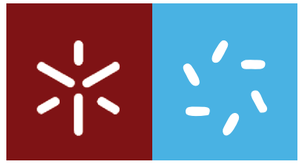
Uncovering the role of acetate in the crosstalk between monocarboxylate transporters and oncogene signalling pathways in colorectal cancer

Sara Gomes

UMinho | 2018



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Escola de Ciências

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Tese de Mestrado

Mestrado em Genética Molecular

Trabalho efetuado sob orientação de:

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Fevereiro de 2018

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Título da dissertação: Uncovering the role of acetate in the crosstalk between monocarboxylate transporters and oncogene signalling pathways in colorectal cancer

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(Sara Daniela Coelho Gomes)

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"Be thankful for what you have; you'll end up having more. If you concentrate on what you don't have, you will never, ever have enough." Oprah Winfrey

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ABSTRACT

Colorectal cancer (CRC) is one of most commonly diagnosed cancer worldwide. A normal human intestine harbours hundreds of different bacterial species which play several roles in human health, such as protection against pathogens, immune system maturation, degradation of toxic substances, digestion of complex carbohydrates and production of short-chain fatty acids (SCFAs). SCFAs, specifically acetate, propionate and butyrate are produced by propionibacteria and constitute a major source of energy for colonocytes. Previous reports from our group showed that acetate inhibits CRC cell proliferation, induces apoptosis, promotes lysosomal membrane permeabilization, increases CRC cell glycolytic phenotype and regulate its own uptake by increasing the expression of monocarboxylate transporters (MCTs). However, the signalling pathways associated to the phenotypic changes induced by acetate have not been characterized. In order to clarify this issue, here we aimed at evaluate the involvement of acetate in the expression levels of KRAS/BRAF oncogene signalling pathways molecules known to be important in CRC cells survival namely PI3K/AKT and MAPK pathways. We also aimed at understanding the role of KRAS and BRAF oncogenes in the regulation of glycolytic metabolism and uncover the role of MCTs in the regulation of the KRAS/BRAF signalling pathways in CRC cells exposed to acetate.

Our data suggest that acetate treatment is able to modulate the expression levels of some signalling molecules namely phosphorylated cRAF and ERK, in a time and dose-dependent manner. Moreover, preliminary results herein presented show that acetate may activate some feedback mechanism to maintain the uptake of SCFAs when there is downregulation of the MCT-1 expression levels.

To the best of our knowledge this is the first work studying the interplay between acetate and two important hallmarks of cancer, namely oncogene signalling activation and metabolism reprogramming in CRC. This study might help in the discovery of new approaches in prevention/therapy of CRC.

Keywords: *Colorectal cancer, acetate, signalling pathways, KRAS, BRAF, glycolytic metabolism, monocarboxylate transporters (MCTs).*

RESUMO

O cancro colorretal (CCR) é um dos tipos de cancro mais comumente diagnosticado em todo o mundo. Um intestino humano normal abriga centenas de espécies bacterianas diferentes que desempenham diversos papéis na saúde humana, como proteção contra agentes patogénicos, maturação do sistema imunológico, degradação de substâncias tóxicas, digestão de carbohidratos complexos e produção de ácidos gordos de cadeia curta (AGCC). Os AGCC, especificamente o acetato, propionato e butirato são produzidos pela propionibacteria e constituem uma importante fonte de energia para os colonócitos. Recentemente, foi demonstrado pelo nosso grupo que o acetato inibe a proliferação de células de CCR, induz apoptose e promove a permeabilização da membrana lisossomal, aumenta o fenótipo glicolítico destas células e regula a sua própria absorção aumentando a expressão de transportadores de monocarboxilatos (MCTs). No entanto, as vias de sinalização associadas às alterações fenotípicas induzidas pelo acetato não foram ainda devidamente caracterizadas. Para clarificar esta questão, nesta tese avaliámos o envolvimento do acetato nos níveis de expressão de moléculas das vias de sinalização dos oncogenes KRAS/BRAF, importantes na sobrevivência das células de CCR, nomeadamente as vias da PI3K/AKT e das MAPK. De seguida, tentamos compreender o papel dos oncogenes KRAS e BRAF na regulação do metabolismo glicolítico e entender o papel dos MCTs na regulação das mesmas vias de sinalização em células de CCR expostas ao acetato.

Os nossos resultados sugerem que o tratamento com acetato é capaz de modular os níveis de expressão de algumas moléculas de sinalização, nomeadamente a cRAF e a ERK fosforilada, de uma maneira dependente da dose e do tempo. Além disso, os resultados preliminares aqui apresentados demonstram que o acetato poderá ativar algum mecanismo de feedback que mantenha a entrada de AGCC quando existe uma redução dos níveis de expressão de MCT-1.

Este constitui o primeiro trabalho no qual se estuda a interação entre o acetato e duas importantes características do cancro, nomeadamente a ativação da via de sinalização oncogénica e a reprogramação do metabolismo energético no CCR. Assim, este estudo poderá ajudar na descoberta de novas abordagens na prevenção/terapia do CCR.

Palavras-chave: *Cancro colorretal, acetato, vias de sinalização, KRAS, BRAF, metabolismo, transportadores de monocarboxilatos (MCTs).*

The work presented in this master thesis resulted in two international articles with peer review and one poster presentation.

International articles with peer review

1. **S. Gomes**, C.S.F. Oliveira, J. Azevedo-Silva, M. R. Casanova, J. Barreto, H. Pereira, S. R. Chaves, L. M. Rodrigues, M. Casal, M. Côrte-Real, F. Baltazar, A. Preto. The role of diet related short-chain fatty acids in colorectal cancer metabolism and survival: prevention and therapeutic implications. Current Medicinal Chemistry, 2017. ***(Under review)***

2. **S. Gomes**, F. Baltazar, C. Calhau, A. Preto. Diet: a double-edged sword in cancer. Frontiers in Nutrition, 2018 (by invitation). ***(In preparation)***

Posters

1. **S. Gomes**, S. Granja, F. Baltazar, A. Preto. "Characterization of the signalling pathways associated with the effect of acetate in colorectal cancer cells". 1st ICVS Open Day. University of Minho, Braga, July 1st, 2017.

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AIF - apoptosis inducing factor

ANT - adenine nucleotide transporter

APC - adenomatous polyposis coli

BVI - blood vessel invasion

CatB - cathepsin B

CatD - cathepsin D

CatL - cathepsin L

CIMP - CpG island methylator phenotype

CMS - consensus molecular subtype

CIN - chromosomal instability

CRC - colorectal carcinoma

CRCSC - CRC Subtyping Consortium

EDTA - ethylenediamine tetraacetic acid

EGF - epidermal growth factor

EGFR - epidermal growth factor receptor

EMT - epithelial-to-mesenchymal transition

ERK - extracellular-signal-regulated kinases

FAP - familial adenomatous polyposis

GDP - guanosine diphosphate

GLUT-1 - glucose transporter 1

GPR - G-protein-couple receptor;

GTP - guanosine triphosphate

HAT - histone acetyltransferase

HDAC - histone deacetylase

HNPCC - hereditary nonpolyposis colorectal carcinoma

HPP - hyperplastic polyposis

HRAS - Harvey rat sarcoma viral oncogene homolog

IBD - inflammatory bowel disease

IDT - Integrated DNA Technologies

IL - interleukin

JNK - c-Jun N-terminal kinase

JPS - juvenile polyposis syndrome

KRAS - Kirsten rat sarcoma viral oncogene homolog

LVI - lymphatic vessel invasion

LMP - lysosomal membrane permeabilization

MAP - MUTYH-associated polyposis

MAPK - mitogen-activated protein kinase

MCT - monocarboxylate transporter

MOMP - mitochondrial outer membrane permeabilization

MSI - microsatellite instability

NF-κβ, nuclear factor-kappa β

NRAS - neuroblastoma rat sarcoma viral oncogene homolog

PBS - phosphate buffer saline

PH - pleckstrin homology

PI3K - phosphatidylinositol 3-kinase

PIP2 - phosphatidylinositol (4, 5)-phosphate

PIP3 - phosphatidylinositol (3, 4, 5)-phosphate

PJS - Peutz-Jeghers syndrome

ROS - reactive oxygen species

RTK - receptor tyrosine kinase

SAPK - stress-activated protein kinase

SCFAs - short chain fatty acids

SCNA - somatic copy number alterations

siRNA - small-interfering RNA

SMCT1 - sodium-coupled monocarboxylate transporter

TCA cycle - tricarboxylic acid cycle

TGF - transforming growth factor

TNM - tumor-node-metastasis

TOM22 - subunit of the outer mitochondrial membrane translocator (TOM complex)

VDAC - voltage dependent anion channel

VMP - vacuolar membrane permeabilization

$\Delta\Psi m$ - mitochondrial membrane potential

5-FU - 5-fluorouracil

LIST OF FIGURES

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Part of this chapter is based on the following publication:

S. Gomes, C.S.F. Oliveira, J. Azevedo-Silva, M. R. Casanova, J. Barreto, H. Pereira, S. R. Chaves, L. M. Rodrigues, M. Casal, M. Côrte-Real, F. Baltazar, A. Preto. *The role of diet related short-chain fatty acids in colorectal cancer metabolism and survival: prevention and therapeutic implications.* (*Current Medicinal Chemistry Journal*, under review)

1.1. Cancer epidemiology

Cancer remains a leading cause of death in both more and less economically developed countries, being considered a major public health problem. Based on GLOBOCAN reports, the occurrence of cancer is increasing not only because of the growth of the population, but also because it has been an increasing prevalence of established risk factors including tobacco use (lung, colorectal, stomach, and liver cancer), overweight/obesity and physical inactivity (breast and colorectal cancer), and infection (liver, stomach, and cervical cancer) [1]. In 2012, there where estimated the occurrence of 14.1 million new cancer cases and 8.2 million cancer deaths worldwide [1]. The three most commonly diagnosed cancers are lung, prostrate and colorectal carcinomas (CRC) in men, and breast, colorectal and lung in women [1]. The most abundant causes of cancer related deaths are lung, liver and stomach cancer in men and breast, lung and colorectal cancer in women (Figure 1) [1]. However, some recent studies have a more encouraging perspective, indicating that cancer mortality rates in European Union tend to decline for both sexes, despite the increase in the number of deaths due to the aging of the European population [2]. This decrease in the mortality rate is related, in the case of men, with the decrease in tobacco consumption, and in the case of women, it is mainly due to the capacity for early diagnosis of cancers amenable to treatment, such as breast and uterine cervix cancer [2].

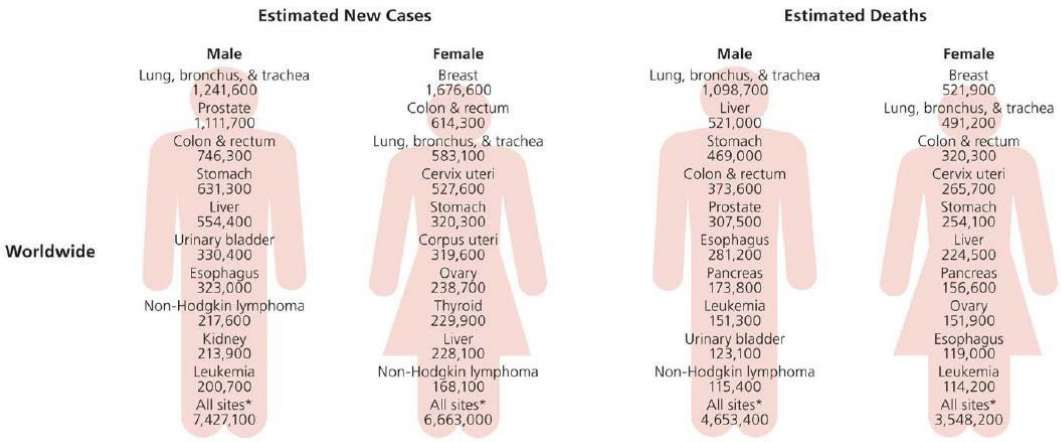


Figure 1 - Estimated new cancer cases and deaths worldwide by sex, excluding non-melanoma skin cancers.

Adapted from GLOBOCAN 2012 [1].

All these advances that have been observed in the prevention, detection and treatment of cancer is due to the fact that the scientific community has been devoted a lot of effort in the comprehension of this disease. Indeed, all over the world research on the field have tried to better understand cancer and to develop more efficient diagnostic tests and targeted therapies, despite this there are still much to be discovered and investigated.

Currently, it is known that carcinogenesis requires molecular, biochemical and cellular changes, and these changes usually occur due to accumulation of genetic mutations in normal cells which affect their proliferation, differentiation and development. Generally, the process of cell substitution is orderly, in which new and healthy cells replace the oldest. However, mutations can induce activation or inactivation of certain genes, enabling cells to divide uncontrollably [3].

Since 2000, Weinberg and his collaborators have been describing a number of characteristics that distinguish cancer cells from the other cells of the body, called "the hallmarks of cancer" (Figure 2) [4]. Nowadays, they have already identified ten main biological capabilities acquired during the multistep development of human tumors, including sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, deregulating cellular energetics, avoiding immune destruction, tumor promoting inflammation and genome instability and mutation [5].

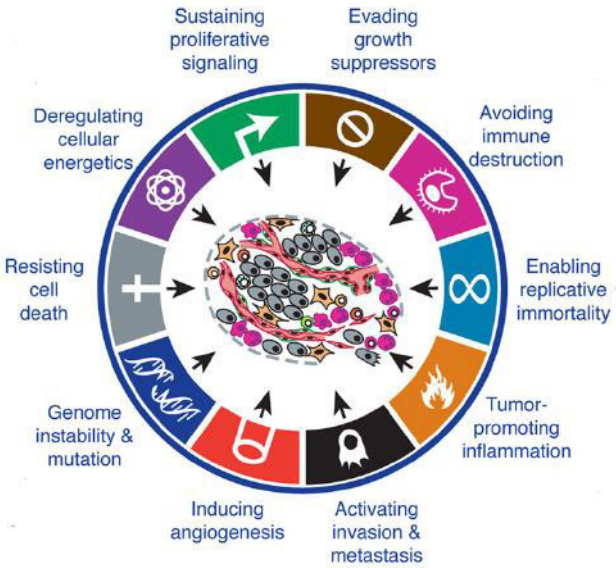


Figure 2 - The Hallmarks of Cancer: acquired capabilities of cancer cells during carcinogenesis. Adapted from Weinberg *et al.* [5].

Looking closer to each capability, one of the fundamental hallmarks of cancer cells involves their ability to sustain chronic proliferation, contrary to normal cells that control the production and release of growth-promoting signals to ensure the homeostasis of cell surface [5]. Furthermore, cancer cells have programs that negatively regulate cell proliferation and that depend on the actions of tumor suppressor genes, being another hallmark the ability to circumvent these programs [5]. The programmed cell death, called apoptosis, is a natural mechanism that prevents cancer development [6]. However, it has been shown that cancer cells have developed the ability to resist apoptosis, enabling tumors to reach more severe malignancy states [7]. Another specific characteristic of cancer cells is the requirement of an unlimited replicative potential, to generate macroscopic tumors. Normal cells, in contrast, have a limited number of successive cell growth-and-division cycles [5]. As tumors are constantly growing, their energetic needs are high, becoming necessary to have processes such as angiogenesis, which consists in the sprouting of new vessels from existing ones, in order to supply cancer cells with nutrients needed to fulfil the proliferation demands [5]. The sixth fundamental hallmark of cancer is related to the invasion and metastasis. In a first step, cancer cells invade surrounding tissues and blood vessels; then, they are transported by the circulatory system to distant sites and, finally, cancer cells reinvade and grow at new location [5]. This carcinoma cell capacity to invade other tissues is closely related with the E-cadherin, a key cell-cell adhesion molecule [8]. In addition to these six hallmarks that have been mentioned, more recently it has been suggested the existence of two enabling characteristics, the tumor promoting inflammation, once the tumor-associated inflammatory response had the unanticipated and paradoxical effect of enhancing tumorigenesis and progression, helping incipient neoplasias to acquire hallmark capabilities and the genome instability and mutation, that can confer selective advantages to cells subclones, allowing their growth and eventual dominance in a local tissue environment [5]. Moreover, they were also proposed two additional hallmarks of cancer, one involved with the capability of cancer cells to promote immunological destruction in order to prevent resistance or eradication of the formation and progression of incipient tumors, tumors in the final stage and micrometastases and the other involved with the capability to modify or reprogram cellular metabolism in order to fuel the uncontrolled cell growth and division [5].

All these biological capabilities that differentiate cancer cells from the rest of the cells have been closely investigated with the aim of recognize new potential therapeutic targets to treat different types of human cancer.

1.2. Colorectal cancer

Colorectal cancer (CRC) is a type of cancer that starts in the colon or rectum. As illustrated in the Figure 3, the colon and the rectum are two different parts of the large intestine, which is the lower part of the body's digestive system.

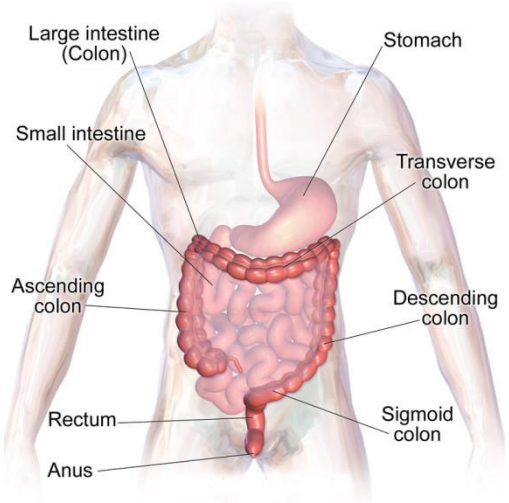


Figure 3 - Illustration of the large intestine.

Front of abdomen showing the different sections of the colon, rectum and anus.

CRC is the third most commonly diagnosed cancer in males and the second in females worldwide, being the highest incidence rates in Australia/New Zealand, Europe, and Northern America and the lowest in Africa and South-Central Asia [1].

The increasing incidence of CRC in young populations has increased the need to disseminate preventive measures, including maintaining a healthy body weight, being physically active, minimizing consumption of red and processed meat and alcohol, and avoidance of smoking. Moreover, it is important the detection of colorectal polyps when they can be removed before they became malignant as well as the detection of cancer at an early stage when treatment is usually less extensive and more successful [1].

In addition to the need for the implementation of new methods of prevention and detection, the increased incidence of CRC that has been observed leads to an increasing need to develop new and more effective therapies. As such, the knowledge of the molecular and biological bases of CRC has been a very important subject of study by scientists all over the world.

1.2.1. Colorectal carcinogenesis

CRC cases are traditionally divided into sporadic and familial or hereditary, having approximately 75% to 80% of colorectal tumors a sporadic origin [9]. Despite the name, it is quite clear that even in sporadic cases, the descendants have a higher risk of suffering CRC.

Considering the hereditary or familial CRCs, only between 2% to 5% of all colon cancers arise in the setting of well-defined inherited syndromes. Some of these well-known syndromes express adenomatous polyps, such as hereditary nonpolyposis colorectal carcinoma (HNPCC), familial adenomatous polyposis (FAP), attenuated FAP and MUTYH-associated polyposis (MAP), others express hamartomatous polyps like Peutz-Jeghers syndrome (PJS) and juvenile polyposis syndrome (JPS) and there are also the hyperplastic polyposis (HPP), which although rare, has a substantial cancer risk [10]. Besides the clinical similarities, each syndrome has distinct cancer risks, characteristic clinical features, and separate genetic etiologies [10].

In the past decades, findings have demonstrated that this process of CRC initiation and development can be subdivided at least in three pathways: chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP). However, more recently, there has been proposed the existence of other alternative pathways such as the inflammatory pathway and another that involved microRNAs [11, 12].

CIN or suppressor pathway

Approximately 70% to 85% of CRCs generally develop via the CIN or suppressor pathway, being the most well described pathway for genomic instability in CRC [12, 13]. It is characterized by the accumulation of structural or numerical abnormalities, preferentially is associated with mutation in adenomatous polyposis coli (APC) or loss of 5q (APC gene), a tumour suppressor gene mutated both in inherited and sporadic CRC forms, followed by activation of the KRAS or BRAF oncogenes and

inactivation of the tumor suppressor gene p53 (Figure 4) [13, 14]. However, just a small group of CIN possesses the complete set of these molecular abnormalities.

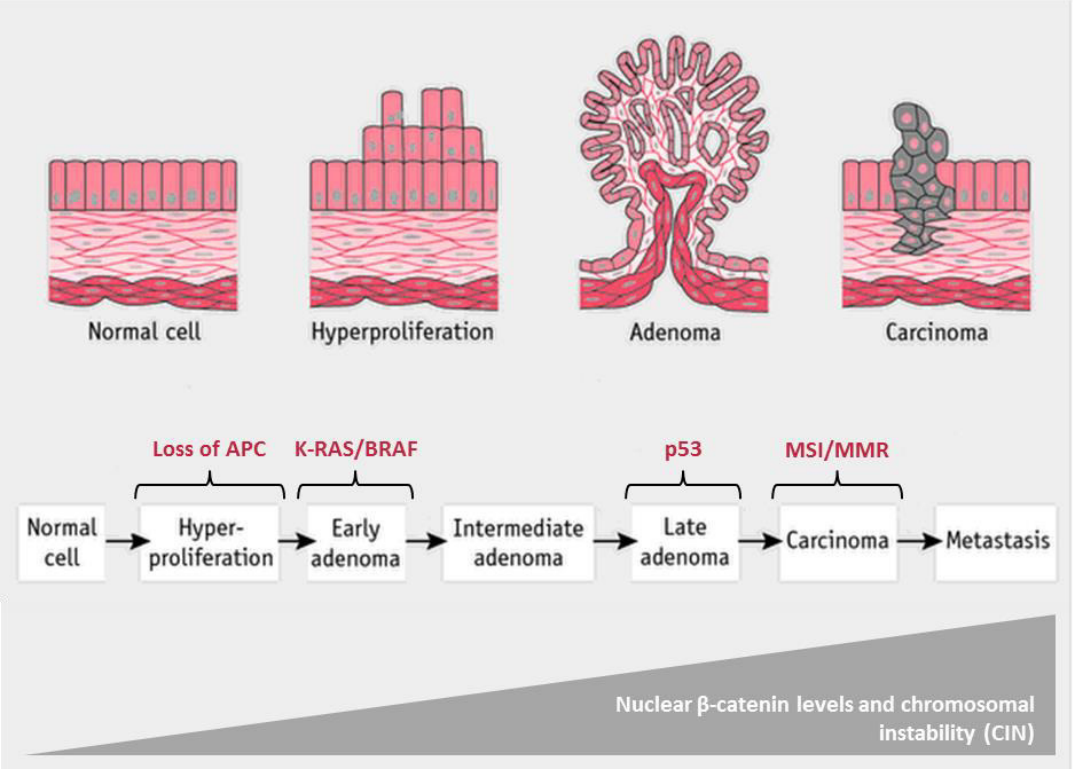


Figure 4 - Colorectal carcinogenesis from normal cell to invasive carcinoma.

The main mutations in CRC development are shown: Adenomatous polyposis coli (APC), K-RAS/BRAF, p53 and microsatellite instability (MSI), mismatch repair (MMR) and chromosomal instability (CIN) (adapted from [15]).

MSI pathway

MSI pathway represents a form of genomic instability involved in the genesis of approximately 15% of sporadic CRC and in almost all of hereditary non polyposis CRC syndrome [12]. This mechanism of tumorigenesis is driven by a failure of the mismatch repair system that interrupts normal review and repair of DNA following replication, leading to the occurrence of mutations in the microsatellite sequences. As such, in these situations, the number of nucleotide repeat regions is different when compared with germline DNA [13].

The colorectal tumors originated from this pathway are normally located in the proximal colon, with a poorly differentiated and a mucinous or medullary histotype. In general, the prognosis and survival of these patients is better and longer than that of patients with CRC originated from the CIN pathway [12]. Furthermore, CRC tumourigenesis depends on mutations in genes that deregulate intracellular signalling pathways, e.g. the EGFR mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways [16]. The most frequently mutated genes in these pathways are KRAS, BRAF and PI3K. As such, these genes have been suggested as prognostic biomarkers, but although examined in many previous studies, the precise prognostic role of mutations in these genes remains unclear. In a recent study, a high concordance rate of KRAS/BRAF mutation status and MSI status was observed between primary CRC and corresponding metastases [17].

CIMP pathway

In 1999, Toyota *et al* have described another mechanism of tumorigenesis, called CIMP pathway [18]. This mechanism of CRC progress consists of the aberrant hypermethylation of CpG dinucleotide sequences localized in the promoter regions of genes involved in cell cycle regulation, apoptosis, angiogenesis, DNA repair, invasion and adhesion, resulting in the inactivation of several tumor suppressor genes or other tumor-related genes [11, 12, 18]. Considering the pathological characterization, CIMP tumors have the high rate of mutations (KRAS or BRAF), wild type p53, proximal colon location, mucinous histological type, higher age at diagnosis, poor differentiation and higher occurrence in female gender and older patients [11]. What concerns to the survival rates of these patients, researches have demonstrated that DNA methylation is associated with a worse outcome in CRC, but this adverse prognostic influence is lost in those methylated tumors showing MSI [11].

1.2.1. Characteristics and types of colorectal cancer

Pathologic characteristics

The initial diagnosis of most colorectal carcinomas are made by an endoscopic biopsy or a polypectomy [19]. The pathological characteristics of the sample taken are the most powerful predictors of CRC. These include pathologic stage and stage-independent prognostic factors such as histopathologic grade, vascular invasion, perineural invasion and tumor border features [20].

Considering the histopathologic analysis, there are several types of CRCs including neuroendocrine, squamous cell, adenosquamous, spindle cell and undifferentiated carcinomas but more than 90% of CRCs are adenocarcinomas with origin on epithelial cells of the colorectal mucosa [19].

Another pathological characteristic of CRC that represents a crucial step in carcinogenesis is the capability of vascular invasion: blood vessel invasion (BVI) and lymphatic vessel invasion (LVI). The blood vessel invasion is associated with the occurrence of lymph node metastases and distant metastases, whereas the prognostic impact of lymphatic invasion is less clear [21]. In 1989, Bruce Minsky *et al* reported that there is an increase in positive BVI with increasing stage and grade, suggesting that patients with positive BVI CRC had a decreased survival compared with those patients with negative BVI tumors. On the other hand, patients with positive LVI tumors had a lower survival rate compared with those who had negative LVI tumors [22].

The perineural invasion is a pathologic process characterized by tumor invasion of nervous structures and spread along nerve sheaths that is known to be associated with more advanced disease in CRC based on its correlation with poor tumor differentiation and higher stage [23].

Despite the configuration of the tumor border be rarely reported in daily diagnostic practice, its evaluation represents an important histomorphological prognostic indicator in solid cancers, namely in CRCs [24]. In aggressive cases, it can be seen a clear loss of a tumor–host interface, called "infiltrative tumor border configuration". This characteristic has been consistently associated with poor survival outcome and early disease recurrence and is frequently associated with presence of adverse clinicopathological features and molecular alterations related to aggressive tumor behaviour including $BRAF^{V600}$ mutation. In contrast, a well-demarcated tumor border is seen frequently in cases with low risk for nodal and distant metastasis [24].

The consensus molecular subtypes

Recently, in order to resolve discrepancies among CRC classifications based on reported gene expression and to facilitate clinical translation, is was performed the CRC Subtyping Consortium (CRCSC) [25]. Based on large-scale data sharing and analysis by expert groups, the members of this consortium proposed a new taxonomy of CRC, the consensus molecular subtype (CMS), which reflects significant biological differences in the gene expression of the different subtypes (Figure 5) [25].

CMS1 MSI immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
BRAF mutations		*KRAS* mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF-β activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

Figure 5 - Proposed taxonomy of colorectal cancer.

CMS – consensus molecular subtype; CIMP – CpG island methylator phenotype; MSI – microsatellite instability; SCNA – somatic copy number alterations [25].

This new classification system stratifies CRC into four biological consensus molecular subtypes (CMS 1-4) with distinctive clinical and molecular markers. CMS 1 tumors are enriched for MSI and overexpress proteins involved in DNA damage repair, consistent with defective DNA mismatch repair. These tumors have a higher prevalence of CpG island methylator phenotype and a widespread hypermethylation status. It was identified a frequent occurrence of BRAF mutations in these type of CRC tumors and, looking at the gene expression data, they are also characterized by increased expression of genes associated with a diffuse immune infiltration and activation [25]. CMS 2 or canonical subtype includes 37% of all CRCs and is characterized by high levels of chromosomal instability (CIN) measured by somatic copy number alterations (SCNA) counts. These tumors are also associated with epithelial differentiation and strong upregulation of WNT and MYC downstream targets that have been implicated in CRC carcinogenesis [25]. CMS 3 tumors are the most different in terms of genomic and epigenomic profile. They have fewer SCNAs, a mixed MSI status and a lower CIMP. This type of cancer is also characterized by an overrepresentation of KRAS mutations that have been described as inducing prominent metabolic adaptations [25]. Lastly, CMS 4 tumors have high levels of SCNAs, a clear upregulation of genes implicated in epithelial-to-mesenchymal transition (EMT) and the activation of transforming growth factor (TGF)-β signalling. Moreover, they show the activation of angiogenesis, matrix remodelling pathways and the complement-mediated inflammatory system. In

addition, their gene expression profile is compatible with stromal infiltration and the overexpression of extracellular matrix proteins.

Considering the survival rates associated with each cancer subtype, CMS 2 are the tumors with a better prognostic and CMS 4 tumors resulted in worse overall survival and worse relapse-free survival [25].

1.2.2. Signalling pathways altered in colorectal carcinogenesis

In human cells, several mechanisms are responsible for the transduction of information from the extracellular environment into molecular signals. These signals are received by the cells through different receptors, which initiate signalling cascades and alter the state of various effector proteins that regulate transcriptional programs and metabolic pathways. In some cases, these cell signalling alterations may result in severe syndromes and diseases such as cancer.

One of the hallmarks that was stated previously was the ability of cancer cells to sustaining proliferative signalling. This means that while normal tissues carefully control the production and release of growth-promoting signals that instruct entry into and progression through the cell growth and division cycle, cancer cells deregulate these signals, becoming controllers of their own destinies [5].

In CRC, there are several molecular alterations associated this capability, such as the epidermal growth factor receptor (EGFR) overexpression and mutations in classical proto-oncogenes like BRAF and KRAS, between others.

EGFR overexpression

EGFR is a 170 kDa transmembrane glycoprotein that belongs to a family of receptor tyrosine kinases and is composed of an intracellular tyrosine-kinase domain, a hydrophobic transmembrane region and an extracellular ligand binding domain, whose main ligands are the epidermal growth factor (EGF) and the transforming growth factor-alpha (TGF-α) [26, 27].

In 1991, Gross *et al* have demonstrated that the overexpression of EGFR in colon cancers may indicate an advanced stage of the disease [28] and, four years later, Radinsky and his collaborators have reported that this overexpression may predict the metastatic potential, what indicates that the

EGF or TGF-α-activated growth regulatory system may play a significant role during colorectal carcinogenesis, possibly via increased EGFR expression [29]. More recently, studies have shown that the overexpression of membrane EGFR is correlated with poor prognosis in several cancers, including colorectal but, on the other hand, there are results that contradict the relationship between EGFR expression and CRC prognosis [30].

It was also shown that EGFR levels are a poor predictor of response to anti-EGFR therapies. Looking to the clinical trials that evaluate the efficacy of cetuximab, the treatment response was not related to the levels of membrane EGFR expression, showing, however, that nuclear EGFR expression contributes to acquire resistance to this drug [30].

PI3K/AKT signalling pathway mutations

PI3K/AKT signalling cascade has been shown to be disturbed at some level in many human cancers (Figure 6). Because this pathway is involved not only in tumor development but also in the potential response to chemotherapy, currently, some of its downstream effectors are considered attractive pharmacological targets.

The activation of this pathway can occur in response of a range of signals, including hormones, growth factors and components of the extracellular matrix. When one of these extracellular ligands bind to a receptor tyrosine kinase (RTK) placed in the plasma membrane, the receptor dimerizes and the tyrosine residues in the intracellular domains of the receptor suffer a cross-phosphorylation. This leads to PI3K association with the receptor and, consequently, allosteric activation of its catalytic subunit [31]. Activated PI3K converts phosphatidylinositol (4, 5)-phosphate (PIP2) into phosphatidylinositol (3, 4, 5)-phosphate (PIP3), which recruits a subset of signalling proteins with pleckstrin homology (PH) domains to the membrane, including PDK1 and AKT. PTEN, is a PIP3 phosphatase, which negatively regulates the PI3K/AKT pathway, by reconverting PIP3 into PIP2. Once phosphorylated, AKT mediates the activation and inhibition of several molecular targets, promoting cellular survival, growth and proliferation through different mechanisms [31-33].

Nowadays, multiple genetic events have been described that lead to activation of the PI3K/AKT pathway in cancer [34]. Activating mutations in PI3K, which encodes the PI3K p110a catalytic subunit, are common in many cancer types [34]. In turn, PTEN is subject to both genomic deletion and small point mutations that inactivate its function and is one of the most commonly mutated cancer genes

overall [34]. Also, AKT is occasionally activated by mutation at a single site [34]. Inactivating mutations in both TSC1 and TSC2 have been identified in cancer at low frequency, as well as activating mutations in MTOR [34].

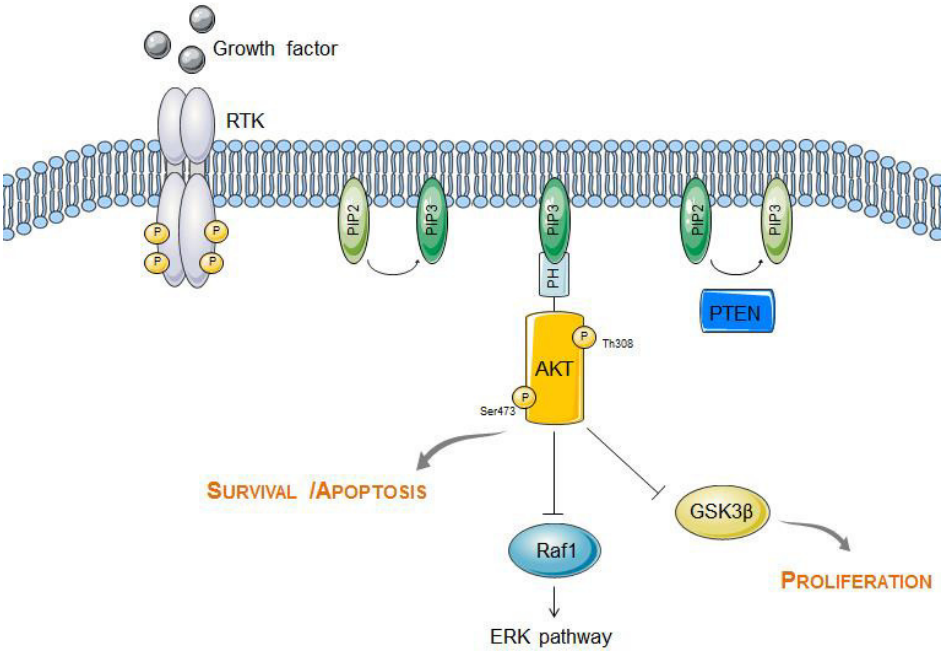


Figure 6 - PI3K/AKT signalling pathway.

RAS/RAF/MAPK signalling pathway

The mitogen-activated protein kinase (MAPK) cascade is a critical cell-proliferation signalling pathway that transduces signals from the cell surface to the nucleus, activated in most of the human cancer and that contributes for cancer cell survival, dissemination and resistance to drug therapy (Figure 7).

The MAPK is subdivided into three major subfamilies: the extracellular-signal-regulated kinases (ERK MAPK, RAS/RAF1/MEK/ERK); the c-Jun N-terminal or stress-activated protein kinases (JNK or SAPK); and MAPK14 [35].

The MAPK/ERK signalling pathway, in particular, is one of the most important for cell proliferation, being involved in the pathogenesis, progression, and oncogenic behaviour of human colorectal cancer [35].This cascade is stimulated both by internal stimuli, such as metabolic stress, DNA damage pathways and altered protein concentrations, as external stimuli, including external growth factors, cell-matrix interactions and communication from other cells [36].

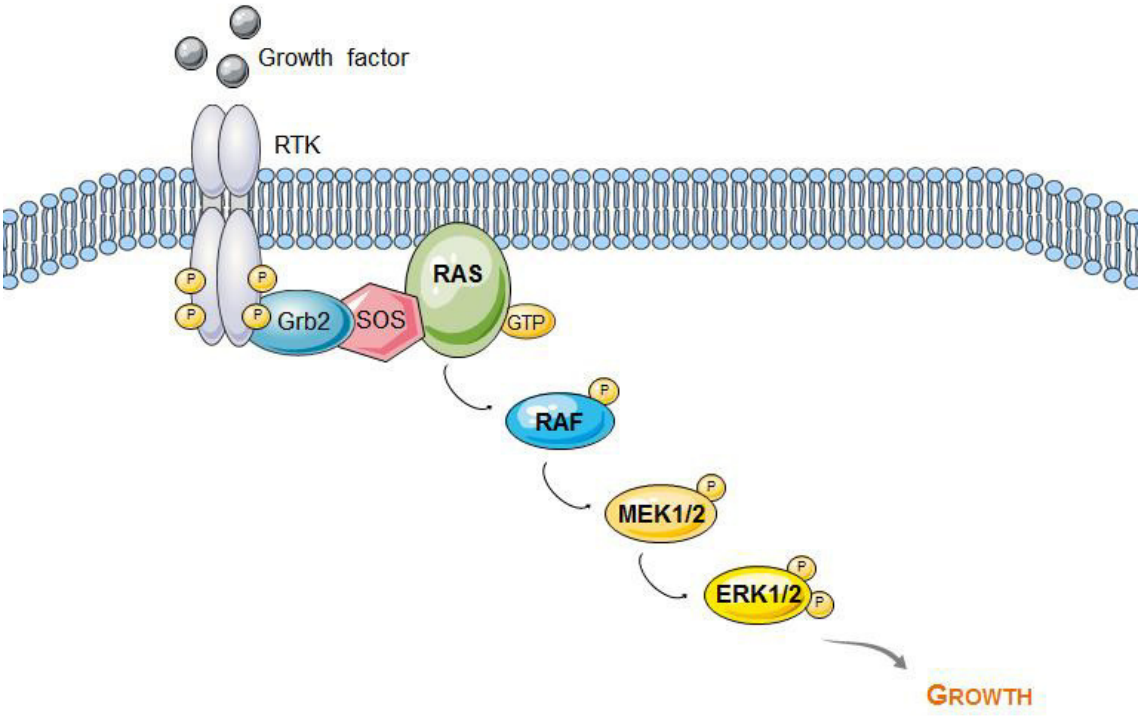


Figure 7 - MAPK/ERK signalling pathway.

KRAS mutations

RAS proteins are small GTPases that control cellular signalling pathways responsible for growth, migration, adhesion, cytoskeletal integrity, survival and differentiation [37].

According to its connection to guanosine triphosphate or guanosine diphosphate (GTP and GDP, respectively) molecules, these proteins alternate between an active and an inactive conformation, where the GTP-bound conformation represents the "on" and GDP-bound the "off" state [37].

Considering the homology levels, the RAS family comprises four major RAS proteins: NRAS (neuroblastoma rat sarcoma viral oncogene homolog), HRAS (Harvey rat sarcoma viral oncogene homolog) and KRAS (Kirsten rat sarcoma viral oncogene homolog) 4A and 4B [38]. KRAS triggers the RAS-RAF-MEK-ERK signalling cascade being, when in the active state, responsible for initiate the activation of an array of signalling molecules allowing the transmission of transducing signals from the cell surface to the nucleus (Figure 7). This affects cell differentiation, growth, chemotaxis, and

apoptosis, eliciting changes in the cytoskeleton and consequently affecting cell shape, adhesion and migration [39].

When KRAS gene suffers an activating mutation, KRAS protein loses the ability to switch between active and inactive states, leading to cell transformation and increased resistance to chemotherapy and biological therapies targeting EGFRs [39]. As such, KRAS, specifically, is considered an oncogene that plays an important role in CRC carcinogenesis.

In this type of cancer, KRAS activating mutations are usually found in exon 2, at codons 12 or 13 and correspond to G→A transitions at the second base of the respective codon resulting in G12D or G13D mutations [40]. Such mutations occur in approximately 40% of CRCs and are maintained throughout carcinogenesis, as evidenced by the nearly perfect concordance of KRAS mutation status in primary and metastatic colorectal cancer what makes the KRAS mutational analysis useful on archived primary tumor specimens in patients with metastatic disease and usually eliminates the need for additional biopsy tissue from a metastatic lesion [41]. However, despite continuous efforts, no successful therapy targeting KRAS has been developed and it remains an elusive target for cancer therapy [42].

BRAF mutations

The RAF family is composed by three serine/threonine-specific protein kinases: ARAF, BRAF and CRAF [43]. BRAF gene, in specific, encodes a protein kinase that is the direct downstream effector of KRAS in the RAS/RAF/MEK/ERK signalling pathway [41].

Mutations in the BRAF gene are found in about 10% of CRC patients and are associated with shorter progression-free and overall survival [44]. Despite BRAF was reported to be mutated at several sites, the clear majority of BRAF mutations is the GTG→GAC substitution at position 1799 of exon 15, which results in the V600E amino acid change (T→A transition). This causes a destabilization of the inactive form of the enzyme, conferring a constitutive kinase activity which in turn results in uncontrolled proliferation and growth of cells [43, 44].

In 2012, Yokota has demonstrated that, in patients with CRC, BRAF mutations constitute a strong negative prognostic marker, since patients have demonstrated very poor response to conventional chemotherapy, especially to anti-EGFR therapy [45].

Emerging evidences suggest that the constitutive activation of the RAS/RAF/MEK/ERK pathway caused by KRAS and BRAF mutations are an important hit in the development of colorectal cancers, supporting a role for BRAF as a genetic marker for prognosis and possibly for predicting response to therapy.

Resistance associated with EGFR KRAS BRAF in cancer therapy

In the past decade, in addition to the traditional 5-fluorouracil (5-FU) treatment, it was approved the use of several chemotherapeutic agents such as capecitabine, oxaliplatin and irinotecan that targets the DNA synthesis; cetuximab and panitumumab that blocks EGFR pathway; bevacizumab and aflibercept that blocks VEGF; and regorafenib that inhibits VEGFR [46]. These chemotherapeutic agents have been used as the first choice for CRC treatment, as a single agent or in combination with other agents. However, despite of the effect of prolonging the median overall survival of CRC patients, these chemical anticancer drugs have serious side effects. As such, given that not all patients respond equally to these different treatments, it has become important to select patients who would benefit from a specific therapeutic approach [46].

Intrinsic mechanisms of resistance, such as RAS mutations, can prevent patients from having a response to anti-EGFR drugs with clinical benefit [47]. However, approximately half of patients with KRAS wild type tumors do not respond to anti-EGFR treatment. This can be explained by other genetic alterations such as the level of expression of EGFR or BRAF mutations that are found in less than 10% of CRC cases [46]. Despite KRAS and BRAF wild type status, there have been approximately 41% of non-responders to anti-EGFR therapy, what suggests the existence of further pathways to acquire resistance to these treatments [46].

1.3. Colorectal cancer energetic metabolism

Another hallmark of cancer that was previously referred in this work was the deregulation of the energetic metabolism. In contrast to the normal differentiated cells, which are mainly based on mitochondrial oxidative phosphorylation (OXPHOS) to generate the required energy for cell's processes, most cancer cells present the called "Warburg effect" (Figure 8) [48]. This is a metabolic adaptation that happens when, even in the presence of sufficient amounts of oxygen, cancer cells use glycolysis instead of the oxidative phosphorylation, in order to satisfy their high energy needs [49]. However, considering the yield of ATP, glycolysis process is less efficient compared to oxidative phosphorylation. The lactate metabolism of glucose generates only two ATP molecules per glucose molecule, whereas oxidative phosphorylation generates 36 ATP molecules per complete oxidation of one glucose molecule [50]. To understand the reason why cancer cells, choose glycolysis instead of oxidative phosphorylation, several studies have been developed. Initially, Warburg proposed the existence of a defect in mitochondria that directs glucose into aerobic glycolysis [48, 51]. However, this hypothesis was refuted by a research that showed that mitochondrial function is normal in most cancer cells [50, 51]. More studies have been developed and it has been proposed that this metabolic adaptation performed by cancer cells is associated with their high demand for nucleotides, amino acids and lipids during proliferation [49]. The metabolism of cancer cells, specifically proliferating cells, is adapted to facilitate the uptake and incorporation of this nutrients into the biomass [48]. Concerning the role of OXPHOS, some studies reported that OXPHOS capacity is reduced in different types of cancer cells [52]. However, some recent researches reveal an upregulation of OXPHOS components and a larger dependency of cancer cells on oxidative energy substrates for anabolism and energy production [52]. This antagonism can be explained by differences in tumor size, hypoxia, and the sequence of oncogenes activated. Evidences suggest that this metabolic pathway may control cancer cells life and proliferation [53].

The "Warburg effect", together with mitochondrial oxidative phosphorylation, is crucial for the rapid production of energy that sustains the high proliferative rate, providing biosynthetic precursors that support cell growth. This metabolic process is reflected in a high level of lactate production in cancer cells that need to be exported to prevent intracellular acidification and, consequently, cell death. As such, cancer cells generally increase the expression of monocarboxylate transporters (MCTs) [7].

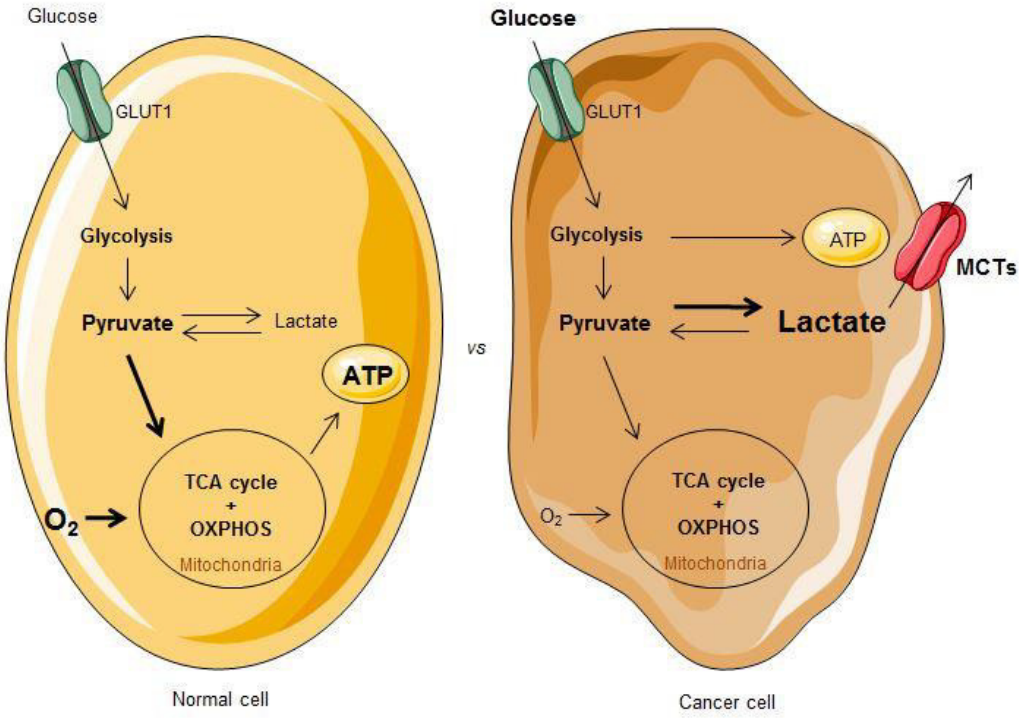


Figure 8 – Glucose metabolism in normal cells *versus* in cancer cells – the Warburg effect.

1.3.1. Monocarboxylate transporters overexpression in colorectal cancer

MCTs are a family of transmembrane transporters composed by 14 members; however, only MCT1 to 4 is known to participate in the uptake and efflux of monocarboxylic acids in a proton-linked mechanism. In cancer cells, such transporters contribute not only to the maintenance of the high glycolytic rates by performing lactate efflux, but also for the preservation of intracellular and extracellular pH by the co-transport of protons [54].

In the past few years, several studies revealed contradictory conclusions about the abnormal expression of MCTs, particularly in solid tumors [55]. For example, while in 2002, Lambert *et al* described a decrease in MCT1 expression during transition to malignancy [56], more recently Pinheiro *et al* have demonstrated an increase in MCT1 and MCT4 in CRC compared with normal colonic epithelium [57]. These contradictory results are probably due to antibody specificity, becoming essential further investigations about the relationship between clinicopathological factors and the immunohistochemical MCTs expression on the plasma membrane of the primary CRC cells.

It is also important that the regulation of the functional expression of MCTs by accessory proteins, such as Cluster of Differentiation 147 (CD147), that are involved in trafficking and anchoring of plasma membrane proteins. This make sense because the contribution of MCTs to the malignant phenotype may not be limited to their own function as lactate transporters and pH regulators, but may also be further enhanced by their role in regulating CD147 expression [55].

In CRC, our group previously analysed the immunoexpression of MCT isoforms 1, 2 and 4, CD147 and glucose transporter 1 (GLUT1) in a series of 126 cases of CRC and the results showed that most proteins studied were overexpressed at the plasma membrane of CRC cells and CRC lymph node, supporting the role of MCT1, MCT4, CD147 and GLUT1 in CRC maintenance and progression [58]. Moreover, this study was also able to prove that MCT1, MCT4, CD147 and GLUT1 expressions are associated with poor prognostic features [58].

Considering the multiple functions performed by MCTs, particularly in cancer cells as a key factor for both cell transformation and progression of the neoplastic process, its targeting may result in promising specific therapies.

1.4. The role of acetate in colorectal cancer metabolism and survival

Understanding the normal colon microbiota environment, how it changes in CRC, as well as the concentration of short chain fatty acids (SCFAs) and the physiological effects of these metabolites in the normal colon, is important. This knowledge might help in finding new adjuvant strategies to prevent or treat CRC.

1.4.1. Impact of diet and microbiota in short chain fatty acid production

The increasing incidence of CRC in young populations has increased the need to disseminate preventive measures, including maintenance of a healthy body weight, physical activity, minimization of red and processed meat and alcohol consumption, and avoidance of smoking [1, 59]. It has been demonstrated that specific changes in human intestinal microbiota (dysbiosis) can also be associated to sporadic CRC [60-62], supporting the hypothesis that colon cancer may be a bacteria-related disease [60, 61], characterized by the involvement of some specific bacterial species entailed in CRC pathogenesis (Figure 9).

Normal Colon Microbiota and Short-Chain Fatty Acids

The human intestine harbors as many as 10^{12} microorganisms composed by 500 - 1000 different bacterial species [61, 63, 64]. Although the intestinal the intestinal microbiome has a very varied composition, in normal symbiotic state (normobiosis), it is predominantly constituted by *Bacteroidetes* species, such as *Prevotella* and *Bacteroides* genera, and Firmicutes species, such as *Eubacterium* and *Streptococcus* genera. *Bifidobacterium*, *Atopobium* and *Fusobacterium* are also bacteria presented in high amounts [63, 65-68] (Figure 9). This intestinal microbiota constitutes a very complex system with numerous beneficial roles to human health, including protection against pathogens, maturation of the immune system, degradation of toxic substances, digestion of complex carbohydrates and production of SCFAs [68, 69].

SCFAs, namely acetate, propionate and butyrate, are produced by anaerobic microorganisms able to ferment polysaccharides, oligosaccharides, proteins, peptides and glycoproteins in the colon [65, 70-72].

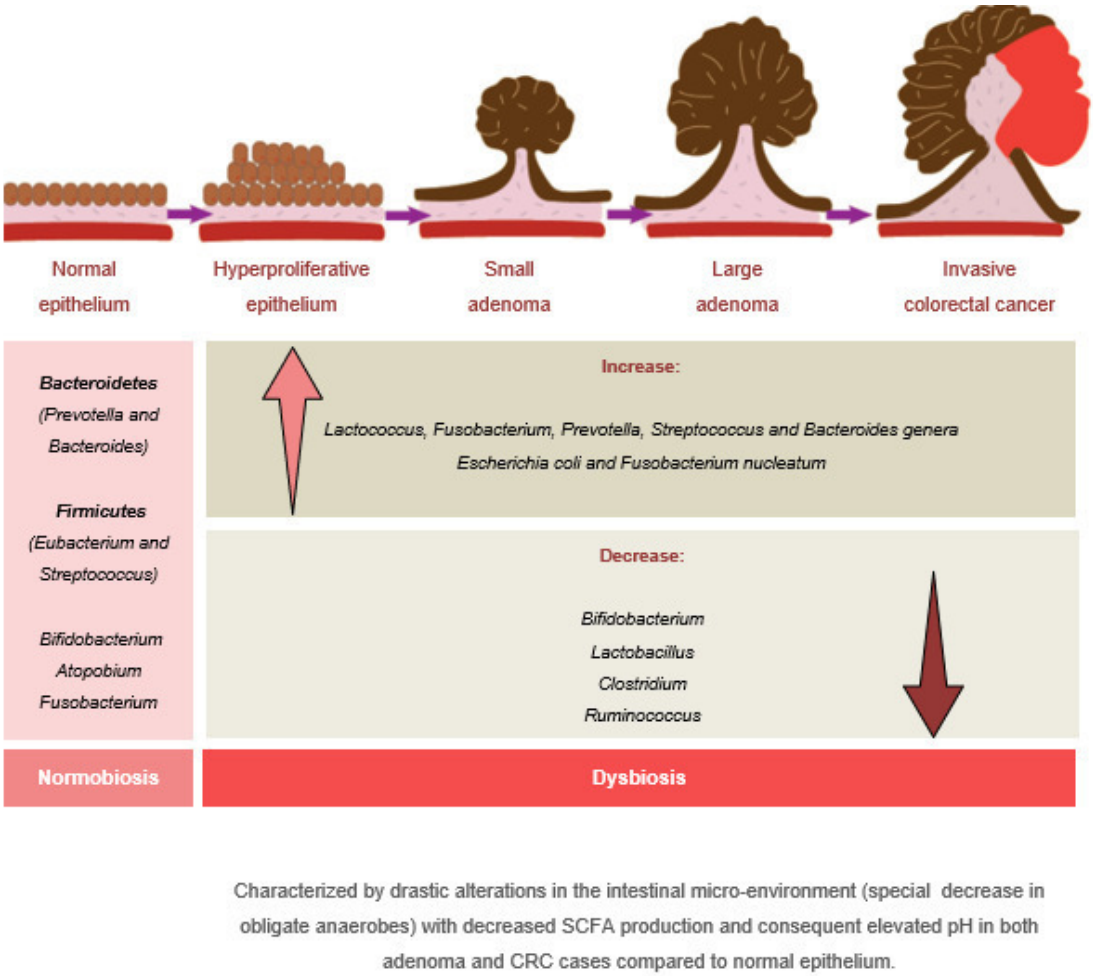


Figure 9 - Predominant composition of the intestinal microbiota in each step of colorectal carcinogenesis.

Acetate, in particular, is produced by acetogenic bacteria such as *Acetobacterium* species, *Clostridium aceticum* and *Propionibacterium,* able to perform reductive acetogenesis from formate [69, 72]. Normally, these bacteria exist in a mutually beneficial symbiotic relationship in the human colon, providing the amount of this SCFA necessary to maintain colon homeostasis [64]. Indeed, it has been shown that some changes in SCFA production are directly associated with alterations in the intestinal microbiota, modulated by numerous extrinsic factors such as diet, age, medication, treatment (drugs, radiation, surgery), stress and diseases [64, 73]. These changes in the normal symbiotic state (normobiosis) can be associated with many intestinal disorders, such as obesity, inflammatory bowel disease (IBD) and CRC [61, 63, 74, 75].

Intestinal Microbiota and Short-Chain Fatty Acids in Colorectal Cancer Patients

Most studies have shown a significant difference between the bacteria genera associated with cancerous and non-cancerous intestinal tissue or fecal samples [60, 61, 76]. It has been demonstrated that some bacteria have a complex arsenal of virulence factors which allow them to colonize and persist in the intestine, inducing chronic inflammation, accumulated mutations by DNA damage through superoxide radicals, genotoxin formation, increased T-cell proliferation and biosynthesis of pro-carcinogenic compounds that interfere with cell cycle regulation, contributing to colorectal carcinogenesis [60, 64, 77-79].

Escherichia coli and *Fusobacterium nucleatum* are the main bacteria associated with CRC and commonly over-represented in many tumor tissues from CRC patients [60, 61, 76-78].

These changes in the intestinal microbiota of CRC patients affect the amount and types of metabolites produced by colonic microbes. In this regard, some studies reported that changes in colon microbiota are associated especially with a decrease in SCFA concentrations accompanied by an elevated pH in patients with CRC compared to healthy individuals [61, 62, 80]. However, it is not yet understood if the changes in colon microbiota are the cause or the consequence of CRC development. In this context, it is important to characterize the possible over- and under-represented bacteria, exploring the relationship between changes in the colon microbiota composition with the production of bacterial metabolites, specifically SCFAs, as well as the tumorigenic process of CRC.

1.4.2. Acetate transporters and receptors

Acetate, propionate and butyrate display pKa values of 4.76, 4.87 and 4.82, respectively. At neutral pH, less than 1% of these fatty acids exist in the protonated form, which means that these compounds do not cross the plasma membrane via simple diffusion [81]. Instead, such for lactate, their transport to the intestinal epithelial cells is mediated by transporter proteins like MCTs [82]. Together with MCT-1, SMCT-1 has been identified as the main monocarboxylate transporter responsible for the uptake of SCFAs across the membrane of intestinal cells [72, 82, 83].

Recently, our group showed that the transport of acetate occurs via SMCT-1 and by passive diffusion via aquaporins in CRC cells. However, MCT-1 is also involved in acetate uptake in cells that overexpress both MCT-1, MCT-4 and CD147, upon culture in medium with acetate [84]. We found that acetate

promotes plasma membrane re-localization of MCT-1 and further triggers changes in glucose metabolism by increasing both glucose consumption and lactate production, thus increasing CRC cell glycolytic phenotype. This work provided new evidence for the role of acetate in the regulation of MCT-1 expression, thus influencing its own transport and increasing the sensitivity of CRC cells to acetate, as well as to other agents that are transported via MCT-1 [84].

Nevertheless, the role of SCFA transporters and receptors in CRC cells are still poorly understood and more studies are needed to grasp how these metabolites can influence the regulation of their own transporters and receptors in CRC cells.

1.4.3. The Specific Role of Acetate on Colorectal Cancer Cell Proliferation and Apoptosis

Several studies have shown that butyrate, propionate and acetate induce apoptosis in CRC cells but not in normal cells [85-90]. The anti-cancer effect of SCFAs is also supported by epidemiological studies suggesting an inverse relationship between the level of dietary fibers and the incidence of CRC [63, 91, 92]. These fatty acids that derive from the diet influence the risk of human colon cancer through diverse mechanisms, such as activation of cell death through different processes depending on their concentration, pH and cell type (Figure 10).

Among the three most relevant SCFAs, acetate has been the least studied. Initially, Jan and co-workers showed that acetate (0 - 40 mM) decreased viability and induced typical signs of apoptosis, including loss of mitochondrial membrane potential, generation of ROS, caspase-3 processing and nuclear chromatin condensation in the colon adenocarcinoma cell line HT-29 [93]. Studies in *Saccharomyces cerevisiae* first demonstrated that acetic acid induces a mitochondria-mediated apoptotic process [94, 95] with several features similar to apoptosis mediated by SCFAs in CRC cells. Indeed, alterations in mitochondria were identified in yeast, including production of ROS, mitochondrial swelling, decrease of mitochondrial membrane potential ($\Delta\Psi m$) [96], mitochondrial fragmentation/degradation [97, 98], mitochondrial outer membrane permeabilization (MOMP) with consequent release of pro-apoptotic factors like cytochrome *c*, yeast apoptosis inducing factor 1 (Aif1p) and Nuc1p (yeast orthologue of EndoG) [98-100]. The yeast orthologues of the mammalian VDAC (voltage-dependent anion channel) and ANT (adenine nucleotide transporter) were shown to play a role in MOMP and cytochrome *c* release during acetic acid-induced apoptosis in yeast [101]. Later, vacuolar

membrane permeabilization (VMP) and release of Pep4p, yeast cathepsin D (CatD), from the lysosome-like vacuole to the cytosol, were observed in yeast cells exhibiting apoptotic cell death induced by acetic acid [102]. In that study, the authors also showed that, once in the cytosol, Pep4p played an important role in mitochondrial degradation through an autophagic-independent process, which protected yeast cells from acetic acid-induced apoptosis. Recently, it was demonstrated that both the protective function of Pep4p and its role in mitochondrial degradation during acetic acid-induced apoptosis in yeast depends on Pep4p proteolytic activity [91], which is complemented by heterologous expression of human CatD [103, 104].

Taking these results into account, our group hypothesized that similar events could occur in response to acetate in CRC cells (Figure 10 and Table 1). We have shown that, acetate treatment in CRC cells (0 - 140 mM and 0 - 220 mM, respectively for HCT-15 and RKO cells) decreased cell proliferation and induced apoptosis [105]. This process was characterized by DNA fragmentation, caspase-3 activation and phosphatidylserine exposure to the outer leaflet of the plasma membrane with appearance of a sub-G1 population [105]. Moreover, we showed that acetate induced lysosomal membrane permeabilization (LMP) with cathepsin D (CatD) release to the cytosol. Importantly, we revealed that CatD, but not CatB and CatL (also overexpressed in CRC cells), has an anti-apoptotic role in acetate-induced apoptosis [105]. We next showed that acetate induces mitochondrial dysfunctions, such as ROS accumulation and an increase in mitochondrial mass accompanied by mitochondrial membrane depolarization [104]. Additionally, we found an increase in the levels of mitochondrial proteins, namely the apoptosis inducing factor (AIF), the voltage dependent anion channel (VDAC1) and a subunit of the outer mitochondrial membrane translocator (TOM22) in CRC cells after acetate treatment [104]. This means that during acetate-induced apoptosis mitochondrial degradation following release of CatD is not enough to counteract autophagy inhibition by acetate. Indeed, inhibition of CatD (with siRNA or pepstatin A) increased mitochondrial mass and enhanced apoptosis associated with higher mitochondrial dysfunction [104].

The protective role of CatD demonstrated by our data can partly explain the CatD overexpression in some CRC clinical cases in comparison to normal colon mucosa as a cell strategy to cope with acetate cytotoxicity. In summary, CatD plays a role in the degradation of damaged mitochondria when autophagy is impaired, protecting CRC cells from acetate-induced apoptosis. CatD inhibitors could

therefore enhance acetate-mediated cancer cell death, presenting a novel strategy for CRC prevention or therapy.

In conclusion, the data from different researchers suggest that the use of acetate, butyrate and propionate, as possible chemopreventive agents, should be considered. However, more studies, especially using these SCFA combinations, that mimic their relative concentrations in the colon, are necessary to support their combined use as a new strategy to potentiate the effects of each one in CRC cells.

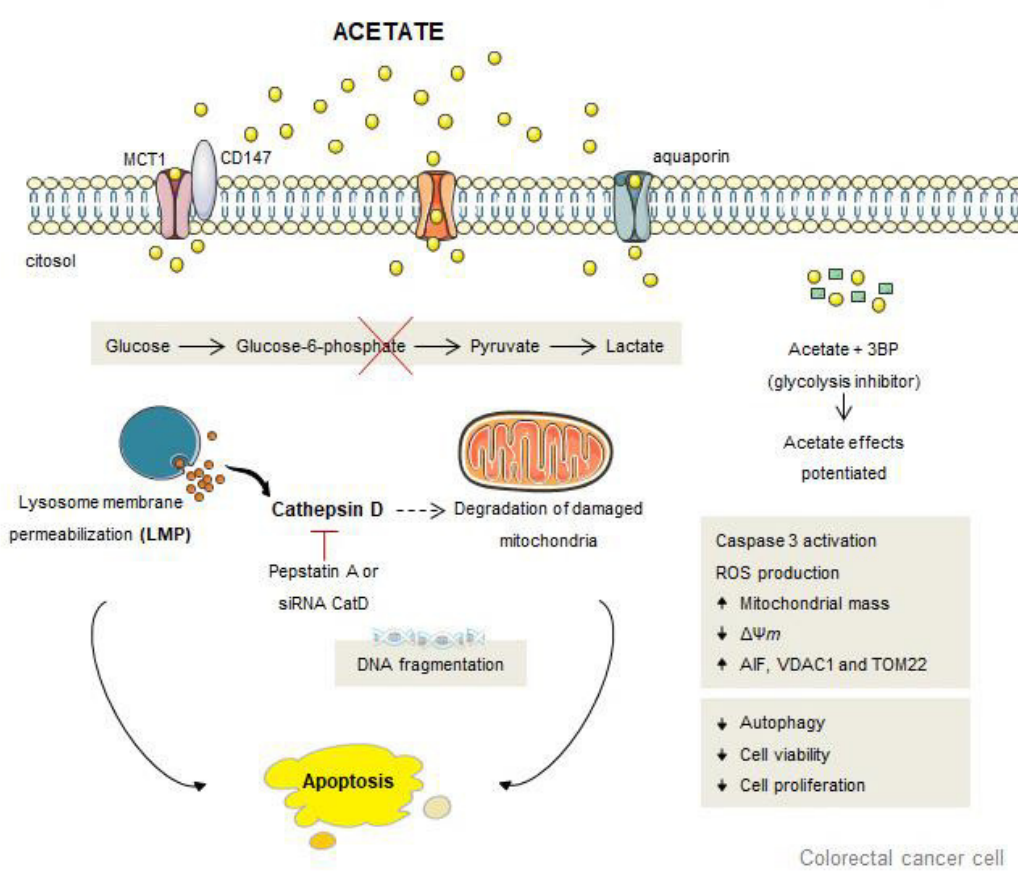


Figure 10 - Schematic representation of different cell processes involved in apoptosis triggered by acetate in colorectal cancer cells.

Table 1 - Effects of acetate in colorectal cancer cells.

Acetate			
CRC cell lines	Concentration	Effects	Reference
HT-29 Caco-2	1.7 – 40 mM	Apoptosis induction with mitochondrial alterations	Jan et al, 2002
HT-29	4 – 32 mM	Ineffective in these concentrations	Comalada, 2006
HT-29	80 mM	Does not induce histone acetylation	Kiefer et al, 2006
Colo320DM	2.4 mM	Inhibition of the NF-¬B pathway	Tedelind et al, 2007
HT-29	15 mM	Apoptosis at pH 7.5 Necrosis at pH 5.5	Lan et al, 2007
RAW264.7·	1 – 1.200 µmol/L	Decrease of pro-inflammatory factors with an increase in the anti-inflammatory cytokine IL-10	Liu et al, 2012
HCT-15 RKO	70 – 140 mM	Induction of apoptosis/Inhibition of proliferation and partial lysosome permeabilization with CatD release to the cytosol	Marques et al, 2013
HCT-15 RKO HCT116	110 – 220 mM	Mitochondrial dysfunction/ inhibition of autophagy and active CatD involved in mitochondrial degradation	Oliveira et al, 2015
HCT-15 RKO	70 – 140 mM	Transport mediated by SMCT-1 or by aquaporins MCTs regulation and co-localization associated with changes in glucose metabolism	Oliveira et al, 2017

The potential use of SCFAs in the prevention and/or as adjuvants to conventional chemotherapy regimens of CRC is currently well supported by the literature.

Several *in vivo* studies have proven that SCFAs act as tumor suppressor agents, promoting apoptosis of CRC cells and enhancing the inhibition of tumor cell growth and proliferation. However, since the concentrations of SCFAs in the colon seem to be low in CRC patients, it becomes important to optimize the production of these agents with specific nutritional diets, specifically with the intake of fibers and appropriate probiotics like *Propionibacteria*.

In conclusion, the state of the art on the field argues in favor of the potential use of SCFAs in prevention and/or as adjuvants to conventional chemotherapy regimens of CRC. Indeed, the use of

nutraceuticals to increase butyrate, propionate and acetate concentrations in the colon might emerge as a novel strategy for prevention/therapy of CRC.

Acetate, together with other SCFA has been implicated in CRC prevention/therapy. SCFAs, specifically acetate, propionate and butyrate are produced by propionibacteria at the intestine and constitute a major source of energy for colonocytes. Our group has been studying for the last years the role of acetate in CRC cells. We showed that acetate inhibits CRC cell proliferation, induces apoptosis, promotes lysosomal membrane permeabilization with cathepsin-D release which has a protective role in CRC cells [15, 84, 104, 106]. Moreover, it was already showed that MCTs are overexpressed in CRC clinical cases [54, 55, 57, 107], and recently we also demonstrated that acetate increases CRC cell glycolytic phenotype and regulate its own uptake by increasing the expression of MCTs [84]. However, the precise role of MCT expression and activity in CRC glycolytic metabolism and metabolic remodelling in the presence of acetate in CRC cells is still poorly understood. BRAF and KRAS are the main oncogenes activated in CRC and recent evidences showed that cells with these mutations exhibited enhanced glucose uptake and glycolysis. Despite recent studies pointing to a crosstalk between oncogene activation and glycolytic metabolism, the mechanisms underlying this crosstalk are still poorly explored. Moreover, we have already shown that the glycolytic phenotype is also affected through MCT regulation in CRC exposed to acetate. However, the signalling pathways associated to the phenotypic changes induced by acetate have not been characterized

Taking this into consideration, our **general aim** was to understand the role of signalling pathways underlying the activation of KRAS and/or BRAF oncogenes in the response to acetate exposure and the crosstalk to the glycolytic metabolism through MCT expression regulation.

Specifically, we aim at:

1 - Study of the involvement of acetate in the expression levels of signalling pathways molecules downstream of KRAS/BRAF oncogenes known to be important in CRC cells survival namely PI3K/AKT and MAPK pathways.

2 - Study the role of KRAS and BRAF oncogene signalling pathway in the regulation of glycolytic metabolism in CRC cells exposed to acetate.

3 - Study the role of MCTs in KRAS/BRAF signalling pathway regulation in CRC exposed to acetate.

CHAPTER 3 – MATERIALS AND METHODS

3.1. Cell lines and culture conditions

In this work, two cell lines derived from human colorectal cancer (CR), RKO and HCT-15, were used. RKO cell line was first described by Michael Brattain [108] and is derived from a primary colonic carcinoma. These cells are microsatellite-unstable and harbour a BRAF mutation [109]. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Biochrom, Germany), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Biochrom, Germany) and a mixture of penicillin-streptomycin at a final concentration of 1% (v/v) (5,000 Units/mL of penicillin and 5,000 μg/mL of streptomycin). HCT-15 cell line is derived from a adenocarcinomas specimen of human colon removed during the normal course of a surgery [110, 111]. They are microsatellite-unstable and harbour a KRAS mutation [109]. These cells were grown in RPMI 1640 medium (with stable glutamine) (Biochrom, Germany) and, as well as for DMEM medium, it was supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Biochrom, Germany) and a mixture of penicillin-streptomycin at a final concentration of 1% (v/v) (5,000 Units/mL of penicillin and 5,000 μg/mL of streptomycin).

Both cell lines were grown and maintained in 25 cm^2 or 75 cm^2 tissue culture flasks at 37 °C under a humidified atmosphere containing 5% CO_2. When cells reached 80% to 100% of confluence, the culture medium was removed, and cells washed with PBS 1x (phosphate buffered saline) and trypsinized (trypsin 0.05 % (v/v) dissolved in phosphate buffer saline - ethylenediamine tetra acetic acid (PBS-EDTA) (Gibco®)) at 37°C, in a 5% CO_2 atmosphere. After 5 minutes, trypsin effect was neutralized with addition of fresh culture medium and the desired volume of cell suspension was transferred to a new 25 cm^2 or 75 cm^2 tissue culture flask, together with the correspondent volume of culture medium.

All cells were manipulated under aseptic conditions in a flow chamber.

3.2. Western blot analysis

Total protein extraction and quantification

RKO and HCT-15 cells were seeded at $5x10^5$, $3x10^5$ and $2.5x10^5$ cells/mL, according to the timepoint of protein extraction (4, 24 and 48 hours, respectively). 24 hours after seeding, cells were exposed to the IC_{30}, IC_{50} and IC $_{intermediate\ values}$ (IC_{int}) of acetate previously determined by our group [106] (Table 2), while control cells were only exposed to the culture medium.

Table 2 – IC_{30}, IC_{50} and IC_{int} of acetate for RKO and HCT-15 cell lines.

The acetate concentrations were previously determined by our group for 48h of treatment [106].

Cell line	IC_{30} of acetate (mM)	IC_{50} of acetate (mM)	IC_{int} of acetate (mM)
RKO	75	110	140
HCT-15	45	70	100

After 4, 24 or 48 hours of incubation, spent media were collected to 15 mL Falcon tubes, cells were washed with PBS 1x and trypsinized. Trypsin was inactivated with addition of culture medium. Samples were centrifuged at 2000 rpm, at 4 °C for 10 minutes, and the supernatant was discarded. Cells were then ressuspended and transferred to 1.5 mL microtubes, followed by a new centrifugation at 1200 rpm, at 4 °C for 5 minutes. The pellets were suspended in RIPPA buffer (Tris HCl 50 mM pH 7.4, NaCl 150 mM, EDTA 2 mM, NP-40 1% (v/v)) supplemented with protease inhibitors (NaF 20 mM, Na_3VO_4 20 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, complete Mini, EDTA-free, protease cocktail inhibitor (Roche™) (4%)) and chilled for 20 minutes. Samples were then centrifuged at 13000 rpm, at 4 °C for 15 minutes and the supernatants containing the protein extract were transferred to new 1.5 mL microtubes.

Protein quantification of the extracts was performed using a commercial kit (BioRad *DC*™ Protein Assay), according to the instructions provided by the manufacturer. A bovine serum albumin (BSA) standard curve with known protein concentration was used to assess the protein content of each sample. The validity of the results was assumed for an R^2>0.98 of the standard curve.

Western blotting analysis

Protein samples (25 μg) were separated on 10% (w/v) polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes (Amersham Biosciences). After that, membranes were blocked with 0.1% Tween, 1% BSA or non-fat dry milk in TBS for 1h and then incubated overnight at 4°C with the primary polyclonal antibodies. In the following day, membranes were washed in TBS-0.1% Tween and incubated with the secondary antibodies (Table 3). The specific protein bands were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA) and densitometry analysis of protein bands was performed using the Quantity One software (version 4.6.9), being the levels of actin used as a normalization control for protein loading.

Table 3 – List of antibodies and correspondent incubation conditions used in western blot analysis.

Antibody	Host	Size of the target	Dilution	Temperature	Incubation time	Manufacturer reference
Akt	Rabbit	60 kDa	1:1000	4 °C	Overnight	Cell signalling (#4691)
Phospho-Akt (Ser473)	Rabbit	60 kDa	1:2000	4 °C	Overnight	Cell signalling (#4060)
Phospho-Akt (Thr308)	Rabbit	60 kDa	1:1000	4 °C	Overnight	Cell signalling (#13038)
Phospho-c-Raf (Ser259)	Rabbit	74 kDa	1:1000	4 °C	Overnight	Cell signalling (#9421)
Phospho-GSK-3β (Ser9)	Rabbit	46 kDa	1:1000	4 °C	Overnight	Cell signalling (#5558)
Phospho-PDK1 (Ser241)	Rabbit	58 to 68 kDa	1:1000	4 °C	Overnight	Cell signalling (#3438)
Phospho-PTEN (Ser380)	Rabbit	54 kDa	1:1000	4 °C	Overnight	Cell signalling (#9551)
p44/42 MAPK (ERK1/2)	Rabbit	42, 44 kDa	1:1000	4 °C	Overnight	Cell signalling (#4695)
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Thr204)	Rabbit	44, 42 kDa	1:2000	4 °C	Overnight	Cell signalling (#4370)
p38 MAPK	Rabbit	40 kDa	1:1000	4 °C	Overnight	Cell signalling (#8690)
Phospho-p38 MAPK (Thr180/Thr182)	Rabbit	43 kDa	1:1000	4 °C	Overnight	Cell signalling (#4511)
BRAF	Mouse	96 kDa	1:1000	4 °C	Overnight	Santa Cruz Biotechnology (sc-5284)
KRAS	Mouse	21 kDa	1:1000	4 °C	Overnight	Santa Cruz Biotechnology (sc-30)
MCT-1	Mouse	45 kDa	1:500	4 °C	Overnight	Santa Cruz Biotechnology (sc-363501)
MCT-4	Rabbit	43 kDa	1:500	4 °C	Overnight	Santa Cruz Biotechnology (sc-30329)
Actin	Mouse	43 kDa	1:5000	4 °C	1h	Sigma - Aldrich® (A5441)
Anti-rabbit	Goat	-	1:5000	Room temperature	1h	Cell signalling (#7074)
Anti-mouse	Goat	-	1:5000	Room temperature	1h	Santa Cruz Biotechnology (sc-2031)

3.3. RNA interference (RNAi) assay for BRAF and KRAS silencing

RKO and HCT-15 cells were plated in 6 well plates at a density of $1.75x10^5$ and $1.8x10^5$ cells per well, respectively. After 24 hours, they reached approximately 70% of confluence and the transfection with small interfering RNA (siRNA) oligonucleotides was made. Both cell lines were transfected with 200 nM siRNA against BRAF (67337963, Integrated DNA Technologies (IDT)) and/or with 200 nM siRNA against KRAS (si02662051, Qiagen), being the transfection monitored using a validated Silencer Select Negative Control (scrambled siRNA control, 1027292, Qiagen). Transfection was performed with 6 and 4 µL Lipofectamine 2000 (Invitrogen Corp., Paisley, UK) in RKO and HCT-15 cells, respectively. After 14 hours, the culture medium was removed and the transfected cells were treated with the correspondent concentration of acetate during 24 hours.

3.4. RNA interference assay for MCT-1 and MCT-4 silencing

HCT-15 and RKO cells were transfected with specific siRNA for MCT-1 (SLC16A1 siRNA, Ambion, catalogue number 4390824)) and MCT-4 (SLC16A3 siRNA, Ambion, catalogue number 4390824) using Lipofectamine RNAiMAX (Invitrogen). Non-silencing siRNA control was used as negative control (Ambion, catalogue number 4390843). siRNA transfection was performed using 2 µL of Lipofectamine RNAiMAX, 20 nM of siRNA for MCT-1 and 20 nM of siRNA for MCT-4 for both cell lines. Knockdown of target genes was confirmed at the protein level by western blot analysis in the fourth day of silencing (previously established in our laboratory).

To evaluate the effect of acetate treatment, after 72h of silencing, the medium of the transfected cells was replaced by medium containing the selected concentration of acetate and after 24h of acetate treatment, protein extraction and the trypan blue exclusion assay were performed.

3.5. Trypan blue exclusion assay

To study cellular viability after treatments, in parallel with the protein extraction protocol, a cell suspension of RKO and HCT-15 cell line was mixed with trypan blue solution in a 1:1 dilution. 10µl of this mixture were loaded in Neubauer counting chambers and the number of stained and total cells

was counted, being the dead cells the ones which present a blue colour. The percentage of cell death was determined according to the formula: *(Number of blue cells ÷ Number of total cells) × 100.*

3.6. Statistical analysis

The results herein presented were obtained from at least three independent experiments. The Image Lab software version 3.0 was used to determine the volume intensity of each western blot band. One-way ANOVA was performed using GraphPad Prism version 7.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com and the results were considered statistically significant according to the following p values: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

CHAPTER 4 – RESULTS

4.1. The involvement of acetate in the regulation of cell signalling pathway in colorectal cancer cells

4.1.1. Acetate influences the expression levels of some signalling molecules in a dose and time dependent manner

To understand the functional role of acetate in the regulation of signalling pathways in CRC cells, the expression levels of several downstream proteins were evaluated by western blot. Key proteins namely pAKT, AKT, pPDK1, pPTEN, pcRAF, pGSK3β, pERK, ERK and p38 were analysed in RKO and HCT-15 cells. We used the doses of acetate corresponding to IC_{50}, IC_{int}, $2xIC_{50}$ for each cell line. RKO cells were treated with 75, 110 or 140 mM of acetate while HCT-15 cells were treated with 45, 70 or 100 mM of acetate and were compared to control cells incubated only with medium. Moreover, protein extracts were collected at three different time points: 4h, 24h and 48h after treatment. Total AKT and total ERK were used as loading control due to a technical problem with the actin antibody. These results are representative of at least three independent experiments (Figures 11-14).

It is evident that the most significant difference for both cell lines is the decrease of the expression levels of phosphorylated cRAF, also known as RAF1, after 24h and 48h of acetate treatment with all the tested concentrations (Figure 12 C and Figure 14 C). Only in HCT-15 cells it is possible to observe a significant decrease of pERK expression after 4 h, when cells were treated with 70 mM or 100 mM of acetate (Figure 12 E). Although not significant, the same tendency is observed in RKO cells (Figure 14 E).

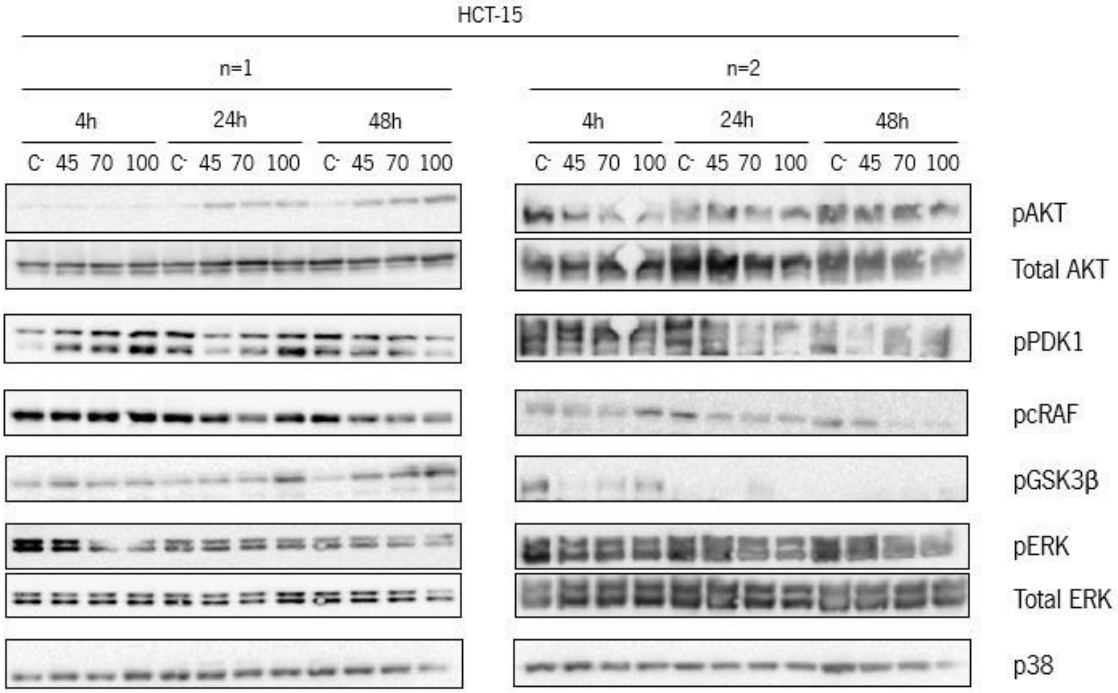


Figure 11 - Acetate treatment influences the expression levels of signalling proteins in HCT-15 cells. Western blot analysis, being the control cells not subjected to any treatment. Protein extracts were collected at three different time points: 4h, 24h and 48h after treatment. Total AKT and total ERK were also used as loading controls.

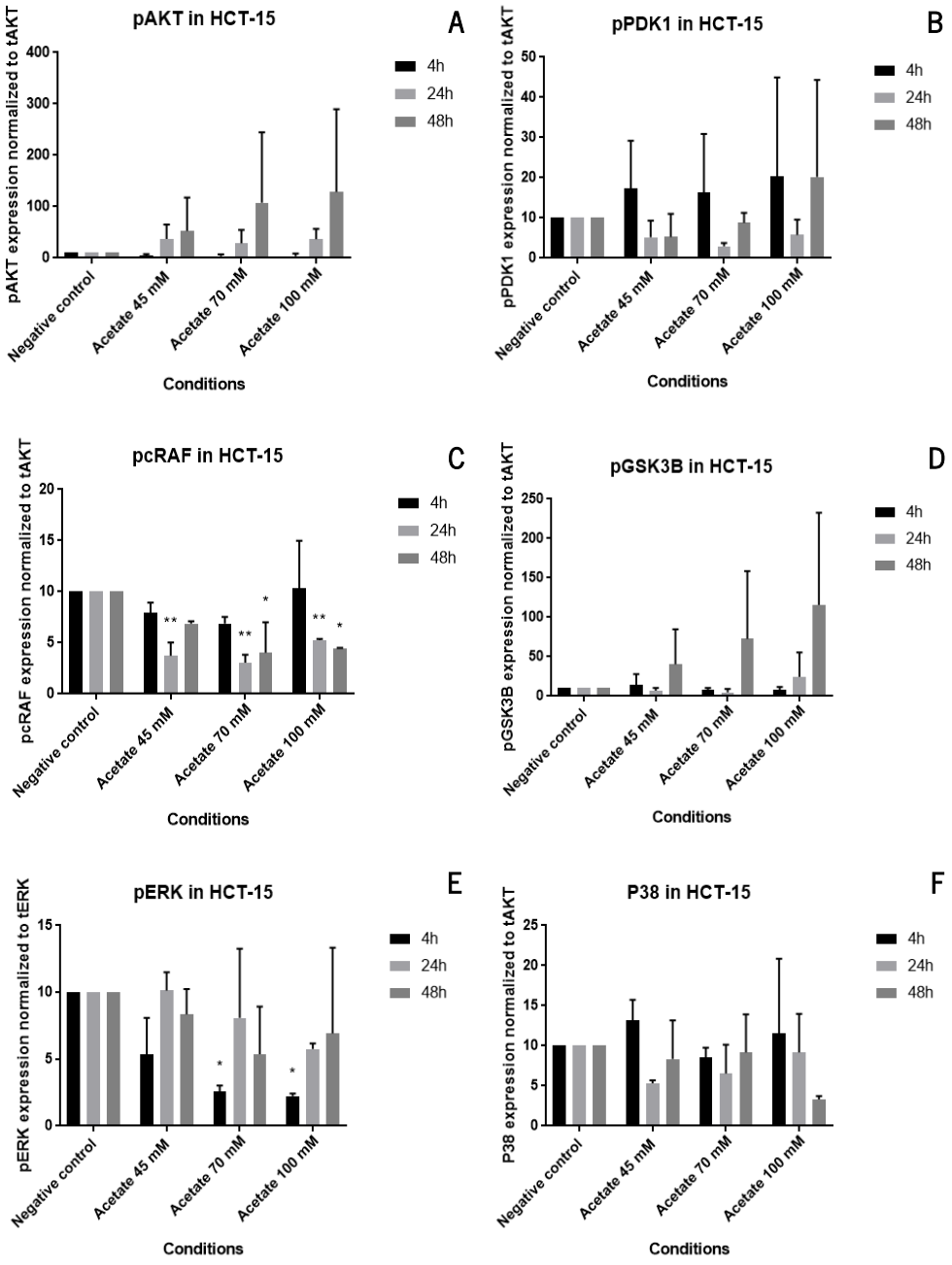


Figure 12 - Acetate treatment influences the expression levels of signalling proteins in HCT-15 cells - quantification analysis.

(A) phospho-AKT expression normalized for total AKT. (B) phospho-PDK1 expression normalized for total AKT. (C) phospho-cRAF expression normalized for total AKT. (D) phospho-GSK3B expression normalized for total AKT. (E) phospho-ERK expression normalized for total ERK. (F) phospho-P38 expression normalized for total AKT. Values represent mean ± SD of at least two independent experiments. * P≤ 0.05; ** P≤ 0.01 compared with negative control.

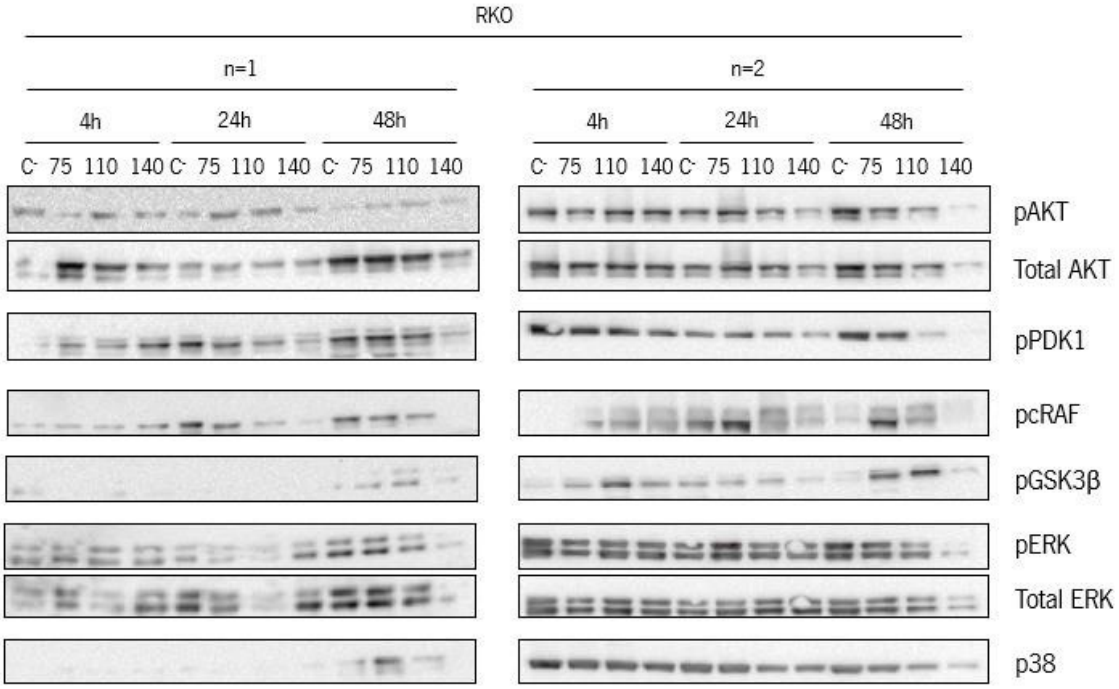


Figure 13 - Acetate treatment influences the expression levels of signalling proteins in RKO cells.

Western blot analysis for pAKT, AKT, pPDK1, pcRAF, pGSK3β, pERK, ERK and P38 of RKO cells. RKO cells were treated with 75, 110 or 140 mM of acetate, being the control cells not subjected to any treatment. Protein extracts were collected at three different time points: 4h, 24h and 48h after treatment. Total AKT and total ERK were also used as loading control.

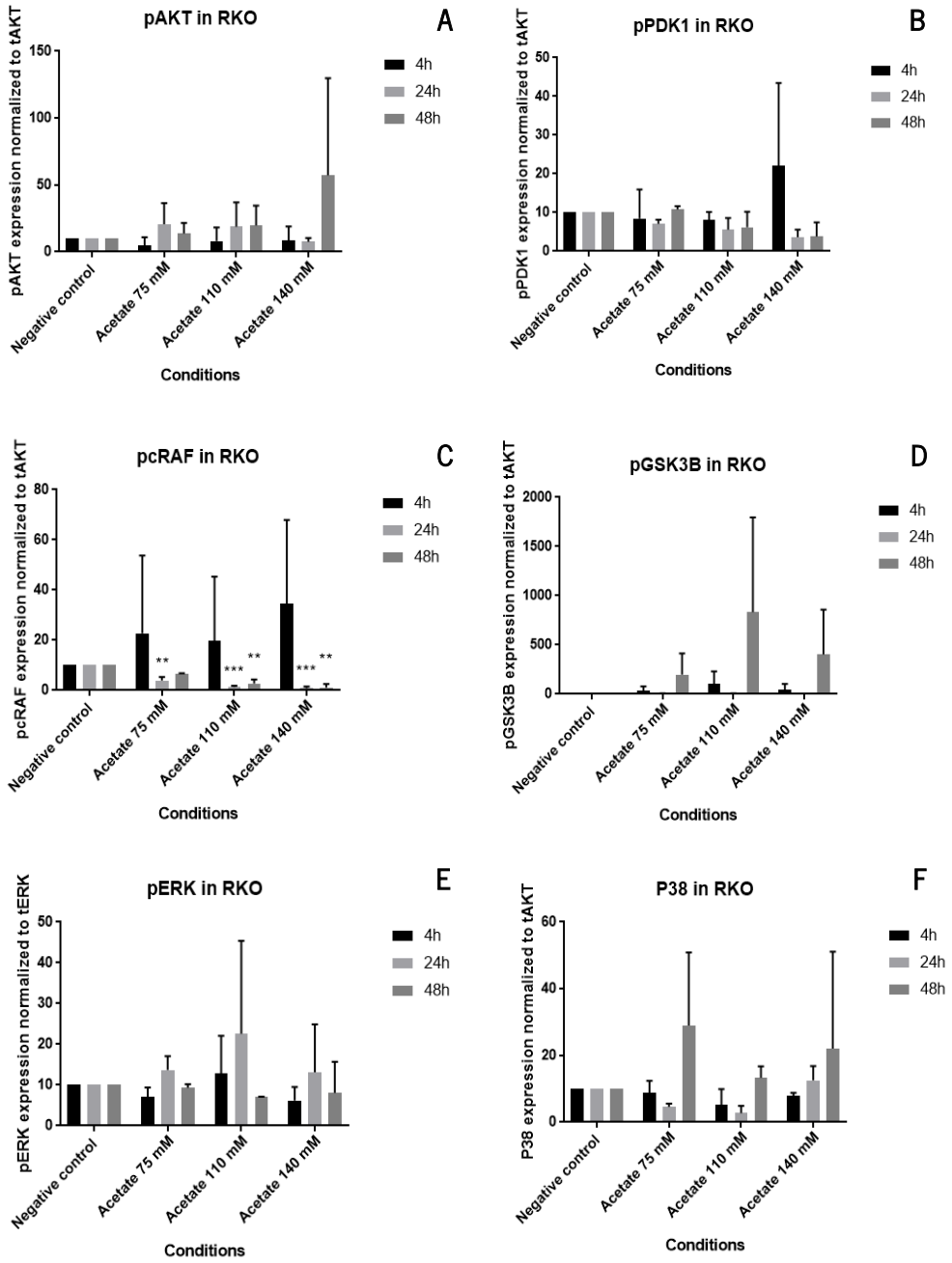


Figure 14 - Acetate treatment influences the expression levels of signalling proteins in RKO cells - quantification analysis.

(A) phospho-AKT expression normalized for total AKT. (B) phospho-PDK1 expression normalized for total AKT. (C) phospho-cRAF expression normalized for total AKT. (D) phospho-GSK3B expression normalized for total AKT. (E) phospho-ERK expression normalized for total ERK. (F) phospho-P38 expression normalized for total AKT. Values represent mean ± SD of at least two independent experiments. ** P≤ 0.01; *** P≤ 0.001 compared with negative control.

4.1. The role of KRAS and BRAF in the regulation of glycolytic metabolism in CRC cells

Being KRAS and BRAF the major oncogenes implicated in sporadic colorectal carcinogenesis, we wanted to understand if the downregulation of one or both oncogenes influenced the effect of acetate in CRC cells. We also aimed to clarify the role of KRAS and BRAF oncogene signalling pathway in the regulation of glycolytic metabolism in cells exposed to acetate by testing the expression of some metabolic markers, such as MCTs (MCT1, MCT2, MCT4) and CD147 (MCT-1 chaperone)).

4.1.1. Optimization of BRAF and KRAS RNA interference conditions

Before starting this part on the work, we needed to perform some experimental tests in order to optimize the BRAF and KRAS RNA interference conditions.

We tested the BRAF RNA interference (RNAi) conditions in both cell lines (RKO and HCT-15), using lipofectamine 2000 as transfection agent, to determine the best concentration to be used. Cells were transfected with 100, 150, 200 and 400 nM of BRAF siRNA and used as controls, the negative control (cells incubated only with medium) and the control scramble (cells transfected with 400 nM scramble siRNA). The protein extracts were collected 48h after transfection, and 25 μg of protein were used to perform a western blot analysis for BRAF expression using β actin the protein as loading control. The results showed that 200 nM seems to be the lowest concentration of BRAF siRNA with the best silencing efficiency at least in HCT15 because in RKO cells the β actin antibody did not work. (Figure 15).

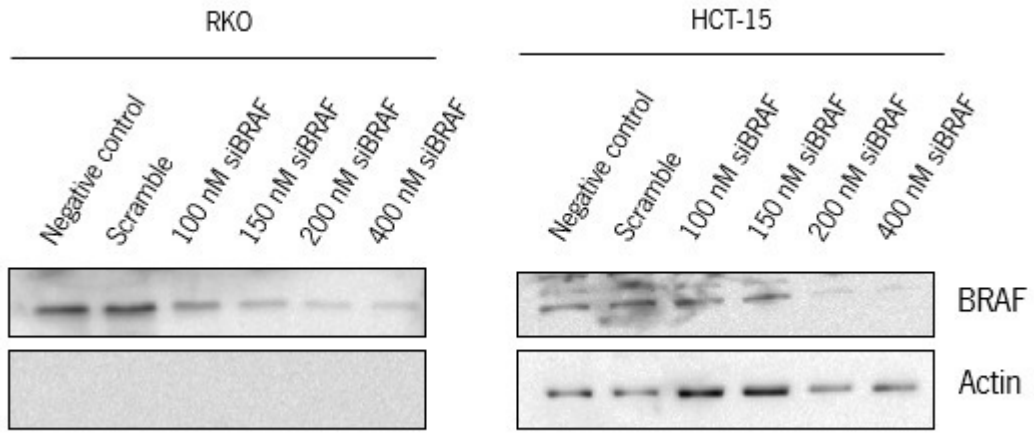


Figure 15 - Test of the BRAF siRNA conditions in RKO and HCT-15 cells.

Western blot analysis for BRAF of RKO and HCT-15 cells, using 25 μg of protein per well. Cells were transfected with 100, 150, 200 and 400 nM of BRAF siRNA, being the control cells not subjected to any treatment or transfected with 400 nM scramble siRNA. Lipofectamine 2000 was used as transfection agent. Protein extracts were collected 48h after transfection. Actin was used as loading control, however, for RKO cells this antibody did not work.

For the optimization of the KRAS RNA interference conditions, RKO cells were transfected with 150 or 200 nM of KRAS siRNA, using the same controls described previously. We tested three different transfection agents: lipofectamine 2000, lipofectamine RNAiMAX and lipofectamine HiPerfect. Protein extracts were collected 48h after transfection and β actin used as loading control. We have faced some technical problems as the staining with the KRAS antibody did not work, despite the perfect bands visualized after incubation with β actin (Figure 16). We thought that maybe KRAS antibody had lost the efficiency, thus we performed another western blot analysis, using 75 μg of protein of the previous samples. β actin antibody for unknown reasons did not work, so the membrane was stained with Ponceau red and used as loading control. The results revealed that KRAS antibody worked and that KRAS siRNA was not working because KRAS is equally expressed in controls and in the transfected cells (Figure 17).

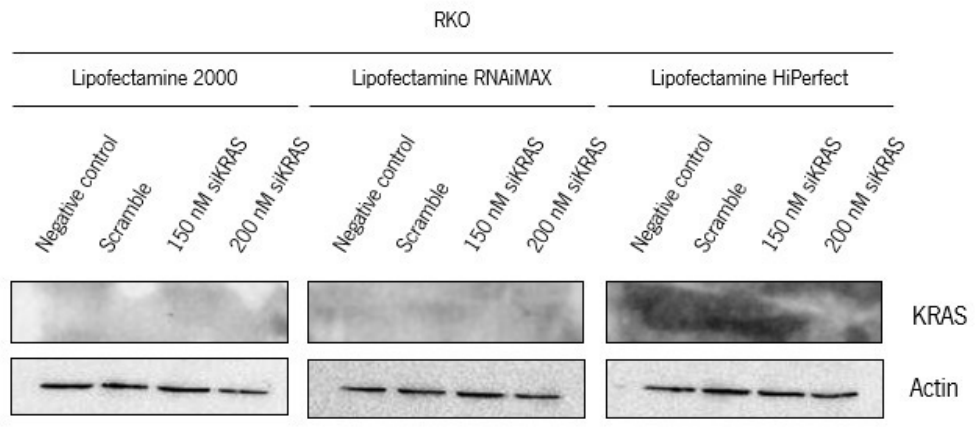


Figure 16 - Test of the KRAS siRNA conditions in RKO cells.

Western blot analysis for KRAS in RKO cells, using 25 µg of protein per well. Cells were transfected with 150 or 200 nM of KRAS siRNA, being the control cells not subjected to any treatment or transfected with 200 nM scramble siRNA. Three different transfection agents were tested: lipofectamine 2000, lipofectamine RNAiMAX and lipofectamine HiPerfect. Protein extracts were collected 48h after transfection. Actin was used as loading control. KRAS antibody did not work.

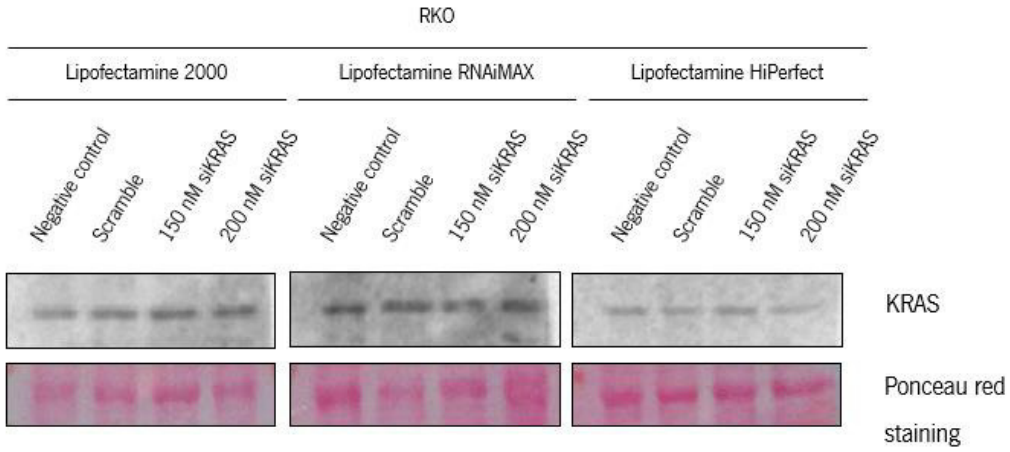


Figure 17 - Test of the KRAS siRNA conditions in RKO cells.

Western blot analysis for KRAS of RKO cells, using 75 µg of protein per well. Cells were transfected with 150 or 200 nM of KRAS siRNA, being the control cells not subjected to any treatment or transfected with 200 nM scramble siRNA. Three different transfection agents were tested: lipofectamine 2000, lipofectamine RNAiMAX and lipofectamine HiPerfect. Protein extracts were collected 48h after transfection. Because actin antibody did not work, the membrane staining with Ponceau red was used as loading control.

However, despite so many limitations, we tried to achieve our aim and decided to transfect RKO cells with 150 nM of KRAS siRNA and/or 200 nM of BRAF siRNA with/without 70 nM of acetate. As it can be seen in Figure 18, the cells achieved a high confluence in the plate wells, however, despite the absence of significant alterations in the morphology, acetate treatment seams to interfere with cell proliferation (Figure 18, conditions 3,6,7 and 9). Western blot analysis of the samples confirmed that KRAS siRNA is not working as well as the BRAF antibody (Figure 19).

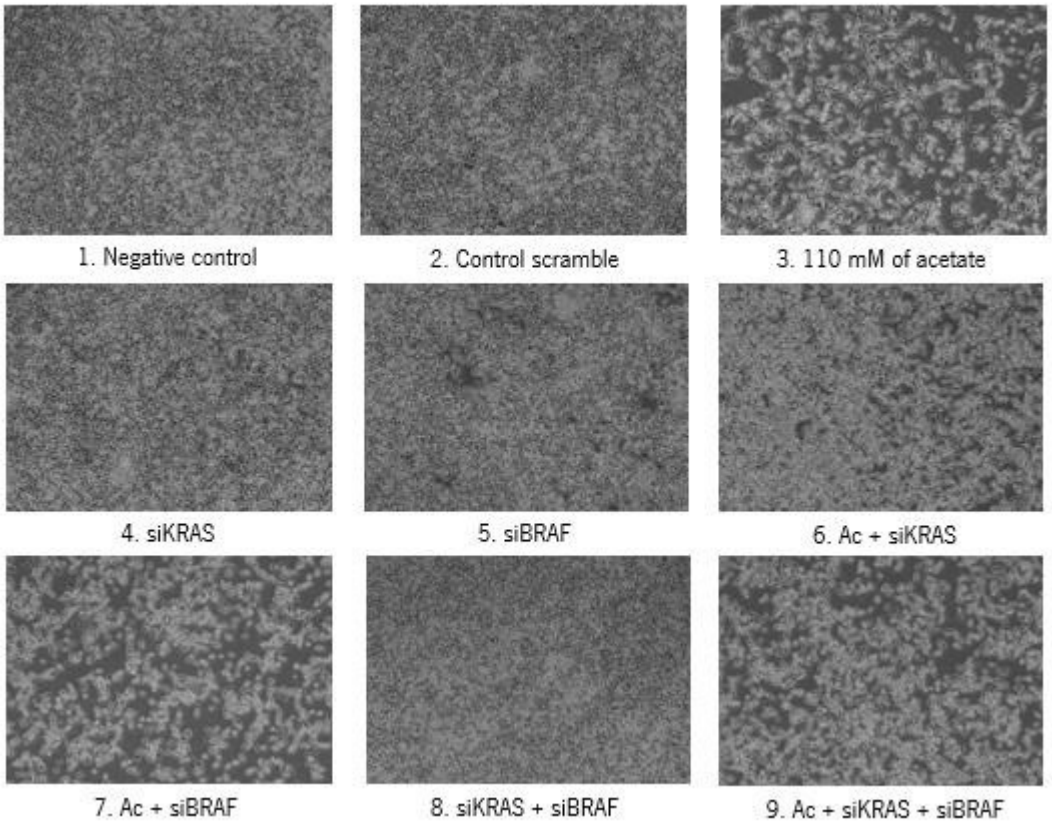


Figure 18 - Effects of acetate treatment in RKO cells when KRAS and/or BRAF are silenced using siRNA – cell morphology and confluence.

Images obtained from an inverted bright field microscope, 100x amplified of RKO cells transfected with 150 nM of KRAS siRNA and/or 200 nM of BRAF siRNA and then treated or not with 70 nM of acetate. (1) The control cells were not subjected to any treatment or (2) transfected with 200 nM of scramble siRNA. (3) There is also a condition were cells were only treated with acetate.

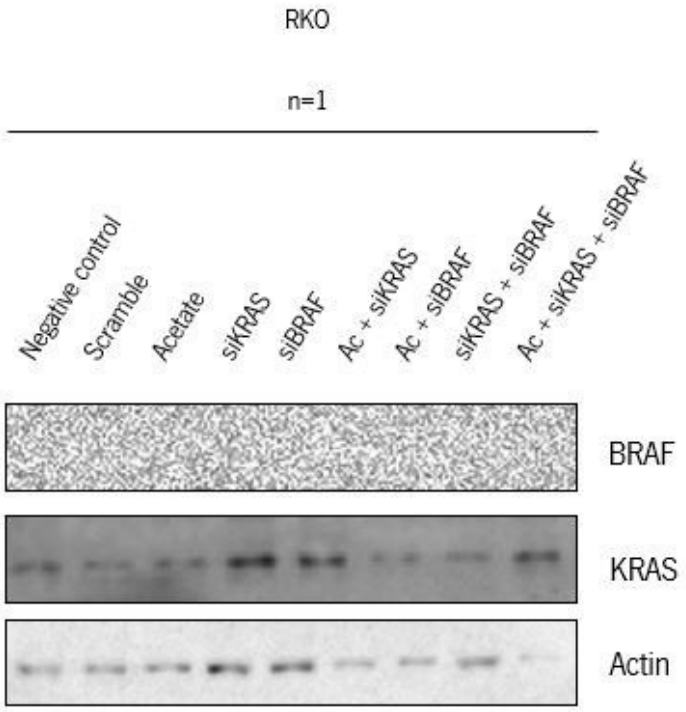


Figure 19 - Effects of acetate treatment in RKO cells when KRAS and/or BRAF are silenced using siRNA.

Western blot analysis for BRAF and KRAS of RKO cells. Cells were transfected with 150 nM of KRAS siRNA and/or 200 nM of BRAF siRNA and then treated or not with 70 nM of acetate. The control cells were not subjected to any treatment or transfected with 200 nM of scramble siRNA. There is also a condition were cells were only treated with acetate. Protein extracts were collected 72h after transfection. Actin was used as loading control. BRAF antibody did not work.

4.2. The role of MCTs in the signalling pathway regulation in CRC cells exposed to acetate

We next decided to test if the downregulation of the MCT-1 and MCT-4 interfere with the effect of acetate in the expression levels of pcRAF signalling molecule. For that, we decided to use HCT-15 cell line in which acetate has more effect in the expression of the pcRAF. We also included another member of the RAF family BRAF.

Firstly, we needed to optimize the concentrations of MCT-1 and MCT-4 siRNA necessary to use in this cell line and with the transfection agent available in our laboratory. After this adjustment, we

proceeded to the inhibition of these to transporters and subsequent treatment of the transfected cells with 70 mM of acetate.

4.2.1. Optimization of MCT-1 and MCT-4 RNA interference conditions

To determine what was the best concentration of MCT-1 and MCT-4 siRNA to use in HCT-15 cells, the first step was to test 10 nM of siRNA because this was the concentration used in previous studies of our group (Figures 20 and 21). However, using this concentration, the efficiency of silencing was low. To improve the efficacy of the MCT-1 and MCT-4 transfection, we decided to double the concentration to 20 nM and proceed the rest of the experiments with this new concentration.

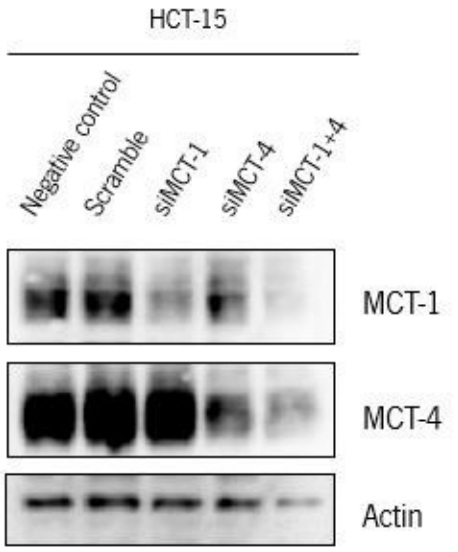


Figure 20 - Test of the MCT-1 and MCT-4 siRNA in HCT-15 cells.

Western blot analysis for MCT-1 and MCT-4 of HCT-15 cells. Cells were transfected with 10 nM of MCT-1 siRNA and/or MCT-4 siRNA, being the control cells not subjected to any treatment or transfected with 10 nM of scramble siRNA. Protein extracts were collected 48h after transfection. Actin was used as loading control.

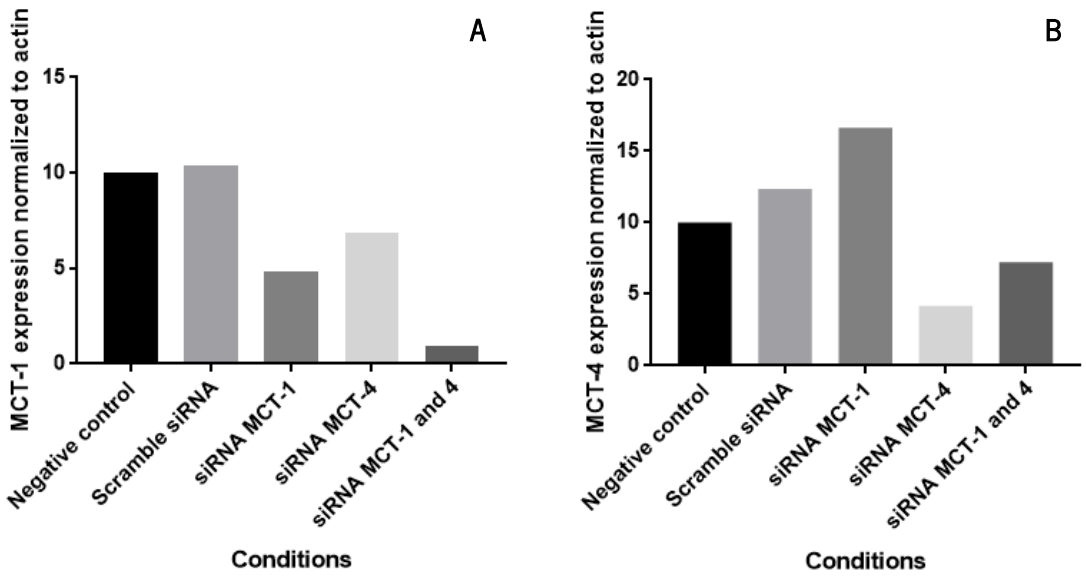


Figure 21 - Test of the MCT-1 and MCT-4 siRNA in HCT-15 cells - quantification analysis.

(A) MCT-1 expression normalized for actin. (B) MCT-4 expression normalized for actin.

4.2.2. Inhibition of MCT-1 and MCT-4 expression in HCT-15 cells using interference RNA

To understand the role of MCTs in the expression of pcRAF and BRAF in CRC cells exposed to acetate, HCT-15 cells were transfected with 20 nM of MCT-1 and/or MCT-4 siRNA with/without 70 mM of acetate solution.

For this experiment we used as controls: cells incubated with medium (negative control), cells transfected with scramble siRNA (scramble). After 72 h of transfection, images of each condition were taken from an inverted bright field microscope, 100x amplification (Figure 22), and a trypan blue assay and protein extraction were simultaneously performed. The protein extracts were used to run western blots, and β actin was used as loading control. The next presented results are representative of one experience, thus very preliminary.

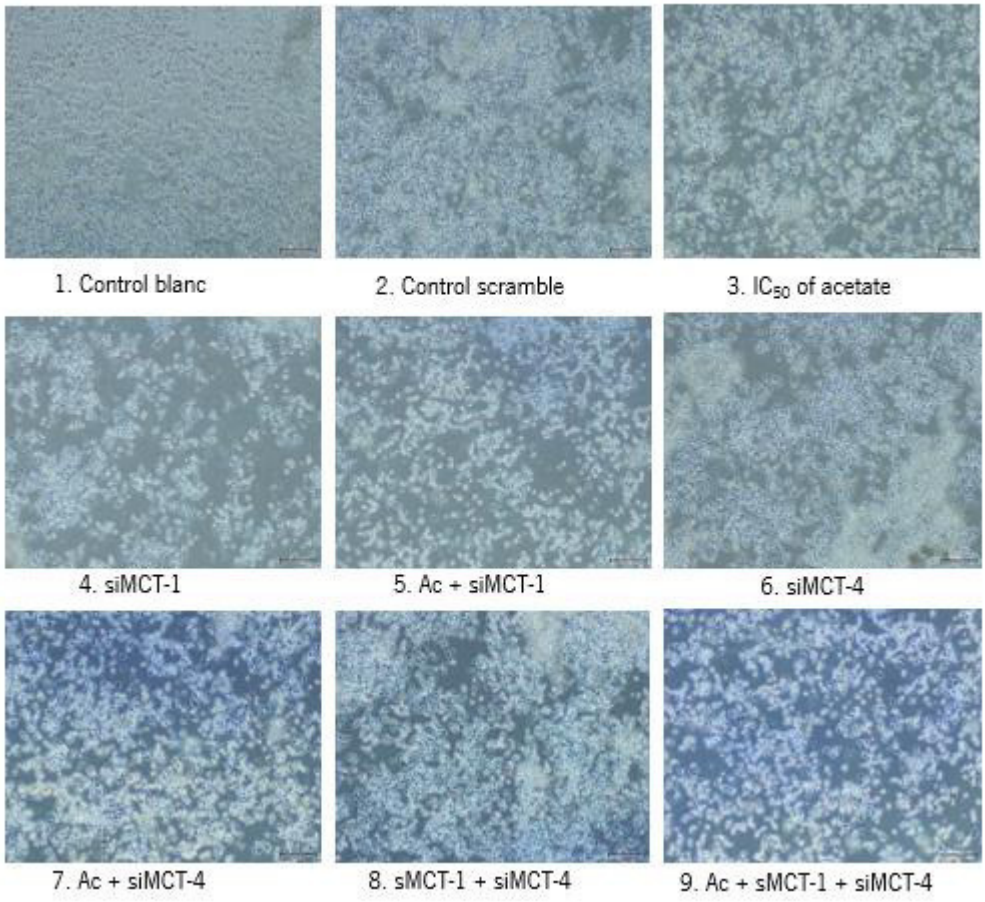
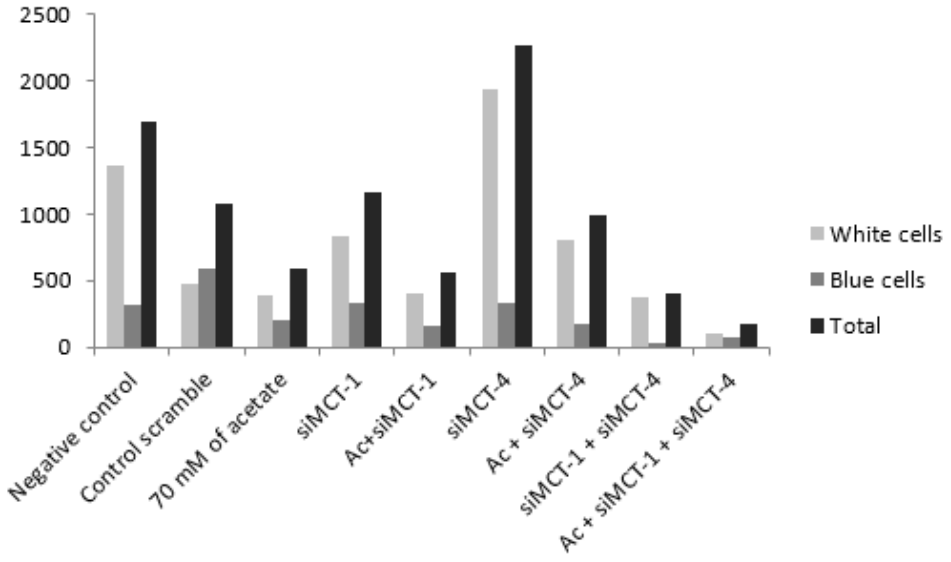


Figure 22 – Analysis of the combination of acetate treatment and MCT-1 and MCT-4 silencing in HCT-15 cells – cell morphology and confluence.

Images obtained from an inverted bright field microscope, 100x amplified of HCT-15 cells transfected with 20 nM of MCT-1 and/or MCT-4 siRNA and then treated or not with 70 nM of acetate. (1) The control cells were not subjected to any treatment or (2) transfected with 20 nM of scramble siRNA. (3) There is also a condition were cells were only treated with acetate.

The microscope images did not reveal significant alterations in the cells morphology or confluence (Figure 22). Also, the trypan blue assay did not indicate a high percentage of cell death (Figure 23). Concerning the western blot analysis, our results showed that the treatment with acetate increases the expression levels of MCT-4 (Figure 24). We could also observe the increase in the levels of MCT-4 protein when MCT-1 is silenced in the presence of acetate, what suggests the existence of some compensation mechanism that ensures the transport of short chain fatty acids when expression of MCT-1 is inhibited (Figure 24).

Furthermore, concerning BRAF protein in conditions where cells are transfected with both MCT-1 and MCT-4 siRNA and then treated with acetate solution, BRAF expression decreased. Compared to the controls (negative control and scramble), we could also observe a decrease in the levels of pcRAF protein in all the conditions, less evident in the conditions where MCT-4 is silenced (MCT-4 siRNA, Ac+ MCT-4 siRNA, Ac+ MCT-1 and 4 siRNA).



	White cells	Blue cells	Total	% of cell death
Negative control	1368	324	1692	19,1
Control scramble	480	596	1076	55,4
70 mM of acetate	386	198	584	33,9
siMCT-1	834	334	1168	28,6
Ac+siMCT-1	398	164	562	29,2
siMCT-4	1934	328	2262	14,5
Ac + siMCT-4	810	178	988	18,0
siMCT-1 + siMCT-4	372	34	406	8,4
Ac + siMCT-1 + siMCT-4	108	70	178	39,3

Figure 23 - Analysis of the combination of acetate treatment and MCT-1 and MCT-4 silencing in HCT-15 cells – Trypan blue assay.

Trypan blue assay of HCT-15 cells transfected with 20 nM of MCT-1 and/or MCT-4 siRNA and then treated or not with 70 nM of acetate. The control cells were not presented to any treatment or transfected with 20 nM of scramble siRNA. There is also a condition were cells were only treated with acetate. The mixture of trypan blue solution with cell suspension was made using a ratio of 1:1.

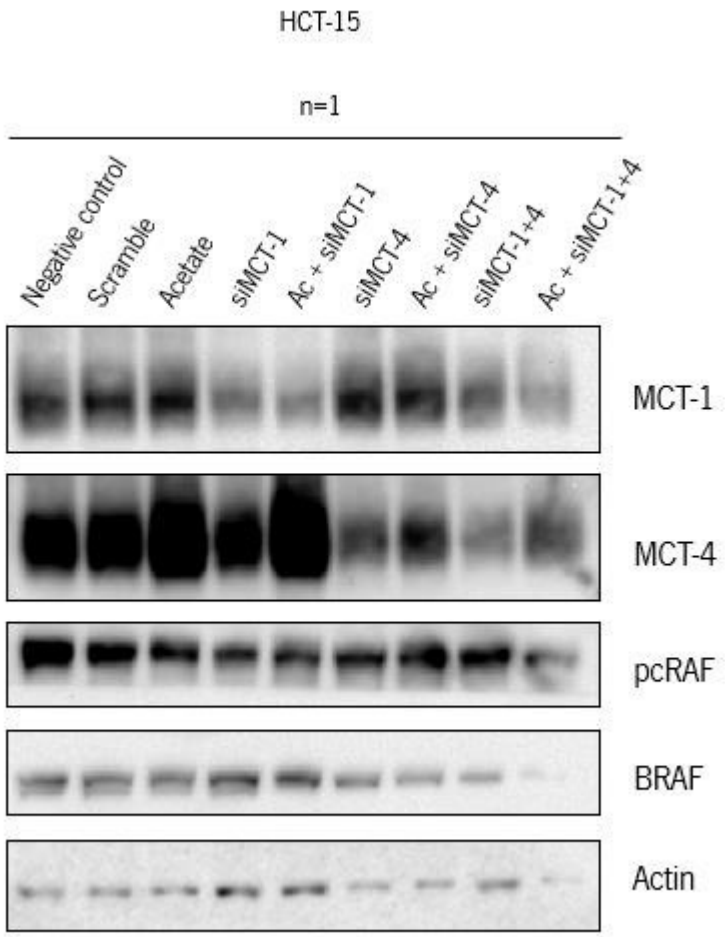


Figure 24 - Acetate treatment alters the MCT-1 and MCT-4 expression in HCT-15 cells.

Western blot analysis for MCT-1, MCT-4, pcRAF and BRAF of HCT-15 cells. Cells were transfected with 20 nM of MCT-1 siRNA and/or MCT-4 siRNA and then treated or not with 70 nM of acetate. The control cells were not subjected to any treatment or transfected with 20 nM scramble siRNA. There is also a condition were cells were only treated with acetate. Protein extracts were collected 72h after transfection. Actin was used as loading control.

CRC is one of the most common solid cancers worldwide, being expected that approximately 4.6% of men (1 in 22) and 4.2% of women (1 in 24) will be diagnosed with CRC in their lifetime [112]. However, CRC incidence varies among different populations according to important risk factors such as dietary habits and lifestyle behaviours, leading to a high mortality rates in Europe. Considering the high CRC incidence, the cancer-related mortality and the variety of risk factors associated with this disease, it becomes crucial to understand its biology and identify specific targets to find new therapeutic approaches for CRC therapy.

Intestinal microbiota can be affected by specific **dietary habits**, being responsible for interacting with the human host and performing many essential functions required for the maintenance of human health. Intestinal microbiota allows the human body to use available energy sources such as carbohydrates and proteins and, in return, the microbiota produce vitamins, synthesizes amino acids, influence the absorption of ions, participate in the conversion of dietary polyphenolic compounds and is involved in the biotransformation of bile acids [79]. There are multiple bacterial species that contribute to CRC, causing alterations in the composition, distribution, and metabolism of the microbiota in the colon what results in changes in homeostasis and consequently promoting inflammation, dysplasia, and cancer [79]. Studies on the protective role of dietary fiber have proven that **SCFAs** namely acetate, propionate and butyrate act as tumor suppressor agents, promoting apoptosis of CRC cells and enhancing the inhibition of tumor cell growth and proliferation.

Despite **acetate** has been the less investigated SCFA, recently, it was reported that it decreases viability and induces typical signs of apoptosis, including loss of mitochondrial membrane potential, generation of ROS, caspase-3 processing and nuclear chromatin condensation. Our group already showed that acetate decreases cell proliferation and induces apoptosis in CRC cells [84]. We also showed that acetate induces lysosomal membrane permeabilization with cathepsin D (CatD) release to the cytosol. Importantly, we revealed that CatD, but not CatB and CatL (also overexpressed in CRC cells), has an anti-apoptotic role in acetate-induced apoptosis. Moreover, we next showed that acetate induces mitochondrial dysfunctions, such as ROS accumulation and an increase in mitochondrial mass accompanied by mitochondrial membrane depolarization [84].

The first aim of this project was to understand the functional role of acetate in the regulation of signalling pathways in CRC cells. We wanted to evaluate the involvement of this agent in the expression levels of several oncogene downstream signalling molecules from two signalling pathways that are known to be altered during cancer development, namely, PI3K/AKT pathway, that is directly related to cellular proliferation, apoptosis and survival, and MAPK pathway, that controls cell growth. To achieve this task, we decided to use two different CRC cell lines, **RKO** and **HCT-15**. While RKO cell line is derived from a primary colonic carcinoma and harbours a $BRAF^{V600E}$ activating mutation, HCT-15 is derived from a specimens of adenocarcinoma of human colon and harbours a $KRAS^{G13D}$ activating mutation [109]. The use of these two different cell lines with mutations in the two major oncogenes associated with colorectal carcinogenesis allow us to understand if the possible effects of acetate treatment could be more efficient in some tumors according to their different genetic background.

The group already determined previously of the half-maximal inhibitory concentration (IC_{50}) of acetate to use for each cell line after 48 h of treatment: 70 mM and 110 mM for HCT-15 and RKO cells, respectively [106]. In our subsequent studies, we decided to compare the effect of three different concentrations, the IC_{50}, the IC_{30} and the $2 \times IC_{50}$. Despite these values have been determined after 48 h of treatment, we wanted to evaluate the effect of the treatment in more than one timepoint to see if by some reason acetate causes some momentary alteration that could not be observed after 48 h. Because of that, we performed protein extraction after 4h, 24h and 48h.

It is already known that mutations of **KRAS and BRAF oncogenes** are frequently found in sporadic CRC and that these mutations lead to constitutive activation triggering the stimulation of the **signalling cascade RAS-RAF-MEK-ERK** [113]. This pathway is responsible for stimulating growth and differentiation of CRC cells, being activated in most of the human cancers and contributing for tumor cell survival, dissemination and resistance to drug therapy [45]. Indeed, it was already described that butyrate and propionate inhibit cell proliferation through the inactivation or downregulation of ERK1/2 in colon cancer cells HT-29 and RKO [114]. Accordingly to previous work from our group that analysed the levels of phosphorylated ERKs in response to acetate treatment and our preliminary results indicated that acetate decreased the levels of phosphorylated ERKs in a dose-dependent manner in both RKO and HCT-15 cell lines after 48 hours of acetate treatment (unpublished data) suggesting a possible role of acetate in the regulation of the RAS-RAF-MEK-ERK signalling pathway [15]. We also considered that it would be interesting to assess the levels of some **PI3K/AKT signalling cascade**

related molecules, that has been shown to be disturbed at some level in many human cancers [115]. This pathway is involved not only in cancer development but also in the potential response to chemotherapy so, some of its downstream effectors are considered attractive pharmacological targets [115].

Taking into consideration what was mentioned, we studied the involvement of acetate in the regulation of phosphorylated AKT, PDK1, PTEN, cRAF, ERK 1 and 2, GSK3β and p38 by western blot, in both cell lines. Our data showed that acetate changes the expression levels of some signalling molecules, in a dose and time dependent manner. We observed a significant decrease in phosphorylated cRAF in both cells lines upon acetate treatment. In fact, our group has already reported that acetate induces apoptosis and inhibits cell proliferation in CRC cell lines so, this result suggests that acetate-induced apoptosis might implicate upregulation of PI3K/AKT signalling pathway and that acetate-inhibition of proliferation might be associated with down-regulation of phosphorylated cRAF. Also, in HCT-15 cells it is possible to observe a significant decrease of pERK expression after 4h, when cells were treated with 70 mM or 100 mM of acetate. This is a tendency that, although not significant, is also observed in RKO cells. The observed decrease in pERK expression levels suggest that acetate might inhibit the growth of CRC cells, what is also visualized in the microscopy images (the wells treated with acetate present a low confluence of cells, when compared with untreated wells). Because HCT-15 cell line harbours a mutation on KRAS oncogene, KRAS oncogene is the main oncogene mutated in CRC and is a main player in ERK pathway, this might be the reason why we observed more markedly the differences in the pERK expression levels in HCT-15 cells. To the best of our knowledge there is no reports in the literature related to the role of acetate in oncogenic signalling pathways.

As it was mentioned before, being KRAS and BRAF the major oncogenes implicated in sporadic colorectal carcinogenesis, one of the objectives of this work was to understand the mechanisms underlying the role of acetate in the crosstalk between the glycolytic metabolism and oncogenic signalling. For that, our propose was to downregulate KRAS and/or BRAF expression by RNA interference in cells exposed to acetate and study the metabolic phenotype by analysing the expression of several metabolic markers as well as analyse the expression of downstream signalling molecules such as phosphorylated cRAF, AKT and ERK. Unfortunately, due to various technical problems, we were not able to respond to this aim. Despite our best efforts, we could not optimize KRAS silencing, since both KRAS antibody and KRAS siRNA did not work.

However, if it is important to understand the biological effects of acetate treatment, it is also crucial to understand how its transport to the intestinal epithelial cells occur. Currently, experimental evidence has already shown that acetate uptake is mediated by secondary transporter proteins, such as the **MCTs**. These transporters are overexpressed in several types of cancer cells and it has been shown that upon butyrate treatment, MCT-1 is the most abundant MCT isoform expressed in the CRC Caco-2 cells [116]. Also, it was already reported that these transporters can also be used to transport of drugs into cancer cells, behaving as "Trojan horses" [7]. Our group already showed that MCTs are able to mediate the entry of the chemotherapeutic agent 3BP in breast cancer cells and that butyrate increased its uptake by inducing the levels of MCT-4 expression [7]. Focusing on acetate, our group has proven for the first time that acetate upregulates specifically MCT-1, MCT-4 and CD147 expression in CRC cells [15, 84]. We have also discovered that, interestingly, other transport mechanisms such as passive diffusion through aquaporins might also contribute to acetate uptake, however, this issue was not explored in this Master thesis [15, 84]. Furthermore, we also showed that acetate promotes plasma membrane re-localization of MCT-1, triggering changes in glucose metabolism by increasing both glucose consumption and lactate production, what contributes for CRC cell glycolytic phenotype [15, 84].

Considering that cancer cells usually express high levels of MCTs to maintain a highly glycolytic profile and that these proteins are involved in acetate uptake, we decided to study the role of MCTs in KRAS/BRAF signalling pathway regulation in CRC cells exposed to acetate. Our aim in this task was to understand if when we downregulate MCT-1 and/or MCT-4 expression using RNA interference, there is some alteration in the effects of acetate in signalling molecules analysed. Importantly, based on the results from the first task, we decided to proceed with this work using only the HCT-15 cell line in which we obtained the most significant differences in pcRAF and ERK. Previous results from our group have showed that upon silencing of MCT-1 and/or MCT-4 no changes were observed in the expression levels of some signalling molecules analysed, namely phosphorylated AKT and ERK (unpublished data, see Figure 26 in Annex). Here, we evaluated the impact of acetate in addition to the silencing of MCT-1 and/or MCT-4 and our preliminary results have shown that acetate treatment increases of the expression levels of MCT-1 and MCT-4, supporting what our group has already reported [84]. Also, for the first time we have seen that when MCT-1 is silenced in the presence of acetate, there is an increase in the levels of MCT-4 protein, suggesting the existence of some compensation mechanism that

ensures the transport of fatty acids even when there is something blocking the expression of MCT-1. Moreover, when cells are transfected with both MCT-1 and MCT-4 siRNA and then treated with acetate solution, BRAF expression seems to decrease. Moreover, we also observed decrease in the levels of pcRAF protein in all the conditions, less evident in the conditions where MCT-4 is silenced. Although preliminary our results suggest for the first time a possible role of MCTs expression in controlling acetate signalling in CRC cells.

In this work, we explored the role of acetate in CRC cells in order to understand its crosstalk with oncogene signalling pathways, glycolytic metabolism and MCTs expression regulation. Despite the technical problems faced during the development of the project, the results herein presented suggest that acetate treatment is able to modulate the expression levels of some KRAS/BRAF downstream signalling proteins, namely phosphorylated cRAF and ERK, in a time and dose-dependent manner (Figure 25). We also showed that acetate might regulate its uptake in CRC cells by the involvement of MCTs expression regulation. Indeed, the most surprising result showed that MCT-1 silencing leads to the overexpression of MCT-4, in the presence of acetate, suggesting that CRC cells might activate some mechanism of compensation to ensure the transport of this SCFA. Moreover, our results also suggest for the first time a possible role of MCTs in the regulation of pcRAF and BRAF expression in response to acetate exposure.

To the best of our knowledge this was the first time that the interplay between acetate and two important hallmarks of cancer namely oncogene activation and metabolism reprogramming, was studied. Although further studies are needed in order to confirm the preliminary results obtained, our study might have relevant implications in the prevention/therapy of CRC.

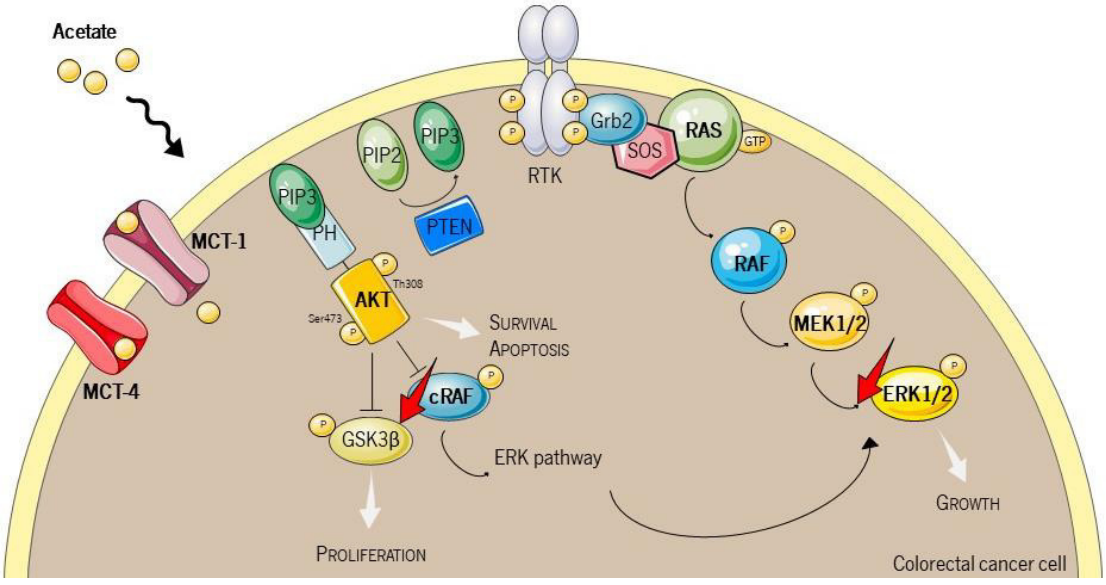


Figure 25 – A schematic representation of the changes caused by acetate exposure on the expression levels of some signalling molecules, namely phosphorylated cRAF and ERK in CRC cell.

This Master thesis is the beginning of an innovative work that still needs to be improved and developed in order to further clarify several aspects. In this project we needed to optimize almost all the experiences and faced several technical limitations and consequently, it was difficult to obtain the expected results. Our first problem was the KRAS RNA interference that always worked in the lab [117] and despite testing two different samples of KRAS siRNA none of them worked. Moreover, the KRAS antibody that has been used in the lab for years also stop working. In fact, we tested several different KRAS antibodies aliquots with different dilutions, different protein concentration and even using different buffers (5% w/v BSA or 5% w/v non-fat dry milk in TBS/tween 20) we did not obtain any result. We believe it might be due to the strength of the antibody that was already discontinued by Santa Cruz that supplied it. Considering the limitations that we faced during the development of the project, and because this is a promising work we intend to finish the established aims and perform the triplicate experiments missing.

We also believe that there are other important questions need to be clarified, thus we propose the following experiments:

- **Test CRC cell uptake of radiolabelled acetate.**

  Several studies on the uptake of radiolabelled acetate in cancer cells have been conducted. For example, Yoshimoto et al. studied the [$^{1\text{-}14}$C] acetate uptake and metabolic fate under normoxia in several cancer cell lines including colon adenocarcinoma, nasal septum quasi-diploid tumor, ovary carcinoma and melanoma [118]. In addition, Hara et al. reported that [$^{1\text{-}14}$C] acetate uptake is accelerated under hypoxia in prostate cancer cells [119]. In this particular case, our idea is to measure the [$^{1\text{-}14}$C] acetate uptake in CRC cells (HCT-15 cells, for example) and compare with cells with silence for MCT-1 and MCT-4 using siRNA. We expect to be able to understand if acetate enters CRC cells via MCTs or if there is some alternative mechanism for its uptake.

- **Search for possible acetate receptors in the membrane of CRC cells.**

  It is already known that GPR43 is a G-protein-coupled receptor for SCFAs that specifically binds to acetate, propionate and butyrate [120]. However, when its expression was compared in a range of 9 different colon cancer cell lines through RT-PCR analyses, it was detected in only

one cell line (HT-29) [120]. This suggests that, contrary to what happens in normal cells, this receptor could be downregulated in colon cancer cells, so, for our work, we should explore more options.

GPR109A is another G-protein-coupled receptor that is expressed in the lumen-facing apical membrane of colonic and intestinal epithelial cells and that the receptor recognizes butyrate as a ligand [121]. Like GPR43, it is silenced in colon cancer in humans, in a mouse model of intestinal/colon cancer and in colon cancer cell lines. In turn, GPR109B has high homology to GPR109A in primary structure but it is expressed almost at normal levels in colon cancer [121]. So, it could be interesting to know if acetate is a possible ligand to GPR109B in colon cancer cells.

In conclusion, the results presented in this Master thesis provide evidence for the involvement of acetate in the regulation of signalling pathways and in MCTs feedback regulation in CRC cells, encouraging the exploitation of this issue in future studies.

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ANNEX

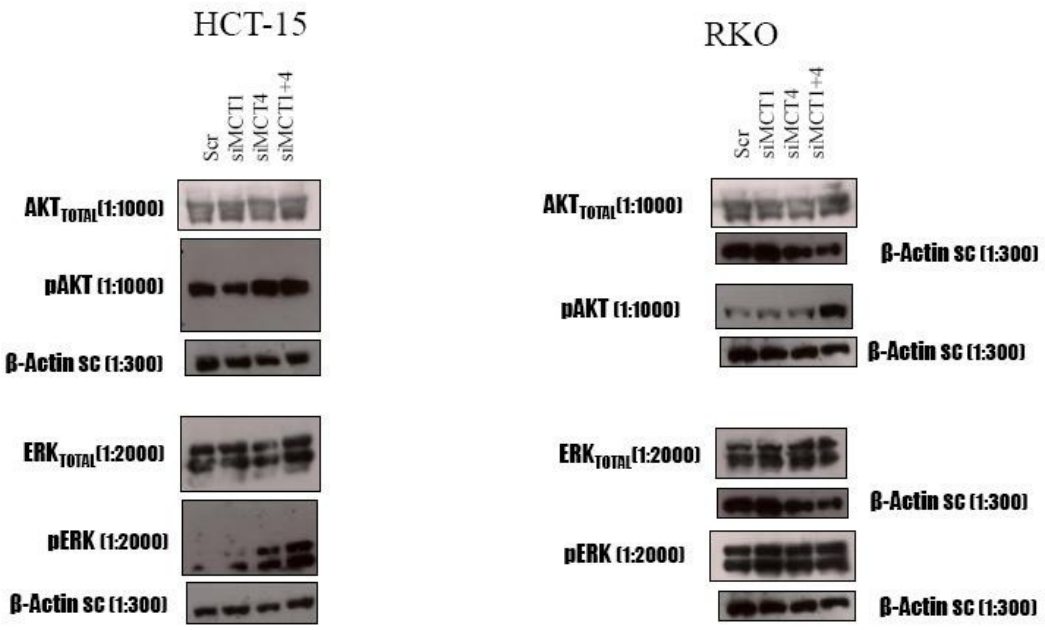

Figure 25 – Unpublished result of a western blot analysis for total AKT, phosphorylated AKT, total ERK and phosphorylated ERK of HCT-15 and RKO cells.

Cells were transfected with 20 nM of MCT-1 siRNA and/or MCT-4 siRNA. The control cells were transfected with 20 nM scramble siRNA. Protein extracts were collected 72h after transfection and β-actin was used as loading control.

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The Role of Diet Related Short-Chain Fatty Acids in Colorectal Cancer Metabolism and Survival: Prevention and Therapeutic Implications.

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Abstract: Colorectal cancer (CRC) is a major cause of cancer-related death worldwide. CRC increased risk has been associated with alterations in the intestinal microbiota, with decreased production of short chain fatty acids (SCFAs). SCFAs produced in the human colon are the major products of bacterial fermentation of undigested dietary fiber and starch. While colonocytes use the three major SCFAs, namely acetate, propionate and butyrate, as energy sources, transformed CRC cells primarily undergo aerobic glycolysis. Compared to normal colonocytes, CRC cells exhibit increased sensitivity to SCFAs, thus indicating they play an important role in cell homeostasis. Manipulation of SCFA levels in the intestine, through changes in microbiota, has therefore emerged as a potential preventive/therapeutic strategy for CRC. Interest in understanding SCFAs mechanism of action in CRC cells has increased in the last years. Several SCFA transporters like SMCT-1, MCT-1 and aquaporins have been identified as the main transmembrane transporters in intestinal cells. Recently, it was shown that acetate promotes plasma membrane re-localization of MCT-1 and triggers changes in the glucose metabolism. SCFAs induce apoptotic cell death in CRC cells, and further mechanisms have been discovered, including the involvement of lysosomal membrane permeabilization, associated with mitochondria dysfunction and degradation.

In this review, we will discuss the current knowledge on the transport of SCFAs by CRC cells and their effects on CRC metabolism and survival. The impact of increasing SCFA production by manipulation of colon microbiota on the prevention/therapy of CRC will also be addressed.

Keywords: Colorectal cancer, short chain fatty acids, microbiota, cell death mechanism, metabolism, cell death, membrane transport.

1. INTRODUCTION

Understanding the normal colon microbiota environment, how it changes in colorectal cancer (CRC), as well as the concentration of short chain fatty acids (SCFAs) and the physiological effects of these metabolites in the normal colon, is important. This knowledge might help in finding new adjuvant strategies to prevent or treat CRC. Here, we focus on the anti-neoplastic role of SCFAs, namely butyrate, propionate and acetate, in CRC cells and discuss how a probiotic-rich diet could increase the levels of SCFAs in the colon.

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1.1. Incidence and risk factors of colorectal cancer: impact of diet and microbiota in short chain fatty acid production

Cancer remains a leading cause of death in both more and less economically developed countries and is thus considered a major public health problem. Based on GLOBOCAN reports, CRC is one of the three most commonly diagnosed cancers in men and women, being the main cause of cancer-related deaths in women [1].

CRC is associated with exposure to environmental factors and to the accumulation of several genetic and epigenetic factors [2]. This type of cancer has the highest incidence rates in Australia/New Zealand, Europe, and Northern America and the lowest in Africa and South-Central Asia [1].

The increasing incidence of CRC in young populations has increased the need to disseminate preventive measures, including maintenance of a healthy body weight, physical activity, minimization of red and processed meat and alcohol

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consumption, and avoidance of smoking [1, 3]. It has been demonstrated that specific changes in human intestinal microbiota (dysbiosis) can also be associated to sporadic CRC [4-6], supporting the hypothesis that colon cancer may be a bacteria-related disease [4, 5], characterized by the involvement of some specific bacterial species entailed in CRC pathogenesis (Figure 1).

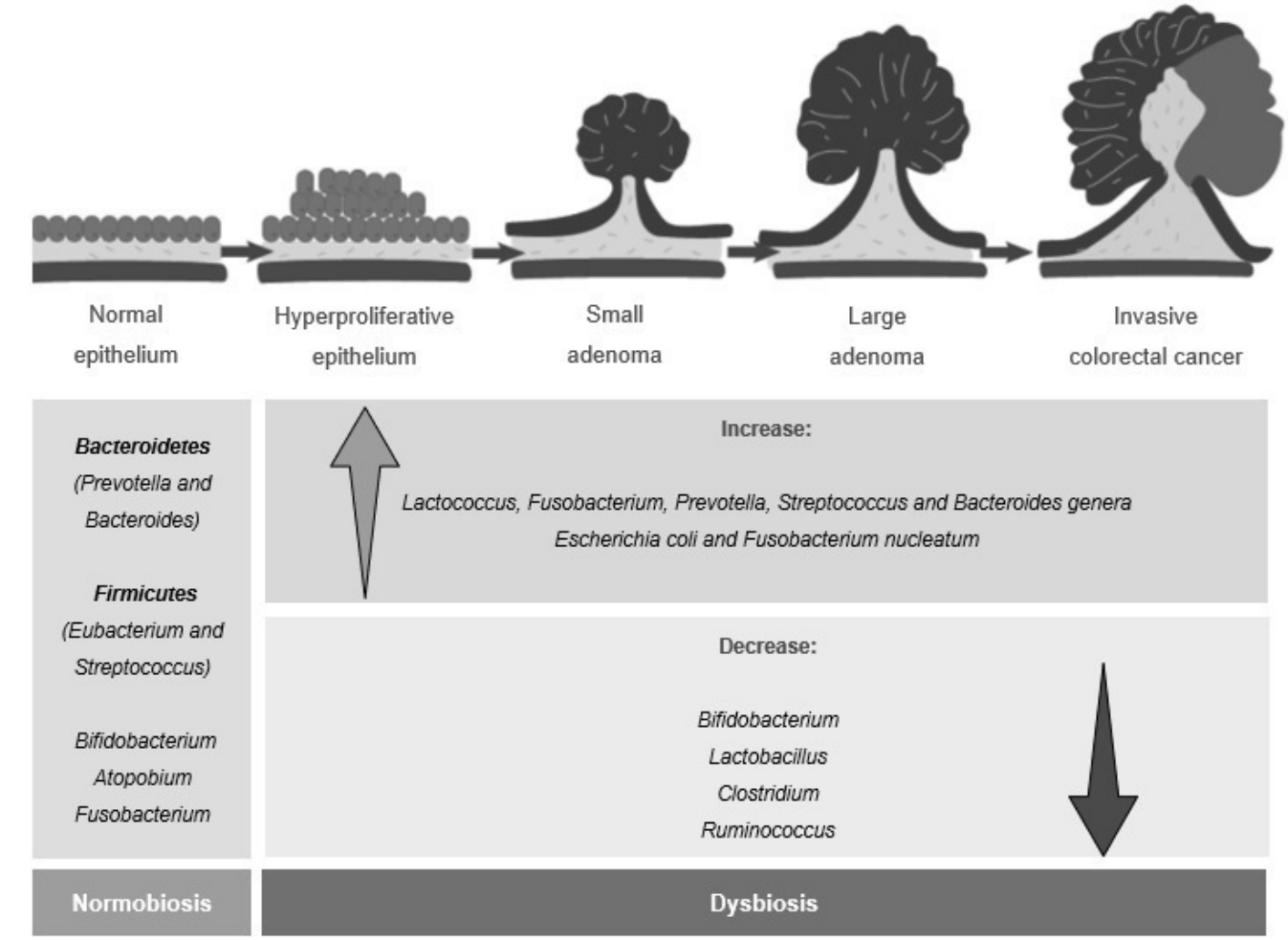

Figure 1 Schematic comparison between the predominant composition of the intestinal microbiota in normobiosis and *during* colorectal carcinogenesis.

The high prevalence of CRC that has been observed has led to an increasing and urgent need to develop new and more effective therapies. As such, the molecular and biological basis of CRC is a current subject of study for several scientists all over the world.

1.1.1. Normal Colon Microbiota and Short-Chain Fatty Acids

The human intestine harbors as many as 10^{12} microorganisms composed by 500 - 1000 different bacterial species [5, 7, 8]. Although the intestinal the intestinal microbiome has a very varied composition, in normal symbiotic state (normobiosis), it is predominantly constituted by *Bacteroidetes* species, such as *Prevotella* and *Bacteroides* genera, and *Firmicutes* species, such as *Eubacterium* and *Streptococcus* genera. Bifidobacterium, *Atopobium* and *Fusobacterium* are also bacterias presented in high amounts [7, 9-12] (Figure 1). This intestinal microbiota constitutes a very complex system with numerous beneficial roles to human health, including protection against pathogens, maturation of the immune system, degradation of toxic

substances, digestion of complex carbohydrates and production of SCFAs [12, 13].

SCFAs, namely acetate, propionate and butyrate, are produced by anaerobic microorganisms able to ferment polysaccharides, oligosaccharides, proteins, peptides and glycoproteins in the colon, including *Firmicutes*, *Clostridium*, *Bifidobacterium, Propionibacterium, Bacteroides* and *Lactobacillus* [10, 14-16]. The ability to produce butyrate requires a specific enzymatic process via butyryl-CoA: acetate CoA-transferase or via butyrate kinase, with *Firmicutes* species able to produce butyrate by both pathways [7, 13, 16]. Propionate is generally produced by *Bacteroides, Firmicutes* and *Propionibacterium* species which involve the succinate, acrylate and propanediol pathways [13, 16, 17], while acetate is produced by acetogenic bacteria such as *Acetobacterium* species, *Clostridium aceticum* and *Propionibacterium,* able to perform reductive acetogenesis from formate [13, 16]. Normally, these bacteria exist in a mutually beneficial symbiotic relationship in the human colon, providing the amount of these SCFAs necessary to maintain colon homeostasis [8]. Indeed, it has been shown that some changes in SCFA production are directly associated with alterations in the intestinal microbiota, modulated by numerous extrinsic factors such as diet, age, medication, clinical treatment (drugs, radiation, surgery), physiological stress and diseases [8, 18]. These changes in the normal symbiotic state can be associated with many intestinal disorders, such as obesity, inflammatory bowel disease (IBD) and CRC [5, 7, 19, 20].

Interesting, despite mucosal immunity functions to protect the host from exogenous, generally microbial threats, it has been proved several benefits provided by our microbiota. As example, it was showed that several species of normal human gut bacteria can induce rapid generation of ROS within epithelial cells, having significant signaling effects on innate immunity, proliferation, and epithelial movement and restitution [21].

1.1.2. Intestinal Microbiota and Short-Chain Fatty Acids in Colorectal Cancer Patients

Most studies have shown a significant difference between the bacteria genera associated with cancerous and non-cancerous intestinal tissue or fecal samples [4, 5, 22]. The intestine microbial composition of CRC patients has been associated with an increase in the *Lactococcus*, *Fusobacterium, Prevotella, Streptococcus* and *Bacteroides* genera (Figure 1) [4, 6, 22]. It has been demonstrated that some bacteria have a complex arsenal of virulence factors which allow them to colonize and persist in the intestine, inducing chronic inflammation, accumulating mutations by DNA damage, genotoxin formation, increased T-cell proliferation and biosynthesis of pro-carcinogenic compounds that interfere with cell cycle regulation, contributing to colorectal carcinogenesis [4, 8, 22-24].

Also, several studies reported that *Escherichia coli* and *Fusobacterium nucleatum* are the main bacteria associated with CRC and commonly over-represented in many tumor tissues from CRC patients (Figure 1) [4, 5, 22-24]. In healthy individuals, *E. coli* and other members from the same bacterial family constitute less than 1% of the gut microbiota. However, in individuals with IBD, in several animal models of gut inflammation and in CRC cases, *E. coli* becomes dominant [24]. *E. coli* strains isolated from individuals with IBD and CRC are often adherent and invasive, displaying several pathogenic properties. It possesses an array of virulence factors involved in host invasion, formation of inflammatory lesions, which then synthesize NO, leading to oxidative stress and genomic instability, supporting an inflammation-carcinoma sequence [22]. Moreover, *F. nucleatum* is an opportunistic bacterium able to induce chronic inflammation and interact directly or indirectly with colonocytes, leading to uncontrolled cell proliferation, dysbiosis and intestinal tumorigenesis [23, 25]. Recently, *F. nucleatum* has been considered as an indicator of disease progression and tumor severity [23].

In contrast, patterns that preserve microbial intestinal homeostasis, such as *Bifidobacterium*, *Lactobacillus*, *Clostridium* and *Ruminococcus* are under-represented in CRC patients compared to healthy individuals (Figure 1) [5, 6, 20, 26].

These changes in the intestinal microbiota of CRC patients affect the amount and types of metabolites produced by colonic microbes. In this regard, some studies reported that changes in colon microbiota are associated especially with a decrease in SCFA concentrations accompanied by an elevated pH in patients with CRC compared to healthy individuals [5, 6, 27]. However, it is not yet understood if the changes in colon microbiota are a cause or a consequence of CRC development. In this context, it is important to characterize the possible over- and under-represented bacteria, exploring the relationship between changes in the colon microbiota composition with the production of bacterial metabolites, specifically SCFAs, as well as the tumorigenic process of CRC.

1.1.3. Short-Chain Fatty Acid Concentrations in the Colon

SCFAs are the major products of bacterial fermentation of undigested dietary fiber and of starch resistant to digestion on the human colon [7, 14]. Thus, diets rich in fiber, resistant starch and complex carbohydrates lead to an increase in the levels of colonic SCFAs [14, 15].

SCFAs are carboxylic acids with aliphatic tails ranging from formic (C1), acetic (C_2), propionic (C_3), butyric (C_4), valeric (C_5) and caproic (C_6) acid [15]. They constitute approximately two-thirds of the colonic anion concentration (~150 mM) and, at physiological pH, they prevail in their dissociated form [28]. The rate and amount of SCFAs produced depend on the composition of the diet, which

determines the type of the fermented substrate, the microbiota present in the colon and the gut transit time [7, 29, 30], being usually generated by carbohydrate fermentation according to the following equation [7] :

$$59\ C_6H_{12}O_6\ (Glucose) + 38\ H_2O = 60\ CH_3COO^-\ (Acetate) + 22\ C_2H_5COO^-\ (Propionate) + 18\ C_3H_7COO^-\ (Butyrate) + 96\ CO_2 + 256\ H^+ + heat + gases$$

Acetate, propionate and butyrate are the three major colonic SCFAs found at considerably high concentrations [31-34]. Although not constant, the percentual concentration values of SCFA in the colonic lumen is approximately 60% acetate, 25% propionate and 15% butyrate [34, 35]. In addition, there are significant differences in the total concentration of these SCFAs along the human intestine as follows: approximately 131 ± 9 mmol/L in the cecum (where the amounts of fermentable substrates are higher), ~ 80 ± 11 mmol/L in the descending colon and ~ 13 ± 6 mmol/L in the rectum [36, 37]. This variation in acid concentration is linked to pH differences along the human colon (Figure 2), being the higher acidity associated with a more active carbohydrate fermentation in the cecum than in the sigmoid/rectum, where the pH is more alkaline [37].

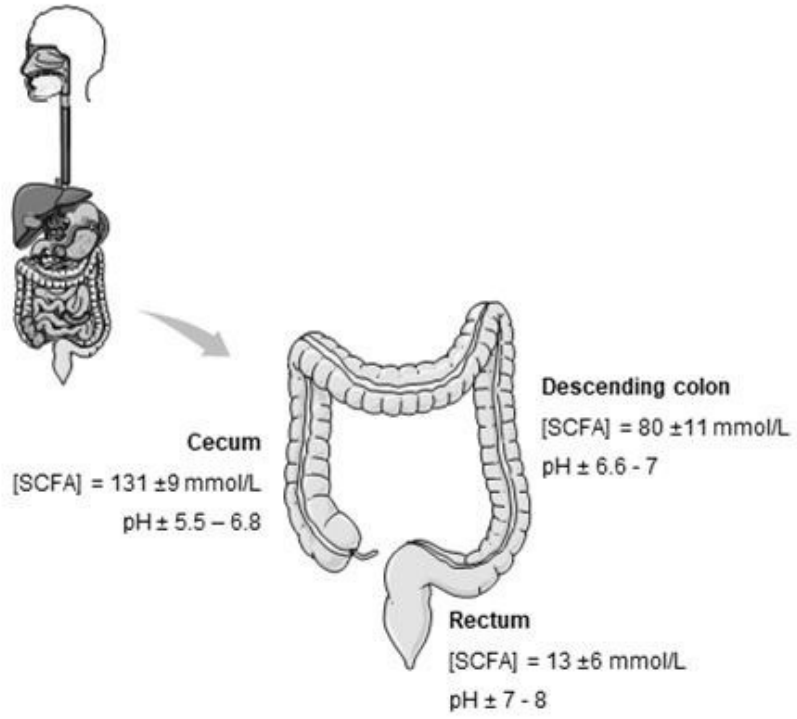

Figure 2 Regions of the colon and rectum with respective SCFA concentrations and pH.

1.2. Short-chain fatty acid transporters and receptors

Production of total colonic SCFAs is difficult to estimate since more than 95% of these SCFAs are water soluble and rapidly absorbed and metabolized by colonocytes with only 5% being excreted in the feces [7, 16, 37]. They are important energy substrates which contribute to up to 70% of the energy requirements of the colonocytes, being the remaining transported through the portal vein into the liver [38].

Distinct mechanisms for SCFA absorption across the plasma membrane of colonocytes in various species have been postulated [28, 39] and most studies show that both intestinal epithelial cells and CRC cells use butyrate as a major energy source [40-42]. However, information regarding transport of the other SCFAs like propionate or acetate is still scarce.

Acetate, propionate and butyrate display pKa values of 4.76, 4.87 and 4.82, respectively. At neutral pH, less than 1% of these fatty acids exist in the protonated form, which means that these compounds do not cross the plasma membrane via

simple diffusion [43]. Instead, their transport to the intestinal epithelial cells is mediated by transporter proteins like monocarboxylate transporters (MCTs) [41]. MCTs belong to the SLC16A family of proteins that comprises 14 members, however, only the first four (MCT-1 to 4) are known to mediate the proton-coupled transport of monocarboxylic acids across the plasma membrane [44, 45]. The main function of MCTs has been associated with the uptake or efflux of monocarboxylates such as, L-lactate, pyruvate, acetate, propionate, D,L-β-hydroxybutyrate and acetoacetate, through the plasma membrane, according to the cell metabolic needs [46]. It was already described an upregulation of MCTs at the plasma membrane of different type of cancer cells, namely in colon, central nervous system, breast, lung, gynecologic tract, prostrate and stomach. The high expression of MCTs has been associated to an adaptive mechanism to allow continuous high glycolytic rates by increasing the lactate transport produced due to the metabolic switch also known as the "Warburg Effect" [47-54]. It is also important to refer that MCTs, specifically MCT-1 is a mediator of lactate uptake, not only in cancer cells but also in stromal cells (such as endothelial cells and fibroblasts) [55].

Several studies have been reported the role of MCT-1 in the transport of the different SCFAs in different models. Broer et al use *Xenopus laevis* oocytes to show that MCT1 mainly contributed to acetate transport [43]. Moreover, based on results of in vivo and in vitro functional studies, it was showed, for the first time, that MCT1 has a direct role in the transepithelial transport and efflux of SCFA across the stratum spinosum and stratum basal of the forestomach toward the blood side [56]. The same research group reported that MCT1 provides a major route for SCFA efflux across the basolateral membrane of the epithelial cells lining the bovine large intestine and that it could play a role in the regulation of intracellular pH [57]. In summary, there are a lot of reports showing the major role of MCT-1 in the transport of SCFAs across the plasma membrane of several cellular models.

Together with MCT-1, SMCT-1 has been identified as the main monocarboxylate transporter responsible for the uptake of SCFAs across the membrane of intestinal cells [16, 41, 58]. SMCT-1 is a Na^+-coupled transporter for a variety of SCFAs, especially butyrate [35, 40], while MCT-1 is a H^+-coupled transporter for SCFAs and related carboxylic acids, which transports these molecules across the plasma membrane depending on the H^+ electrochemical gradient, which contributes to the regulation of intracellular and extracellular pH [16, 47]. MCT-1 is widely expressed in many different cell types and has been characterized as the primary butyrate transporter in the colon epithelial cells [35, 59].

MCT-1 also exports lactate to the extracellular milieu, which is a potentially cytotoxic metabolic by-product of glycolysis. This indicates that MCT-1 plays a dual role in CRC considering that when lactate is exported it promotes cell survival, whereas when it imports butyrate, it induces cell death [59].

It has also been shown that CRC cells silence SMCT-1 by DNA methylation [40-42, 60, 61], which confers a selective advantage to escape butyrate-induced cell death through limitation of butyrate uptake [40, 62].

Recently, our group showed that the transport of acetate occurs via SMCT-1 and by passive diffusion via aquaporins in CRC cells. However, MCT-1 is also involved in acetate uptake in cells that overexpress both MCT-1, MCT-4 and CD147, upon culture in medium with acetate [63]. We found that acetate promotes plasma membrane re-localization of MCT-1 and further triggers changes in glucose metabolism by increasing both glucose consumption and lactate production, thus increasing CRC cell glycolytic phenotype. This work provided new evidence for the role of acetate in the regulation of MCT-1 expression, thus influencing its own transport and increasing the sensitivity of CRC cells to acetate, as well as to other agents that are transported via MCT-1 [63].

In addition to SCFA transporters, their receptors constitute a new and rapidly growing field of research as more functions of SCFA receptors have been discovered. SCFAs are able to activate cells through several cell-surface G-protein-coupled receptors (GPRs), especially GPR41 and GPR43 [64, 65], involved in immune response regulation [64]. These two GPRs are expressed not only by intestinal epithelial cells where SCFAs are produced, but also at multiple other sites considered to be metabolically important such as adipose tissue and pancreatic islets [15]. Recent studies have identified the cell-surface receptors GPR41, GPR43 and GPR109A as essential for the biologic effects of SCFAs in the colon [40, 64]. GPR41 (also called free fatty acid receptor 3; FFA3) expressed in colon cells is activated by SCFAs, primarily by butyrate and propionate [15, 65], while GPR43 (free fatty acid receptor 2; FFA2) has a higher affinity for propionate and acetate [15, 40]. In addition, GPR109A (hydroxycarboxylic acid receptor 2), a receptor for niacin and vitamin B3, is also a receptor for butyrate in colon cells [16, 65]. Nevertheless, the role of SCFA transporters and receptors in CRC cells are still poorly understood and more studies are needed to grasp how these metabolites can influence the regulation of their own transporters and receptors in CRC cells.

1.3. Metabolism of short-chain fatty acids in normal colonocytes versus colorectal cancer cells

SCFAs derived from the microbial metabolism are energy substrates for epithelial cells, and are converted by colonocytes into glucose, ketone bodies, and amino acids [42, 58, 66]. Previous studies showed a preferential use of SCFAs by colonocytes as follow, butyrate > propionate > acetate, being butyrate the major energy source for colonocytes and

also for liver and muscle tissues [7, 13, 14, 67]. Butyrate is usually metabolized to acetyl-CoA by colonocytes [68] while propionate and acetate can modulate lipogenesis and gluconeogenesis [69]. It has been demonstrated that propionate is largely metabolized in the liver [13] and also acts as substrate for gluconeogenesis inhibiting cholesterol synthesis in the hepatic tissue [14, 70]. Acetate can be oxidized in the tricarboxylic acid (TCA) cycle, being used as a substrate for synthesis of cholesterol, long-chain fatty acids or as a co-substrate for glutamine and glutamate synthesis by colonocytes [7, 14, 58]. This compound also enters the systemic circulation and can be used by many tissues, including heart, fat, kidney, liver and muscle [58, 70].

Butyrate is the primary energy source of normal colonocytes, however, due to the Warburg effect, cancerous colonocytes rely on glucose as their primary energy source (Figure 3) [68]. The fact that butyrate can have opposing effects on the growth of normal versus cancerous colonocytes is a paradox that could be explained based on this metabolic adaptation. Butyrate may stimulate the growth of normal colonocytes by functioning as an oxidative energy source, since it is metabolized by β-oxidation followed by the tricarboxylic acid cycle. In cancerous colonocytes, butyrate may inhibit the growth of these cells because, it is metabolized inefficiently due to the Warburg effect, it accumulates in the nucleus, and functions as an HDAC inhibitor, upregulating the expression of downstream target genes [68]. However, to better understand this paradox, additional studies are needed to understand how the metabolic differences between normal and cancerous colonocytes can be used to inhibit tumorigenesis in CRC.

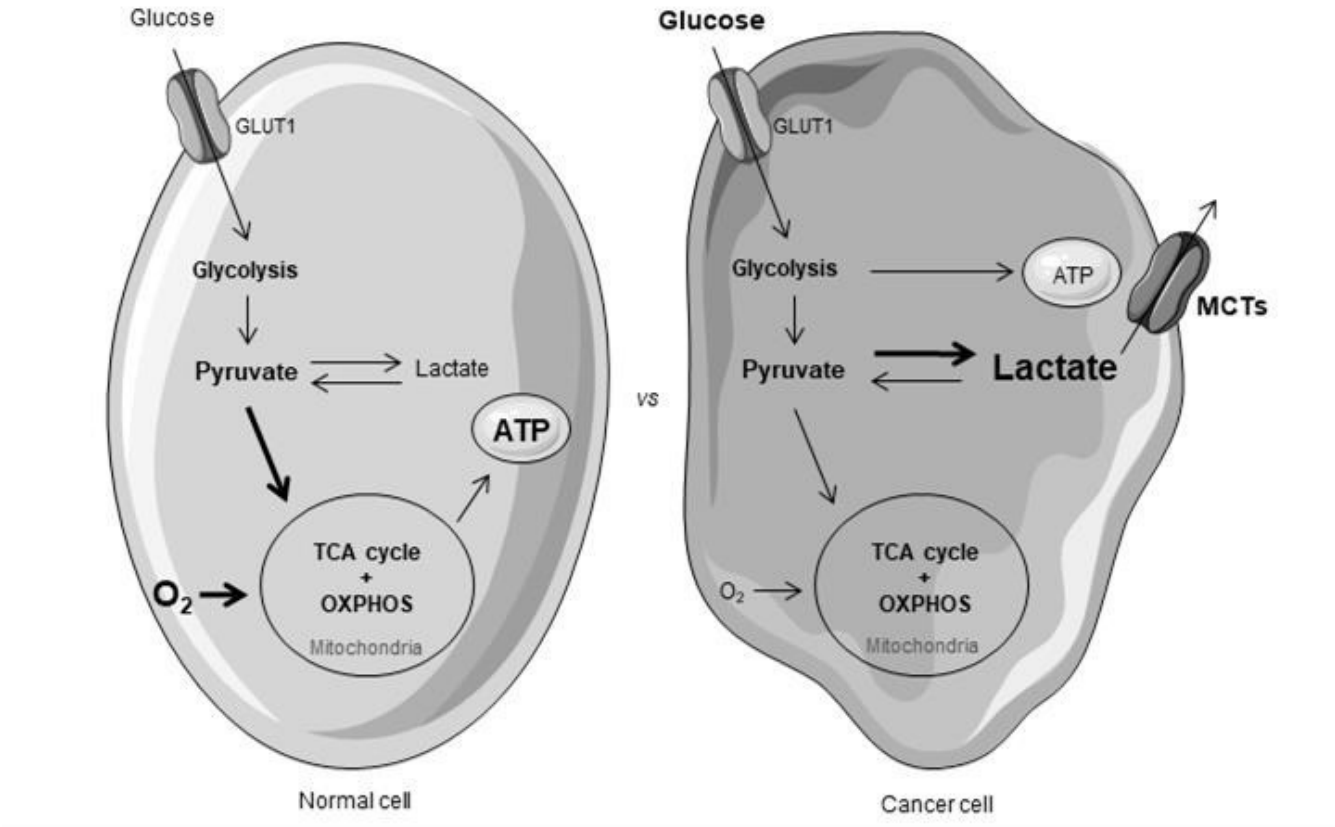

Figure 3 Glucose metabolism in normal cells versus in cancer cells – the Warburg effect.

1.4. Mechanisms of action of short-chain fatty acids in normal colon and in colorectal cancer cells

Several studies have shown that butyrate, propionate and acetate induce apoptosis in CRC cells but not in normal cells [69, 71-75]. The anti-cancer effect of SCFAs is also supported by epidemiological studies suggesting an inverse relationship

between the level of dietary fibers and the incidence of CRC [7, 34, 76]. These fatty acids that derive from the diet influence the risk of human colon cancer through diverse mechanisms, such as activation of different types of cell death, depending on their concentration, pH and cell type (Figures 4-6).

Among the three most relevant SCFAs, acetate has been the least studied. Butyrate, in particular, is considered the most potent of the SCFAs regarding prevention and inhibition of colon carcinogenesis, and its antitumor effects have been more extensively studied than those of the other SCFAs [34, 41, 75, 77, 78]. Although propionate exerts anti-neoplastic effects in CRC cells and has a mechanism of action similar to butyrate, there are fewer published studies on this SCFA [17, 79].

1.4.1. Role of Butyrate on Cancer Cell Proliferation and Apoptosis

The protective effects of butyrate against human colon cancer cells involve inhibition of cell differentiation, promotion of cell-cycle arrest and apoptosis and modulation of histone acetylation (Figure 4 and Table 1) [59, 68, 69, 75, 77, 80].

Previous studies on CRC cell lines showed that induction of apoptosis and cell cycle arrest at G0-G1 or G2-M by butyrate could be *via* p53-dependent or p53-independent pathways [77, 81, 82]. Zhang and co-workers analyzed the cytotoxicity mechanism of butyrate (0-40 mM) and showed that butyrate induced a strong growth inhibitory effect against human colon cancer cells (RKO cells), activating the intrinsic apoptosis pathway, characterized by DNA fragmentation and activation of caspase-9 and caspase-3 [75]. Expression of the anti-apoptotic protein Bcl-2 decreased, whereas the apoptotic protein Bax increased in a dose-dependent manner during butyrate-induced apoptosis [75]. Moreover, oncogene signaling molecules that are involved in the regulation of cell proliferation, cell migration and apoptosis, such as extracellular signal-regulated protein kinase 1/2 (ERK1/2), C-Jun N-terminal kinase (JNK) and p38 MAPK (p38) were also studied in butyrate-induced apoptosis [75]. High levels of ERK1/2 activation/expression were associated with cell proliferation and survival in various cancer cells, including CRC [75, 83]. However, it was demonstrated that butyrate-induced growth inhibition occurs with inactivation or downregulation of ERKs in RKO and HT-29 cells, respectively [75, 83]. In addition, activation of the JNK MAPK pathway played an important role in butyrate-induced apoptosis in RKO cells [75].

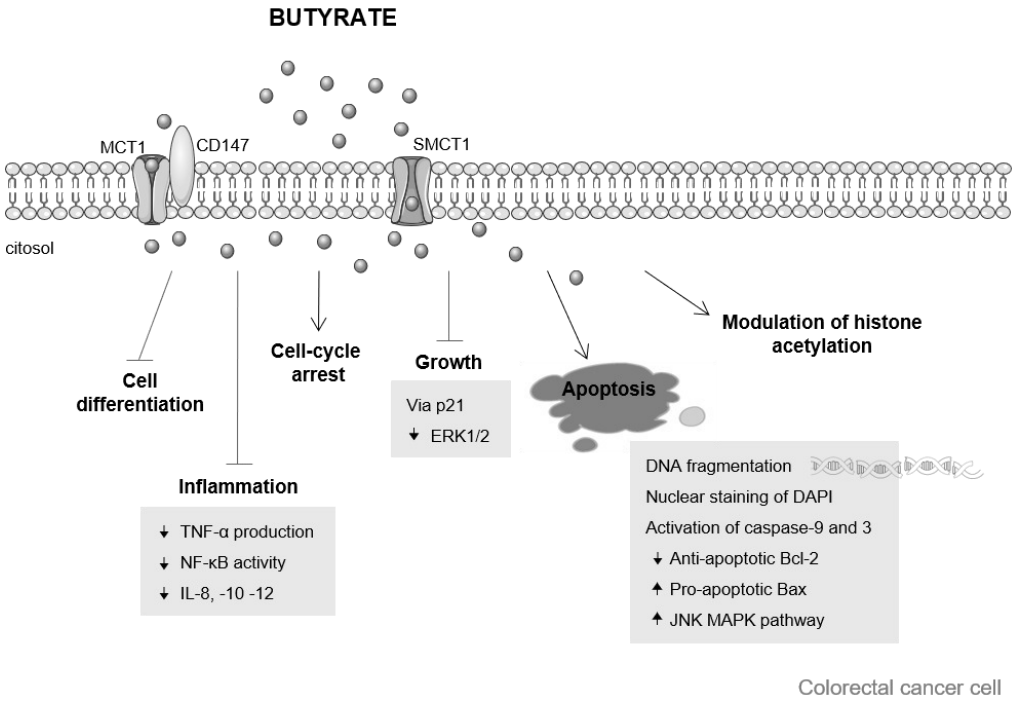

Figure 4 Schematic representation of different cell processes triggered by butyrate in colorectal cancer cells.

Goncalves et al have reported a clear antiproliferative and proapoptotic effect of butyrate in Caco-2 cells [84]. Moreover, in order to understand the role of butyrate in these cellular events, they tested a combination of polyphenols with butyrate and concluded that this combination did not significantly modify the changes in proliferation, differentiation, viability and apoptosis induced by butyrate alone [84].

In 2012, Matthews *et al* showed that butyrate (5 mM) induces apoptosis and G2-M arrest, mediated by alterations in the oxidative pentose pathway, reduction in glutathione availability and glucose consumption, as well as increased levels of reactive oxygen species (ROS) [77]. The same group observed that downregulation of glucose transporter 1 (GLUT-1) expression was associated with apoptosis induced by butyrate (5 mM) in HT-29 cells, also correlated with an increase in the expression and activity of MCT-1 as a mechanism to maximize intracellular availability of butyrate [85]. Other authors showed that butyrate inhibits CRC cell proliferation and induces cell death due to its inefficient metabolism associated with the Warburg effect and nuclear accumulation in transformed colonocytes, where it acts as a HDAC inhibitor [68, 86, 87]. Robert Li and co-workers confirmed that the accumulation of acetylated histone 3 is due to butyrate treatment in CRC cells [88]. Butyrate further contributes to hyperacetylation through conversion to acetyl-CoA and stimulation of histone acetyltransferase (HAT) activity [88, 89]. However, the cell metabolic state influences the levels of intranuclear butyrate and acetyl-CoA, determining whether butyrate functions as an HDAC inhibitor or stimulates HATs, epigenetically regulating the expression of different target genes [68, 81].

Table 1 - Effects of butyrate in colorectal cancer cells.

Butyrate			
CRC cell lines	**Concentration**	**Effects**	**Reference**
HCT116 HT-29	0.1 – 20 mM	Growth arrest mediated by p21	Archer et al, 1998
Caco-2 SW620	2 – 10 mM	Cell cycle arrest and apoptosis	Mariadason et al, 2001
HT-29	-	Growth inhibition by downregulation of ERK1/2	Davido et al, 2001
HCT116 HT-29	1 or 5 mM	Cell growth arrest, differentiation, apoptosis, induction of histone H4 hyperacetylation	Hinnebusch et al, 2002
HT-29	1 – 8 mM	Inhibition of cell proliferation, induction of differentiation and apoptosis	Comalada, 2006
HT-29	5 – 40 mM	Modulation of histone acetylation	Kiefer et al, 2006
HT-29	5 mM	Induction of apoptosis mediated by downregulation of GLUT-1	He et al, 2007
LT92 HT-29	0.5 – 50 mM	Induction of GSTs as a possible mechanism of chemoprevention	Scharlau et al, 2009
RKO	1 – 40 mM	Inhibition of cell proliferation, induction of apoptosis via caspase-9 and 3	Zhang et al, 2010
Caco-2	-	Antiproliferative and proapoptotic effect	Martel et al, 2010
Caco-2	5 mM	Induction of apoptosis, G2-M arrest, alterations in the oxidative pentose pathway	Matthews et al, 2012
RAW264.7*	1 – 1.200 µmol/L	Decrease of pro-inflammatory factors with an increase in the anti-inflammatory cytokine IL-10	Liu et al, 2012

Deregulation of the expression or activity of HATs and HDACs may lead to alterations in gene expression profiles, associated with reactivation or silencing of important genes for cancer progression, differentiation and apoptosis [90-92]. In this context, Archer *et al* (1998) showed that butyrate (0-20 mM) increased p21 expression through a process involving

histone hyperacetylation, and that p21 was required for butyrate-mediated growth arrest in colon cancer cells (HT-29 and HCT116) [93].

The main conclusion of the aforementioned studies is that exposure of the human colon to butyrate might protect against CRC by reducing survival and inducing cell death in CRC cells through several mechanisms. Importantly, cells that metabolize butyrate at higher rates are usually less susceptible to its apoptosis-inducing effects. This may explain why normal colonocytes are unaffected by high levels of this SCFA in the colon, as they preferentially use butyrate as an energy source, in contrast to CRC cells which seem to prefer glucose and accumulate higher levels of butyrate.

The cellular effects of butyrate are dependent on its intracellular concentration, so in this context it is crucial to understand how butyrate transport is modulated by extrinsic factors such as therapeutic drugs, substances present in diet and bacteria. There are several nutrients and xenobiotics known to modulate butyrate transport in CRC cells, interfering with its anticarcinogenic or procarcinogenic effect, including myricetin, catechin, caffeine, tetrahydrocannabinol, 3,4-methylenedioxymethamphetamine (MDMA) also known as ecstasy, as well as high-protein diets. Furthermore, different effects of butyrate can be related to differences in MCT1 and SMCT1 expressions between normal colonocytes and cancer cells. Compared with normal colonic epithelial cells, colon cancer cells show a decrease in SMCT1 protein expression leading butyrate to be taken up by these cells through MCT1. Consequently, this causes intracellularly accumulation of butyrate due to the fact that glucose becomes the primary energy source of these cells [94].

A specific study showed the involvement of MCT1 in the apical uptake of ^{14}C-butyrate by Caco-2 cells and suggest that MCT1-mediated transport is modulated by either acute or chronic exposure to some pharmacological agents and drugs of abuse (acetaldehyde, acetylsalicylic acid, indomethacin, caffeine, theophylline, tetrahydrocannabinol (THC) and MDMA). Moreover, it was described that the acute and chronic effects of the compounds upon ^{14}C-butyrate uptake may be distinct and that the effect of the compounds upon variations in the intraluminal concentration of butyrate, such occur in CRC, may also be distinct [95].

1.4.2. Role of Propionate on Cancer Cell Proliferation and Apoptosis

Jan and co-workers showed that propionate (10 - 40 mM) induces typical signs of apoptosis in human CRC cell lines (Figure 5 and Table 2), with loss of mitochondrial membrane potential, generation of ROS, cytochrome *c* release, caspase-3-processing and nuclear chromatin condensation [96]. Matthews and co-workers demonstrated that propionate at lower doses (5 mM) also induces apoptosis in Caco-2 cells, characterized by elevated ROS production and decreased glucose oxidation after 48 h of treatment [77]. Tang *et al* showed that propionate treatment (3 - 10 mM) induces the generation of ROS and loss of mitochondrial membrane potential in HCT116 cells, along with induction of autophagy. In this case, propionate-triggered autophagy therefore serves as an adaptive strategy to retard mitochondria-mediated cell death in CRC cells [97].

Histone post-translational modifications play an important role in the regulation of gene expression, including those involved in cancer development and progression. It is known that gene expression increases due to histone acetylation and this is associated with increased proliferation of tumor cells. It has been demonstrated that propionate, like butyrate, also acts as an inducer of histone acetylation in CRC cells, which can be the reason of a modulated growth of tumorigenic lesions in the gut [16, 17, 75]. In this regard, Kiefer *et al* showed that propionate alone (2.5 - 40 mM) or in combination with butyrate or acetate induced significant histone acetylation in HT-29 cells [98]. In addition, another study demonstrated that growth inhibition induced by propionate, like butyrate, involves downregulation of ERK1/2 in HT-29 cells [83].

Taken together, these findings suggest that propionate could be effective in the prevention and treatment of some colon alterations, including CRC.

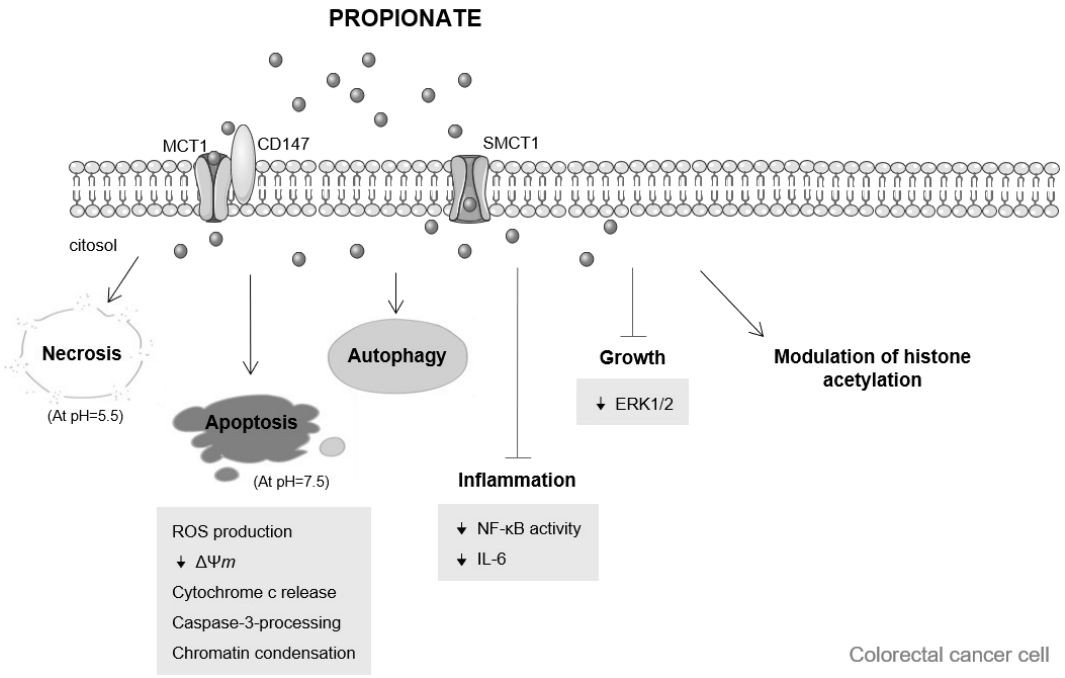

Figure 5 Schematic representation of different cell processes triggered by propionate in colorectal cancer cells.

Table 2 - Effects of propionate in colorectal cancer cells.

Propionate			
CRC cell lines	**Concentration**	**Effects**	**Reference**
HT-29	2-15 mM	Growth inhibition	Davido et al. 2002
HT-29 Caco-2	10 – 40 mM	Decrease of viability/apoptosis with mitochondrial alterations	Jan et al. 2002
HT-29	20 – 40 mM	Modulation of histone acetylation	Kiefer et al, 2006
HT-29	2 – 16 mM	Antiproliferative effect	Comalada, 2006
HT-29	30 mM	Apoptosis at pH 7.5 Necrosis at pH 5.5	Lan et al, 2007
Colo320DM	0.3 – 30 mM	Inhibition of the NF-$_{k}$B pathway	Tedelind et al, 2007
HCT116	1 – 10 mM	Mitochondrial defects and autophagy	Tang et al, 2011b
HCT116 SW480	1 – 3 mM	Induction of autophagy and apoptosis	Tang et al, 2011a

1.4.3. Role of Acetate on Cancer Cell Proliferation and Apoptosis

Initially, Jan and co-workers showed that acetate (0 - 40 mM) decreased viability and induced typical signs of

apoptosis, including loss of mitochondrial membrane potential, generation of ROS, caspase-3 processing and nuclear chromatin condensation in the colon adenocarcinoma cell line HT-29 [96]. Studies in *Saccharomyces cerevisiae* first demonstrated that acetic acid induces a mitochondria-mediated apoptotic process [99, 100] with several features similar to apoptosis mediated by others SCFAs in CRC cells. Indeed, alterations in mitochondria were identified in yeast, including production of ROS, mitochondrial swelling, decrease of mitochondrial membrane potential ($\Delta\Psi m$) [101], mitochondrial fragmentation/degradation [102, 103], mitochondrial outer membrane permeabilization (MOMP) with consequent release of pro-apoptotic factors like cytochrome *c*, yeast apoptosis inducing factor 1 (Aif1p) and Nuc1p (yeast orthologue of EndoG) [103-105]. The yeast orthologues of the mammalian VDAC (voltage-dependent anion channel) and ANT (adenine nucleotide transporter) were shown to play a role in MOMP and cytochrome *c* release during acetic acid-induced apoptosis in yeast [106]. Later, vacuolar membrane permeabilization (VMP) and release of Pep4p, yeast cathepsin D (CatD), from the lysosome-like vacuole to the cytosol, were observed in yeast cells exhibiting apoptotic cell death induced by acetic acid [107]. In that study, the authors also showed that, once in the cytosol, Pep4p played an important role in mitochondrial degradation through an autophagic-independent process, which protected yeast cells from acetic acid-induced apoptosis. Recently, it was demonstrated that both the protective function of Pep4p and its role in mitochondrial degradation during acetic acid-induced apoptosis in yeast depends on Pep4p proteolytic activity [91], which is complemented by heterologous expression of human CatD [108, 109].

Taking these results into account, our group hypothesized that similar events could occur in response to acetate in CRC cells (Figure 6 and Table 3). We have shown that, acetate treatment in CRC cells (0 - 140 mM and 0 - 220 mM, respectively for HCT-15 and RKO cells) decreased cell proliferation and induced apoptosis [110]. This process was characterized by DNA fragmentation, caspase-3 activation and phosphatidylserine exposure to the outer leaflet of the plasma membrane with appearance of a sub-G1 population [110]. Moreover, we showed that acetate induced lysosomal membrane permeabilization (LMP) with cathepsin D (CatD) release to the cytosol. Importantly, we revealed that CatD, but not CatB and CatL (also overexpressed in CRC cells), has an anti-apoptotic role in acetate-induced apoptosis [110]. We next showed that acetate induces mitochondrial dysfunctions, such as ROS accumulation and an increase in mitochondrial mass accompanied by mitochondrial membrane depolarization [109]. Additionally, we found an increase in the levels of mitochondrial proteins, namely the apoptosis inducing factor (AIF), the voltage dependent anion channel (VDAC1) and a subunit of the outer mitochondrial membrane translocator (TOM22) in CRC cells after acetate treatment [109]. This means that during acetate-induced apoptosis mitochondrial degradation following release of CatD is not enough to counteract autophagy inhibition by acetate. Indeed, inhibition of CatD (with siRNA or pepstatin A) increased mitochondrial mass and enhanced apoptosis associated with higher mitochondrial dysfunction [109].

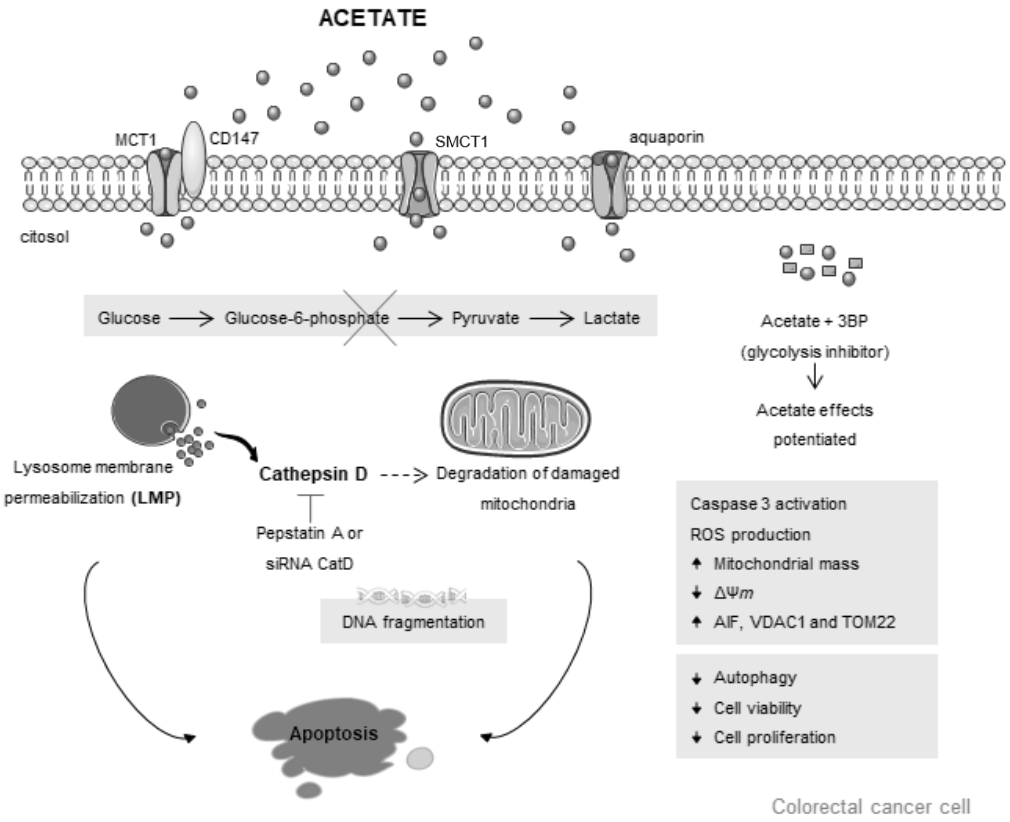

Figure 6 Schematic representation of different cell processes involved in apoptosis triggered by acetate in colorectal cancer cells.

The protective role of CatD demonstrated by our data can partly explain the CatD overexpression in some CRC clinical cases in comparison to normal colon mucosa as a cell strategy to cope with acetate cytotoxicity. In summary, CatD plays a role in the degradation of damaged mitochondria when autophagy is impaired, protecting CRC cells from acetate-induced apoptosis. CatD inhibitors could therefore enhance acetate-mediated cancer cell death, presenting a novel strategy for CRC prevention or therapy.

In conclusion, the data from different research groups suggest that it should be considered the use of acetate, butyrate and propionate, as possible chemopreventive agents. However, more studies, especially using these SCFA combinations, that mimic their relative concentrations in the colon, are necessary to support their combined use as a new strategy to potentiate the effects of each one in CRC cells.

1.4.4. Butyrate, Propionate and Acetate as Anti-Inflammatory Agents

Butyrate exhibits strong anti-inflammatory properties, and this effect is mediated mainly by inhibition of TNF-α production, nuclear factor-kappa B (NF-κB) activity and IL-8, 10 -12 expression in immune and colonic epithelial cells [7, 16, 111].

Despite butyrate being the most studied SCFA, the studies on propionate suggest that this SCFA displays a comparable effect to butyrate as an anti-inflammatory agent in the gut [16, 111, 112]. Tedelind *et al* (2007) observed that the anti-inflammatory activity of propionate (0.3 - 30 mM) involves inhibition of the NF-κB pathway and suppresses IL-6 release in Colo320DM cells [113].

Table 3 - Effects of acetate in colorectal cancer cells.

Acetate			
CRC cell lines	**Concentration**	**Effects**	**Reference**
HT-29 Caco-2	1.7 – 40 mM	Apoptosis induction with mitochondrial alterations	Jan et al, 2002
HT-29	4 – 32 mM	Ineffective in these concentrations	Comalada, 2006
HT-29	80 mM	Does not induce histone acetylation	Kiefer et al, 2006
Colo320DM	2.4 mM	Inhibition of the NF-$_k$B pathway	Tedelind et al, 2007
HT-29	15 mM	Apoptosis at pH 7.5 Necrosis at pH 5.5	Lan et al, 2007
RAW264.7*	1 – 1.200 µmol/L	Decrease of pro-inflammatory factors with an increase in the anti-inflammatory cytokine IL-10	Liu et al, 2012
HCT-15 RKO	70 – 140 mM 110 – 220 mM	Induction of apoptosis/Inhibition of proliferation and partial lysosome permeabilization with CatD release to the cytosol	Marques et al, 2013
HCT-15 RKO HCT116	70 – 140 mM 110 – 220 mM 100 – 200 mM	Mitochondrial dysfunction/ inhibition of autophagy and active CatD involved in mitochondrial degradation	Oliveira et al, 2015
HCT-15 RKO	70 mM 110 mM	Transport mediated by SMCT-1 or by aquaporins MCTs regulation and co-localization associated with changes in glucose metabolism	Oliveira et al, 2017

Despite these recent advances on the involvement of butyrate and propionate in inflammation, the mechanism whereby acetate mediates anti-inflammatory effects is poorly understood. It has already been shown that acetate also reduces the production of pro-inflammatory factors, including TNF-α, IL-1β, IL-6, while enhancing the production of the anti-inflammatory cytokine IL-10 [16, 111, 112] and inhibition of the NF-κB pathway in CRC cells [111].

Although it has been demonstrated that SCFAs can be involved in the immune response in the colon, more studies are necessary to understand the interaction between SCFAs and the intestinal microbiota, host immune cells, colonocytes and CRC cells.

1.4.5. Combined Effects of Short Chain Fatty Acids

Because none of the SCFAs are available alone, as the sole metabolites present in the human colon, their combined effect should be taken into consideration.

Tang et al 2011, using a combination of butyrate and propionate, showed for the first time that treatment with SCFAs, below their survival IC50 values (1–3 mM) towards cancer cells (HCT116 and SW480), did not induce evident apoptosis but rather induced extensive morphological alterations characteristics of autophagy [114]. Combined butyrate and propionate treatment induced autophagy to dampen apoptosis, whereas inhibition of autophagy potentiated SCFA-induced apoptosis [71]. Matthews and co-workers demonstrated that the combination of butyrate and propionate (5 mM each) induced cell cycle arrest at G2-M associated with a rapid and extensive apoptosis with changes in redox state and D-glucose metabolism in Caco-2 cells compared to the treatment using the SCFA alone [77]. In addition, Kiefer et al. showed that mixtures of acetate, butyrate and propionate (at molar ratios 75:11:14, 69:16:15 and 43:24:33, respectively) in colorectal cancer cells (HT-29) which mimic the relative physiological concentrations occurring in the gut, modulated histone acetylation [98]. However, they concluded that histone acetylation was mainly due to additive effects of butyrate and propionate, but not to acetate [79]. Lan and co-workers showed that acetate and propionate (15 mM and 30 mM, respectively) produced by a propionibacteria decreased proliferation and induced cell cycle arrest in G2/M, followed by a sequence of cellular events characteristic of apoptosis at pH 7.7 [115]. However, necrosis was induced at pH 5.5 in colon cancer cells (HT-29)

[115]. This study demonstrated for the first time the impact of the extracellular pH prevailing within the colon (from 5.5 to 7.5) on the mode of cell death triggered by propionibacteria-produced SCFA in CRC cells [115].

Although there are few studies on the combined effects of SCFAs, it seems that they have a greater effect in CRC cells as compared to the treatment using a sole SCFA.

1.5. Implication of the levels of short chain fatty acid production by colon microbiota on the prevention/therapy of colorectal carcinoma according to the diet

The potential use of SCFAs in the prevention and/or as adjuvants to conventional chemotherapy regimens of CRC is currently well supported by the literature.

In order to understand the effect that different types of diet have in the production levels of SCFAs, a recent study compared acetate, propionate and butyrate levels between control rats and rats that were subjected to a specific diet based on boiled, grilled and fried food [116]. The results showed that the three dominant SCFAs present in rats subjected to the specific diet were acetate, propionate, and butyrate. However, while acetate levels were significantly decreased during 6 to 18 weeks of feeding and propionate was markedly reduced after 18 weeks of feeding, butyrate showed a modest increase [116]. This variety of results shows that this kind of diet might have distinct effects on the production of different types of SCFAs suggesting that it is possible to regulate their levels through the consumption of specific types of food.

In order to evaluate the potential of different red wine pomace seasonings in chemoprevention of human colorectal adenocarcinoma, Del Pino-García *et al* have assessed their antiproliferative and antigenotoxic effects against oxidative stress in HT-29 cells [117]. Their results showed that the highest levels of SCFAs were detected in seedless wine pomace, and the lowest in isolated seeds, being acetate the main SCFA produced and butyrate the lowest [117]. Studies of cell viability showed that the seedless wine pomace exhibited the highest ability to reduce cell viability, which indicated that high levels of SCFAs exhibit important cancer suppressing effects [117].

Moreover, our group was able to successfully adapt the *Propionibacterium freudenreichii* to digestive stress by culturing cells in a simulated digestive stress media with acidic pH and bile salts concentrations that mimic the digestion environment. The results indicated that the adapted propionibacteria produce higher levels of SCFAs when compared to the respective control, being this improvement more pronounced in acetate production. As acetate is a potential nutraceutical against CRC that inhibits proliferation and induces apoptosis, these recent results have led our group to propose the potential use of a commercially relevant bacterium as a probiotic in the prevention and/or treatment of CRC [Casanova *et al*, unpublished data].

Studies comparing fecal microbiota of CRC patients with healthy individuals revealed that there is a significant decrease in fecal SCFA concentrations in CRC patients and a significant increase in fecal pH compared to the healthy individuals [27, 95].

A study that compares the dietary fiber intake (production of SCFA by fermentation) and the intestinal microbiota composition between advanced colorectal adenoma patients and healthy individuals, have demonstrated a difference in fecal SCFA concentrations and a structural imbalance in the gut microbiota, represented by the reduction of butyrate-producing bacteria and increased number of opportunistic pathogens in CRC patients [118].

In summary, since the concentrations of SCFAs in colorectal cancer are proven to be low, the generation of SCFAs in the colon increased by a specific nutritional diet, such as the intake of fibers and appropriate probiotics, may have an interesting protective and therapeutic effect against CRC.

CONCLUSION

Several *in vivo* studies have proven that SCFAs act as tumor suppressor agents, promoting apoptosis of CRC cells and enhancing the inhibition of tumor cell growth and proliferation. However, because the concentrations of SCFAs in the colon seem to be low in CRC patients, it becomes important to optimize the production of these agents with specific nutritional diets, specifically with the intake of fibers and appropriate probiotics like *Propionibacteria*.

In conclusion, the state of the art on the field argues in favor of the potential use of SCFAs in prevention and/or as adjuvants to conventional chemotherapy regimens of CRC. Indeed, the use of nutraceuticals to increase butyrate, propionate and acetate concentrations in the colon might emerge as a novel strategy for prevention/therapy of CRC.

LIST OF ABBREVIATIONS

AIF, apoptosis inducing factor; ANT, adenine nucleotide transporter; CRC, colorectal cancer; CatB, cathepsin B; CatD, cathepsin D; CatL, cathepsin L; ERK, extracellular signal-regulated protein kinase; $\Delta\Psi m$, mitochondrial membrane potential; GLUT-1, glucose transporter 1; GPR, G-protein-couple receptor; HAT, histone acetyltransferase; HDAC, histone deacetylase; IBD, inflammatory bowel disease; IL, interleukin; JNK, C-Jun N-terminal kinase; LMP, lysosomal membrane permeabilization; MCT, monocarboxylate transporter; MOMP, mitochondrial outer membrane permeabilization; NF-κB, nuclear factor-kappa B; SMCT-1,

sodium dependent monocarboxylate transporter 1; TCA cycle, tricarboxylic acid cycle; TOM22, subunit of the outer mitochondrial membrane translocator (TOM complex); ROS, reactive oxygen species; SCFAs, short chain fatty acids; VDAC, voltage dependent anion channel; VMP, vacuolar membrane permeabilization.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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