present the ability to reduce the ASCT2 expression (108). ASCT2 presents an important role in cancer cells, not only in the glutamine uptake, but also in essential activities such as proliferation, apoptosis and metastasis formation (106). Studies using xenograft models demonstrated that the inhibition of ASCT2 expression can reduce the uptake of glutamine and inhibit tumor cell proliferation and the mTOR pathway (74).

Furthermore, it has been reported that downregulation of ASCT2, can suppress proliferation and induce apoptosis of CRC cell lines (74). ASCT2 can also modulate the migration capacity of CRC tumor cells, being the overexpression of ASCT2 associated with poor prognosis (74).

As mentioned above, liver metastasis are recognized as the most common site of CRC spread(41). Cumulative evidences show that LAT1 and ASCT2 are overexpressed in hepatocellular carcinoma (HCC), being additionally described that in HCC the cells have a 10-to-20 fold increase in glutamine uptake when compared to fetal human hepatocytes (100, 106, 109, 110). In fact, Namikawa and co-workers report that LAT1 and ASCT2 play an important role in the development and progression of HCC, being the overexpression of both transporters related with metastases establishment and aggressiveness (109).

Over the years, the study for new biomarkers and new therapeutic targets focused in CRC metabolomics has been developed. Recently, Rajasinghe and colleagues, reported that inhibition of glutamine uptake in proliferating cells, by inhibition of glutamine transporters (LAT1 and ASCT2) results in the inhibition of cell proliferation and induction of apoptosis via downregulating the mTOR pathway (111). In fact, according to literature, a two-step AAT mechanism regulates mTORC1 (112). The ASCT2 regulates the intracellular concentration of L- glutamine, which in turn can initiate EAA entry through the heterodimer LAT1/CD98. The complex uses the intracellular L-glutamine as an efflux substrate, with the aim of regulating the uptake of extracellular L-leucine, leading to an activation of mTORC1 signal and promoting cell growth (figure 7) (112, 113). Thus, taking this into account, LAT1 and ASCT2 expression levels could be potential CRC prognostic biomarkers and therapeutic targets.

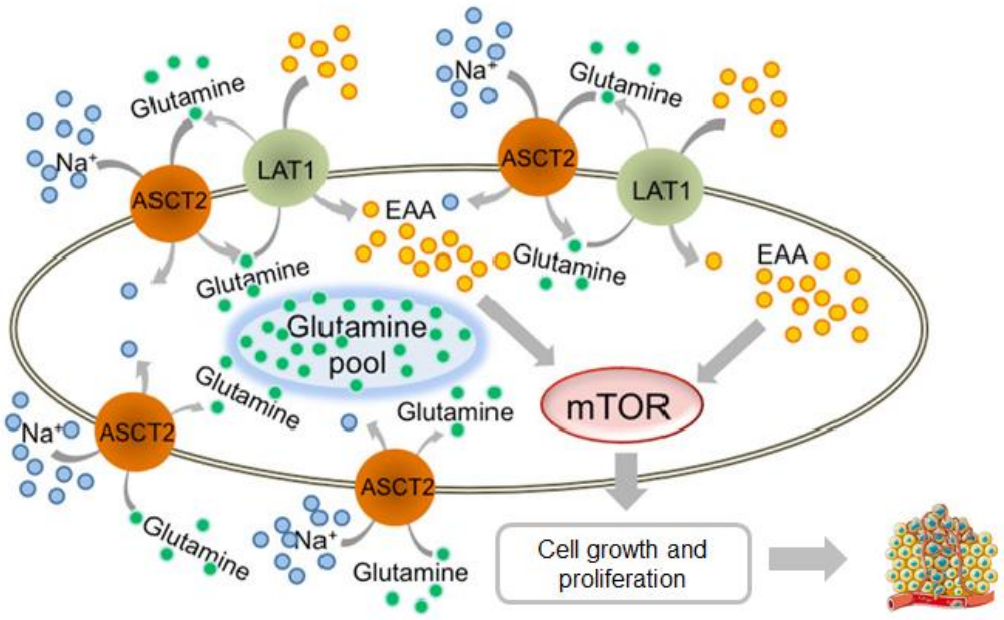

Figure 7. ASCT2 play a role in storage glutamine intracellular levels by co-transport with Na^+, which later, LAT1 uses glutamine in EAA uptake. Subsequently, the mTOR pathway which is regulated by essential amino acids will be active and promotes cell growth and proliferation (Adapted from Yazawa *et al.* (112)).

1.4. Extracellular vesicles as cell-to-cell communication vehicles

In order to coordinate the development and promote their environmental adaptation and function, cells of all multicellular organisms need to communicate with each other (114).

In the last two decades, one of the mechanisms of cell communication that has emerged is the communication by extracellular vesicles (EV's) (115, 116). EV's are microvesicles surrounded by a membrane composed by a lipid bilayer and hydrophilic proteins (117). They are released by numerous cells types, both in physiologic and pathologic conditions (117, 118). EV's are mainly classified into three subsets: exosomes, microvesicles and apoptotic bodies depending on their size, origin and the protein markers expressed in the plasma membrane (119). The inward invagination of endosomal membranes generates intraluminal vesicles in cytosolic multivesicular bodies (MVBs), containing vesicles called exosomes (120). Exosomes range from 30-150 nm in diameter and 1.13-1.19 g/mL in density and are secreted upon fusion of these MVBs with the plasma membrane (118, 121, 122). On the other hand, the direct blebbing of the plasma membrane generates microvesicles (150-1000 nm in diameter) or large apoptotic bodies

(> 1000 nm in diameter) when the host cell is dying (123). According to literature, it is estimated that normal human blood contains approximately 2000 trillion of EV's. However, in the systemic circulation of cancer patients a higher number (~4000 trillion) is found (120, 124). Relatively to protein markers, exosomes express transmembrane proteins such as CD9, CD63 and CD81 and other proteins associated with the plasma membrane (125). The microvesicles contain mainly cytosolic and plasma membrane associated proteins, including the tetraspanins and others proteins such as cytoskeletal proteins, heat shock proteins and proteins containing post translational modifications including glycosylation and phosphorylation (125). Lastly, the apoptotic bodies demonstrate higher levels of proteins associated with the nucleus (histones), mitochondria heat shock protein 60 (HSP60), endoplasmic reticulum (GRP78) and Golgi apparatus (125, 126).

EV's can shuttle several types of molecules between cells, including proteins, lipids, transcriptional factors, and genetic information (DNA, RNA, microRNAs) (119). Furthermore, several studies support that EV's participate in the regulation of several processes such as regulation of cell differentiation and tissue development, being also associated with pathological processes including tumorigenesis, cancer progression and pre-metastatic niche formation (figure 8) (120, 122).

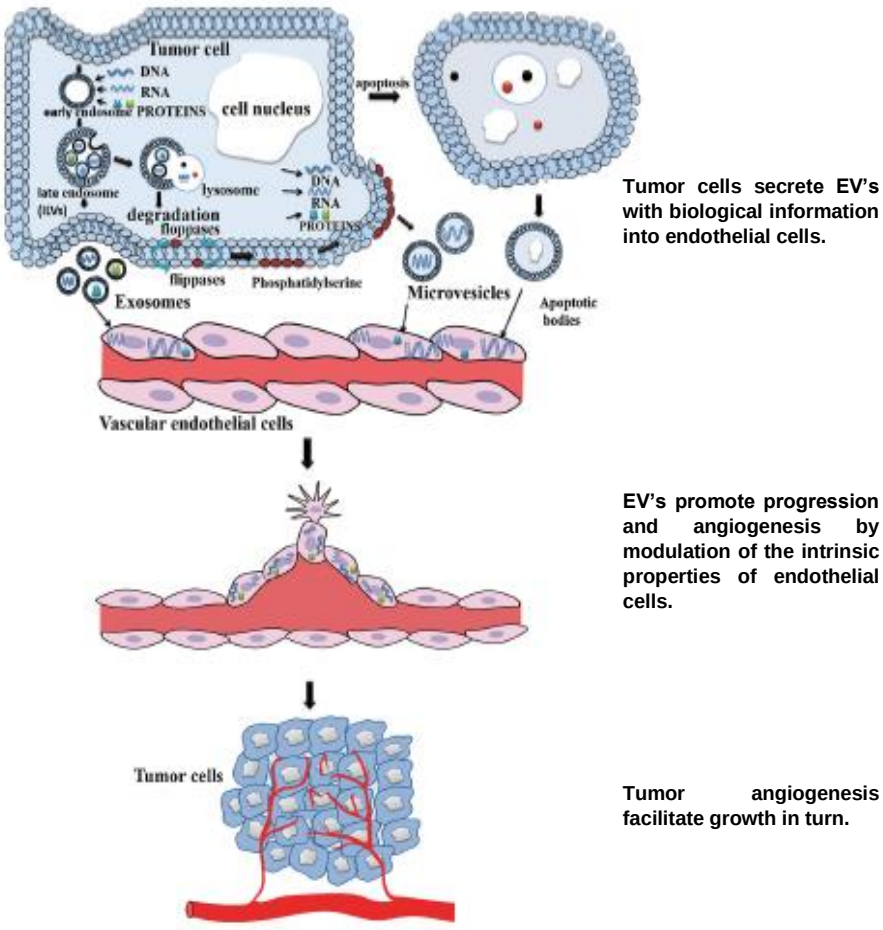

Figure 8. Capacity of tumor-derived EV's in circulate in systemic circulation and promote angiogenesis (Adapted from *Song et al*. (127)).

Tumor progression depends on the active crosstalk between the tumor cells and the microenvironment, being the EV's described as biological mediators, with the ability to modify the phenotype and functions of target cells (117, 127, 128). One pioneer study of how EV's can mediate the communication between cells used breast cancer and glioblastoma cells. Briefly, after exposure to cancer cell-derived exosomes with cargo such as oncogenic RNAs and pro-angiogenic proteins, epithelial cells with non-malignant phenotype acquired malignant characteristics (119, 129).

EV's derived from tumor cells with high metastatic potential are able to mediate critical processes that underline cancer evolution including angiogenesis, migration, invasion and EMT (epithelial-mesenchymal transition) (115, 130–132). In fact, according to the literature, EV's can influence the metastatic capacity of cells. In the study of Li and co-workers, the increased expression of exosomal miR-21 secreted by hypoxic oral

squamous cell carcinoma (OSCC) cells downregulated the expression level of E-cadherin (130, 133–135). On the other hand, the extracellular microenvironment also plays an important role in EV's secretion (130). It was observed that the presence of an alkaline extracellular pH causes a decrease of EV's secretion as well as EV's protein and RNA transportation (130).

In the last decade, several studies supported the use of EV's and specifically exosomes as diagnosis, early detection, chemoresistance, therapy response and prognosis biomarkers (133, 136). Examples are the study of Wang and co-workers which reports that the exosomal miR-125-3p was upregulated in CRC compared to health individuals, and the study of González and colleagues that reports the presence of high levels of the exosomal miR-19a in CRC patients and its association with tumor infiltration, lymph node and liver metastasis (137, 138). Moreover, Chiba and co-workers described that the EV's secreted from human CRC cell transfer RNAs into liver cells and, furthermore, they can regulate the expression of target genes and induce invasion, intravasation and metastasis in recipient cells (139). These studies demonstrate the power that EV's have on "re-educating" the recipient cells and reinforce the idea that further studies are needed in order to validate the applicability of the EV's content analysis as biomarkers of cancer prognosis and aggressiveness.

Considering the key role of EV's in cell modulation phenotype and the effect of AAT alteration during the tumor progression, it will be of great interest to analyze the presence of *LAT1* and *ASCT2* mRNAs in CRC derived EV's in order to establish their role on recipient cell phenotype modulation. In the future, this could help in the definition of new prognosis biomarkers, as well as new potential therapeutic targets.

2.Objectives

2.1. Main Objective

The aim of this study is the evaluation of an EV's-derived mRNA profile associated with CRC development and disease aggressiveness.

Specific Objectives

- Validate the excretion of EV's carrying *LAT1, ASCT2* and other aggressiveness related mRNAs by a colorectal cancer cell line (HCT 116 cell line);

- Evaluate the effect of HCT 116-EV's uptake in a hepatocarcinoma cell line (SK-HEP-1) and in a normal renal proximal tubule epithelial cell line (HKC-8), with focus on the mRNA expression profile modulation and phenotypic changes in recipient cell lines.

3.Materials and Methods

3.1. Cell line characterization and cell culture

Three cell lines were used for the study: HCT 116, SK-HEP-1 and HKC-8 (Figure 9). The HCT 116 is a colorectal cancer cell line, the SK-HEP-1 is a human hepatic adenocarcinoma cell line and HKC-8 is a human-derived normal renal proximal epithelial tubular cell line that was used as an example of a normal epithelial phenotype cell line. The HCT 116 and SK-HEP-1 were provided by Professor Patrício Soares da Silva from Biomedicine Department of Faculty of Medicine of Porto University and HKC-8 were provided by Professor Klaas Kok from the Department of Genetics of University Medical Center of Groningen.

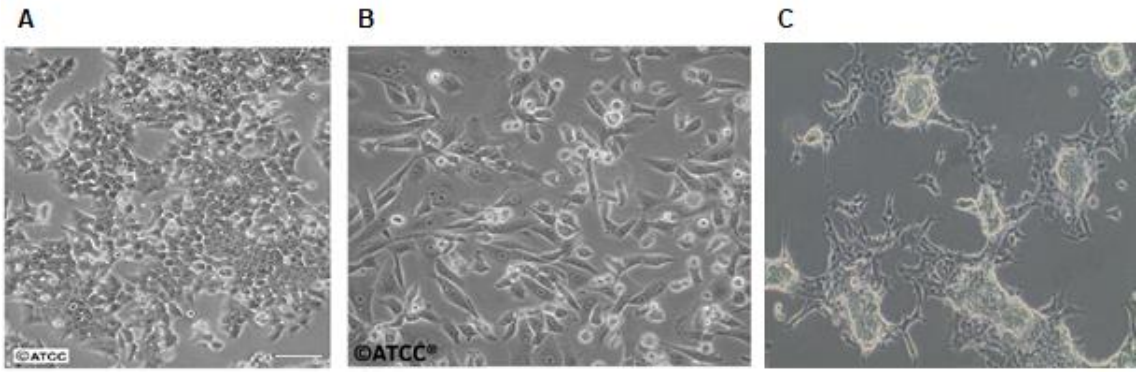

Figure 9. Microscope images of the (A) HCT 116 (ATCC©), (B) SK-HEP-1 (ATCC©) and (C) HKC-8 cell lines.

Firstly, a cryopreserved vial of each cell line was thawed. HCT 116 cells were maintained in MacCoy´s 5A Medium *(Sigma-Aldrich®)*, supplemented with Sodium bicarbonate (*Merck®*), Hepes *(Sigma-Aldrich®)*, 10% of FBS (Fetal Bovine Serum) *(Gibco®)* and 1% of Pen-Strep (penicillium-streptomycin mixture) *(Gibco®)*. SK-HEP-1 cells were maintained in RPMI Medium *(Sigma-Aldrich®)*, supplemented with Sodium bicarbonate (*Merck®*), Hepes *(Sigma-Aldrich®)*, 20% of FBS (Fetal Bovine Serum) *(Gibco®)* and 1% of Pen-Strep (penicillium-streptomycin mixture) *(Gibco®)*. HKC-8 cells were maintained in DMEM/F12 medium *(Gibco®)*, supplemented with ITS (Insulin-Transferrine-Selenum) *(Sigma-Aldrich®)*, Pen-Strep *(Gibco®)*, EGF (Epidermal Growth Factor) *(Sigma-Aldrich®)*, Hepes buffer *(Gibco®)* and Hydrocortisone *(Sigma-Aldrich®).* The three cell lines were kept in an incubator with the following conditions: 37ºC of temperature, 5% CO2 and humid atmosphere.

3.2. Isolation and characterization of EV's

Since HCT 116 cell line is derived from colorectal carcinoma, these cells were used for EV's production. EV's were isolated by a precipitation method using Total Exosome Isolation Reagent *(Thermo Fisher Scientific®).* Initially, the cells were seeded in a T25 flask and maintained until they reached 60-70% of confluence. Thereafter, the medium was replaced by MacCoy´s 5A with exosome-depleted FBS (MacCoy´s Exo-Free) during 48 hours. Then, the cell culture medium was harvested (5mL) and centrifuged at 3500 rpm, for 30 minutes at 4ºC in order to remove cells and debris. The supernatant was filtered with a 0.22 µm filter (GE Healthcare Whatman™). The purified supernatant and the EV's isolation reagent were mixed in a 2:1 proportion and incubated overnight at 4ºC. After overnight incubation, the mixture was centrifuged at 10.000 g for 1 hour and the pellet containing the enriched EV's fraction was resuspended in filtered PBS and stored at -80ºC until further analysis. The HCT 116-EV's were characterized by transmission electron microscopy (TEM) and flow cytometry.

3.2.1. CFSE-labeled EV's analysis by flow cytometry

The flow cytometry analysis was performed in the Immunology Department of IPO-Porto. EV's in our isolates were quantified using a CFSE (Carboxyfluorescein succinimidyl ester) (ab113853 - Abcam®) staining protocol optimized for a conventional flow cytometer. CFSE is a cell and EV's permeant, non-fluorescent pro-dye. Intracellular esterases in the EV's cleave the acetate groups which results in the green fluorescent molecule carboxyfluorescein that is now membrane impermeant. The succinimidyl ester group reacts indiscriminately with intracellular free amines to generate covalent dye-protein conjugates, resulting in EV's with an intravesicular fluorescent label. CFSE-labeled EV's were analyzed using a BD FACS Canto II flow cytometer. Due to their small size, EV's analysis requires working conditions close to the size-related sensitivity limit of the cytometer and some additional steps are necessary in order to better distinguish the EV's from the background. Thus, before acquisition, the cytometer is washed with distilled water during approximately 3 hours to remove debris and potential contaminants. Afterwards, we defined the cytometer settings for EV's analysis using Megamix-Plus SSC beads (BioCytex) which consist of a mix of fluorescent beads of varied diameters that cover a variety of microparticle size range (0.16 to 0.40 µm), and use side scatter (SSC) as a size-related parameter.

The EV's suspension was stained with CFSE at a final concentration of 2 μM and incubated in the dark at 37ºC during 45 minutes. After incubation, the EV's acquisition in the cytometer was made at low flow rate, using a threshold adjusted to FITC fluorescence channel, with a value of 700. The flow cytometry analysis allowed us to conclude that there were approximately $1.3X10^6$ EV's /mL.

The samples were acquired and analyzed with the FACSDiva software.

3.3. mRNA isolation

The mRNA extraction, from the cells and EV's, was performed using the GRS Total RNA Kit- Blood & Cultured Cells (*Grisp*®) according to the manufacturer protocol. When the cells (T25 flask) reached a confluence of 80-90%, the medium in which the cells were being maintained (HCT 116: MacCoy's Exo-Free; SK-HEP-1: RPMI with exosome-depleted FBS (RPMI Exo-Free); HKC-8: DMEM/F12) was collected for an EV's isolation and, subsequently, the cells were used for mRNA extraction. The cells were trypsinized using 1X 0.05% trypsin-EDTA *(Gibco®)* and counted using Tripan-Blue dye (*Gibco*®) and an EVE™ Automated Cell Counter (*NanoEnTek*®). For each mRNA extraction it was used approximately two million of cells. This procedure was replicated three times for each cell line used in the study.

3.4. cDNA synthesis and Real-time PCR relative quantification

The mRNA samples were used as templates for complementary cDNA (cDNA) synthesis using a *High Capacity cDNA Reverse Transcription Kit* (*Applied Biosystems*®) according to the manufacturer protocol. For polymerase chain reaction (PCR) amplification, the thermal conditions used were 25ºC for 10 min, followed by 37ºC for 120 minutes and 85ºC for 5 minutes for mRNA.

The mRNA expression levels were analyzed by quantitative Real-Time PCR. The reactions were carried out on a StepOne™qPCR Real-Time PCR machine, containing 1X fast advanced master mix (*Applied Biosystems*®), with 1X probes (*TaqMan® mRNA Expression Assays,* LAT1: Hs01001186_m1, ASCT2: Hs01056542_m1, HIF-1α (Hypoxia-Inducible Factor 1α): Hs00153153_m1, EGFR: Hs01076078_m1, VEGFA (Vascular e Endothelial Growth Factor): Hs00900055_m1, CXCR4: (C-X-C motif Chemokine Receptor 4): Hs00607978_s1, cDNA sample (≈ 50 ng), B2M (Beta-2- Macroglobulin) endogenous control for mRNA data normalization (B2M: Hs99999907_m1). All quantification reactions were performed in duplicate and in each run it was done negative controls. Data analysis was made using StepOne™ Sofware v2.2 (*Applied Biosystems*®) with the same baseline

and threshold set for each plate, in order to generate quantification cycle (Ct) values for all the mRNAs in each sample.

3.5. EV's uptake studies

3.5.1. Study 1 - Ev's fluorescence analysis

The HCT 116-EV's were labeled with 0.5 µl of CFSE membrane-permeable fluorescent dye. Then, the labeled HCT 116-EV's were resuspended in cell medium and added to the SK-HEP-1 and HKC-8 cell lines, cultured in cover slips in a 6-multi well plate, overnight. Subsequently, the medium was removed, and each well was washed with PBS 1X, three times. Then, the cells were incubated with 4% paraformaldehyde (PFA 4%) during 15 minutes, and then the cells were washed with PBS 1X. After, 1 mL of DAPI (4',6'-diamino-2-fenil-indol) *(Thermo Fisher Scientific®)* work solution (1:50 dilution of DAPI intermediate solution which consist in 2.3 µL of DAPI in 100 µL of 1X PBS) was added during 5 minutes to cells, and thereafter the cells were washed again with PBS 1X. Finally, was added Kaiser's glycerol gelatine (Merck®) to the cover slip and done slides, for a visualization in Olympus® IX51 microscope.

3.5.2. Study 2 – cell phenotypic assays

In order to analyze the effect of HCT 116 derived EV's, we designed two uptake studies using SK-HEP-1 and HKC-8 cell lines.

For these studies, 200 000 cells of SK-HEP-1 and HKC-8 were maintained in the conditions previously described. When the cells reached 60-70% of confluence, the HCT 116-EV's were administrated in two concentrations: 1) $1.3X10^6$ EV's (HCT 116-EV's 1) and 2) $3.93X10^6$ EV's (HCT 116-EV's 2). The phenotypic effect of the EV's uptake was evaluated through cell proliferation analysis, wound healing assay and mRNA expression and protein levels analysis. The mRNA expression analysis was performed according with the procedures described in sections 3.3 and 3.4.

3.5.2.1. Cell proliferation assay

Water soluble tetrazolium salt-1 (WST-1) assay (Abcam®) was used for the cell proliferation quantification of SK-HEP-1 and HKC-8. Briefly, cells were plated in a 96 multi-well plate, with 20 000 cells per well (6 replicates for each condition) and after 24 hours of cell adherence, the HCT 116-EV's were administered in the same volumes described in section 3.5.2 and incubated during 24 hours. After the incubation the WST-1 reagent was added and incubated during 1 hour. Then the absorbance was read in a *Biochrom™ EZ Read 400* microplate reader at 450 nm.

3.5.2.2. Wound healing assay

The wound healing assay was used to evaluate cell migration capacity. 200 000 cells of SK-HEP-1 and HKC-8 were cultured according the previously conditions using a 6-multi well plate. After 24 hours, the medium was removed, and it was done a scratch in the confluent cell monolayer using a 200-pipette tip, in order to remove the cells from the area. Then, the cells were washed with 1X PBS and incubated with 1.5 mL of the corresponding culture medium (SK-HEP-1: RPMI Exo-Free and HKC-8: DMEM/F12) with HCT 116-EV's. The wounded areas were photographed by phase contrast microscopy (Olympus® IX51 microscope) in the different time points: 0 hours, 7 hours and 24 hours for SK-HEP-1 and 0 hours, 3 hours, 24 hours and 48 hours for HKC-8, considering the growth kinetic of each cell line.

The scratch distances and wound closure was evaluated using the beWound software (beWound-Cell Migration tool (Version 1.5)). The relative migration distances were calculated according to the followed formula: % of wound closure= $100x(d_0-d_t)$ /mean of d_0, where d_0 is the width of cell wounds at time point 0 hours, and d_t is the width of cell wounds at different time points.

3.5.2.3. Protein Extraction and Quantification

Cells were plated according to protocols described in section 3.1 and 3.5.2. When the cells reach a confluence of 80-90%, they were trypsinized and centrifuged 10 minutes, 2500 g, 4ºC. The pellet was lysed with 150 µL of RIPA Lysis buffer (Radioimmunoprecipitation Assay Buffer) (*Santa Cruz Biotechnology®*) and supplemented with 1.5 µL of phosphatase inhibitor cocktail *(Thermo Fisher Scientific®).* Cell lysates were

centrifugated for 15 minutes, at 14 000 g and 4ºC, and the supernatant was recovered for protein quantification using a DC Protein Assay (*BioRad Laboratories*®).

The protein concentration was performed by measuring solution absorbance at 750 nm, in iMark™ Microplate Absorbance Reader (*BioRad Laboratories*®).

3.5.2.4. Western Blot

The expression of LAT1 and ASCT2 proteins were analyzed by Western Blot. Electrophoretic separation of proteins (40 µg) was performed in Mini-Protean TGX Gels (4-20%) (*BioRad Laboratories*®). The separated proteins were electrotransferred to polyvinylidene difluo-ride (PVDF) membranes (*BioRad Laboratories*®) using a Trans-Blot Turbo System (*BioRad Laboratories*®), for 7 minutes at 25V.

The PVDF membranes were blocked for non-specific interactions using 5% (% w/v) Albumin Bovine Fraction V (BSA) (*nzytech®)* in Tris-buffered saline with Tween20 (TBS-T) (20 mM Tris, 150 mM NaCl, pH 7.6, 0,05% (v/v) Tween 20) for 1 hour, at constant agitation, followed by three washes for 5-10 minutes, in TBS-T. Incubation with primary antibodies (LAT1, ASCT2, GAPDH) (table 1) were performed overnight at 4ºC.

Then, membranes were washed for 5-10 minutes in TBS-T, three times, at room temperature and incubated with conjugated secondary antibodies for 1 hour at room temperature. Finally, the membranes were washed, three times, for 5-10 minutes in TBS-T.

Antibody-labeled proteins were detected using Clarity™ Western ECL Substrate (*BioRad Laboratories*®), according to manufacturer´s instructions. A ChemiDoc XRS imaging system (ChemiDoc™ Gel Imaging System) (*BioRad Laboratories*®) and Bio-Rad Image Lab Software (*BioRad Laboratories*®) were used for the detection and analysis of band intensity.

Table 1. Antibodies and respective dilutions used in Western Blot technique.

Antigen	Primary antibody	Dilution	Source
LAT1	Mouse	1:200	Santa Cruz Biotechnology
ASCT2	Rabbit	1:800	Cell Signaling Technology
GAPDH	Mouse	1:500	Santa Cruz Biotechnology
Anti-Mouse	-	1:8000	Santa Cruz Biotechnology
Anti-Rabbit	-	1:8000	Santa Cruz Biotechnology

3.6. Statistical Analysis

Statistical analysis was performed using IBM®SPSS®Statisticals for Windows v23. The $2^{-\Delta\Delta Cq}$ method (140), along with the Student t' test were used in order to evaluate any statistical differences in the normalized expression levels of the mRNAs here explored.

4.Results

4.1. Characterization of HCT 116, SK-HEP-1 and HKC-8 cell lines

According to the results, there is a statistically significant difference in the intracellular levels of *LAT1* (*P*=0.001), *ASCT2* (*P*=0.001), *HIF-1α* (*P*=0.017) *CXCR4* (*P*<0.001), and *VEGFA* (*P*<0.001), when comparing HCT 116 and SK-HEP-1 cell lines. In fact, we found higher levels of these transcripts in HCT 116 cell line compared to SK-HEP-1 (Figure 10). Relatively to *EGFR* mRNA, we also found higher levels in HCT 116 cell line. However, this difference is not statistically significant (*P*=0.999). Concerning the HKC-8 cells, we found a statistically significant increase of *LAT1* mRNA (*P*<0.001) and decrease of *HIF-1α* mRNA (*P*=0.020) when compared to SK-HEP-1 cells.

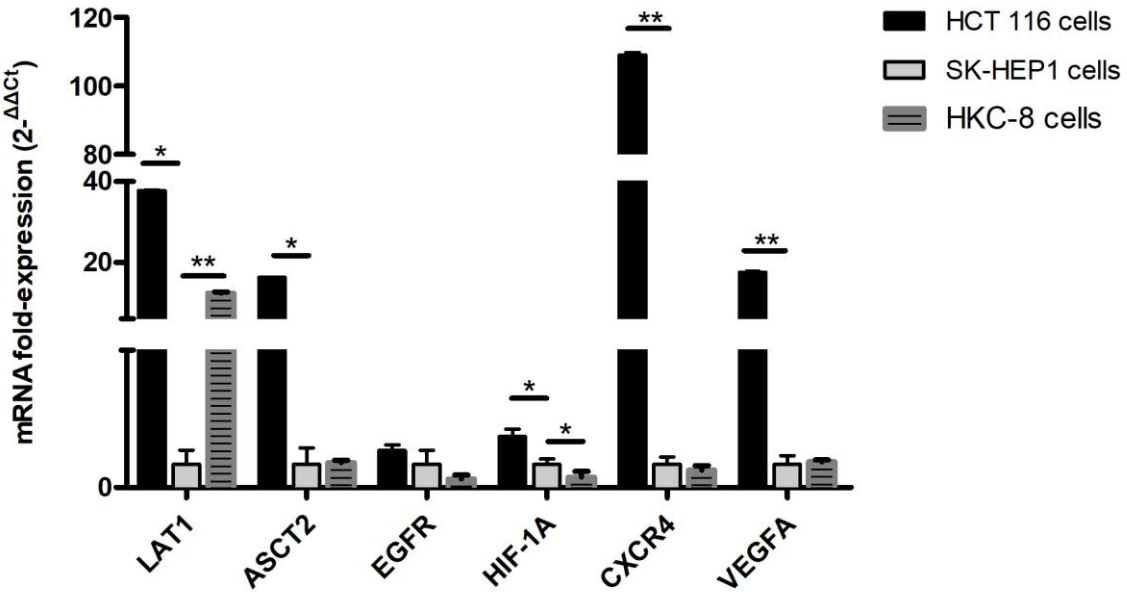

Figure 10. Fold-change of *LAT1, ASCT2, EGFR, HIF-1α, CXCR4 and VEGFA* mRNAs levels in HCT 116, SK-HEP- and HKC-8 cell lines. * p<0.05. **p<0.01.

Considering the protein levels, there is an increase in the levels of LAT1 and ASCT2 in HCT 116 when compared to SK-HEP-1 cell line (Figure 11). Furthermore, according to the image, it is suggested that in HCT 116 and SK-HEP-1 cells, the ASCT2 level is mostly expressed in their glycosylated form, contrary to HKC-8 cells, which presents a stronger non-glycosylated ASCT2 expression level.

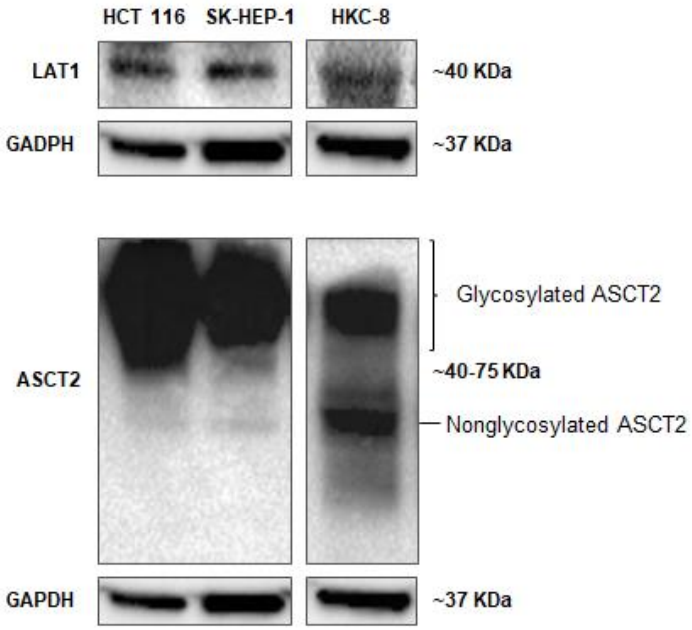

Figure 11. LAT1 and ASCT2 protein levels, in HCT 116 and SK-HEP-1 cell lines.

4.2. Characterization of EV's derived from HCT 116 cell line

The TEM image shows the variability of sizes and morphology of the HCT 116 EV's isolates (Figure 12A). The EV's used in this study were characterized according to size, shape, purity and mRNA content. The flow cytometry analysis showed us that the EV's analyzed presented sizes below 160 nm (Figure 12B- D). We also characterized the EV's content in terms of *LAT1, ASCT2, EGFR, HIF-1α, CXCR4* and *VEGFA* mRNA expression and we observed that all mRNAs that were detected in the HCT 116 cell fraction were also detected in the EV's fraction (Figure 12E), which allowed us to validate that these mRNAs are encapsulated and excreted in EV's by the HCT 116 cell line.

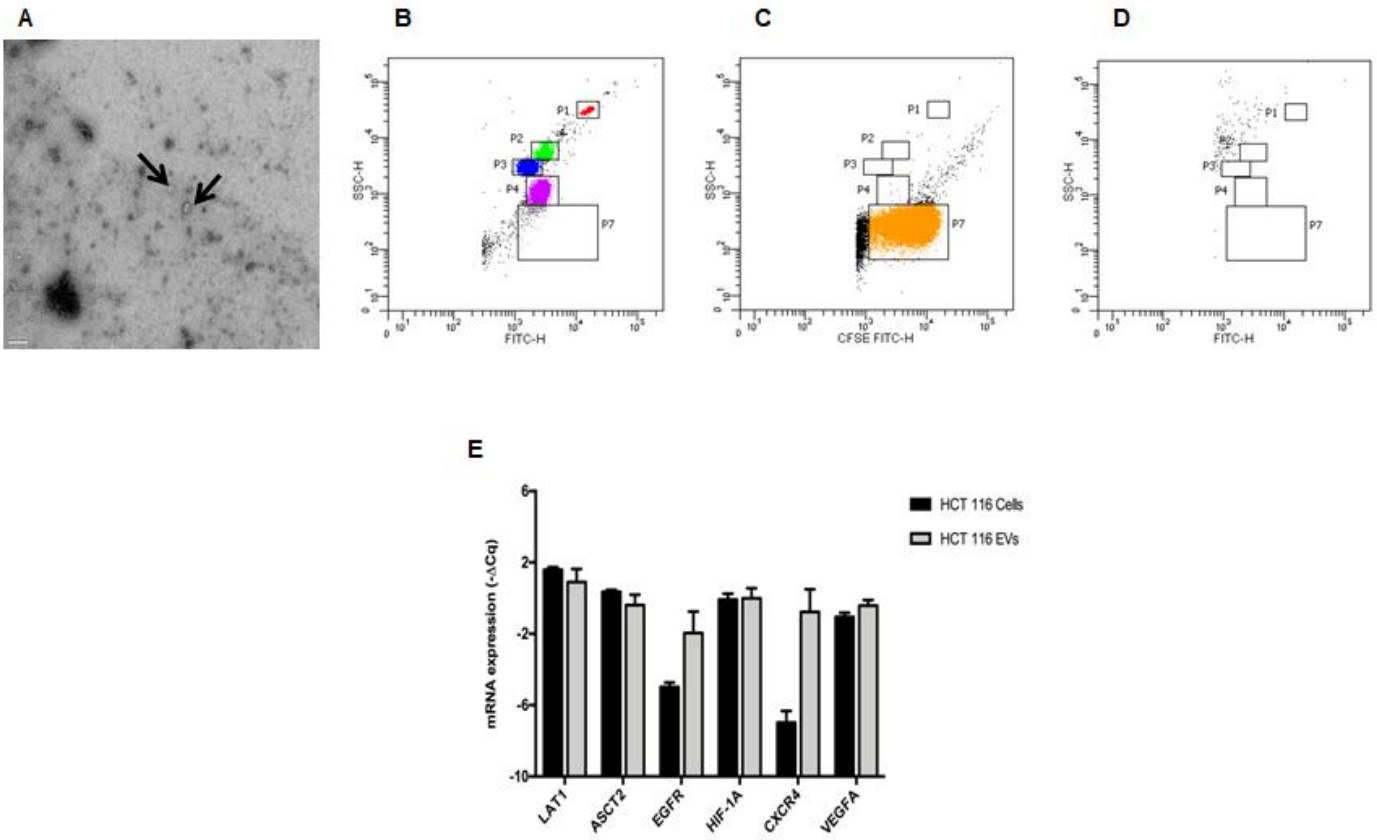

Figure 12. (A) Transmission electron microscopy (TEM) of EV's derived from HCT 116 cell line (scale 200 nm). The TEM image was acquired in the Histology and Electron Microscopy platform from I3S Porto using a Transmission Electron Microscope Jeol JEM 1400. (B) Megamix-Plus SSC beads used to define the cytometer settings for EV´s acquisition, with the following diameters following diameters: 0.5 µm (gate P1), 0.24 µm (gate P2), 0.20 µm (gate P3) and 0.16 µm (gate P4); (C) Fluorescent EV's (approximately $2{,}6x10^5$ /200 µM) as observed by flow cytometry from a sample of EV's isolate derived from HCT 116 cell line stained with CFSE (gate P7); (D) Negative control of EV's isolate derived from HCT 116 cell line without previous staining (gate P7). The flow cytometry analysis was made at the Immunology Department of IPO-Porto. (E) The intracellular and EV's mRNA expression levels (*LAT1, ASCT2, EGFR, HIF-1α, CXCR4* and *VEGFA)* of HCT 116 cell line.

4.3. HCT 116-EV's phenotypic effect in SK-HEP-1 cell line

When co-cultured with SK-HEP-1 cells, the labeled HCT 116-EV's were internalized by SK-HEP-1 cells. Thus, the conditions used, in time of exposure and quantity of administrated EV's, was enough for uptake by SK-HEP-1 cells (Figure 14A).

After 24 hours of HCT 116-EV's incubation in SK-HEP-1 cells, we observed that the color of the culture medium changed. In fact, we detected a pH change in the uptake condition 1 and 2 compared to control. In the control condition the pH is 7.4 and after HCT 116-EV's administration the pH is 7.1 and 8.8 in condition 1 and 2, respectively (Figure 13).

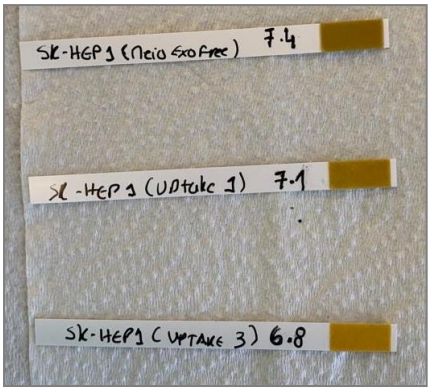

Figure 13. pH values in the different conditions tested in SK-HEP-1 after 24 hours.

Regarding the migration capacity, we observed that after 7 hours, the SK-HEP-1, HCT 116-EV's 1/ SK-HEP-1 and HCT 116-EV's 2/ SK-HEP-1 migrated and covered approximately 44.34%, 46.25% and 37.26%, respectively, of the scratch area. Furthermore, at 24 hours, the SK-HEP-1, SK-HEP-1 with HCT 116-EV's 1 and SK-HEP-1 with HCT 116-EV's 2 migrated and covered approximately 76.74%, 86.77% and 72.69%, respectively, of the scratch area. Thus, we can find that the uptake of HCT 116-EV's 1 by the SK-HEP-1 cells induces migration advantage with a significantly higher percentage of wound closure when compared with the control (P=0.002). On the other hand, we can observe that the uptake of HCT 116-EV's 1 induces a higher capacity of migration of wound closure, comparatively to the internalization of HCT 116-EV's 2. However, this difference is not statistical significant (P=0.331) (Figure 14B-C).

Additionally, we also observed higher proliferation rates in SK-HEP-1 cells submitted to the effect of HCT 116-EV's 1 (P<0.001) and HCT 116-EV's 2 (P<0.001) (Figure 14D).

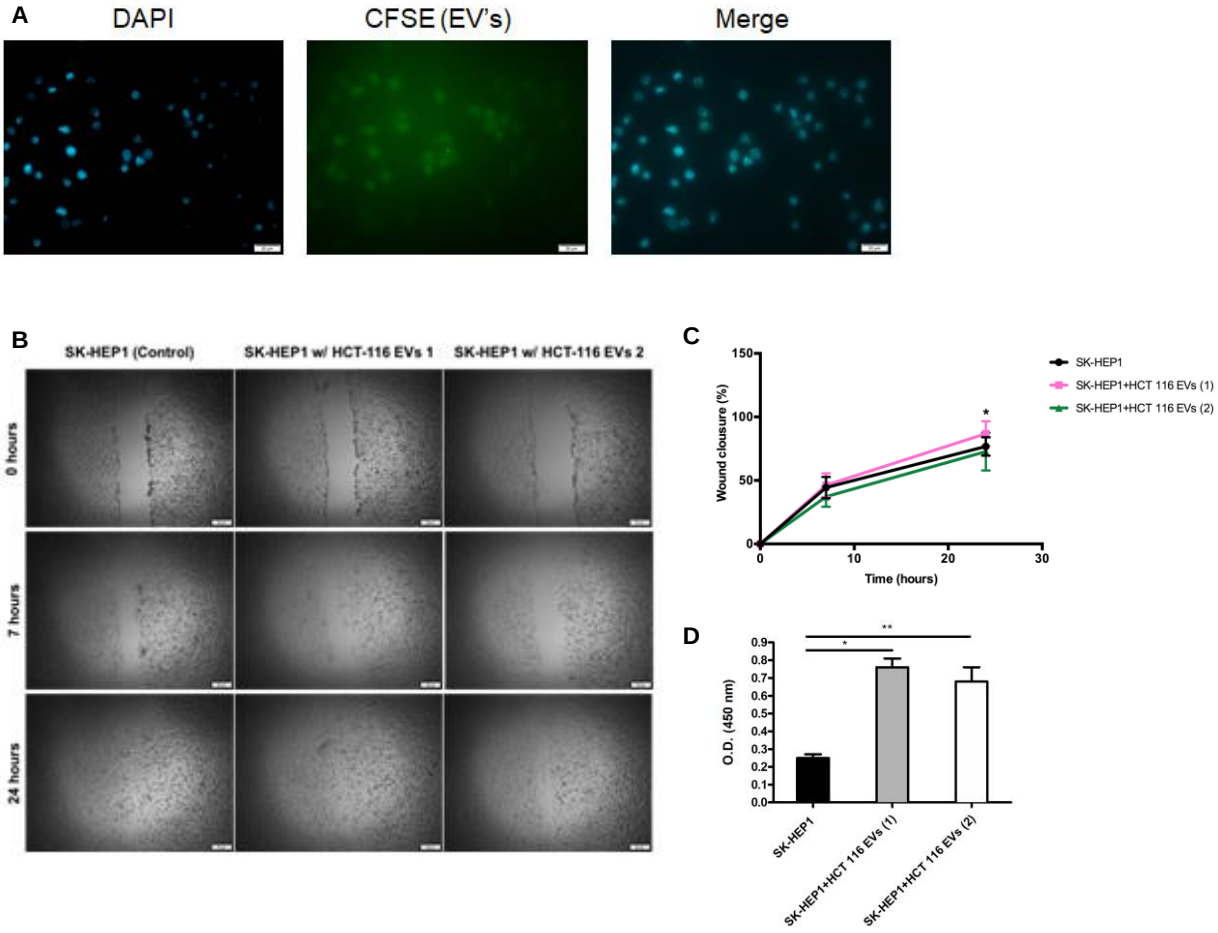

Figure 14. (A) Internalization of HCT 116-EV's by SK-HEP-1 cells. The EV's were stained with CFSE (green) and the cell nucleus with DAPI (blue). (B) cell migration capacity of receptor cells in different time points after stimulus with 1.3X10^6 EV's (HCT 116-EV's uptake 1) and 3.93X10^6 EV's (HCT 116-EV's uptake 2). (C) The stimulus with HCT 116-EV's decreases the gap closure time in the SK-HEP-1 cells (P=0.002). (D) the HCT 116-EV's uptake is also associated with a higher proliferation rate (HCT 116-EV's 1 *P*< 0.001; HCT 116-EV's 2 *P*<0.001). * p<0.05. **p<0.001.

Concerning the effect of the EV's uptake in the transcripts levels of the molecules included in the study, we found an increase of the expression levels of *LAT1*, *ASCT2, EGFR* and *HIF-1α* mRNAs levels and a decrease of *CXCR4* and *VEGFA* mRNAs levels. However, only the differences observed for *LAT1* (*P*=0.049) and *VEGFA* (*P*<0.001) present statistically significant differences. In fact, we found a 3.41 fold-increase of *LAT1* mRNA levels associated with the effect of HCT 116-EV's 1 and a 0.43 decrease of *VEGFA* mRNA levels associated with HCT 116-EV's 2 (Figure 15).

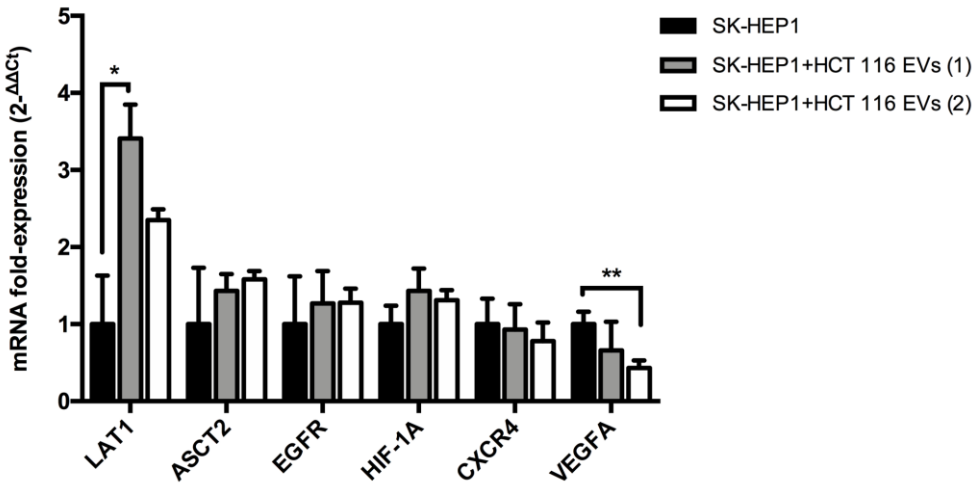

Figure 15. Fold-change of *LAT1, ASCT2, EGFR, HIF-1α, CXCR4 and VEGFA* mRNA levels in SK-HEP-1 cells after points after stimulus with $1.3X10^6$ EV's (HCT 116-EV's uptake 1) and $3.93X10^6$ EV's (HCT 116-EV's uptake 2). *p<0.05. **p<0.001.

We also found an increase of LAT1 protein level in SK-HEP-1 cell line associated with the HCT 116-EV's 2 when compared to control (figure 16). Concerning ASCT2, we observed a difference in the glycosylation status. When submitted to the effect of HCT 116-EV's (1 and 2), the SK-HEP-1 presented protein expression in their non-glycosylated form, contrary to what happens in basal cell line which presents ASCT2 protein expression in their glycosylated form.

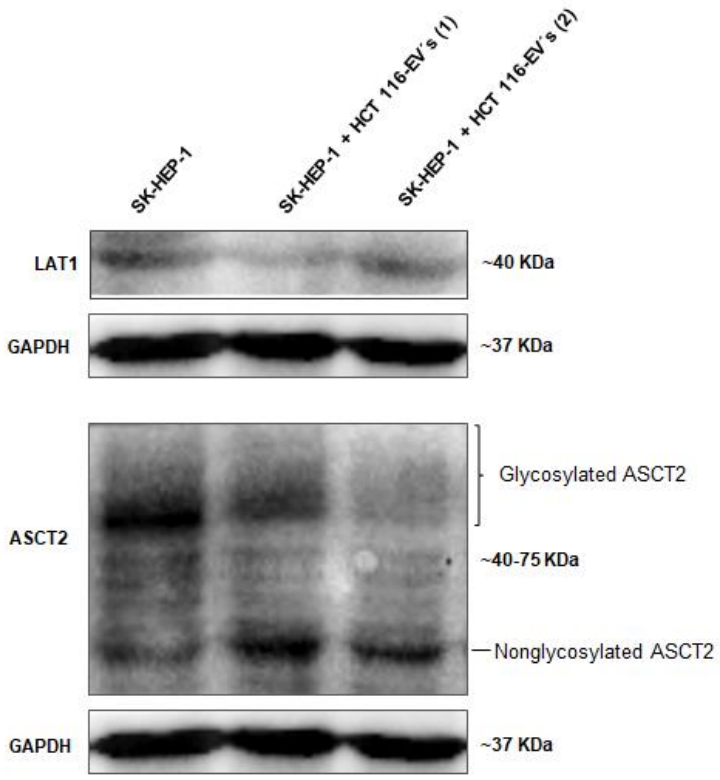

Figure 16. LAT1 and ASCT2 protein levels in SK-HEP-1, SK-HEP-1 + HCT 116-EV's (uptake 1) and SK-HEP-1 + HCT 116-EV's (uptake 2).

4.4. HCT 116-EV's phenotypic effect in HKC-8 cell line

Similarly to SK-HEP-1 cells, we also observed the internalization of HCT 116-EV's in HKC-8 cells (Figure 18A). Moreover, we also detected pH changes in cell culture after cell incubation with HCT 116-EV's (Figure 17). After 48 hours incubation of HKC-8 cell with HCT 116-EV's, we observed a decrease of pH to 6.0 (uptake 1 and 2) compared to control condition (pH 7.0).

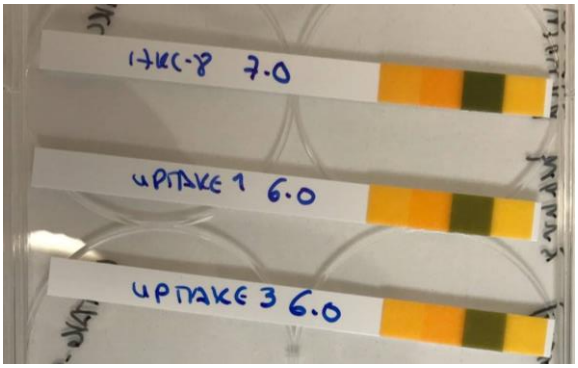

Figure 17. pH values of different cell condition tested after 48 hours of HKC-8 incubation with HCT 116 EV's.

Regarding the migration capacity, we verified that after 3 hours, the HKC-8, HCT 116-EV's 1/HKC-8 and HCT 116-EV's 2/HKC-8 migrated and covered, respectively, approximately 8.83%, 13.70% and 9.54% of the area quantified in the time zero (Figure 18B-C). After 48 hours, the HKC-8, HCT 116-EV's 1/ HKC-8 and HCT 116-EV's 2/HKC-8 migrated and covered, approximately and respectively, 29.90%, 39.30% and 31.17%. According to these results, the internalization of HCT 116-EV's 1 in HKC-8 cells increases the migration capacity of HKC-8 cells (P=0.0017). Moreover, it is observed that the uptake of HCT 116-EV's 2 also induces a minimal migration advantage of the cells comparatively to HKC-8 cells. However, this difference is not statistical significant (P=0.678).

Regarding cell proliferation, we observed that HKC-8 presented a higher proliferation after incubation with HCT 116-EV's 2, when compared to HKC-8 control condition (P=0.002) (Figure 18D).

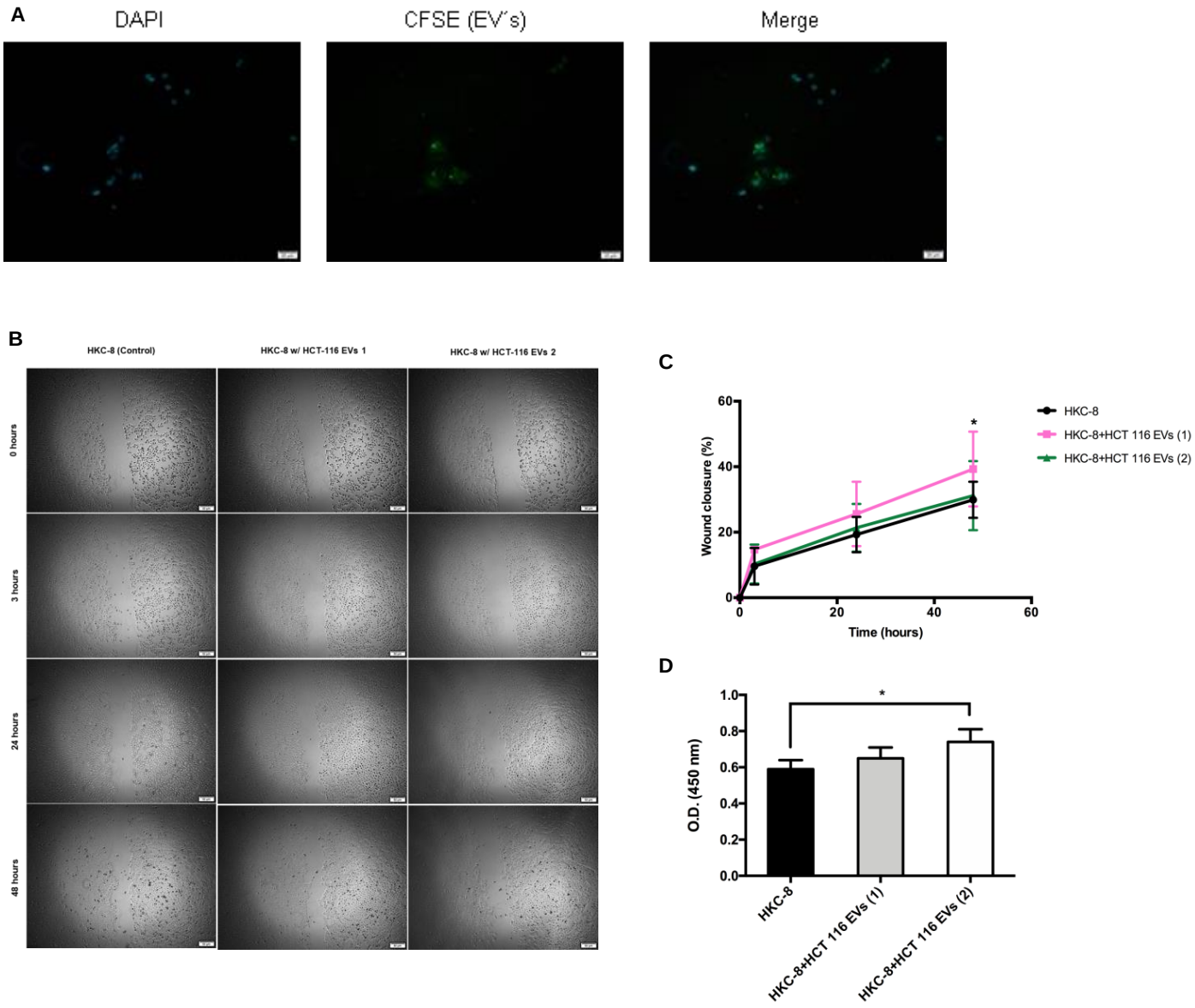

Figure 18. (A) Internalization of HCT 116-EV's by HKC- cells. The EV's were stained with CFSE (green) and the cell nucleus with DAPI (blue). (B) cell migration capacity of receptor cells in different time points after stimulus with 1.3X10^6 EV's (HCT 116-EV's uptake 1) and 3.93X10^6 EV's (HCT 116-EV's uptake u2). (C) The stimulus with HCT 116-EV's uptake 1 decreases the gap closure time in the HKC-8 cells (P=0.0017). (D) HCT 116-EV's uptake by HKC-8 cells is also associated with a higher proliferation rate (HCT 116-EV's uptake 2 *P*<0.001). * p<0.05. **p<0.001.

Concerning the effect of the EV's uptake in the transcripts levels of the target included in the study, we found that the expression level of *ASCT2* (*P*=0.982*), EGFR* (*P*=0.841), *HIF-1α* (*P*= 0.129) and *VEGFA* (*P*=0.183) increased in HCT 116-EV's 1/HKC-8

when compared to the basal cell line. However, this increase is not statistically significant. On the other hand, after the HCT 116-EV's 2/HKC-8 uptake, we found higher levels of *LAT1 (P*=0.011*)* and *HIF-1α* (*P*=0.019) mRNAs (Figure 19).

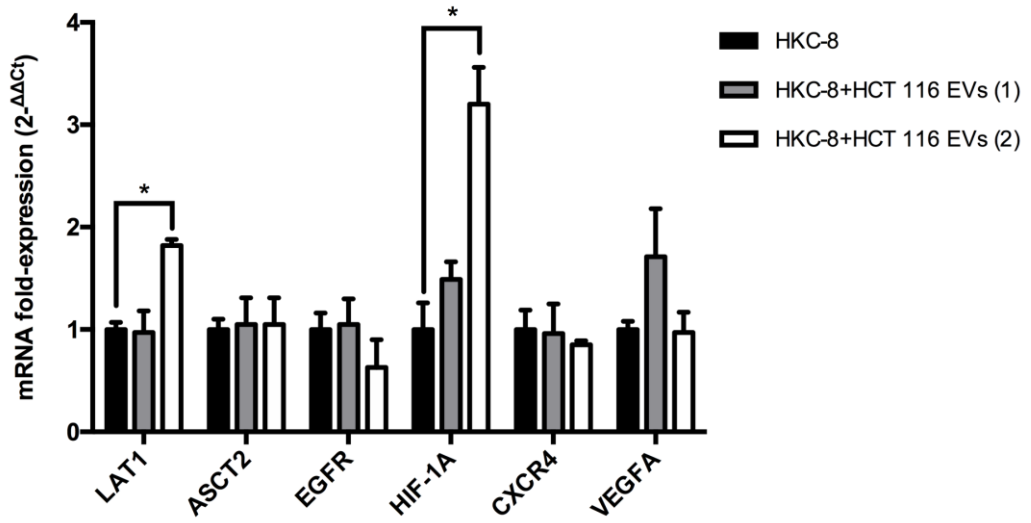

Figure 18. Fold-change of *LAT1, ASCT2, EGFR, HIF-1α, CXCR4 and VEGFA* mRNAs levels in HKC-8 cell line after stimulus with $1.3X10^6$ EV's (HCT 116-EV's uptake 1) and $3.93X10^6$ EV's (HCT 116-EV's uptake 2). * $p<0.05$.

Considering the protein levels (Figure 20), we observed that the uptake of HCT 116-EV's increased the LAT1 levels when compared to control condition. Concerning the ASCT2 protein levels, it is suggested an increase of ASCT2 after stimulus with HCT 116-EV's (2). Furthermore, we observed that in HKC-8 in their basal condition and with the addition of HCT 116-EV's (1 and 2), the ASCT2 expression is mostly expressed in its non-glycosylated form.

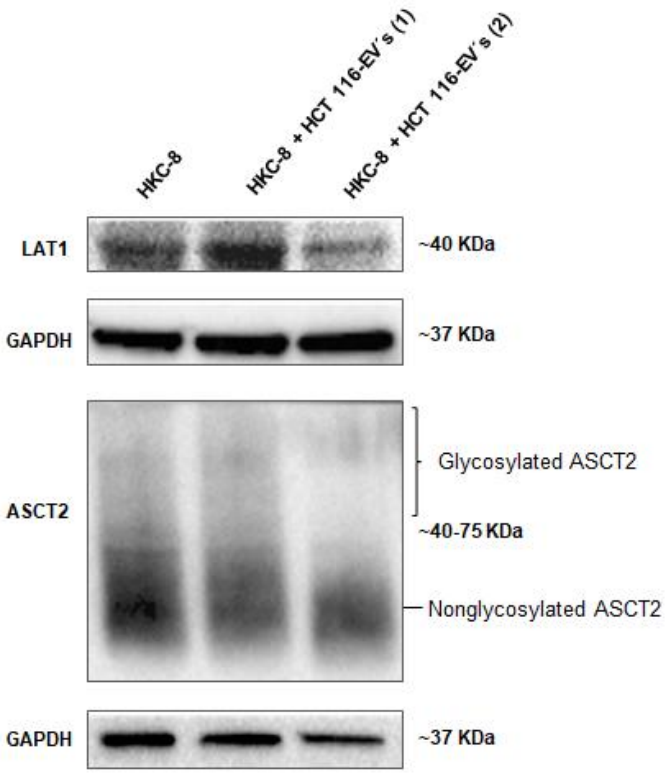

Figure 19. LAT1 and ASCT2 protein levels in HKC-8, HKC-8 + HCT 116-EV's (uptake 1) and HKC-8 + HCT 116-EV's (uptake 2).

5.Discussion

The role of EV's as mediators of cell communication has been documented over the last years (141). It is well established that EV's are important mediators facilitating the acquisition of cancer-associated hallmarks through the modulation of tumor microenvironment, since EV's are able to shuttle biomolecules between cells (141). Thus, the EV's transfer of functional cargo to different recipient cell types modulates different processes, namely cell proliferation, migration, invasion and angiogenesis, which promotes tumor progression (142).

Regarding CRC, Lugini and colleagues described that the EV's derived from tumor cells are able to induce a tumor-like behavior in mesenchymal stromal cells, suggesting that the inflammatory microenvironment initiated by cancer EV's promotes tumor growth and invasiveness (143, 144). Additionally, Chiba and co-workers observed that EV's secreted from human CRC cells transfer mRNAs into liver cells, which is interesting since the liver is known to be one of the most common sites for metastasis formation (31, 145). These studies highlight the fact that the molecular cargo of EV's, such as mRNAs, can be used as potential predictive biomarkers that could help to improve the prognosis of patients and treatment choice.

In the present study, we aimed to evaluate the impact of CRC EV's-derived mRNA profile in disease aggressiveness, namely the *LAT1* and *ASCT2* mRNAs. In CRC, these two AAT are associated with tumor progression through the increase of amino acid uptake by cancer cells and with the aggressiveness of disease (74, 88, 98, 106). In the study of Hayase and co-workers, LAT1 was highly expressed in >70% of the investigated cases of CRC, being associated with the depth and venous invasion (98). Additionally, the study of Huang and colleagues, proved that the ASCT2 overexpression in CRC is associated with tumor stage (146). However, in the literature, the study of these mRNAs in CRC derived EV's remains unclear. Since tumor cells have a unique metabolic demand for AA in order to satisfy the rapid growth and proliferation, and the expression of AAT is elevated compared with normal tissues, these have a potential role as emerging targets for cancer therapy. As previously mentioned, Endo and co-workers, observed that the inhibition of LAT1 had a strong suppressive effect on cancer growth in animal models (94). In fact, a phase I clinical trial involving a LAT1 inhibitor showed that this treatment was well tolerated both in CRC patients' and BTC patients' (104).

In the present study, we validated for the first time the presence of *LAT1* and *ASCT2* mRNAs in HCT 116-EV's, that we recovered from HCT 116 cell line. Moreover, we also found the presence of *EGFR, HIF-1α, VEGFA* and *CXCR4* mRNAs in HCT 116-EV's.

Dong and co-workers report the melanoma antigen family A3 (*MAGEA3*) mRNA and keratin associated protein 5-4 (*KRTAP5-4*) mRNA in the serum exosomes of CRC

patients, being the quantity of *KRTAP5-4* mRNA significantly lower in CRC than in controls (147). Furthermore, Sun and colleagues describe the plasma exosomal copine III (CPNE3) in CRC. This exosomal protein was significantly elevated in CRC patients when compared to healthy controls, being a potential diagnostic candidate for CRC (148). The study of Campanella and co-workers showed that Hsp60 protein in exosomes isolated from of CRC patients may be a promising diagnostic biomarker. The authors demonstrated that that levels of Hsp60 differed before and after surgery in CRC patients', being the level of exosome-derived Hsp60 before surgery significantly higher compared to the levels after surgery (149, 150). Regarding the EV's study in CRC models, Wang and colleagues demonstrated that the HT-29 CRC cell line produced EV's that can modulate the migration capacity of Caco-2 cells, which is a liver cell line (151).

Furthermore, as previously mentioned, we also found other molecules within HCT 116-EV's, the *HIF-1α*, *VEGFA*, *EGFR* and *CXCR4* mRNAs. HIF-1α activity is regulated by oxygen, and plays a major role in the metabolism that supports the survival of hypoxic tumor cells regulating gene expression for tumor angiogenesis, glucose metabolism and resistance to oxidate stress (17). In CRC, it is described that HIF-1α expression is correlated with aggressiveness through the activation of VEGF (also known as VEGFA) (17, 152). VEGFA is a cytokine with angiogenic, mitogenic and vascular permeability power, giving to tumor cells the opportunity to enter the circulation (153). In CRC carcinogenesis, George and co-workers described increased levels of *VEGFA* mRNA levels compared to other angiogenic cytokines, being the expression higher in tumors with lymph nodes metastases, compared to non-metastatic tumors (153). Additionally, the results of Mohamed and colleagues, showed that VEGFA was associated with higher tumor grade, high incidence of lymph node metastases and advanced stage of disease, being associated to poor prognosis (154, 155). Another cytokine deregulated in CRC is CXCR4, being correlated with poor histological differentiation, distant metastasis, lymph node metastasis, being the CXCR4 high levels associated with poor prognosis of CRC patients CRC (156, 157). On the other hand, EGFR is also correlated with an aggressive tumor phenotype and poor prognosis in several types of cancer, included the CRC (158, 159). In fact, Spano and co-workers' reported that EGFR overexpression is associated with tumor stage. However, it seems to not influence CRC patients' overall survival (158, 159). Thus, these mRNAs play a key role during CRC progression and metastasis formation.

Moreover, considering the uptake studies, we found that HCT 116-EV's were able to induce an increase of *LAT1* mRNA levels, with consequent protein increase, and a decrease of *VEGFA* mRNA levels in SK-HEP-1 recipient cells. Furthermore, this uptake of HCT 116-EV's also had the capacity of stimulate cell migration capacity and proliferation

of the recipient cells. Hafliger and co-workers described a correlation between LAT1 and VEGFA expression in adenocarcinoma patients, since VEGFA was significantly higher in patients with a positive LAT1 expression (102, 160)**.** The study performed by Elias and co-workers showed that, microenvironmental changes, such as pH variations induce variations in the *VEGFA* splicing pattern (161). One of the changes that we observed after HCT 116-EV's stimulus was the decrease of pH. Thus, we can hypothesize that we may be inducing an alternative splicing pattern of VEGFA and consequently the TaqMan probe that we used to quantify this transcript was not able to detect it efficiently.

Regarding the HKC-8 cell line, the uptake of HCT 116-EV's led to an increase of *LAT1* mRNA levels, with consequent protein increase, and an increase of *HIF-1α* mRNA levels. Similarly to SK-HEP-1, the uptake of HCT 116-EV's also had an impact in migration capacity and proliferation in HKC-8 recipient cells. A study by Kaira and colleagues describes that LAT1 expression is significantly associated with hypoxic markers such as HIF-1α, in non-small cell lung cancer (160). Additionally, Shi and colleagues described that the overexpression of LAT1 is correlated with hypoxia, in CRC (162). This suggests that the addition of HCT 116-EV's can modulate the phenotype of target cell, by increasing the uptake of amino acids and, consequently, supporting cancer cell growth and promoting a hypoxic microenvironment.

Moreover, according to our results, we observed that the HCT 116-EV's, not only present the ability to transfer *LAT1* and *ASCT2* mRNAs into recipient cells, but these mRNAs are also translated into proteins, increasing the protein levels of these AAT in recipient cells. These evidences support the hypothesis that EV's can transfer key molecules involved in CRC progression, creating a microenvironment that promotes the metastatic niche establishment.

In the present study, we uncovered an additional role of EV's in the CRC metastasis establishment through the transfer of ATT mRNAs, especially *LAT1* mRNA, with a phenotypic impact on the increase of cell proliferation and migration capacity of recipient cells. Thus, further studies are needed to validate the biomarker and therapeutic potential of AAT in CRC.

6.Conclusions and Future Perspectives

Although the mortality rates have declined due to the improvement in diagnosis and treatment, CRC still represents one of the most lethal cancer types, due to its high metastatic potential, mostly to the liver.

It is well documented that cancer cells-derived EV's have the power to modulate the recipient cells, "reeducating" them, since they carry genetic information with ability to induce alterations in the phenotype of target cell. Thus, by transporting and transferring functional cargo to multiple recipient cells types, cancer cell-derived EV's serve as nucleic acid delivery vehicles stimulating various cellular processes such as endothelial cell proliferation, migration, invasion, angiogenesis, and consequently promoting tumor progression.

According to our results, the *LAT1* and *ASCT2* mRNAs and other aggressiveness mRNAs markers of disease reported in CRC progression are encapsulated in CRC-derived EV's, and more importantly, when added to hepatocarcinoma and normal renal cells, have the ability to induce phenotypic alterations, namely an enhanced migratory and proliferative behavior in the recipient cells. These phenotypic alterations will contribute to the establishment of a pre-metastatic niche in CRC, but further studies are needed in order to replicate these observations and validate this hypothesis.

One possible approach to validate our hypothesis would be the study of the inoculation of HCT 116-EV's in an animal model and monitor the cell traffic establishment. This type of study would allow us to understand if these EV's only present tropism for liver and kidney cells or if they are able to affect the proliferation and migration capacity of cell from other organs as well. Additionally, it would also be interesting to validate our findings in CRC patients' plasma EV's to evaluate the biomarker potential of CRC EV's-derived mRNA, especially *LAT1,* in patients' prognosis and follow-up. The patients' study should include a prospective cohort of patients with localized disease and a group of patients with metastatic disease so that we could evaluate the CRC EV's enrichment in our mRNAs of interest during the course of the disease. The validation of the biomarker potential of CRC EV's-derived mRNAs would be useful for liquid biopsies implementation and also for the development of new therapeutic approaches for CRC.

7.References

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8.Supplementary Material

Submitted manuscript

Interplay of LAT1 and ASCT2-related microRNAs in colorectal cancer progression: potential new therapeutic agents

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Influência de mRNA LAT1 derivado de vesículas extracelulares na progressão do cancro colorretal

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Introdução: O cancro colorretal (CCR) é uma das principais causas de morte por cancro na população ocidental, sendo a nível mundial o terceiro cancro mais frequente. Estudos demonstram que aproximadamente 50% dos casos desenvolvem doença metastática hepática no decurso da doença, o que traduz a necessidade de novos alvos moleculares associados à oncobiologia do CCR, com potencial de serem usados como biomarcadores de prognóstico. Recentemente a literatura evidencia que transportadores de aminoácidos (TAA) são capazes de regular a via de sinalização PI3K/Akt/mTORC1 - central na regulação da proliferação e metabolismo celular de CCR, estando também envolvidos na reprogramação metabólica. Paralelamente, o aumento da expressão dos TAA, LAT1 e ASCT2 tem sido observado em diferentes modelos tumorais, associado ao crescimento, proliferação celular e angiogénese. Atualmente, admite-se que as vesículas extracelulares (VEs) medeiam a comunicação celular e exercem diferente atividade biológica por troca de informação molecular (ácidos nucleicos, proteínas...) a células recetoras, podendo modificar fenótipos celulares e condicionar a progressão tumoral.

Objetivos: Analisar o conteúdo de mRNAs derivados de VEs de uma linha celular de CCR e o seu potencial na modificação do fenótipo celular de uma linha celular de fígado.

Material e Métodos: Como modelo *in vitro* foram usadas as linhas celulares HCT116 (CCR) e SK-HEP1 (hepatocarcinoma). O isolamento de VEs de HCT116 (VEs-HCT116) foi realizado pelo *Total Exosome Isolation Reagent (Thermo Fisher Scientific*®) e validado por TEM. A quantificação dos níveis de mRNA (celulares; Evs-HCT116) de *LAT1, ASCT2, EGFR, HIF1A, CXCR4, VEGFA* foi realizada por PCR quantitativo em tempo real. Avaliou-se a expressão dos níveis proteicos de LAT1, ASCT2 e dos níveis de mRNAs do perfil acima mencionado na linha celular SK-HEP1 após *uptake* de VEs-HCT116. O efeito funcional ao nível da proliferação celular (*WST-1 assay kit- ABCAM*®) e *wound healing* foram analisados na linha SK-HEP1.

Resultados: A HCT116 apresenta um aumento de expressão de LAT1 e ASCT2 comparativamente à SK-HEP1, verificando-se níveis aumentados de mRNA *LAT1* (*P*=0,001), *ASCT2* (*P*=0,001), *HIF1A* (*P*=0,017), *CXCR4* (*P*<0,001) e de *VEGFA*

(*P*<0,001). Verificamos a presença dos mRNAs *LAT1*, *ASCT2*, *EGFR*, *HIF1A*, *CXCR4* e *VEGFA* nas VEs-HCT116, sendo que a adição destas originou um aumento da expressão de LAT1 e dos níveis de mRNA de *LAT1* (*P*=0,049) e *VEGFA* (*P*<0.001) na SK-HEP1 após 24 horas de incubação. Este *uptake* resultou também num aumento da proliferação, verificado pelos ensaios de *WST-1* e *wound healing*.

Discussão/Conclusão: Observamos a presença de TAA em VEs-HCT116 e de mRNAs associados a um fenótipo de agressividade, com capacidade de modificar o fenótipo celular da linha recetora. Deste modo, os transportadores LAT1 e ASCT2 analisados poderão ser potenciais novos alvos terapêuticos no CCR.