Universidade de Trás-os-Montes e Alto Douro

Study of a Germline Variant in Lymphotoxin Alpha (*LTA*) Gene in Colorectal Cancer

Master's degree in Biotechnology for Health Sciences

Fátima Cátia Leite Pinheiro

Orientador: Professora Doutora Estela Maria Bastos Martins de Almeida

Coorientador: Professor Doutor Ricardo Jorge Teixeira Ribeiro

Vila Real, 2019

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Composição do Júri:

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Vila Real, 2019

It is declared under an honor commitment that this work was expressly prepared by the author, as an original dissertation, for the purpose of obtaining a master's degree in Biotechnology for Health Sciences, at University of Trás-os-Montes and Alto Douro. All non-original contributions were properly identified with indication of the source.

“Somewhere, something incredible is waiting to be known”

Carl Sagan.

Agradecimentos

Para a realização deste trabalho, foram várias as pessoas que ajudaram, direta ou indiretamente, com o seu apoio e disponibilidade, às quais quero mostrar o meu honesto agradecimento.

À **Universidade de Trás os Montes e Alto Douro**, em nome do **Magnifico Reitor, professor Doutor António Fontainhas Fernandes,** pela disponibilização das instalações e material necessários para a realização deste trabalho.

A todos os **Professores da Licenciatura e Mestrado** por todo o apoio que nos deram durante estes anos e por todos os ensinamentos. Em particular, um obrigada muito especial à **Professora Doutora Estela Bastos** por ter aceitado, uma vez mais, ser minha orientadora, pela oportunidade concebida na realização deste trabalhado, por tudo aquilo que me ensinou, pela compreensão, disponibilidade a nível laboral e pessoal e apoio na execução desta dissertação.

Ao **Doutor Ricardo Ribeiro** por ter aceitado ser meu coorientador e por todo o apoio e disponibilidade para a realização e redação desta dissertação.

À **Professora Maria dos Anjos** e ao **José** um grande obrigada por nos terem ajudado com a parte da imunohistoquímica e à **Professora Maria dos Anjos** por estar sempre disponível para ajudar quando alguma dificuldade surgisse.

Ao **Centro Hospitalar de Trás-os-Montes e Alto Douro** por permitir a recolha de material biológico, e um especial obrigada à **Enfermeira Alexandra** e à **Doutora Mariana** por toda ajuda que sempre disponibilizaram. Aos pacientes que aceitaram fazer parte deste estudo, um enorme obrigada, pois sem eles nada disto teria sido possível.

À **Beatriz Cardoso**, obrigada por me acompanhares nesta jornada de trabalho, não poderia ter pedido melhor colega de trabalho.

Às pessoas do laboratório, à Marlene, à Márcia, à Sónia e à Leonor do laboratório de molecular, e à dona Glória, dona Ana e dona Lígia do laboratório da patologia por terem sido tão simpáticas e por estarem disponível por ajudar quando as dúvidas surgiam.

Inês Pereira, Andreia, Inês Pacheco, Érica, Rodolfo, Márcia, Dina, Hugo e Eurico, que apesar de já não estarmos todos juntos como antigamente, foram um grande apoio e, por isso, um obrigada por continuarem a ser a minha família de Vila Real. À **Inês Pereira** obrigada

por seres a minha companheira de séries e por estares ao meu lado neste último ano. À **Érica** por todos os momentos e almoços partilhados e por nos dares a conhecer, gastronomicamente, a Madeira. Ao **Rodolfo**, um obrigada por estes anos de amizade e apoio quando mais precisava. À **Márcia,** apesar de já não estar tão presente, um obrigada por ter "um botão" nos momentos difíceis e atender o telemóvel sempre que fosse necessário. Tenho saudades.

À **Clara, Daniela, Patrícia e Anicia** pela amizade, jantares e bons momentos que proporcionaram ao longo destes dois anos.

Ao **Miguel** por ter sido como um irmão ou um afilhado chato, mas que tornou a minha vida muito mais divertida, mais interessante e me tornou mais culta.

À **Filipa** por ter sido uma ajudante indispensável com muitas ou poucas amostras, mas que esteve sempre presente. Um obrigada pela tua amizade.

Às minhas amigas de infância, **Cátia e Andreia** por terem crescido comigo e, também, à **Adriana e à Sofia (Fifi),** a todas obrigada por não desistirem de mim apesar da distância e do, por vezes, pouco tempo tinha para vocês.

À minha família pelo apoio que sempre me deram.

Um obrigada muito especial ao meu **Pai** e à minha **Mãe** que sem eles nada disto era possível. Por todo o esforço e sacrifícios. Nunca conseguirei agradecer tudo o que fizeram por mim.

A TODOS, UM GRANDE OBRIGADA!

Resumo

O cancro colorretal (CRC) representa a quarta causa de morte relacionada com o cancro no mundo. Sendo causada por diversos fatores de risco como a dieta, história familiar, doenças inflamatórias, o cancro colorretal é influenciado por alterações em genes importantes para a função celular.

A linfotoxina alfa (*LTA*) pertence à superfamília do fator de necrose tumoral. Esta citocina é expressa pelos linfócitos T e B, células dendríticas e linfócitos NK. LTA pode ligar-se ao recetor linfotoxina-beta e aos recetores fator de necrose tumoral. Após a ligação aos seus recetores nas células no microambiente tumoral, a proteína LTA pode regular a apoptose, proliferação, sobrevivência e a diferenciação. No cancro, a sua influência ainda não está bem explicada. Foram realizados alguns estudos, contudo os resultados não são conclusivos. A LTA pode estar associada com atividade anti-tumoral tendo efeitos citotóxicos nas células de cancro pelo recrutamento de células NK para a lesão. Contudo, outros estudos têm revelado que LTA pode promover o crescimento celular e a adesão das células de cancro.

Desta forma, os objetivos deste estudo foram caracterizar e analisar se os genótipos do polimorfismo funcional rs1041981 do *LTA*, particularmente a sobrevivência global e a sobrevivência livre de progressão em pacientes com cancro colorretal e avaliar os macrófagos e linfócitos T em tecidos CRC, por imunohistoquímica, e a sua associação com os dados dos genótipos.

Para realizar o estudo, foram recolhidas amostras sanguíneas em 172 pacientes sobreviventes com CRC no departamento de Oncologia, Centro Hospitalar de Trás-os-Montes e Alto Douro (CHTMAD) pelos clínicos. As amostras foram transportadas para o departamento de Genética e Biotecnologia na UTAD, para separação em soro, plasma e *buffy coat.* Foi otimizado um protocolo de extração de DNA, e foi realizado PCR em tempo-real para descriminação alélica usando sondas Taqman e subsequente confirmação por sequenciação de um amplicão específico. O alelo C e o alelo A apresentaram, respetivamente, frequências alélicas de 70 % e 30 %. As frequências genotípicas do CC, CA e AA foram 49%, 42% e 9%, respetivamente.

Considerando os parâmetros clinico-patológicos e a associação com os dados genéticos, foi possível colocar em evidência que as variáveis que influenciam a sobrevivência dos pacientes com CRC nesta população foram a percentagem de linfócitos no sangue, a localização e o lado do tumor, terapia adjuvante e o genótipo CA/AA pelo modelo dominante. O alelo A parece ter um efeito protetor para os pacientes com cancro colorretal nesta população para o *endpoints*

primário e secundário, respetivamente a sobrevivência global e a sobrevivência livre de progressão. Os pacientes com a asparagina na proteína LTA parecem ter um melhor prognóstico que os pacientes com a treonina na proteína LTA na mesma posição.

Os resultados para a possível associação entre TAMs e TILs no cancro colorretal e a variante *LTA* rs1041981 revelaram diferenças significativas no rácio TILs/TAMs entre os indivíduos CC e CA no estudo.

Este gene, como demostrado neste trabalho, parece ter uma importante função no cancro colorretal. A compreensão do impacto deste polimorfismo na evolução da doença pode trazer novas informações na regulação do microambiente tumoral.

Palavras-chave: cancro colorretal, *LTA*, SNP, células inflamatórias.

Abstract

The colorectal cancer (CRC) represents the fourth cause of cancer death in the world. Being caused by several risk factors as diet, family history, inflammatory diseases, the colorectal cancer can be influenced by alterations in important genes to cell function.

Lymphotoxin alpha (*LTA*) belongs to tumor necrosis factor superfamily. This cytokine is expressed by T and B lymphocytes, dendritic cells and NK lymphocytes. LTA can bind to lymphotoxin-beta receptor and tumor necrosis factor receptors. After binding to its receptors in tumor microenvironment cells, LTA protein can regulate apoptosis, proliferation, survival and differentiation. In cancer, its influence is not well explained. Some studies were already performed; however, the results are not conclusive. The LTA may be associated with anti-tumor activity having cytotoxic effects on cancer cells by recruitment of NK cells to lesion. However, other studies have revealed that LTA can promote the cell growth and adhesion of cancer cells.

Thus, the objectives of this study were to characterize and analyze whether genotype of *LTA* functional polymorphism rs1041981 influences clinicopathological parameters, particularly overall and progression-free survival in colorectal cancer patients and evaluate the macrophage and T lymphocytes in the CRC tissues, by immunohistochemistry, and its association with genotype data.

To perform the study, blood samples of 172 survivor patients with CRC were collected in the Department of Oncology, Centro Hospitalar de Trás-os-Montes e Alto Douro (CHTMAD) by clinical researchers. The samples were transported to the Department of Genetics and Biotechnology at UTAD, to separate in serum, plasma and buffy coat. A DNA extraction protocol was optimized, and the allele discrimination was performed by real time-PCR using Taqman probes and subsequent confirmation by sequencing of a specific LTA amplicon. The C and A alleles presented, respectively, allelic frequency of 70 % 30 %. CC, CA and AA genotypic frequencies were 49%, 42% and 9%, respectively.

Considering the clinicopathological parameters and the association with the genetic data, it was possible to put in evidence that the variables that influence the survival of CRC patients under study were blood lymphocytes percentage, tumor side and localization, adjuvant chemotherapy and the genotype CA/AA, by a dominant model. The A allele appears to be a protective factor for patients with colorectal cancer in this population for primary and secondary endpoints, respectively overall survival and progression-free survival. The patients with

asparagine in LTA protein appear to have a better prognosis than patients that have the threonine in the same position.

The results of possible association between TAMs and TILs in colorectal tumors and rs1041981 *LTA* variant revealed statistical differences in TILs/TAMs ratio between CC and CA individuals under study.

This gene, as evidenced in this work, appears to have an important role in colorectal cancer. The understanding of the impact of this polymorphism on the evolution of the disease can bring new information on tumor microenvironment regulation.

Keywords: Colorectal cancer, *LTA*, SNP, inflammatory cells.

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Abbreviations, acronyms and symbols

µ

µL (microliter)
µm (micrometers)

A

AFAP (Attenuated FAP)
AP cells (Antigen-Presenting Cells)
APC gene (WNT signaling pathway regulator)
ASB 4 (Ankyrin repeat and SOCS Box protein 4)

B

BMI (body mass index)
bp (base pairs)
BRAF (B-Raf proto-oncogene, serine/threonine kinase)
BUB1 (BUB1 mitotic checkpoint serine/threonine kinase)

C

CAFs (cancer-associated fibroblasts)
CHTMAD (Centro Hospitalar de Trás-os-Montes e Alto Douro)
cIAP (cellular Inhibitor of Apoptosis)
CIMP (CpG island methylator phenotype)
CIMP-H (CIMP-high)
CIN (chromosomal instability)
CMS (Consensus Molecular Subtypes)
COLCA1-2 (Colorectal Cancer Associated 1 and 2)
CpG (Cytosine phospho-Guanine)
CRC (Colorectal Cancer)
CSC (Cancer Stem Cells)
CTL (cytotoxic T lymphocyte)
CTLA-4 (Cytotoxic T-Lymphocyte Associated protein 4)

D

DAB (3,3'-diaminobenzidine tetrachloride)
dbSNP (NCBI database)
DCs (Dendritic Cells)
DD (Death Domain)
DMSO (dimethyl sulfoxide)
DNMTs (DNA Methyltransferases)
dNTP (deoxyribonucleotide)

E

EGFR (epidermal growth factor receptor)
EMT (Epithelial-Mesenchymal Transition)

F

FADD (FAS-Associated Domain)
FAP (Familial Adenomatous Polyposis)
FFPE (Formalin-Fixed and Paraffin-Embedded)
FOXp3 (Forkhead box P3)

G

GALT (Gut-Associated Lymphoid Tissue)

H

H_2O_2 (hydrogen peroxide)
HNPCC (Hereditary Nonpolyposis Colorectal Cancer)
HVEM (Herpes-Virus Entry Mediator)

I

IBD (Inflammatory Bowel Disease)
IFN-γ (Interferon Gama),
IKK (IκB Kinase)
IL-2 (Interleukin-2)
ILC (Innate Lymphoid cells)
iMCs (immature Myeloid Cells)
IHC (immunohistochemistry)
iNOS (inducible Nitric Oxide Synthase)
IQR (inter-quartile range)

J

JNK (Jun N-terminal Kinase)

K

KRAS (KRAS proto-oncogene, GTPase)

L

LGR 5 (Leucine-rich repeat-containing G-protein coupled Receptor 5)
LHAP (Laboratory of Histology and Anatomical Pathology)
LINE- 1 (Long Interspersed Element-1)
LPS (Lipopolysaccharide)
LTA (Lymphotoxin Alpha)
LTi (Lymphoid Tissue inducer)
LTβ (Lymphotoxin Beta)
LTβR (Lymphotoxin-beta Receptor)

M

MAP3K (Mitogen-Activated Protein-3 Kinase)
MCs (Mast Cells)
MDSCs (Myeloid-Derived Suppressor Cells)
MHC (Major Histocompatibility Complex)
min (minutes)
miRNA (microRNA)
mL (milliliters)
MLH1 (mutL homolog 1)
MMR (mismatch repair)
MSH2 (mutS homolog 2)
MSH6 (mutS homolog 6)

MSI (microsatellite instability)
MSI-H (MSI-high)
MSI-L (MSI-Low)
MSS (Microsatellite Stable)
MUTYH (mutY DNA glycosylase)

N

NCBI (National Center for Biotechnology Information)
ncRNA (Non-coding RNA)
NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells)
NIK (NF-kappa-B-inducing kinase)
NK (Natural Killer)
nm (nanometers)
NTC (no template control)

O

OS (overall survival)

P

PAMPs (Pathogen-Associated Molecular Patterns)
PBS (phosphate-buffered saline)
PD-1 (Programmed Death 1*)*
PD-L1 (Programmed Death Ligand 1)
PFS (progression-free survival)
PMS2 (PMS1 homolog 2, mismatch repair system component)
PTMs (Post-Translational Modifications)
PVR (Proliferative Vitreoretinopathy)

R

RIP-1 (Receptor-Interacting Protein 1)
ROS (Reactive Oxygen Species)
rpm (revolutions per minute)

S

SCNA (Somatic Copy Number Alterations)
SD (standard deviation)
SEM (standard error of mean)
SNP (single nucleotide polymorphism)

T

T2K (TRAF2-associated kinase)
TAMs (Tumor Associated Macrophages)
TANK (TRAF family member Associated NF-kB activator)
TANs (Tumor Associated Neutrophils)
TCR (T-cell Receptor)
TGFβ (Transforming Growth-Factor Beta)
Th cells (T Helper cells)
TILs (Tumor Infiltrating Lymphocytes)
TME (Tumor Microenvironment)
TNFR (TNF Receptor)
TNFSF1 (Tumor Necrosis Factor Ligand Superfamily member 1)
TNF-α (Tumor Necrosis Factor Alpha)
TNFβ (Tumor-Necrosis Factor Beta)
TP53 (tumor protein p53)
TRADD (TNFR-Associated Domain)
TRAF (TNF Receptor-Associated Factor)
Treg cells (T Regulatory cells)
TNFRS19 (TNF Receptor Superfamily member 19)

V

VEGF (Vascular Endothelial Growth Factor)

I- Introduction

1 – Colorectal Cancer

1.1 – Epidemiology

Colorectal Cancer (CRC) is one of the most prevalent human cancers; it is the second cause of cancer death in Europe (Ferlay *et al.*, 2012 cit. Roseweir *et al.*, 2017) and the fourth in the World (Kuipers *et al.*, 2015). The highest incidence is observed in Australia, New Zealand and in the developed countries in Europe and Northern-America, whereas less-developed regions such as African countries have the least incidence (Kuipers *et al.*, 2015).

Frequently, the initial lesion that may led to CRC is designated as polyp, which is characterized by the proliferation of colonic mucosa on the inner lining of the colon or rectum creating pedunculated or sessile outgrowths. The transformation of a benign lesion (adenomatous polyp with dysplastic cells) into malignancy usually requires several years (Grady and Markowitz, 2015). Epigenetic and somatic genetic alterations in the DNA of mucosal cells have been described to play a key role in CRC initiation, specifically in oncogenes, tumor suppressor genes, and in cell cycle regulation and mismatch repair genes (Migliore *et al.*, 2011). Some lesions can acquire additional mutations during years and originate colorectal cancer (Kuipers *et al.*, 2015). Often, this lesion does not cause symptoms, and takes years or decades to become clinically evident (Bardhan and Liu, 2013 cit. Mahasneh *et al.*, 2017). The first signs are more frequently rectal bleeding, change in bowel habits and abdominal pain (Brooks *et al.*, 2008 cit. Moore and Aulet, 2017; Kuipers *et al.*, 2015). Other symptoms emerge with worsening of the disease, such as fatigue, weight loss and anemia (Kuipers *et al.*, 2015). Notably, about 20-25 % of patients with colon cancer and 18 % of patients with rectal cancer are diagnosed in advanced disease stages, where metastases are already installed, preferentially at the liver or lung (Leufkens *et al.*, 2011; Kekelidze *et al.*, 2013 cit. De Rosa *et al.*, 2015).

During the last years, diagnostic and surgical techniques have improved, and new therapies developed. Nevertheless, the survival rate, in 5-years is, according to Bupathi and Wu, (2016 cit. Mahasneh *et al.*, 2017) frequently, only 50%, if the tumor is detected in an early stage (when cancer has not capability to spread or symptoms have not yet begun to manifest). Otherwise, if CRC is detected in metastasis phase, the survival rate decreases to 12%, in 5-years (Bupathi and Wu, 2016 cit. Mahasneh *et al.*, 2017).

Categorization of risk in CRC patients as average-, increased- and high-risk has been recently proposed by Levin *et al.* (2008 cit. Moore and Aulet, 2017). Average-risk group include those without personal history of CRC, polyps or Inflammatory Bowel Disease (IBD) and without any relative with CRC or genetic syndromes. In this group, screening is recommended at age 50 (Levin *et al.*, 2008 cit. Moore and Aulet, 2017, Rex *et al.*, 2017). Patients with increased risk have personal and relatives with history of polyps or CRC and history of IBD, but without hereditary syndromes. In this group the time of screening varies according to size, quantity and histology of surgical specimens (National Comprehensive Cancer Network cit. Moore and Aulet, 2017). In case of family history, screening should start 10 years before the age at which the relative was diagnosed. The recommended screening in the case of patients who had inflammatory bowel diseases is influenced by duration of disease (National Comprehensive Cancer Network, Levin *et al.*, 2008 cit. Moore and Aulet, 2017). The high-risk people have family and IBD history and hereditary syndromes. In case of hereditary syndromes, screening starts 10 years before the earliest diagnosis of the relative or at age 20 to 25 (Levin *et al.*, 2008 cit. Moore and Aulet, 2017).

About 90% of cases of CRC in the USA are older than 50 years (American Cancer Society, 2014 cit. Choi *et al*., 2017[a]). However, CRC is increasingly being diagnosed at earlier ages, due to hereditary syndromes carrying germline mutations that predispose to colorectal cancer, as well as environmental factors such as diet, sedentarism and obesity (Connel *et al.*, 2017).

1.2 – Risk factors

Many factors have been described as related with development of CRC, including genetic, epigenetic, and environmental factors (Oines *et al.*, 2017).

1.2.1 – Aging and gut microbiome

The aging itself modifies the gastrointestinal system changing the digestion, absorption or excretion of nutrients (Baker and Blakely, 2017). Even these alterations might be responsible for transforming microflora colonization towards a growth promoting state of host-microbes, which has been recently associated with the development of CRC (Chung *et al*., 2016). *Helicobacter pylori*, *Bacteroides fragilis*, *Fusobacterium spp.* and *Escherichia coli* have been associated with risk for colorectal cancer initiation and progression. Although the exact mechanisms remain under research, it has been hypothesized that genotoxicity, oxidative stress and inflammation might mediate the pro-carcinogenic effects of digestive endoluminal bacteria

in CRC carcinogenesis (Gagnière *et al.,* 2016). Notably, other microbial agents have been related with protection for developing CRC, namely *Faecalibacterium prausnitzii* due to its anti-inflammatory properties (Gao *et al.,* 2017), thus suggesting that the resulting impact of microbiome in CRC is liable to a disequilibrium between pro- and anti-cancer bacteria species. Nevertheless, microbial imbalance is not the only player in CRC development.

1.2.2 – Obesity, sedentarism and diet

Sedentary lifestyle and unbalanced diet have a large impact on development of colorectal cancer, which can be related to the increase and hypertrophy of adipocytes and increase of adiposity, leading to obesity (Mahasneh *et al.,* 2017). Lack of exercise and excess adiposity likely contributes to the development and aggressiveness of colorectal cancer, through the dysregulation of sex hormones levels, mild chronic inflammation and insulin resistance (Neilson *et al*, 2009; Lynch, 2010 cit. Cong *et al.,* 2014).

In fact, obesity, sedentarism and diet are all part of a same continuum, contributing individually and combined to the development of CRC. Center and colleagues (2009) demonstrated that the increase in body mass index (BMI) and lack of exercise was associated with increased incidence of cancer. Furthermore, a recent meta-analysis from Theodoratou and co-workers (2017), concluded that tobacco, alcohol and red and processed meat were related with predisposition for having CRC. Conversely, it was found that a lower incidence of CRC was liable to a diet based on high calcium supplements, high total fiber, vegetables, whole grains and dairy products (Teodoratou *et al.,* 2017).

1.2.3 – Other diseases

Inflammatory bowel diseases such as ulcerative colitis is pointed as a predisposing condition linked with carcinogenesis, through an inflammation-associated mechanism (Yashiro, 2015).

1.2.4 – Genetic factors

Somatic and germline genetic alterations are also important players in CRC development. Most of the CRC are sporadic (90-95%) without apparent evidence of hereditary association, but around 5-10% have family history (Wells and Wise, 2017). Recognizing hereditary markers allow early identification of at risk individuals, thus representing an important preventive measure with impact in mortality (Vasen *et al.,* 2015). Symptomatic patients are submitted to colonoscopy, which allows observation of rectum and colonic endoluminal mucosa searching

for lesions that might be biopsied as needed. Definitive diagnosis is given by histopathological analysis of suspected biopsied tissue specimens (Moore and Aulet, 2017). Hereditary ones can be divided into syndromes with and without polyposis. The most common and most studied hereditary syndrome is Familial Adenomatous Polyposis (FAP) and Attenuated FAP (AFAP). They are caused by mutations in the WNT signaling pathway regulator (*APC)* gene (Nieuwenhuis *et al.,* 2009 cit. Vasen *et al.,* 2015). Another syndrome with polyposis is associated with mutations in mutY DNA glycosylase (*MUTYH*) gene, which is involved in base excision repair pathway (Nielsen *et al.,* 2011, Sampson and Jones, 2009 cit. Vasen *et al.,* 2015). The most frequent syndrome without polyposis is the Lynch syndrome, which is caused by mutations in DNA mismatch repair genes such as mutL homolog 1 (*MLH1*), mutS homolog 2 (*MSH2*), mutS homolog 6 (*MSH6*) and PMS1 homolog 2, mismatch repair system component (*PMS2*) (Martin-Lopez and Fishel, 2013 cit. Vasen *et al.,* 2015).

1.2.5 – Epigenetic factors

Epigenetics influence cancer tumorigenesis. The epigenetic profile leads to alteration of gene function without modification of the DNA sequence transmitted during cell division (Choudhuri, 2011 cit. Lardenoije *et al.,* 2015). The main epigenetic mechanisms are mediated through methylation, chromatin remodeling, histone modification and histone variants and non-coding RNA (You and Jones, 2012 cit. Sandoval-Basilio *et al.,* 2018), which have been shown to have a role in colorectal carcinogenesis (Puccini *et al.,* 2017).

- DNA methylation

DNA methylation consists in the addition of a methyl group by DNA Methyltransferases (DNMTs), primarily, in cytosine at Cytosine phospho-Guanine (CpG) dinucleotides (Lao and Grady, 2011). This process can, also, occur in adenine or guanine (Thomas *et al.,* 2013 cit. Lardenoije *et al.,* 2015).

The level of methylation can control the expression of genes. Methylation in CpG island of gene promoter is associated with repression of transcription, therefore, the high level of methylation is related with silencing of the genes (Ye *et al.,* 2017). The methylation can, also, occur in gene-body of DNA (portion of gene that is transcript) being associated with transcriptional activation and gene expression (Ball *et al.,* 2009). Nevertheless, DNA methylation is also important during human development because it is involved, by repression

of transcription, in processes as X chromosome inactivation and genomic imprinting (Paluch *et al.*, 2016).

Generally, in cancer cells, the hypermethylation can lead to inactivation of tumor suppressor genes, contributing to cancer initiation through suppression of apoptosis and DNA damage repair pathways (Herman and Baylin, 2003 cit. Paluch *et al.*, 2016). Hypomethylation is also associated with initiation and progression of colorectal cancer, particularly the hypomethylated DNA is the Long-Interspersed Element-1 (LINE- 1) that has the ability to copy itself and move to other positions in the genome (Danese and Montagnana, 2017).

- Chromatin Remodeling, Histone modification and Histone Variants

There are several processes of chromatin dynamic that are critical to control the transcription level by changing the chromatin structure restricting the accessibility of enzymes and transcription factors to DNA (Strahl and Allis, 2000 cit. Biterge and Schneider, 2014). These processes are known as histone modification, histone variants and ATP dependent chromatin remodelers (Swygert and Peterson, 2014). In histone modification, the amino acids of these histones can undergo Post-Translational Modifications (PTMs). These PTMs are generally reversibly and are related with transcription level by modification of accessibility of DNA by modification of condensation level of chromatin (Iwasaki *et al.*, 2013 cit. Paluch *et al.*, 2016). There are already 22 PTMs described (Arnaudo and Garcia, 2013, Dai *et al.*, 2014, Rothbart and Strahl, 2014, Zhao and Garcia, 2015), being the most studied methylation, acetylation, phosphorylation, ubiquitination and SUMOylation (Zhang and Pradhan, 2014). These histone modifications have been related with initiation and progression of colorectal cancer. Acetylation of histone's amino acids can dysregulate the oncogenic pathways and promote the colorectal carcinogenesis (Pelàez *et al.*, 2010 cit. Puccini *et al.*, 2017). In colorectal cancer cells, it was suggested that promoter of genes with CpG islands hypomethylated were associated with histone acetylation, which relates with an open chromatin and increased transcription (Rezasoltani *et al.*, 2017). The study from Cai *et al.* (2014) described the relationship between methylated DNA and initiation of colon cancer.

- Non-coding RNA

Currently, it is accepted that nonfunctional DNA is transcribed into non-coding RNA , considered a functional product, important for regulation of gene expression. There are multiple types of ncRNA that can be grouped in two groups (Lardenoije *et al.*, 2015): small non-coding

RNA (< 200 nucleotides) and the long non-coding RNA (> 200 nucleotides). MicroRNAs (miRNA) are a type of small non-coding RNA (20-25 nucleotides) and single-strand molecules. Their function is to silence target genes through binding mRNA, thereby causing its degradation or inhibiting the translation (Kita *et al.,* 2014). In healthy cells, the regulation of these short RNAs is performed by hyper and hypomethylation and can lead to cancer development. In colorectal cancer, it was shown that hypermethylation of some miRNAs is related with tumorigenesis, since they are involved in specific pathways that regulate cancer cells, like WNT, RAS/MAPK and PI3K/AKT pathways (Puccini *et al.,* 2017).

1.3 – Workup, Diagnosis and Prognosis Markers

The most precise method to identify suspect colorectal lesions is the colonoscopy (Choi *et al.,* 2017[a]), which allows biopsy sampling during the procedure when suspect lesions are detected. However, this method is invasive and can be uncomfortable to the patient (Rex *et al.,* 2012 cit. Liu *et al.,* 2014). Other methodologies, with lower sensitivity and specificity, from blood-based tests (genetic) and hemoglobin detection tests through imagiological approaches, are being used in colorectal cancer screening to guide the use of other more expensive methods (colonoscopy) **(**Church *et al.,* 2014 cit. Choi *et al.,* 2017[a]; Pignone *et al.,* 2001 cit. Moore and Aulet, 2017; Hol *et al.,* 2010 cit. Moore and Aulet, 2017; Choi *et al.,* 2017[a]; Labianca and Merelli, 2010 cit. Das *et al.,* 2017; Whitlock *et al.,* 2008 cit. Das *et al.,* 2017; Moore and Aulet, 2017). The definite diagnosis is determined by histopathological analysis of tissue samples representative of the tumor, either surgical specimen or biopsies collected during colonoscopy.

The use of molecular biomarkers in CRC is less specific, despite lower invasiveness when compared with colonoscopy (Ling *et al.,* 2001 cit. Liu *et al.,* 2014). Mutations and methylation in *APC*, tumor protein p53 (*TP53),* epidermal growth factor receptor (*EGFR)* and *MLH1* genes are examples of prognostic molecular biomarkers (Das *et al.,* 2017), whereas detection of mutations in mismatch repair (MMR) genes are important biomarkers to early detection of polyps and can be an indicator of Hereditary Nonpolyposis Colorectal Cancer (HNPCC) (Kulendran *et al.,* 2011 cit. Das *et al.,* 2017). Predictive biomarkers contribute towards personalized treatment. Mutations on KRAS proto-oncogene, GTPase (*KRAS)* and polymorphisms in *COX2* were able to predict the response to therapy and the risk for advanced stage CRC, respectively (Gonzalez-Pons and Cruz-Correa, 2015, Umar *et al.,* 2004 cit. Das *et al.,* 2017).

1.4 – Pathogenesis of CRC

Each new genetic alteration confers further advantage for cancer progression (Rajagopalan and Lengauer, 2004), while the microenvironment surrounding malignant cells contribute to increment genetic and epigenetic alterations that induce cancer development and aggressiveness (Hoeijmakers, 2004). Intestinal polyps emerge in mucosa and may undergo modifications over time, becoming morphologically different: adenomas or serrated polyps. Adenomas are considered a pre-cancerous condition since cells are dysplastic and might become precursors of malignancy, with increased capability for dividing.

A traditional view explaining initiation and progression of colorectal cancer has been described earlier by Fearon and Vogelstein (1990). In their perspective initiation occurs with the formation of adenoma by inactivation of *APC* gene and is associated with chromosomal instability, followed by accumulation of genetic and epigenetic alterations and modification of tumor microenvironment and the microbiome, which taken together allow progression for adenocarcinoma and invasive carcinoma (Grady and Markowitz, 2015) (Figure 1). Few years later, an alternative mechanism has been proposed, named the serrated pathway (Jass and Smith, 1992), since different genetic alterations were detected, such as initial mutational and methylation burden in B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) proto-oncogene (Leggett and Whitehall, 2010) (Figure1). Alternatively, a third pathway has been reported to occur in gut-associated lymphoid tissue (GALT), a lymphoid tissue responsible for eliminating invasive agents in the gastrointestinal tract. This lymphoid tissue recognizes antigens, microorganisms and macro-molecules arising from the lumen, presenting them to immune cells, therefore having a rapid response. However, the mechanisms behind colorectal carcinogenesis in association with GALT mucosal domain remain unknown (Rubio *et al.*, 2018).

1.5 – Molecular pathways in CRC

Colorectal cancer usually develops due to molecular alterations that result from chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP). Ultimately, these pathways contribute to initiation and progression of tumors leading to transformation in pathologic lesion and progression to malignancy (Gonzalez-Pons and Cruz-Correa, 2015). Patients with MSI show genetic hyper mutability

resulting from impaired DNA mismatch repair. These patients often have a better prognosis if MSI is associated with high CIMP (Tahara *et al*., 2014).

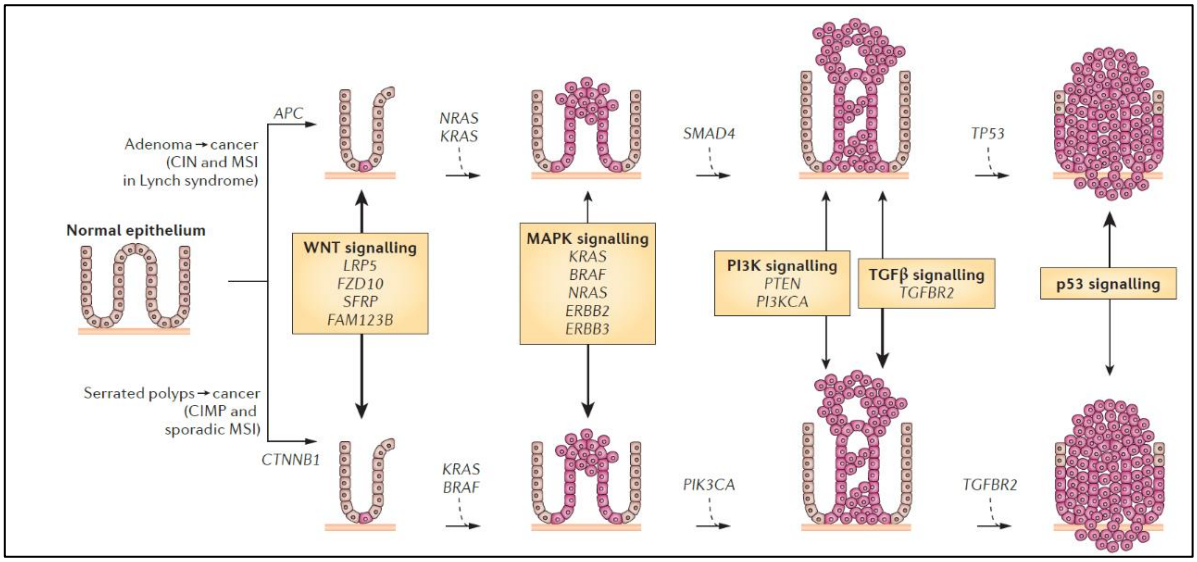


Figure 1 - Initiation and progression of colorectal cancer. Cancer aggressiveness is related with accumulation of mutations in important genes that regulate, e.g. the cell cycle, leading to disruption of homeostasis and over-growth of epithelial cells in colon and retum (adapated from Davies *et al.,* 2005).

1.5.1 – Chromosomal Instability

CIN is the most common form of genomic instability, being found in 85% of CRCs (Grady and Carethers, 2008). The underlying mechanism remains incompletely understood but it has been recognized to promote cancer progression by increasing clonal diversity (Hermsen *et al.*, 2002). CRC with this instability may exhibit aneuploidy or polyploidy and could be defined as having numerical chromosome changes or multiple structural chromosomal aberrations. Dysregulation of mitotic spindle checkpoint regulators, such as *BUB1* mitotic checkpoint serine/threonine kinase (*BUB1*), or centrosome amplification have been associated with gains and losses of arms or chromosomes, whereas the dysregulation of double strand DNA break repair mechanisms was related with smaller gains or losses. These alterations occur later, after the first alterations (Shin *et al.*, 2003, Anderhub *et al.*, 2012). Genomic instability, DNA hypomethylation and telomere shortening can be induced by oncogenic stress[1], which has relevant role in the progression of colorectal cancer (Gilad *et al.*, 2010, Roger *et al.*, 2013).

These molecular pathways are associated with *APC*, *KRAS* and *TP53* mutations or chromosome loss (Grady, 2004). Mutations in *APC* usually result in proteins unbound to β-

[1] Oncogenic stress is observed when there is an imbalance between the expression of oncogenes and tumor suppressor genes, which can trigger apoptosis or a tumor process (Lopes, 2017).

catenin, ultimately leading to continuous activation of Wnt pathway (Cadigan and Liu, 2006). This pathway is important to regulate cell growth, apoptosis and differentiation (Kuhnert *et al.,* 2004). Dysregulation interfere with regulation of mitosis and contribute to chromosomal instability (Fodde *et al.,* 2001). The Ras-MAPK signaling pathway can be affected by mutations in *KRAS*. *KRAS* codes for a GTP-binding protein with activity in that pathway (Leslie *et al.,* 2002). Mutations in *TP53* gene are associated with advanced histological stage and with transition from adenoma to adenocarcinoma (Worthley and Leggett, 2010).

1.5.2 – Microsatellite Instability

Another molecular pathway involved in CRC is related with MSI. It consists of differences in the number of nucleotide repeats in microsatellite sequences dispersed across somatic and germline DNA. MSI is responsible for increasing genetic errors implicated in carcinogenesis (Worthley and Legget, 2010). Often, CRC presents elevated microsatellite instability (MSI-H), which corresponds to, at least, 2 unstable makers from a panel of 5 loci (Boland *et al.,* 1998 cit. Copija *et al.,* 2017). It is also common the identification of CRC cells lacking DNA mismatch repair system activity, resulting in failure to repair errors in microsatellite DNA repeats (Bacher *et al.,* 2004). The tumors that present 10-29% unstable loci can be classified as MSI-low (MSI-L) and have distinct characteristics being considered a subclass of CRC (Grady and Carethers, 2008). The mechanism underlying microsatellite instability has been uncovered in recent years, involving aberrant DNA methylation or somatic mutations that induce inactivation of DNA mismatch repair family genes (Grady, 2004). Lynch syndrome patients develop, almost exclusively, MSI colorectal cancer since they carry germline mutations in one of the *MMR* genes (*MLH1, MSH2, MSH6* and *PMS2*) (Grady, 2004). Conversely, sporadic cancers often lose *MMR* activity through aberrant methylation and silencing of *MLH1*, in order to develop colorectal cancer wit, MSI (Kane *et al.,* 1997). This instability might be detected through analysis of expression in MLH1, MSH2 and MSH6 proteins by immunohistochemistry and by PCR (Kurzawski *et al.,* 2004 cit. Roseweir *et al.,* 2017).

1.5.3 – CpG Island Methylator Phenotype

Hypermethylation of CpG island exist in almost all CRC patients. This is an epigenetic instability feature and manifests itself as hypermethylation of loci which contains CpG islands and in all DNA hypomethylation. However, 10-20 % of CR tumors present extremely high proportion of aberrant methylated CpG loci characterized as CIMP phenotype (Grady and

Markowitz, 2015). The underlying mechanism remains unknown, although it is thought that the overexpression of DNA methyltransferases may be a possible mechanism. Alternatively, the mechanism might rely on inactivation of mechanisms that avoid the methylation of normally unmethylated CpG islands (Issa *et al.*, 2005 cit. Grady and Markowitz, 2015). The epigenetic instability can explain this phenotype, when during the aging process a tumor suppressor gene is methylated, promoting clonal outgrowth of tumorigenic cells (Issa, 2014). In addition, another explicative hypothesis regards to genes involved in chromatin remodeling and histone modification status, which may be implicated in CIMP (Tahara *et al.*, 2014). However, besides these mechanistic hypotheses, it is known that environmental exposures, such as tobacco contribute to methylation of CpG islands (Limsui *et al.*, 2010). Some studies suggest that CIMP might serve as a prognostic marker due to its association with lower survival (Vedeld *et al.*, 2017). Concerning hypomethylated genes in CRC, albeit mechanisms are unknown, it has been recognized that LINE-1 and SAT-α are affected. It has been described that they induce the expression of oncogenes or CIN (Karpf and Matsui, 2005; Grady and Markowitz, 2015).

1.6 - Molecular classification of Colorectal Cancer

Colorectal cancer represents a heterogeneous group of malignant diseases, which begin in the proximal colon up until the rectum, that have anatomical and molecular specificities, therefore stratification is important for appropriate staging and determining therapeutics (Mahasneh *et al.*, 2017). A recent consensus classification emerged from a consortium that proposed a classification based on data from 18 CRC databases (Guinney *et al.*, 2015), the Consensus Molecular Subtypes (CMS) for colorectal cancer: i) CMS1 is the MSI Immune, ii) CMS2 is the Canonical, iii) CMS3 is the Metabolic, (iv) CMS4 is the Mesenchymal. The last three subtypes are characterized by high levels of Somatic Copy Number Alterations (SCNA) (Muller *et al.*, 2016). The authors also described some samples with mix features that represent intra tumoral heterogeneity or transition phenotype (Guiney *et al.*, 2015).

1.6.1 – CMS1: MSI Immune

The CMS1 subtype is characterized by high levels of gene mutations with frequent *BRAF* mutations, high microsatellite instability, high-CIMP (CIMP-H), strong intratumor inflammation and low SCNA (Guiney *et al.*, 2015). The hypermutation status is due to overexpression of DNA damage repair, hypermethylation and *MLH1* silencing. The majorities

of MSI tumors are sporadic and are associated with worse prognosis (Roseweir *et al*., 2017). However, patients bearing MSI positive have better prognosis with this phenotype than patients MSS. However, if BRAF mutations are present, survival decrease (Ogino *et al.*, 2009). CIMP is the most accepted molecular pathway explaining why hypermethylated *MLH1* give rise to sporadic MSI. This occurs since the tumor suppressor and DNA repair genes are silenced if DNA promoter-associated CpG islands are hypermethylated (Boland and Goel, 2010). The main characteristic of CMS1 is a strong immune activation, and this subtype is associated with cytotoxic lymphocyte genes activation which was recognized to be important in intra tumoral inflammation (Becht *et al.*, 2016). The tumor microenvironment is characterized by an increase of tumor infiltrating lymphocytes (TILs). The peritumoral area has increased cytotoxic T-lymphocytes and macrophages when compared to Microsatellite Stable (MSS) tumors (De Smedt *et al.*, 2015). It has been observed that patients with increased count of intratumor cytotoxic T-lymphocytes have increased survival (Deschoolmeester *et al.*, 2011). Helper T ($CD4^+$) lymphocytes promote proliferation of cytotoxic T $CD8^+$ lymphocytes, which eliminate tumor cells. TILs are found either in the invasive margin of tumors and within the cancer cell layers of slowly growing and invasive tumors (Deschoolmeester *et al.*, 2011). Therefore, TILs help characterize CMS1 patients and concomitantly are strong prognostic factors and immunotherapeutic targets (Galon *et al.*, 2006, Park *et al.*, 2016; Singh *et al.*, 2015). CMS1 subtype is more frequently diagnosed in female patients and in right-sided lesions. After relapse, the patients with this subtype have a poorer survival rate (Guiney *et al.*, 2015).

1.6.2 – CMS2: Canonical

The molecular CMS2 subtype is designated as canonical, and the most prominent genomic alteration is epithelial chromosomal instability and somatic copy number alterations (Guiney *et al.*, 2015). SCNA are changes in DNA may cause gain or loss in genome during meiosis (Wang *et al.*, 2016). These alterations in chromosomes can lead to progression of cancer by dysregulating WNT and MYC pathways, being associated with survival (Muller *et al.*, 2016). WNT pathway is key for initiation of colorectal neoplasia; due to APC and other mutations the non-invasive polyp cells progress and originate the CRC (Najdi *et al.*, 2011). Commonly, patients with this subtype have increased survival after relapse as well as long-term survival (Guiney *et al.*, 2015).

1.6.3 – CMS3: Metabolic

The metabolic subtype has less SCNA, but higher hypermutation ratios than CMS2 and CMS4. In this case, the *KRAS* mutations and CIMP-low phenotypes are prevalent, besides the evident metabolic dysregulation (Muller *et al.,* 2016). When Guiney and co-workers (2015) analyzed its genetic and epigenetic features, they found characteristics that are particular of this type of tumors: fewer SCNA, 30% were hypermutated coinciding with MSI status and higher prevalence of CIMP-low. *KRAS* mutations, which are overrepresented in CMS3 tumors, have been associated with development of lung metastasis in advanced disease (Ghidini *et al.,* 2016). Furthermore, *KRAS* mutations are associated with response to EGFR inhibitors, guiding the usefulness of therapeutics towards this target (Al-Shamsi *et al.,* 2015). Cancer cells with CMS3 subtype have an evident metabolism dysregulation that associates with KRAS mutational landscape (Muller *et al.,* 2016).

1.6.4 – CMS4: Mesenchymal

The CMS4 mesenchymal subtype has a high number of SCNA and increased expression of Epithelial-Mesenchymal Transition (E-M-T) genes including transforming growth-factor beta 1 (TGFβ1) (Muller *et al.,* 2016). Another characteristic of this subtype is the overexpression of proteins implicated in stromal invasion and angiogenesis, activation of matrix remodeling pathway and complement inflammatory system, particularly associated with the presence of cancer-associated fibroblasts (Guiney *et al.,* 2015, Colangelo *et al.,* 2017). The increased stromal invasion upregulated TGF-β1 production and consequently metastasis (Calon *et al*., 2012). The CMS4 tumors are associated with advanced stages and patients with this subtype are prone towards a shorter overall survival and relapse-free survival (Muller *et al.,* 2016).

1.7 – Overview of CRC therapeutics

The adequate treatment depends of several factors, from anatomical variables to staging, molecular subtyping, moment in the natural history of the disease, as well as previous therapies. In the case of the rectal cancer the treatment is made by surgical excision, usually involving removal of surrounding lymph nodes. In colon cancer, surgery allows removal of tumor and if necessary the nearby lymph nodes (Brenner *et al.,* 2014). Before surgery, in specific cases, chemotherapy might be adequate to shorten the dimension of tumor burden, in order to down-stage the disease. After surgery, adjuvant chemotherapy and/or radiotherapy intends to

complete the removal of micrometastatic focuses either at distance or locally, respectively, thereby preventing tumor relapse. When colorectal cancer metastasizes into the liver or lungs, options are surgical metastasectomy (when achievable) and/or palliative chemotherapy for most patients (Brenner *et al.,* 2014).

1.7.1 – Immunotherapy for Colorectal Cancer

Chemotherapy, radiotherapy and surgery are major ways currently in use for treating CRC. Nevertheless, the development of targeted therapies allowed a rational therapeutic approach, envisaging, for example, the inhibition of the vascular endothelial growth factor (VEGF) or the EGFR pathways (Brenner *et al.,* 2014; Pernot *et al.,* 2014), which despite improvements in survival are far from curing cancer or eradicating all cancer cells. These treatment strategies have been shown to synergize with chemotherapy and are now used in the clinical setting together with cytostatic drugs (Boland and Ma, 2017).

The most recently developed weapon for cancer armamentarium was immunotherapy. Initial findings demonstrated a strong impact on survival in several oncological models such as lung, bladder and renal cancers, while clinical research on CRC is ongoing (Bever and Le, 2017). The first results in colorectal patients showed best results in patients with MMR system deficiency (Noel, 2017).

In immunotherapy, the immune system is triggered to attack tumor cells by recognition of tumor-specific antigens or targeting immune checkpoints (Koido *et al.,* 2013). These targets are associated with inhibitory pathways that maintain self-tolerance, reduce the duration of the immune response in peripheral tissues and attenuate the duration and strength of signaling between Antigen-Presenting Cells (AP cells) and T cells (Amin and Lockhart, 2015). There are now evidences supporting either cancer cells' tumor-specific antigens mutational profile that originates immune evasion, particularly of cytotoxic T cells, and tumor-induced downregulation effect through binding T-cells (Schreiber *et al.,* 2011). The rationale for checkpoint inhibitors (anti-PD-L1, anti-PD-1) and anti CTLA4 drugs is precisely based on the fact that immune checkpoint interacts with ligand-receptor for initiation. The drugs currently on clinical trials for colorectal cancer are ipilimumab (anti-CTLA4), pembrolizumab and nivolumab (anti-PD1) and atezolizumab (anti-PD-L1).

- Cytotoxic T-Lymphocyte Associated protein 4

Cytotoxic T-Lymphocyte Associated protein 4 (CTLA-4) is a protein that modulate the second T-cell Receptor (TCR) signal, working as co-inhibitor by decreasing the immune response and through intensification of the first signal (interaction between TCR and co-receptors CD4 or CD8 with the Major Histocompatibility Complex (MHC)/peptide of APC). The CTLA-4 protein is homologous of CD28 (and belongs to its family) competing to bind to B7 receptor, with CTLA-4 having higher affinity to receptor (Buchbinder and Desai, 2016). When CD28 binds to B7, it stimulates the immune response, by inducing the proliferation of immune cells and Interleukin-2 (IL-2) production (figure 2A). When CTLA-4 interacts prevalently with B7 the negative signals are dominant and result in reduced T cell proliferation and decreased IL-2 production (figure 2B) (Buchbinder and Desai, 2016). Blocking CTLA-4, the B7 receptor is free to bind CD28, thus stimulating the immune response to tumor (figure 3) (Blank and Enk, 2014).

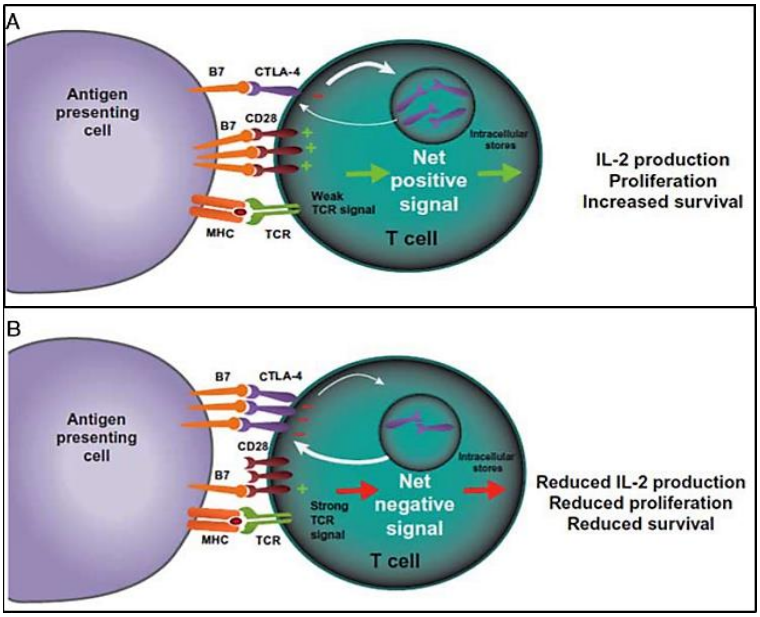


Figure 2 - CTLA-4-molecular pathway. (A) When the connection of CD28 receptor to receptor of AP cell is prevalent, the result is IL2 production, proliferation and increased survival of immune cells. (B) However, the IL-2 production, proliferation and survival decrease when the resulting prevalent signal is the binding of CTLA-4 to B7 receptor (Buchbinder and Desai, 2016).

- Programmed Death 1 and Programmed Death Ligand 1

The Programmed Death 1 (PD-1) protein is involved in the late phase of immune response in peripheral tissues (Buchbinder and Desai, 2016). It is expressed by T and B cells, while Programmed Death Ligands (PD-L1 and PD-L2) are expressed in other cellular populations, including neoplastic cells. The connection between PD-1 and its ligands induces decrease of

the immune response when cells are already engaged with T-cell (Wherry, 2011 cit. Buchbinder and Desai, 2016). The decrease in tumor immunoreactivity is established by different pathways: when PD-1 interacts with PD-L1 the immune response is affected by inhibition of expression of cytokines and reduced proliferation and survival of T-cells. Thus, when one of these proteins is inhibited, the immune function of T-cell is restored (figure 3) (Keir *et al.*, 2008 cit. Buchbinder and Desai, 2016).

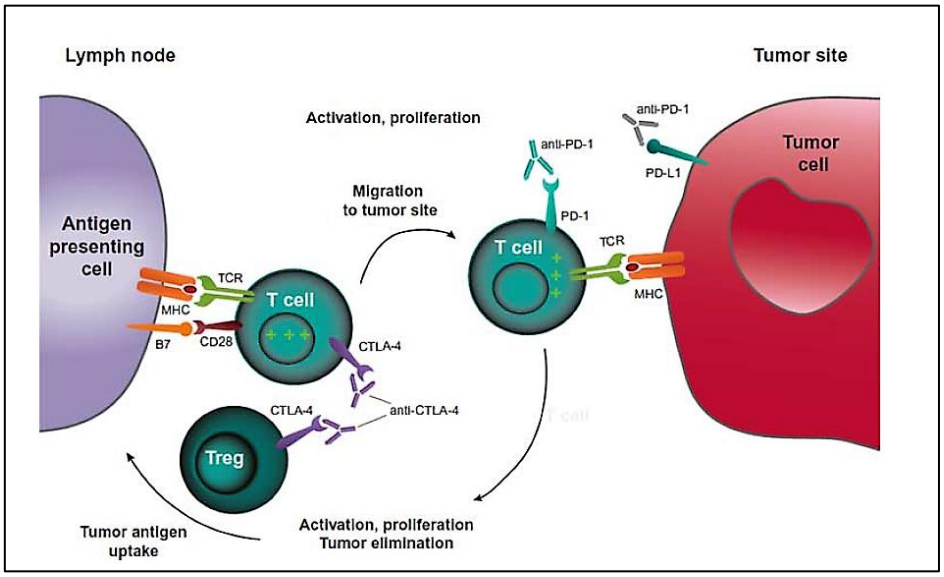


Figure 3 – Mechanisms of cancer immunotherapy. The blocking of CTLA-4 allow the binding of CD28 to AP cell receptor leading to activation, proliferation and migration of T cell to tumor site. Blocking PD1 or its ligand, PD-L1, trigger the activation and proliferation of T cell, resulting in tumor cell elimination. As these pathways occur in different moments of immune response, some studies suggest that to assure an efficient therapy, these two approaches can be used together (Buchbinder and Desai, 2016).

- Potential target to immunotherapy

Recently, a new target for immunotherapy emerged, allowing to discriminate cancer stem cells from non-cancer stem cells, thereby useful for oncology and particularly for colorectal cancer (Miyamoto *et al.*, 2018). Cancer Stem Cells (CSC) have the ability to escape chemo and radiotherapy effects, through mechanisms that activate drug transporters and damage the checkpoint pathways (Bao *et al.*, 2006, Li *et al.*, 2008, Diehn *et al.*, 2009 cit. Miyamoto *et al.*, 2018). The gene encoding the ankyrin repeat and SOCS Box protein 4 (ASB4) , has been shown in mice to be expressed during development, while in adults is expressed only in cancer stem cells. In colorectal cancer stem cells, this ASB4 antigen can promote the activation of cytotoxic T lymphocyte (CTL) by connection with its peptide IV-9. After stimulation of these cytotoxic cells, cancer stem cells can be eliminated and prevent resurgence of new tumors (Miyamoto *et al.*, 2018).

2 – CRC Tumor Microenvironment

2.1. Overall

The tumor microenvironment (TME) is characterized by cellular and non-cellular components that include malignant cells and its neighboring, contributing to the acquisition of some cancer hallmarks, such as resistance to apoptosis, ability to invade new tissues and angiogenesis. In tumor microenvironment, players such as extracellular matrix, fibroblasts, immune cells, endothelial, pericytes, platelets and adipocytes exert interactive cell-to-cell modulation, ultimately impacting malignant cells. The role of these cells in colorectal cancer is not well understood, while some have a role in promoting aggressiveness, others support the immune system in the fight against cancer progression. As depicted in figure 4, cells that support cancer progression increase its number in tumor microenvironment, thereby the cells that fight and eliminate tumor cells decrease in this milieu (Wang *et al.*, 2017).

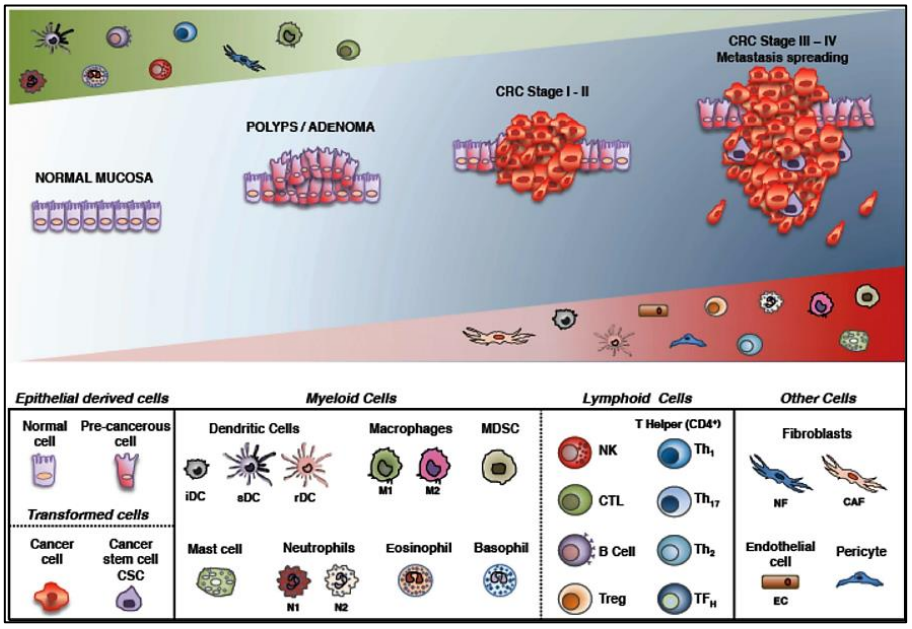


Figure 4 - Tumor microenvironment in colorectal cancer. In different phases of CRC, cells in tumor microenvironment are different. Number of some cells decrease with advance of stage and malignancy of the tumor (green triangle). Otherwise, the cells which promote the progression of cancer increase its number (red triangle). With progression of aggressiveness of tumor, the number of cancer cells increase, and, in late stages, emergence of cancer stem cells is seen (Colangelo *et al.*, 2017).

In normal colonic mucosa, fibroblasts are the major stromal population. The cooperation between those cells and the epithelial compartment is essential to reassure tissue integrity; being also responsible for synthesis and renewal of components of the cellular membrane

(Karagiannis *et al.,* 2012). During tumorigenesis, fibroblasts are transformed into cancer-associated fibroblasts (CAF) through different pathways, including induction by interleukins or growth factors secreted to the microenvironment or overproduction of reactive oxygen species (ROS) (Cirri and Chiarugi, 2011). CAFs are considered the major cellular component of tumor stroma and are responsible by secretion of growth factors. In colorectal cancer, CAFs have the ability to promote tumorigenesis and tumor survival, supporting growth, migration, and metastasis dissemination (Karagiannis *et al.,* 2012). Furthermore, CAFs are able to dedifferentiate colorectal cancer cells into CR cancer stem cells (Todaro *et al.,* 2014), which are responsible for tumor heterogeneity, metastasization, persistence and relapse of the disease (Mathonnet *et al.,* 2014). Thus, CAFs are associated with worst prognosis and clinical outcomes in colorectal cancer patients (Colangelo *et al.,* 2017).

Endothelial cells are the surface layer of vessels, very important to the development and normal function of blood and lymph vessels. They create a continuous and uniform monolayer through cytosolic projections. In the case of overexpression, the monolayer become irregular in shape and size as observed in tumors. Pericytes, cells located instead at the basal layer of the vessels are also important for the formation and function of blood vessels by directly communicating with endothelial cells (Colangelo *et al.,* 2017). These cellular components of TME usually contribute to aggressiveness and metastasis of colorectal cancer (Raza *et al.,* 2010; Cima *et al.,* 2016). Metastasis, tumor recurrence, and patient mortality are related with high vascularity in colorectal cells with ability for invasion ((Raza *et al.,* 2010).

Adipocytes store fat, representing an important energy source, as well as participate in inflammation, recruiting immune cells. In obese individuals, adipocytes may be related to tumor progression, as they secrete a wide range of adipokines with pro-tumoral potential. Besides, in obese individuals, the high proportion of adipose-derived stem cells are responsible for increasing the number and activity of macrophages and monocytes which promote a proinflammatory microenvironment (Wang *et al.,* 2017), and for contributing towards its migration homing for tumors, where they might differentiate as endothelial or fibroblastic precursors.

2.2 - Immune cells

Immune cells can be divided into two groups: innate immune system cells (mast cells, neutrophils, eosinophils, basophiles, monocytes-macrophages, dendritic cells, myeloid derived

suppressor cells and natural killer cells) and adaptive immune system cells (B and T lymphocytes). Immune cells, together with tissue and organs form a complex aiming at defending the organism against foreign antigens. The direct contact between these cells and tumor cells is a complex crosstalk that can result either in cancer elimination or its survival and metastasis (Colangelo *et al.*, 2017).

2.2.1 – Mast Cells

Activated mast cells (MCs) are responsible for the production of cytokines and growth factors that are involved in the inflammatory process, allergic reaction, response to pathogens, autoimmune diseases and wound healing (Stone *et al.*, 2010). They may be involved in cancer, and in colorectal cancer, are associated with upregulated angiogenesis, aggressiveness, resulting in reduced survival (Malfettone *et al.*, 2013).

2.2.2 – Granulocytes: neutrophil, eosinophil and basophil

Neutrophils are important in both acute and chronic phase's responses of inflammation, once they migrate to inflammation areas by chemotaxis (Kolaczkowska and Kubes, 2013) and diapedesis, leading to the elimination of intra and extracellular pathogenic agents by phagocytosis (Arosa *et al.*, 2012). The tumor microenvironment has the ability to transform neutrophil in Tumor Associated Neutrophils (TANs), which can be subdivided into two subgroups: N1 and N2. While the N1 phenotype secrete cytokines and chemokines that participate in immune response and have the ability to kill tumor cells, conferring an anti-tumor effect, the N2 has pro-tumor effects, secreting low levels of proinflammatory/proneoplasic agents. The N2 phenotype is related with increased TGF-β availability, whereas when this cytokine is blocked the N1 phenotype prevails (Granot and Jablonska, 2015). In CRC, the role of neutrophil is not well elucidated, although the activated neutrophil can induce tumor angiogenesis by production of oncostatin M, CXCL 1 and 8 and VEGF, thus associating them with poorer clinical outcome. However, neutrophil can also have anti-tumor properties, by producing cytokines, proteases and ROS that promote the death of tumor cells (Peddareddigari *et al.*, 2010). Eosinophils are less abundant in blood compared to neutrophil but can be found with a higher density infiltrated in gastrointestinal tissue. They are involved in inflammatory response, but having low phagocytic capability (Singh *et al.*, 2010). In CRC, eosinophils are associated with better prognostic outcome, which may be related with higher abundance of Colorectal Cancer Associated 1 and 2 (COLCA1-2) transmembrane proteins in eosinophils of

tissue stroma than in bone marrow (Prizment *et al.,* 2016). In the case of basophils, its importance in CRC is not yet elucidated (Colangelo *et al.,* 2017). These cells are involved in hypersensitive reactions, without ability to develop phagocytic responses, producing active substances like heparin and histamine (Arosa *et al.,* 2012).

2.2.3 – Macrophages and Tumor Associated Macrophages (TAMs)

Monocytes in circulation are recruited towards tissues by chemotaxis and then differentiate into macrophages. Macrophages have improved phagocytic ability, increased number of lysosomes with hydrolytic enzymes, which represent an improvement of metabolism and microbicide potential. Macrophages are also cells that present antigens to T lymphocytes (Arosa *et al.,* 2012**)**. In tumor microenvironment, two populations of mature macrophages have been identified, M1 or M2 (Chanmee *et al.,* 2014). The M1 phenotype can be activated by Interferon Gama (IFN-γ) or by microbial products as Lipopolysaccharide (LPS). Upon stimulation, they produce interleukins and stimulate the expression of MHC class II and its costimulatory molecules. M2 macrophages are associated with down-regulation of MHC II expression, and through cytokines and differentiation of regulatory T-cell, they promote tumor progression (Martinez and Gordon, 2014). The M2 macrophages are also able to promote angiogenesis by secretion of growth factors, and afford resistance to chemotherapy (Stockmann, *et al.*, 2011). Different phenotypes of macrophages play different roles in cancer. M1 are associated with absence of metastasis, anti-cancer properties (Chanmee *et al.,* 2017) and better prognosis, while M2 are associated with progression of cancer and poorer prognosis (Erreni *et al.,* 2011). Apparently, in colorectal cancer, the role of these cells in tumor biology is influenced by its localization in TME. When located in the edge of the tumor, they promote Fas-ligand associated apoptosis of cancer cells, but if placed in the invasive front of the tumor, apoptosis is reduced (Colangelo *et al.,* 2017).

2.2.4 – Dendritic Cells

Dendritic cells (DCs) are potent antigen presenting cells, including to T *naive* cells (Arosa *et al.,* 2012). In tumors, the microenvironment can influence the specialization of the mature dendritic cells. If they are exposed to proinflammatory signals, such as pathogen-associated molecular patterns (PAMPs), they differentiate into stimulatory dendritic cells which promote an immune response by secretion of Tumor Necrosis Factor Alpha (TNF-α) and interleukins, leading to survival of tumor cells (Blanco *et al.,* 2008). Otherwise, DC can differentiate into

regulatory DCs, if stimulated by tolerogenic signals, such as prostaglandins, and stimulate the differentiation and proliferation of the T-regulatory (T-Reg) cells, thus promoting tumor progression (Schmidt *et al.*, 2012). In colorectal cancer, when the elevated number of tumor-infiltrating dendritic cells associates with reduced tumor size, the odds for tumor metastasis is low and relates with better clinical outcome. However, sometimes this correlation is inconsistent, perhaps due to different populations of DCs or its plasticity (Colangelo *et al.*, 2017).

2.2.5 – Myeloid-Derived Suppressor Cells

These cells normally arise from immature myeloid cells (iMCs) with immune-suppressive activity, originating mature granulocytes, macrophages or dendritic cells. However, in cancer, iMCs may also originate myeloid-derived suppressor cells (MDSCs) due to the effect of various factors produced by tumor cells. These factors have the ability to promote the survival, proliferation (Schmid and Varner, 2010) and activation of MDSCs by stimulation of STAT6, STAT1 and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways (Colangelo *et al.*, 2017). In colorectal cancer, upon activation of MDSCs, they can suppress the immune activity by increasing the production of ROS and expression of immune suppression factors, such as arginase and inducible nitric oxide synthase (iNOS). These pathways are capable of reducing the activity of T and natural killer cells, therefore, MDSCs are associated with advanced stage of disease (Vasquez-Dunddel *et al.*, 2013).

2.2.6 – Innate Lymphoid Cells

Innate lymphoid cells (INL) are part of the innate immune system, being a family of cells where Natural Killer (NK) cells, lymphoid tissue inducer (LTi) and non-cytotoxic ILC cell populations belong. NK are a family of cells related to lymphocytes, sharing the same precursor and the cytotoxic effects (Owen *et al*, 2013). These cells that have a limited set of no rearranging receptors do not depend of antigen recognition by MHC molecules (Owen *et al.*, 2013). They can directly eliminate tumor cells which blocked MHC class I molecules (Vitale *et al.*, 2014) or present altered expression of surface receptors. The NK are also responsible for secreting IFN and proinflammatory cytokines (Gras Navarro *et al.*, 2015). In colorectal cancer, NK cells are related with good prognosis, survival and low recurrence of metastasis due to its cytotoxic effect, despite they exhibit only limited capability to infiltrate CRC (Sconocchia *et al.*, 2014).

Non-cytotoxic innate lymphoid cells (ILC1, ILC2 and ILC3) exhibit similar function to lymphocyte T helper due to cytokine release (Artis and Spits, 2015). All express different surface molecules and are crucial to biological processes such as chronic inflammation (ILC1 and 3), and metabolic (ILC2) and intestinal homeostasis (ILC3) (Artis and Spits, 2015). IL-23 receptor is expressed on ILC surface and its ligand by colorectal cancer cells. In transgenic mice the induction of systemic expression of IL-23 leads to development of adenomatous tumors (Chan *et al.,* 2014). IL-22 is also expressed by ILC3 and is important to colonic epithelial cell repair. Its effects are controlled by IL-22BP. In transgenic mice, when the control is not made, IL-22 is constitutively produced contributing for tumor development (Huber *et al.,* 2012).

2.2.7 – B lymphocytes

B cells are involved in humoral responses of adaptive immunity. When differentiated to plasma cells produce antibodies or immunoglobulins and memory cells. They can be activated either directly through the interaction with Th2 or independently of T helper cells (Hoffman *et al.,* 2016). In CRC, B cells have been associated with good prognosis by a mechanism of specific immune response against tumor cells. However, there is a subpopulation of B cells, the regulatory B cells, which are associated with advanced tumor stages and metastases due to its immuno-suppressive potential (Berntsson *et al.,* 2016).

2.2.8 – T lymphocytes

T lymphocytes also originate in the bone marrow, but their maturation occurs in the thymus. Functionally, they are composed of three subpopulations: i) cytotoxic T lymphocytes (CTL, $CD8^+$), (ii) T helper (Th, $CD4^+$) cells and (iii) T regulatory (Treg $Foxp3^+$) cells.

Cytotoxic T cells, usually associated to a CD8 molecule are activated by MHC class I molecules, triggering its cytotoxic effects (Arosa *et al.,* 2012), and related with CRC survival (Calon *et al.*, 2015).

T helper lymphocytes interact directly with MHC class II molecules and identified by the presence of CD4 (Arosa *et al.,* 2012). Upon stimulation, T helper cells secrete cytokines that are responsible for stimulating different pathways, in order to aid other cells, such as $CD8^+$ T, macrophages and B cells. After activation, these cells can differentiate into different subpopulations, including Th1, Th2, Th17, Th follicular and Th regulators (or Treg with Foxp3). Depending of the subgroup, the T cells secrete different cytokines which are responsible for inducing immune responses (Arosa *et al.,* 2012). The different subgroups also

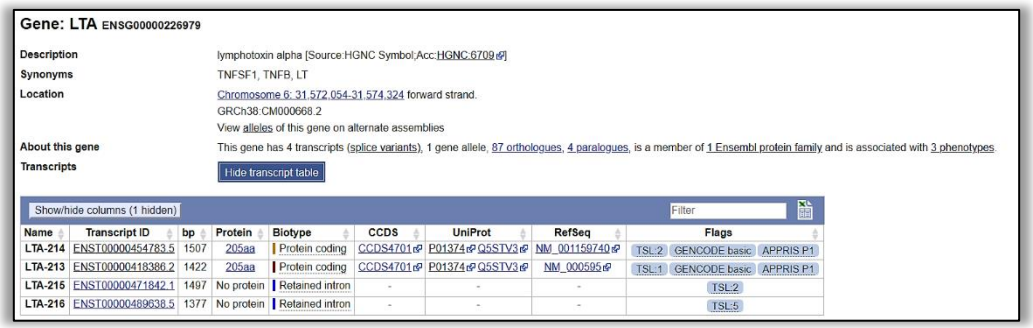
exhibit different roles in tumorigenesis. Th1 cells are associated with better prognosis due to its anti-tumor profile that includes secretion of IFNγ and recruitment of macrophages to eliminate tumor cells (Tosolini *et al.,* 2011), whereas Th17 cells are related with worst survival (Housseau *et al.,* 2016).

T regulatory cells, which are essential to promote the tolerance and immune suppression, modulate the immune response of multiple cells such natural killer, B and T cells and may have CD4 or CD8 molecules in the surface (Arosa *et al.,* 2012). In tumors, such as endometrial and breast cancer, the Treg are associated with the promotion of angiogenesis, but in CRC, the prognostic is still uncertain. However, recently, a classification of Treg cells based on the expression of Forkhead box P3 (FOXp3) in their surface has emerged. Accordingly, patients with high $FOXp3^+$ T cells infiltration have poor prognosis, while patients with infiltration of low $FOXp3^+$ T cells have better prognosis probably due to reduced immune suppressive activity promoted by this class of cells (Saito *et al.,* 2016).

3 - Lymphotoxin Alpha Gene

Lymphotoxin alpha (*LTA*) (ENSG00000226979), also known as Tumor-Necrosis Factor Beta (TNFβ) or Tumor Necrosis Factor Ligand Superfamily member 1 (TNFSF1), is located on human chromosome 6 in p21.3 position in the forward strand with 2 270 base pairs (bp). This gene is located in to the class III region of MHC loci (Remouchamps *et al.,* 2011). The *LTA* gene is expressed by NK, T and B cells, in ILCs and DCs (Koroleva *et al.,* 2018). In organs, the LTA is mostly expressed in the lymph nodes, human appendix, spleen, bone marrow, testis and urinary bladder. In the colon, duodenum and small intestine, its expression is smaller, according to National Center for Biotechnology Information (NCBI) database.

Based on the information located on the Ensembl database, the *LTA* has four transcripts, but only two code for protein coding (ENST00000454783.5 and ENST00000418386.2). These proteins have both 205 amino acids, but the transcript has 1507 bp and 1422 bp, respectively (figure 5).



Gene: LTA ENSG00000226979

Description	lymphotoxin alpha [Source:HGNC Symbol;Acc:HGNC:6709]
Synonyms	TNFSF1, TNFB, LT
Location	Chromosome 6: 31,572,054-31,574,324 forward strand. GRCh38:CM000668.2 View alleles of this gene on alternate assemblies
About this gene	This gene has 4 transcripts (splice variants), 1 gene allele, 87 orthologues, 4 paralogues, is a member of 1 Ensembl protein family and is associated with 3 phenotypes.
Transcripts	Hide transcript table

Show/hide columns (1 hidden) — Filter

Name	Transcript ID	bp	Protein	Biotype	CCDS	UniProt	RefSeq	Flags
LTA-214	ENST00000454783.5	1507	205aa	Protein coding	CCDS4701	P01374 Q5STV3	NM_001159740	TSL:2 GENCODE basic APPRIS P1
LTA-213	ENST00000418386.2	1422	205aa	Protein coding	CCDS4701	P01374 Q5STV3	NM_000595	TSL:1 GENCODE basic APPRIS P1
LTA-215	ENST00000471842.1	1497	No protein	Retained intron	-	-	-	TSL:2
LTA-216	ENST00000489638.5	1377	No protein	Retained intron	-	-	-	TSL:5

Figure 5 - Descriptive data of *LTA* gene. This gene has four transcripts: two give rise to proteins and, the other, are non-coding. The alternative name and symbols of *LTA* gene, as well as its localization on chromosome 6, are shown.
(http://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000226979;r=6:31572054-31574324, consulted in December 13th, 2017).

LTA protein belongs to the Tumor Necrosis Factor Superfamily and its structure and function is related with TNF-α (Lu and Browning, 2014). The proteins of this family are type II transmembrane proteins with N-terminal intracellular and C-terminal extracellular. This TNF protein family is synthetized as membrane bound protein, however, some of them may be cleaved by proteases and assume the soluble form (Bodmer *et al.*, 2002). In the case of LTA, this can be released by activated lymphocytes as: (i) homotrimer (LTα3), located outside the cell membrane and usually binding to the cell TNF Receptor (TNFR); (ii) membrane bound complex, originating a LTA heterotrimer, binding to a second protein, the Lymphotoxin Beta (LTβ), to form LTα/β complex. This protein complex can be presented in two different structures (Albarbar *et al.*, 2015): (i) LTα1β2 binding exclusive a Lymphotoxin-beta Receptor (LTβR); (ii) LTα2β1 binding to TNFR and LTβR. However, LTα/β membrane complex can also change to a soluble form by proteolytic cleavage and binding to TNF receptor in distant cells (Young *et al.*, 2010).

The LTA is recognized as a pro-inflammatory cytokine and mediates several functions such as inflammation, immunostimulation, participate in viral response and organogenesis of secondary lymphoid organs during fetal development (Koni *et al.*, 1997 cit. Morishige *et al.*, 2013). In mouse experiments where this protein and other associated proteins are blocked, the mice show absence of lymph nodes and Peyer's patch and disturbance on lymphoid organs architecture such as thymus and spleen (Koroleva *et al.*, 2018). Furthermore, this cytokine is also involved in apoptosis (Aggarwal *et al.*, 2012) and induces the expression of chemokines and adhesion molecules in endothelial cells. In cancer cells, this product of activated T cells,

helps in the communication between lymphocytes and stromal cells, resulting in the triggering of a cytotoxic effect (Huang *et al.,* 2013).

3.1 – Signaling pathway

All cytokines produced only have effector effects when linked to a specific receptor. The LTA protein is able to bind to different receptors forming a complex network. This ligand has the capability to bind to TNFR1, TNFR2, LTβR, Herpes-Virus Entry Mediator (HVEM) and TROY or TNF Receptor Superfamily member 19 (TNFRS19) (figure 6) (Remouchamps *et al.,* 2011). These receptors have extracellular, transmembrane and cytosolic domains. The extracellular domain is common to all receptors having cysteine-rich domain. The intracellular domain differs between receptors. The intracellular domain of TNFR1 contains a Death Domain (DD), while the TNFR2, LTβR, HVEM and TROY do not have a death domain, but contains a TNF Receptor-Associated Factor (TRAF) binding site (Remouchamps *et al.,* 2011). The TNFR1 (also known as p55 or TNFRSF1A) is expressed in almost all nucleated cells and tissue of the body, and, generally, signals to the cell death (Albarbar *et al.,* 2015). This receptor mediates downstream signaling through its DD. The connection between LTA and TNFR1 triggers the caspase-8-dependent cell death by recruitment the adaptors like the TNFR-Associated Domain (TRADD) or FAS-Associated Domain (FADD) (Dempsey *et al*., 2003). The TNFR2 (also called p75 or TNFRSF1B) is expressed in more restricted cells types, such as immune and endothelial cells (Aggarwal *et al.,* 2012). Usually, this receptor is associated with cell survival by stimulation of NF-kB signaling pathway (Faustman and Davis, 2013). The LTβ receptor or TNFRSF3 is constitutively expressed in myeloid cell lineage and in the stroma of thymus and secondary lymphoid organs (Ware, 2005). The ligands that can bind to this receptor are LTα/β complex and TNFSF14 [or homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpes virus entry mediator, a receptor expressed on T lymphocytes (LIGHT)] (Schneider *et al.,* 2004). Upon binding, the activation of downstream signaling pathways, such as NF-κB, is triggered (Dempsey *et al.*, 2003).

HVEM acts as receptor and ligand, causing activation or inhibition of the pathways. This protein can engage with LTA and LIGHT promoting inflammation and immune response. The binding of HVEM ligand with BTLA or CD160 triggers an inhibitory response (Cheung *et al.*, 2009).

TROY is a tumor necrosis factor receptor superfamily which interact with LTα3. It was identified in 2000 by Kojima and colleagues and it is expressed in developing hair follicle, embryonic skin and adult central nervous system (Kojima *et al.*, 2000). The interaction between LTα3 and TROY results in the activation of NF-kB pathway (Hashimoto *et al.*, 2008). Furthermore, it was identified as an important receptor to Wnt signaling in the intestine, by interaction with Leucine-rich repeat-containing G-protein coupled Receptor 5 (LGR 5) (Fafilek *et al.*, 2013).

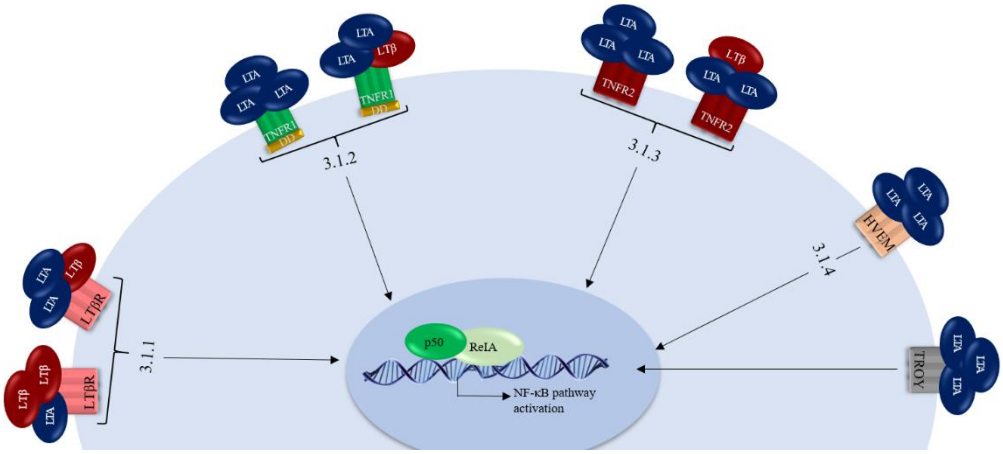


Figure 6 - LTA molecular mechanisms. The molecular pathways activated by LTα2β1 and its receptors have the same results when these receptors are activated by LTα3 or LTα1β2. The connection between LTα3 with TROY leads to NF-κB pathway activation such as TNFR1. The main pathways are described below in detail on indicated items.

3.1.1 – LTα1β2 binding to LTβR

The connection between LTβ receptor with its ligands may activate the classical or canonical and alternative or noncanonical NF-κB pathways by recruitment of TRAF proteins. In first pathway, the binding of LTα1β2 to LTβ receptor induce its trimerization and recruitment of TRAF2 and TRAF5 which connects directly to LTβR in cytosolic tail. By ubiquitination and phosphorylation of the IκB Kinase (IKK) complex, IκBα is degraded and release p50/p65. In the nucleus, NF-κB activate gene expression (Remouchamps *et al.*, 2011) (figure 7).

In the case of alternative pathway, upon LTα1β2 binding to receptor, LTβR is internalized through dynamin-2-dependent route, exposing the tail of receptor and facilitating the competition between the receptor and NF-kappa-B-inducing kinase (NIK) to binding to TRAF and cellular Inhibitor of Apoptosis (cIAP), in the complex form TRAF2/TRAF3/cIAP1/cIAP2. Upon binding LTβR to complex, the NIK degradation decreases. The accumulation of NIK leads to activation of IKKα by phosphorylation and, in its turn, phosphorylation of p100. This

protein is also ubiquitinated and processed in p52. The ReIB/p52 dimer is translocated to the nucleus and stimulates gene expression (figure 7) (Choi *et al.*, 2017[b]).

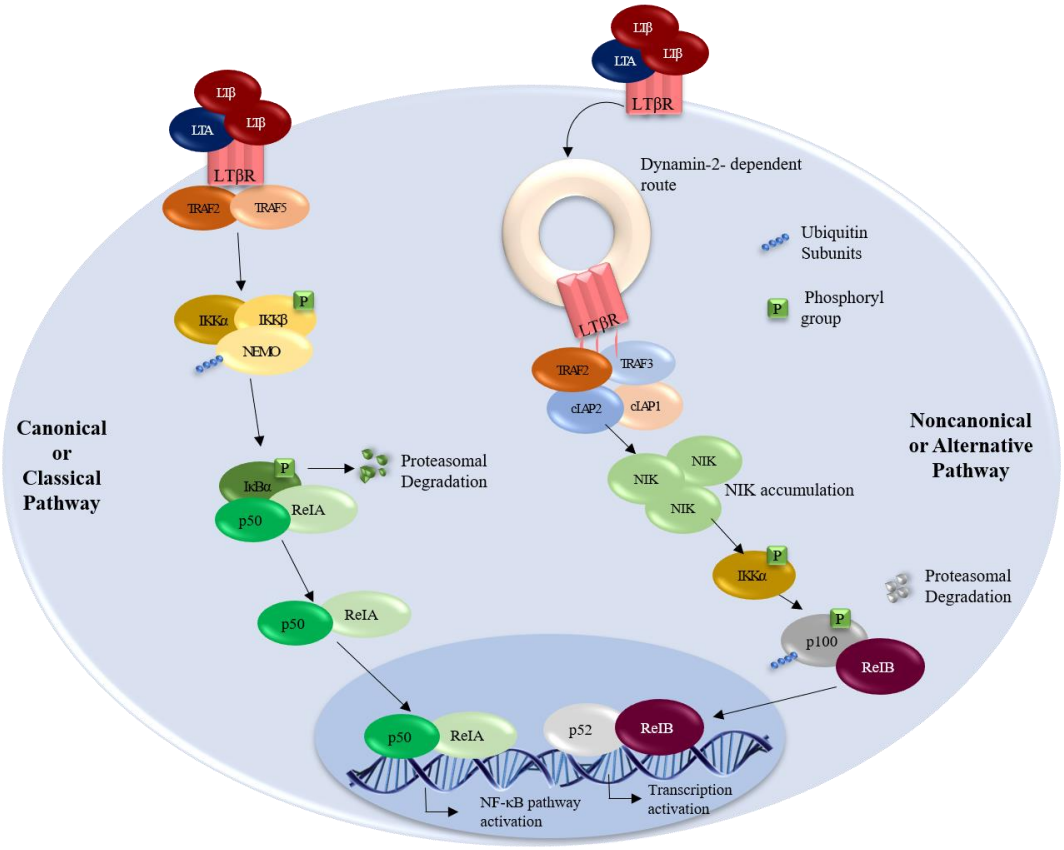


Figure 7 - LTβ receptor intracellular signaling pathway. The LTβ receptor has the capability to activated canonical pathway through TRAF2 and TRAF5, resulting in released and translocation to the nucleus of the transcription factors p50/p65. Noncanonical pathway is activated by binding of LTα1β2 to LTβR. This connection leads to internalization of receptor and recruitment of TRAF2, TRAF3, and cIAPs, leading to ubiquitination of NIK and, consequently, activation of IKKα complex. The result of this activation is the translocation of p52/Re1B to nucleus and stimulation of the gene expression.

3.1.2 – LTα3 binding to TNFRI

The stimulation of TNFR1 leads to activation of NF-κB by canonical signaling, triggering gene expression to proliferation, cell survival, differentiation and apoptosis (Bauer *et al.*, 2012). In this pathway, the complex I is formed when LTα3 binds to TNFR1 and this attachment is responsible for the recruitment of elements of complex, TRADD, TRAF2, TRAF5, cIAP and ubiquitinated Receptor-Interacting Protein 1 (RIP-1) which form complex I (Vandenabeele *et al.*, 2010). This complex induce activation of second complex inhibitor of IκB kinase. This complex consists in IKKα, IKKβ and NEMO/IKKβ and for its activation it is necessary the ubiquitination of NEMO and phosphorylation of IKKβ. This last subunit lead to phosphorylation and consequently proteasomal degradation of IκBα. With its degradation, NF-

κB1 (p50/Re1A) is release and translocated to the nucleus, where it mediates proinflammatory and pro-survival signaling by stimulation of gene expression (Remouchamps *et al.*, 2011) (figure 8). When the canonical pathway is shut down, the stimulation of TNFR1 induce the formation of complex II (formed by TRADD, FADD, caspase-8, RIP1 and RIP3) and result in caspase-8 mediated apoptosis by caspase 8 cleavage of RIP1 and RIP3 or, in case of the blocking caspase-8, occurs the necroptosis[2] mediated by phosphorylation of RIP1/3 (figure 8) (Micheau and Tschopp, 2003, Vandenabeele *et al.*, 2010).

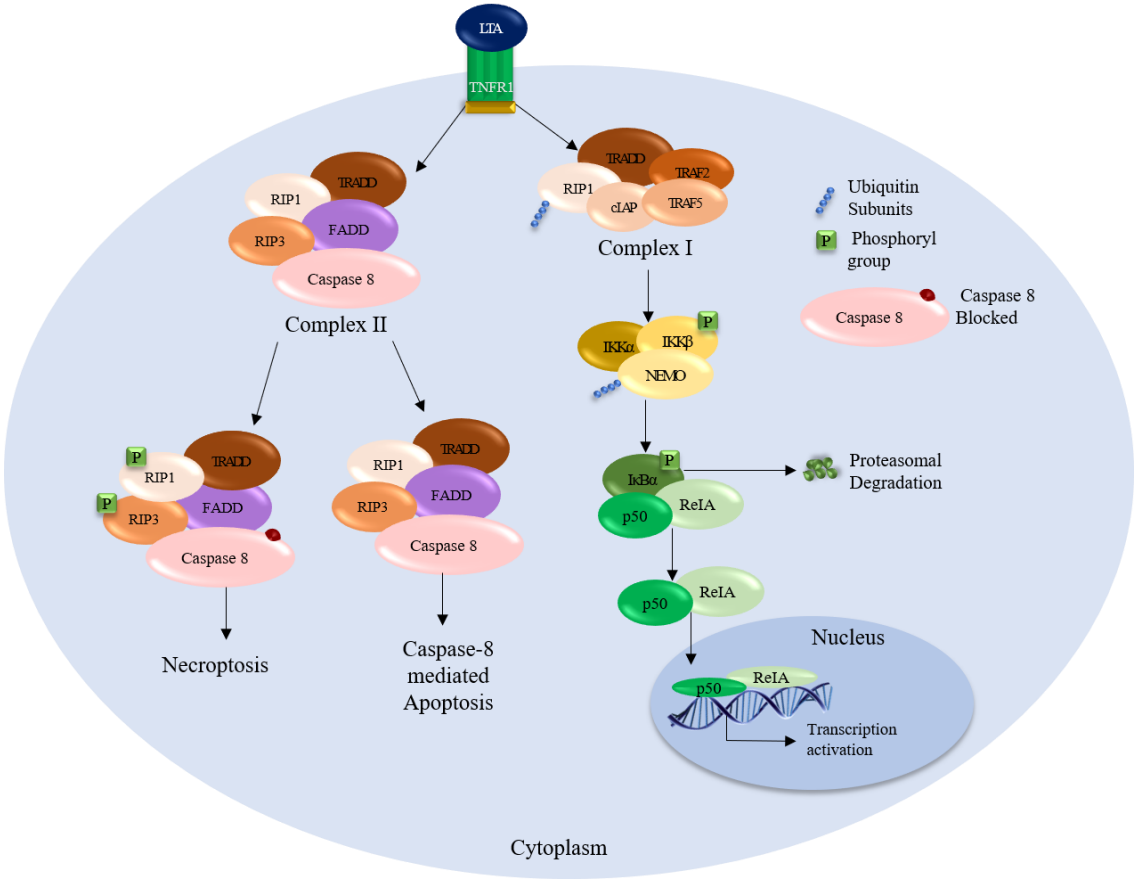


Figure 8 - TNFR1 signaling pathway. The binding of LTα3 to TNFR1 induce the recruitment of complex I and activation of IKK complex by ubiquitination of NEMO and phosphorylation of IKKβ. Its activation lead to degradation of IkBα and release of p50/Re1A. In the nucleus, this complex induces gene expression, inducing the cell survival. In the case of shut down of canonical pathway, the apoptosis or necroptosis are triggered by caspase 8-dependent pathway. If caspase 8 cleave the RIP1 and RIP3 apoptosis occurs. However, these proteins can be phosphorylated when caspase 8 is blocked and, in this case, the necroptosis is triggered.

[2] Necroptosis is a manner to cell death triggered by the same molecules than necrosis, but manifest characteristics from necrosis and sometimes from apoptosis. It is a programmed necrotic cell death (Vandenabeele *et al.*, 2010).

3.1.3 – LTα3 binding to TNFR2

This receptor does not have a death domain, therefore, TRAF proteins have to associate directly with cytoplasmic tails of TNFR (Brenner *et al.*, 2015). This signaling pathway can promote the cell survival by triggering of NF-κB or c-Jun (Mak and Yeh, 2002). Thus, when LTα3 binds to TNFR2, TRAF2 associates with tail of the receptor and stimulates the binding of TRAF3, TRAF5, cIAP1 and cIAP2. These proteins stimulate other proteins for activate NF-kB pathway mediated by NIK, and, in the nucleus, active a gene expression that ensure the cell survival (Faustman and Davis, 2013). This NF-kB signaling pathway can also be activated independently of the IKK, when TRAF2-associated kinase (T2K) binds with TRAF2, with help of TRAF family member Associated NF-κB activator (TANK). The gene expression and cell survival can also be stimulated by c-Jun pathway when Mitogen-Activated Protein-3 Kinase (MAP3K) family members associate with TRAF2, which activates Jun N-terminal Kinase (JNK) (figure 9) (Mak and Yeh, 2002).

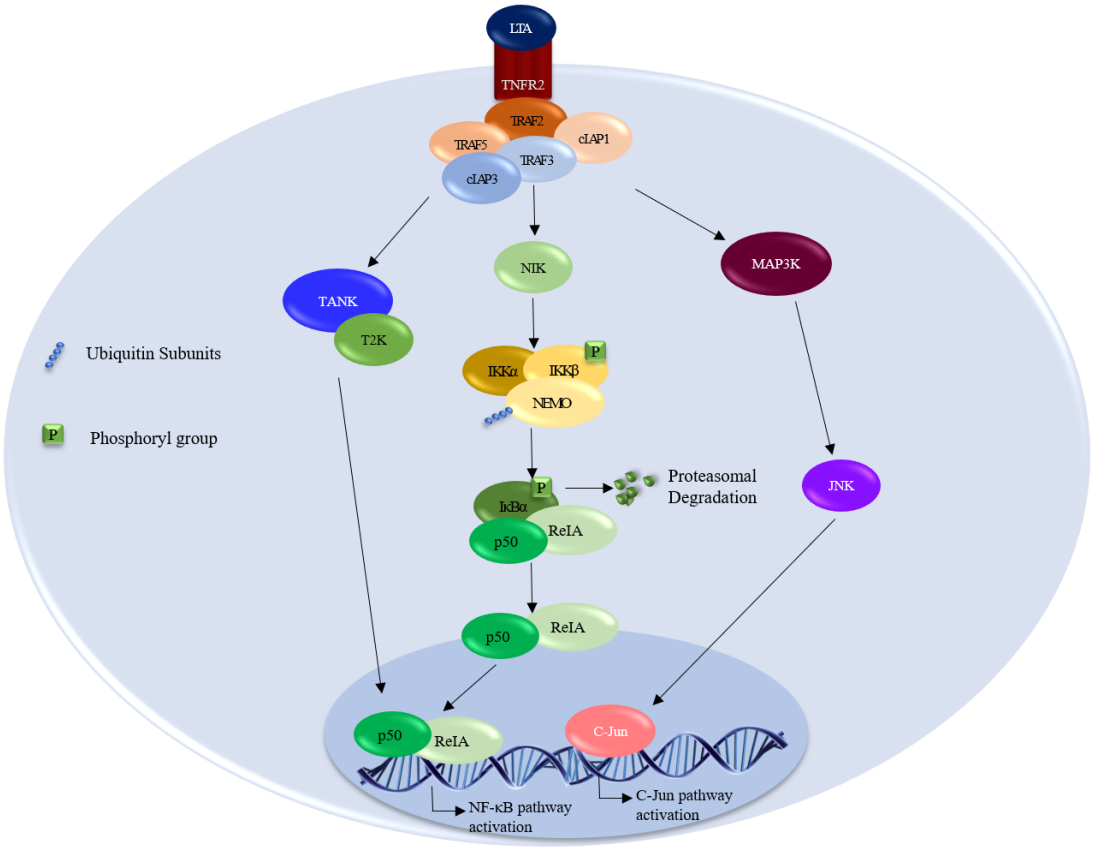


Figure 9 - TNFR2 signaling pathway. To this receptor can bind to TNF α and β (LTα). The triggered pathway results in cell survival by activation of NF-κB, c-Jun and TANK/T2K pathways. After binding of the ligand to the receptor, a complex formed by TRAF2, TRAF1, TRAF3 and cIAPs is recruited. This complex, through NIK, activate the IKK complex and transcription factor NF-κB. If MAP3K proteins are activated, the transcription of genes is stimulated by JNK. It is an alternative pathway which stimulate NF-κB pathway mediated by TANK and T2K.

3.1.4 – LTα3 binding to HVEM

The HEVM represents a third receptor of LTα3, though with low affinity (Ware, 2005). Upon binding of ligand with receptor, TRAF2, TRAF3 and cIAP1 are recruited and activate NF-kB pathway (Ward-Kavanagh *et al.*, 2016). As in TNFR1 signaling pathway, IKK complex is activated and NEMO is ubiquitinated and IkBα is phosphorylated. These alterations lead to degradation of IkBα and release p50/Re1A (NF-kB1). This dimer is translocated to the nucleus and, as transcription factors, promote the transcription of genes to cell survival (Scheneider *et al.*, 2004).

3.2 – Single Nucleotide Polymorphisms

The single nucleotide polymorphisms (SNPs) are variations at a base pair across the genome. Occasionally, these SNPs are responsible for yielding different phenotypes and might contribute to the individual variability of the genome. However not all SNP originate a different phenotype. When SNP occurs in the gene or near of the gene regulatory region, this may affect the gene's function, acting as a biological marker. Furthermore, the SNP may also play an important role in response to drugs, risk of developing a disease and susceptibility to environmental factors (Pierce, 2012).

According to NCBI database (dbSNP), *LTA* gene has 89 SNPs in the coding region in a total of 525 (entire gene). From these 89, only three are cited in Pubmed database. They have missense alterations, leading to amino acid change despite the alteration in protein is tolerated according to the SIFT database. The SNP identifiers are rs2229092, rs2229094 and rs1041981. The *LTA* rs2229092 variant, a modification from adenine-to-cytosine, leads to histidine-to-proline substitution at the codon 2. This missense alteration has 11 citations on Pubmed and, according to Ensembl database, do not have a phenotype associated and its variability in human population is very low (A allele is present in 97 % of world population).

Relatively to rs2229094, the transition of thymine (present in 73 % of world population) to cytosine leads to a conversion of cysteine-to-arginine. This variant has been related with predisposition to development of Proliferative Vitreoretinopathy (PVR) after retinal detachment surgery (Rojas *et al.*, 2010 cit. Pastor-Idoate *et al.*, 2017). This SNP is, also, associated with increased risk of adenocarcinoma in Asians but not in European or North American by recessive model (Huang *et al.*, 2013). The C allele was correlated with change of white blood cell count and lymphocytes percentage of white cells in study of Astle *et al*. (2016).

In the present work, the SNP under study is the variant with higher number of citations in Pubmed, with 77 papers. It is located on gene forward strand, in exon 3 and is identified as rs1041981. This missense alteration (a transversion from cytosine-to-adenine, ENST00000418386.2:c.179C>A) leads to a substitution of threonine-to-asparagine at codon 60 (ENSP00000413450.2:p.Thr60Asn) (Takei *et al.,* 2008). The C allele is considered the ancestral and is present in 61 % in all population, in 69 % is European population, in 49 % of African population and 74 % of South Asian population (information provided by Ensembl 1000 genomes project phase 3, consulted in May 12th, 2018).

3.3 – Relationship with Colorectal Cancer

The *LTA* gene is associated with susceptibility to psoriatic arthritis (Balding *et al.,* 2003), myocardial infarction (Ozaki *et al.,* 2002) and leprosy type 4 (Alcais *et al.,* 2007). Particularly, the specific variant in study (T60N) is indicated for increased risk of type 2 diabetes (Upadhyay and Fu, 2013) and myocardial infarction (Li *et al.,* 2014).

The influence of lymphotoxin in initiation or progression of cancer is not well explained. Some studies were already performed but the results are not conclusive (Ito *et al.,* 1999; Hehgans *et al.,* 2002; Haybaeck *et al.,* 2009; Ammirante *et al.,* 2010). The LTA may be associated with anti-tumor activity having cytotoxic effects on cancer cells. Initially, this protein was isolated based on the anti-tumor properties due its capability to kill the tumor cells (Aggarwal, 2003). But, now it is recognized that LTA protein has immune, inflammatory effects and capability to recruit natural killer cells to lesion (Takei *et al.,* 2008). Other studies revealed that LTA can promote cell growth and adhesion of cancer cells due to bioactive alleles such as A in rs1041981 variant promoting tumor growth (Takei *et al.,* 2008). This polymorphism was associated with CRC by Sainz *et al.* (2012), where the A allele was associated with decreased risk in women and increased risk in men (Sainz *et al.,* 2012). It was proved that amino acid change (T60N) influence the LTA transcription level which may induce maturation and activation of lymphocytes (Huang *et al.,* 2013).

The activation of one receptor of LTA was also studied to verify a possible influence in CRC. The LTβ receptor, whose ligands are LTα1β2 and LIGHT, when activate demonstrate restrain of cancer lines cells growth from CRC in mice because when ligands from T cells, NK cells or dendritic cells bind to receptor they induce cytotoxic effects preventing the tumor growth (Fernandes *et al.,* 2016).

Therefore, more studies are necessary in order to explain the role of LTA in the initiation and progression of CRC or the role of its variant rs1041981 for improving the diagnosis or treatment of this disease.

II- Objectives

The main objective of this research was to contribute to the study of the molecular epidemiology and genetics of colorectal cancer. Based on the possibility of this disease being influenced by genetic factors, several interesting candidate genes arise, possibly involved in patient's survival.

In detail, the proposed objectives were:

- Characterize the *LTA* functional polymorphism rs1041981 in a CRC population;

- Analyze whether this SNP influences clinicopathological parameters, particularly overall survival and progression-free survival in colorectal cancer;

- Evaluate macrophage and T lymphocytes infiltration in tumor CRC tissue, by immunohistochemistry, and its association with genotypes and clinicopathological parameters.

III - Material and Methods

1 – Sample collection

To perform the study, blood samples were collected on survivor patients with colorectal cancer at any time in the natural history of disease, with more than one-year follow-up after diagnosis. The collections were made in the Department of Oncology, Centro Hospitalar de Trás-os-Montes e Alto Douro (CHTMAD) by clinical researchers. The patients, who accepted to take part in this study, signed an informed consent and the work was conducted in strict conformity with the Declaration of Helsinki (annex I). In total of 173 patients, patient's clinicopathological and demographic information were collected by clinical researchers from CHTMAD through revision of clinical story (stage; histopathology; obesity at diagnosis; blood lymphocyte, monocyte and neutrophil counts at the time of diagnosis; treatments, with beginning-ending dates - surgery, radio, and chemotherapy; diagnosis, relapse and death dates).

Two blood samples were collected at the same time by venipuncture during routine clinical analyses (6 milliliters (mL) in a EDTA tube). Blood samples were stored immediately at 4 ˚C and transported to the Department of Genetics and Biotechnology at UTAD. In order to separate serum, plasma and buffy coat, a centrifugation was performed [2500 revolutions per minute (rpm), 10 ºC and 10 minutes (min) using Eppendorf, centrifuge 5804R (A-4-44)]. Buffy coat and plasma were separated and distributed by different tubes. Aliquots of samples were then stored at -20 ˚C for further DNA extraction.

2 – DNA extraction

DNA extraction was performed using the buffy coat obtained from blood. This fraction of blood contains white blood cells, which in mammals means cells with nucleus and DNA. These cells are usually used for clinical studies once the DNA from these cells show correlation with the individual physical and immune conditions (Huang *et al.,* 2017).

For DNA extraction three protocols were tested (the selected protocol is described below and the other two are described in annex II) to determine the best procedure to obtain DNA with quality and quantity required for the ensuing phase. The tested protocols were: Isolate II Genomic DNA Kit (Ref.: BIO-52065, Bioline), ExtractME® DNA blood Kit (Ref.: EM05-050, BLIRT) and Invisorb® Spin Blood Mini Kit (Ref.: 1031100200, STRATEC Molecular). The DNA concentration and purity were evaluated with NanoDrop ND-1000 spectrophotometer.

Quality was evaluated by polymerase chain reaction using housekeeping gene primers, histone H4, as suggested by Pineau *et al.* (2005).

2.1 – Protocol followed

The protocol optimized and selected among the three initially tested was the ExtractME® DNA blood Kit (Ref.: EM05-050, BLIRT) and is detailed below.

1. Transfer 350 microliter (µL) of buffy coat sample to sterile 1.5 mL microtube.
2. Add 350 µL of the RBC Lysis Buffer and invert the tube to mix until a clear red solution is obtained.
4. Centrifuge for 4 min at 9 000 rpm.
5. Discard carefully the supernatant from over pellet (white blood cells).
6. Add 375 µL of the BL Lysis Buffer.
7. Add 6 µL of the Proteinase K and mix in vortex.
8. Incubate at 55 ºC for 10 min and shake the tube every 3 min.
9. Add 400 µL of the BB Buffer and mix thoroughly.
10. For 20 secs, vortex vigorously.
11. Transfer 500 µL of the lysate onto purification minicolumn placed in a collection tube. Centrifuge for 1 min at 11 500 rpm.
12. Discard the filtrate and reuse the collection tube. Transfer the remain lysate onto purification minicolumn. Centrifuge for 1 min at 11 500 rpm.
13. Transfer the purification minicolumn to a new 2 mL collection tube.
14. Add 600 µL BW1 Buffer and centrifuge for 30 secs at 11 500 rpm. Discard the filtrate and reuse the collection tube.
15. Add 400 µL BW2 Buffer and centrifuge for 30 secs at 11 500 rpm. Discard the filtrate and reuse the collection tube.
16. Centrifuge for 1.5 min at 13 700 rpm to remove the residual ethanol.
17. Discard the collection tube and carefully transfer the purification minicolumn to a sterile 1.5 mL microcentrifuge tube.
18. Add 100 µL of the Elution Buffer, which was pre-heated to 70 ºC, directly onto the purification minicolumn membrane and incubate at room temperature for 2 min.
20. Centrifuge at 11 500 rpm for 1 min.

21. Remove the minicolumn and recover the DNA, storing it at -20 ºC until use.

2.2 – Evaluation of DNA quality – PCR conditions

To evaluate the DNA quality, a PCR was performed, carrying out a reaction mixture which is described in table 1. The MyTaq™ HS Red Mix (Bioline, BIO-25047) was used. This mix already contains in its constitution *Taq* DNA polymerase, $MgCl_2$ and deoxyribonucleotides (dNTPs) necessary for the reaction to occur. The histone H4 primers were used (H4F2s, 59-TSCGIGAYAACATYCAGGGIATCAC-39 and H4F2er, 5'-CKYTTIAGIGCRTAIACCACRTCCAT-39, Pineau *et al.*, 2005). During the deposition of the PCR products in the agarose gel with GreenSafe Premium, which is a substitute for ethidium bromide, the master mix already contains the loading buffer.

Table 1 – PCR conditions. Reagents and volumes used to evaluate DNA quality.

Reagent	Volume (µL)
MyTaq™ HS Red Mix 2x	7.0
Water	3.8
Primer F (100 ng/µL))	0.6
Primer R (100 ng/µL)	0.6
DNA	2.0
Final Volume	14.0

The temperature cycle programmed in the thermal cycler was:

95 ºC, 1 min
95 ºC, 15 secs
55 ºC, 15 secs } 35 cycles
72 ºC, 10 secs

3 – Real-time PCR

The real-time PCR is based on the activity of DNA polymerase and double-strand DNA-binding dye. For that, it is necessary to use specific primes and probes directed to the sequence of interest and labelled with fluorescent molecules. These probes bind to DNA sequence between primers (Matsuda, 2017). One example of this technique is TaqMan assay, used in the present work. In this case, there are two molecules, reporter and quencher, which are attached to the probe on 5' and 3'-end, respectively. When the probe is intact and bind to DNA sequence, the fluorescence is not detected because the two molecules are too close, and quencher absorb the reporter's fluorescence. In the extension phase of PCR, due to the 5'-3' exonuclease activity of DNA polymerase, the probe is degraded and fluorescence, emitted by reporter, is detected by the equipment (Navarro *et al.*, 2015).

The SNP detection is possible by using probes labelled with different fluorescent dyes. In real-time PCR reaction for detection SNPs, there are three types of fluorophore, two different for discriminate the possible alleles of SNP and the third is to detect the baseline fluorescence (Matsuda, 2017).

In resume, the real-time PCR has been useful to genetic and clinical investigations and been profitable to SNP genotyping (Navarro *et al.*, 2015, Matsuda, 2017).

3.1 – Procedure

Table 2 describes the reagents and respective volumes used in the first reaction of real-time PCR. To optimize this reaction, the volumes of assay, genotyping buffer and final volume were reduced to 0.7 µL, 7.5 µL and 15 µL, respectively. The genotyping buffer used was SensiFAST™ Hi-ROX Genotyping Mix (Ref.: BIO-35020, Bioline). This master mix already contains the *Taq* enzyme, $MgCl_2$, dNTP and ROX which is used as reference dye. The specific assay (c_7514870_20, Thermo Fisher Scientific) has specific primers to amplify the *LTA* gene and two probes to discriminate the alleles. The real-time PCR reaction was performed in Mx3005PM™, STRATAGENE.

Table 2 – Real-time PCR conditions. Reagents and volumes used in the first test of real-time PCR genotyping using Taqman probes.

Reagent	Volume (µL)
SensiFAST™ Hi-ROX Genotyping Mix 2x	10.0
Water	8.0
Assay 20x	1.0
DNA	1.0
Final Volume	20.0

The probes were labelled with FAM (allele C) and VIC (allele A) dyes. Since the HEX has a similar emission profile as VIC, in the thermocycler HEX was selected instead of VIC. The real-time PCR temperature conditions are described below in table 3. The fluorescence was detected in the last phase, after annealing/extension step.

Table 3 - Real-time PCR thermal conditions.

Step	Temperature	Duration	Cycles
Polymerase activation	95 ºC	3 minutes	1
Denaturation	95 ºC	10 seconds	40
Annealing/extension	60 ºC	45seconds	

3.2 – Results confirmation

In order to confirm the real-time PCR genotyping results, 5 % of the DNA samples were genotyped using the Sanger sequencing technique. As a first step for this sequencing reaction, it was necessary to select specific primers, amplify *LTA* fragments by PCR and purify the PCR products.

3.2.1 – Primers design

The specific primers to amplify exon 3 *LTA* were designed using the bioinformatic tool, Primers3Plus. In the input box, the human exon 3 *LTA* sequence with SNP as target was introduced. In the general settings we selected 701-850 base pairs (bp) as the product size range. The remain characteristics, displayed by default, were kept. The selected primers are shown in figure 10.

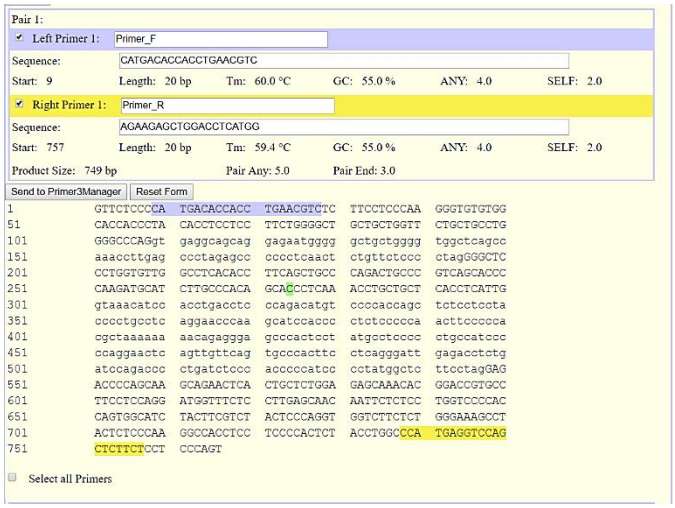


Figure 10 – Primer3Plus output. Representation of the human exon 3 *LTA* gene sequence used for choosing the specific primers. Primer forward is evidenced in blue and reverse in yellow. The SNP of interest is show in green.

3.2.2 – PCR conditions

The optimal volumes of the different reagents and the temperature conditions are detailed below. In table 4 the PCR conditions are described (reagents and volumes) to specific *LTA* amplification. It is not described, but the different alterations performed to optimize the protocol conditions were made regarding the annealing temperature and addition of 5 % dimethyl sulfoxide (DMSO).

Table 4 - Reagents and volumes used in conventional PCR. Reaction performed to confirm the real-time PCR genotyping results.

Reagent	Volume (µL)
Dream Taq ™ PCR Master mix (2x)	10.0
Water	7.0
Primer Forward (16.6 µM)	1.0
Primer Reverse (16.2 µM)	1.0
DNA	1.0
Final Volume	20.0

The optimal temperature cycle used to amplify *LTA* exon 3 was:

95 ºC, 1 min
95 ºC, 15 secs
60 ºC, 15 secs } 40 cycles
72 ºC, 10 secs

3.2.3 – Purification

To purify the PCR products, the ExoProstar ™ 1-Step Illustra ™ kit, U577702 from GE HealthCare was used. This step is important to eliminate primers and dNTPs that were not used in the reaction, assuring that they do not interfere in the sequencing process. To purify, two enzymes are used:

- Exonuclease I: degrades single-stranded residual primers that were not used, and single stranded DNA produced during PCR;
- Phosphatase Alkaline: hydrolyzes the dNTPs that remain in the PCR product.

– Protocol

1. Identify tubes for each sample;
2. Remove the tube from the kit and keep it on ice;
3. Add 10 µL of PCR product into the respective identified tube;
4. Add 3 µL of ExoProStar ™ 1-Step and homogenize;
5. Incubate for 15 minutes at 37°C for enzymatic reaction;
6. Incubate at 80°C for 15 minutes for inactivation of the enzymes;
7. Store in freezer at -20 °C until sent for sequencing.

After purification, the PCR products were sent to STAB Vida (10 µL of the PCR product and 5 µL per sample of each primer with a concentration of 10 µM).

4 – Immunohistochemistry

Based on tissue antigen identification by the immune complex formation through the specific antibody binding the antigen in the tissues, the immunohistochemistry (IHC) technique is an important tool in the diagnosis of several diseases, agents and molecules identification in association with the organ/tissues morphology recognition. The visualization of the results is allowed by the use chromogen substance as 3,3'-diaminobenzidine tetrachloride (DAB) in

photonic microscope, or with fluorochromes by fluorescence microscopy (Ramos-Vara and Miller, 2014).

For a specific and reproducible results general procedures must be followed, and the critical points must be avoided.

The tissues must be rapidly preserved to prevent the disarrangement of normal architecture and autolysis. The most common fixative is neutral buffered formalin solution at 10 %. This reagent promotes proteins cross-link leading to preservation of tissue (Ramos-Vara and Miller, 2014). These tissues are embedded in paraffin, that allows the long-term storage and thin section of sample. This tissue is often referred as Formalin-Fixed and Paraffin-Embedded (FFPE) (Ramos-Vara and Miller, 2014). The next step is to cut in thin sections [usually 2-3 micrometers (µm)] to a glass slide, and this is stained by hematoxylin and eosin to perform the routine diagnosis, or to use to make the IHC; all of this are, at the end, mounted with a resin Entellan® for long time preservation. All the steps of the IHC technique were performed on the slide and at end the slides were counterstained with Gill's hematoxylin, resin mounting and stored in a dark place for prevent the antigenicity loss (Ramos-Vara and Miller, 2014).

The IHC is made in an aqueous medium, so the section placed on the slide needs to be completely free of paraffin (removed by xylol), to allow the reaction between antigens and antibodies. Furthermore, as the samples were fixed in formalin, it is also mandatory to perform antigen retrieval which usually is made by heating or by enzymatic-based technique. This step is performed to facilitate antigen detection because the bridges of cross-link made by the fixation can hide the antigen and avoid the antibody binding (Ramos-Vara and Miller, 2014).

Another important step is the endogenous peroxidases blocking (with hydrogen peroxide) and nonspecific cross reactions targets by incubation with a normal serum, rich in non-specific immunoglobulins. The non-specific targets have similarity with our interested targets and may induce to false positive link and misinterpretation and increase the background staining (Shi *et al.*, 2003, Nambiar *et al.*, 2016).

The immunohistochemistry may be performed by direct or indirect methods. To allow the reaction detection, antibodies are labelled with reporter molecules which are bind to fluorescent molecules, metals or enzymes. The reaction between antibody and antigen is detected by chromogenic or fluorescent molecules (Lucocq *et al.*, 1985, Ramos-Vara and Miller, 2014). The most common chromogen used is DAB giving a brown color reaction (Ramos-Vara, 2005). Gill's hematoxylin is used to counterstaining the nucleus of all the cells, allowing a better visualization of target (Ramos-Vara and Miller, 2014) and the negative reactions. The final

stage is mounting the slide with resin media, that seals the sample, prevent the degradation and allow the visualization at the end of the procedure (Kalyuzhny, 2016).

4.1 – Samples

Were used 34 samples from the same PCR survivor patients with colorectal cancer that have histopathological analysis in the Pathological Laboratory of the CHTMAD. This samples, only one slide per each, were choose by the Pathologist that collaborate with this project.

4.2 – Procedure

The procedure used during the present work was adapted from the Laboratory of Histology and Anatomical Pathology (LHAP) of UTAD and is an indirect enzymatic protocol. The anti-Human CD68 (monoclonal antibody against macrophages, ab955, Abcam) and the anti-human CD3 (polyclonal antibody against T cell, A0452, DAKO) were used as primary antibodies. The specific procedures for each primary antibody are described in table 5.

1. Deparaffinize slides in xylol for 15 min.
2. Transfer the slides to 100 %, 95 %, 80 % and 70 % alcohol for 5 min each to hydrate the slides.
3. Wash the slides with distilled water.
4. Thermal treatment to antigen retrieval with citrate buffer (pH = 6.0 ± 0.2) (table 5).
5. Allow the slides to cool for 30 min in the same citrate buffer (thermal treatment in microwave) or, after antigen retrieval, put immediately the slides in cold phosphate-buffered saline (PBS) (thermal treatment in pressure cooker).
6. Incubate the slides in 3 % hydrogen peroxide (H_2O_2) for 30 min to block the endogenous peroxidases.
7. Wash the slides with PBS.
8. Incubate the slides with polyvalent blocking serum (Ultra V Block ®, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) for 5 min to block non-specific targets.
9. Remove excess of universal serum.
10. Apply the primary antibody and incubate at room temperature in a humidified chamber, for a specific time depending on the antibody (table 5).
12. Wash the slides with PBS.

13. Incubate the slides with Biotinylated Goat Polyvalent Plus® antibody (Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA), for 10 min.

14. Wash the slides with PBS.

15. Add Streptavidin-peroxidase Plus® (Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) and incubate for 10 min.

16. Wash the slides with PBS.

17. Incubate for 12 min the slides with DAB substrate solution, pre-activated with 30 % H_2O_2.

18. Remove the excess of DAB and wash the slides for 10 min in running water.

19. Counterstain the slides by immersing in Gill's hematoxylin for 2 min.

20. Wash the slides in running tepid water for 10 min.

21. Dehydrate the slides with 95 %, 95 %, 100 % and 100 % alcohol, 5 min each.

22. Clear the tissues slides in xylene for 10 min.

23. Coverslip using Entellan® mounting media.

Table 5 – Detailed conditions of immunohistochemistry protocol for each primary antibody used.

Primary antibody	Antigen retrieval method	Dilution	Incubation time
CD68 (macrophages)	3 min, pressure cooker	1:50	2 hours
CD3 (T lymphocytes)	3 cycles, 5 min each, microwave	1:50	3 hours

5 – Data analysis and statistical calculation

5.1 – Real-time PCR results analysis

To analyze the real-time PCR results, for each sample, the respective amplification plot was observed (Mx3005P Allele Discrimination/SNP's Real-Time) and the genotype was determined.

5.2 – Sequencing results analysis

To perform a detailed analysis, editing and correction of the sequences, the ChromasPro version 1.7.7 and GeneDoc version 2.7.000 softwares were used. With ChromasPro, it was possible to analyze and edit the electropherograms and obtain a sequence in FASTA format allowing sequence alignment.

The multiple alignment between sequencing results and Human exon 3 *LTA* gene sequence present in the ENSEMBL (Ensembl: ENSG00000226979) was performed using Clustal Omega program available on the EMBL-EBI platform, saving the information in MSF format. To analyze and edit the multiple alignment the GeneDoc program was used, detecting the primers Forward and Reverse and interest SNP localizations (annex III).

5.3 – Immunohistochemistry results analysis

To perform the analysis of immunohistochemistry results, in each slide, 10 hot-spots areas in the tumor or surrounded stroma were selected, where the labeled lymphocytes and macrophages were in greater number. The CD3 and CD68 cells were counted manually with the help of the ImageJ Software (version 1.52a). The results were given in total number per 724 mm^2.

5.4 – Statistical analysis

Descriptive analyses included absolute count and frequencies, median with respective inter-quartile range (IQR) and mean ± standard deviation (SD) or standard error of mean (SEM). Departure from normality was tested using the Shapiro-Wilk test. Comparison of tumor macrophage and lymphocyte counts between genotypes were conducted using Kruskall-Wallis or Mann-Whitney tests.

The primary end-points were overall survival (OS) and progression-free survival (PFS). Survival analyses included initial empirical time-to-event and comparison of Kaplan-Meier estimates, in order to test robustness of *LTA* SNP and clinicopathological variables to explain the outcomes. The Kaplan-Meier results are presented in months of survival. Then, only variables with $P < 0.10$ on univariate analysis were included in multivariate Cox regression proportional hazards to define whether the resulting model would include *LTA* genetic information. Another Cox regression analysis was conducted to estimate the best fitting Cox

regression models for PFS and OS end-points in a subgroup analysis including patients stratified as lymphopenic (<$1.0x10^3$ lymphocytes/μL) and normal lymphocyte count.

Statistical analyses were conducted in SPSS 17.0 and STATA 12.0.

IV- Results

The main objective of this work was the study of possible association between *LTA* polymorphism (rs1041981) and clinicopathological parameters. The individual's genotype was determined by real-time PCR using Taqman probes, followed by statistical analysis to assess the possible association genotype-phenotype. Immunohistochemistry approaches were performed to search for possible associations between tumor infiltration cells TAMs and TILs and rs1041981 *LTA* variant genotypes.

The results obtained are presented below.

1 – DNA protocol optimization

After the DNA extraction performed by three different protocols, the quantity and purity of DNA were determined by NanoDrop ND-1000 spectrophotometer, and these results are presented in annex IV, which contains the Kit's name, volume of buffy coat used for extraction, the volume of elution buffer, the yield and the absorbance ratio 260/280 which is a purity indicator. Considering the data, the best extraction kit, that combine a good yield and purity, were ExtractME® DNA blood Kit (Ref.: EM05-050, BLIRT) and Invisorb® Spin Blood Mini Kit (Ref.: 1031100200, STRATEC Molecular).

After DNA quantification, the extracted DNA was amplified by PCR using histone H4 primers to assess the DNA quality, following the PCR conditions described in 2.2 – Evaluation of DNA quality – PCR conditions. The Figure 11 is the representation of a 2 % agarose gel with 7 µL of the PCR products.

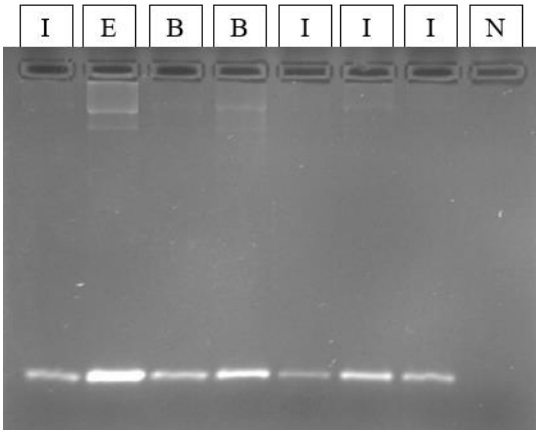


Figure 11 - Representation of 2 % agarose gel with PCR amplification products obtained by using histone H4 primers. This agarose gel presents the comparison of DNA quality extracted by three kits: (I) Invisorb® Spin Blood Mini Kit (Ref.: 1031100200, STRATEC Molecular), (B) Isolate II Genomic DNA Kit (Ref.: BIO-52065, Bioline) and (E) ExtractME® DNA blood Kit (Ref.: EM05-050, BLIRT). N represents the negative control.

Considering the result of figure 11, the highest intensity band was the result of amplification of DNA extracted with ExtractME® DNA blood Kit.

2 – *LTA* genotyping

2.1 – Real-time PCR using Taqman probes

After real-time PCR reaction using Taqman probes, the individuals' genotypes were determined by observation of the amplification curves using software Mx3005P Allele Discrimination/SNP's Real-Time. Figure 12 illustrates these possible curves. In the homozygotic condition, fluorescence from one fluorochrome was detected resulting in an amplification plot with only one curve, referent to the allele amplified. In the heterozygotic condition, the fluorescence of both fluorochromes was detected, generating two curves in the amplification plot. In the case of no template control (NTC) or without amplification, no fluorescence was detected.

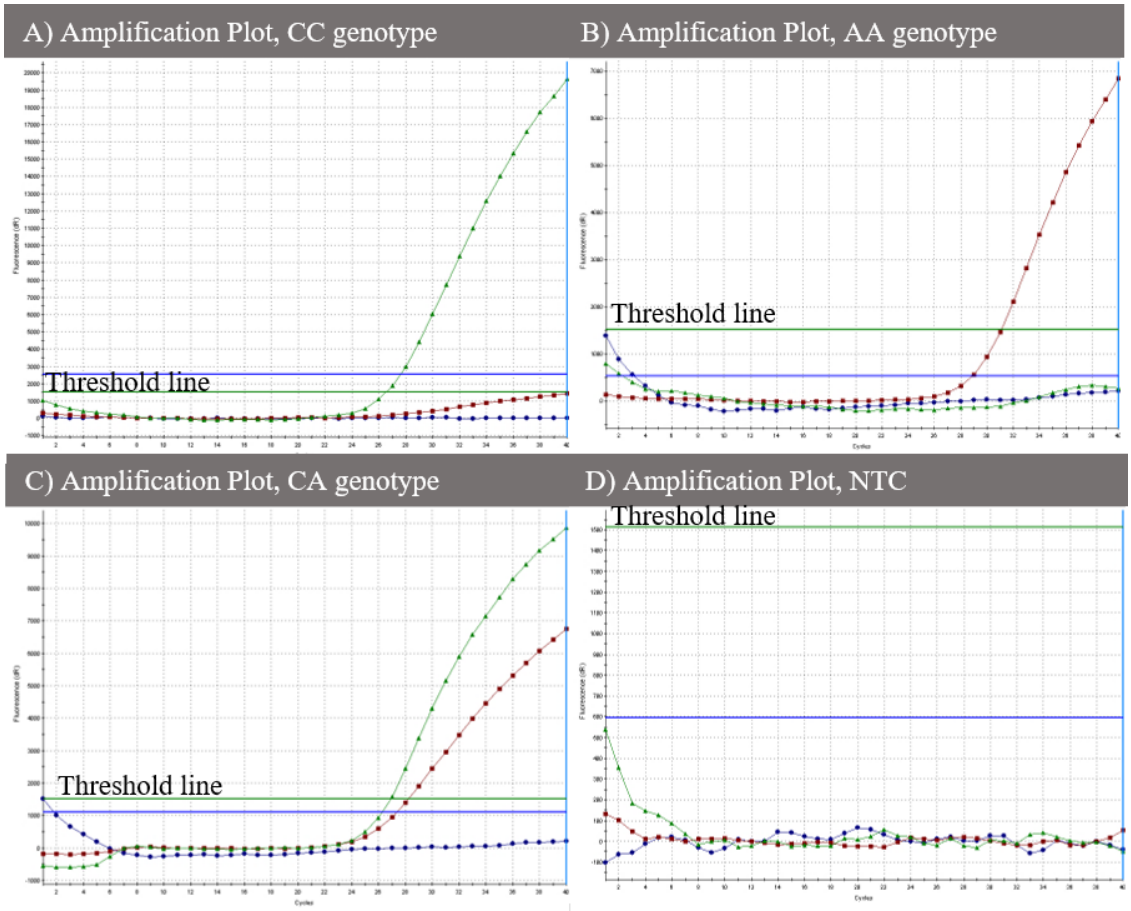


Figure 12 - **Representation of the amplification plots of three possible genotypes of the rs1041981 polymorphism.** A) represents CC genotype, B) the CA genotype, C) shows the AA genotype and no template control (D). The X-axis represents the PCR cycle number and the Y-axis represents the fluorescence from the amplification reaction. The green line represents the threshold line (baseline-subtracted fluorescence).

The genotyping results may be expressed in Dual Color Scatter Plot, where in the Y-axis is the FAM fluorescence index and in the X-axis is the VIC/HEX fluorescence index. Based on this information, whenever there are only FAM fluorescence detection (CC genotypes) there are points at the Y-axis level. The AA genotypes only have VIC/HEX fluorescence detection and the points are at of the X-axis level. The heterozygotic individuals (CA genotype) have detection of fluorescence of both fluorochromes and the points of the genotypes are in the middle of both axes (figure 13).

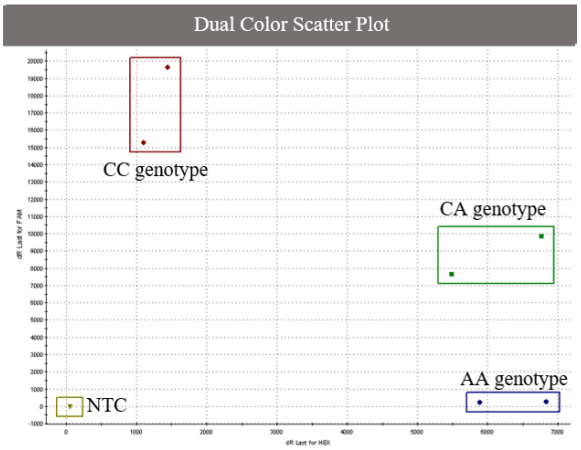


Figure 13 - **Representation of Dual Color Scatter Plot.** FAM fluorescence is measured on Y-axis and the VIC/Hex fluorescence is measured on X-axis.

The characterization of the allelic and genotypic frequencies in 172 CRC patients, showing that C and A alleles presented the allelic frequencies of 70 % and 30 %, respectively. The CC, CA and AA genotypes presented the genotypic frequencies of 49%, 42% and 9%, respectively.

2.2 – Conventional PCR and Sequencing

As described, to confirm the real-time PCR results 5 % of total samples (9 samples) were genotyped by direct sequencing. So, it was necessary to optimize a PCR protocol to amplify the exon 3 from *LTA* gene and sequence the fragments. The PCR products were analyzed in a 2 % agarose gel to confirm whether amplification of a fragment of 749 bp was obtained, as predicted by prior in silico analysis. Figure 14 presents the amplification products obtained after the PCR protocol optimization, using different conditions.

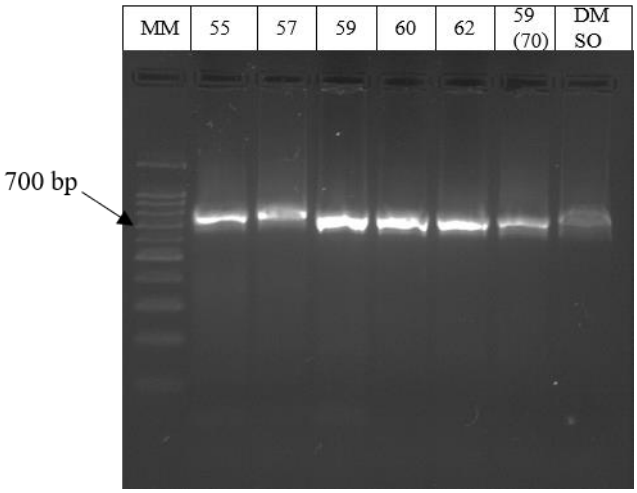


Figure 14 - Agarose gel with amplification products from exon 3 of *LTA* gene. MM: molecular marker 100bp Plus DNA Ladder (C: 304105, BIORON), **55**: representation of amplification with an annealing temperature of 55 ºC. **57**: PCR product amplified with annealing temperature of 57 ºC. **59**: PCR product amplified with 59 ºC as annealing temperature. **60**: result of amplification with annealing temperature of 60 ºC. **62**: amplification result annealing temperature of 62 ºC. **59 (70)**: PCR product amplified with annealing temperature of 59 ºC. **DMSO**: PCR product amplified with annealing temperature of 59 ºC and 5 % of DMSO.

Facing the agarose gel results, the annealing temperature chosen was 60 ºC.

The confirmation of genotypes was performed after the alignment and edition of the sequences and comparing the sequencing results with real-time PCR curves.

In order to analyze the results, it is important to state that the SNP under study is the variant rs1041981 that results in a transversion of a cytosine to adenine.

2.2.1 – Homozygotic wild type genotype

In literature and databases, the C allele is considered the ancestral allele being the allele most common in the world population. In real-time PCR individuals with CC genotype have only amplification signal resulting from FAM. In figure 15 an amplification plot of a CC genotype and a part of the electropherogram of the same sample are shown.

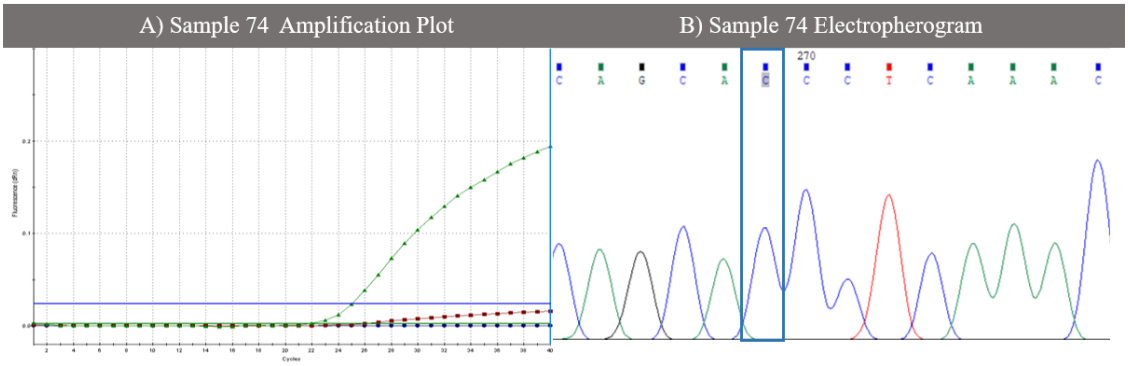


Figure 15 – Exemplification of real-time PCR results and its confirmation by sequencing of a CC genotype. A) Amplification plot of sample 74. B) electropherogram which allow to confirm the CC genotype (indicated in blue) once only a C signal was detected.

The analysis of sample 74 electropherogram allowed the confirmation of genotype CC for this type of real-time PCR amplification plot. According to this result, this type of curve is typical of individuals with the CC genotype.

2.2.2 – Homozygotic AA genotype

In real-time PCR, AA genotypes present only amplification signal resulting of fluorescence emission from VIC/HEX fluorochromes. Figure 16 shows an amplification plot of AA genotype and a part of the electropherogram obtained for the same sample.

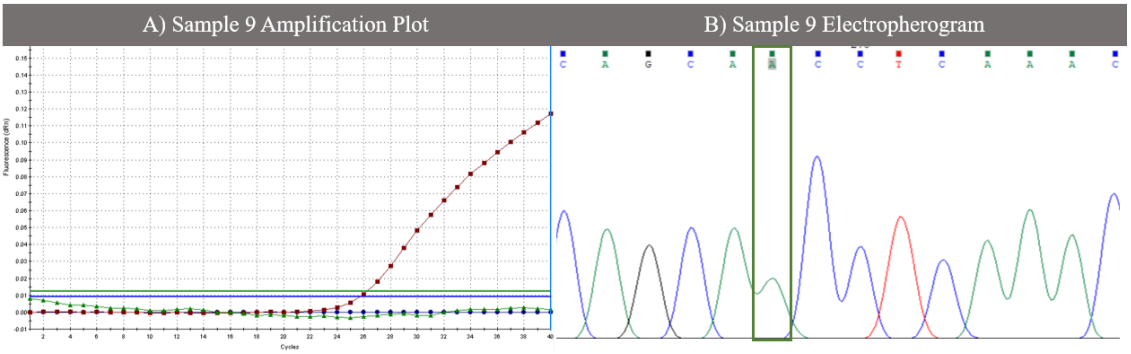


Figure 16 - Exemplification of real-time PCR results and its confirmation by sequencing of a AA genotype. A) Amplification plot of sample 9. B) Electropherogram which allow to confirm the AA genotype (indicated in green) once only a A signal was detected.

The analysis of sample 9 sequencing result allowed the confirmation that this type of curve is indicative of AA genotype observed after the analysis of real-time PCR amplification plot.

2.2.3 – Heterozygotic genotype

The real-time PCR results derived from CA genotypes have two amplification signals resulting of fluorescence emission from both probes VIC/HEX and FAM. Figure 17 presents an example of the amplification plot of a CA genotype and a part of the electropherogram of the same sample.

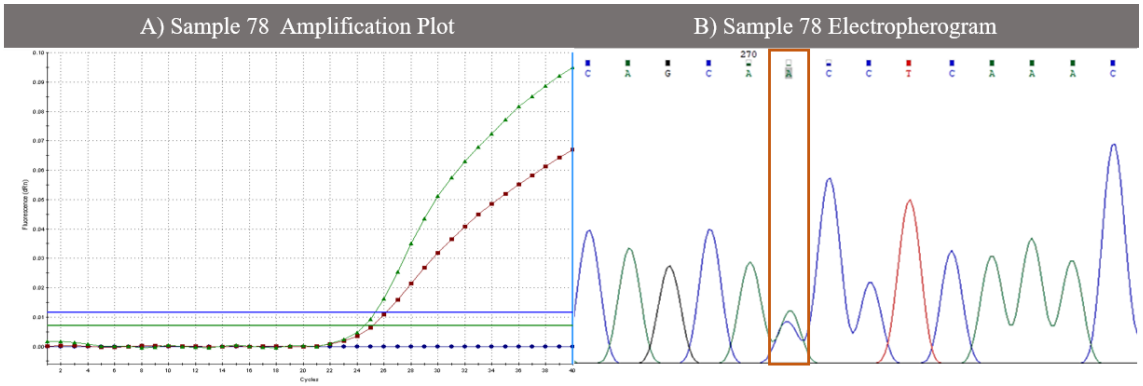


Figure 17 - Exemplification of real-time PCR results and sequencing result of genotype CA for its confirmation. A) Amplification plot of sample 78. B) Confirmation of real-time result (indicated in orange) by sequencing, indicating CA genotype.

The amplification plot with two curves, where the FAM fluorochrome had a higher fluorescence emission is typical of a CA genotype which was confirmed by the analysis of the electropherogram.

During the real-time PCR reactions, sample 62 revealed a heterozygotic result (CA) with VIC/HEX having a higher fluorescence detection (as shown in figure 18.A). This real-time PCR reaction was also evaluated in dual color scatter plot (figure 18.B). This sample was sequenced, and the result is shown in figure 18.C.

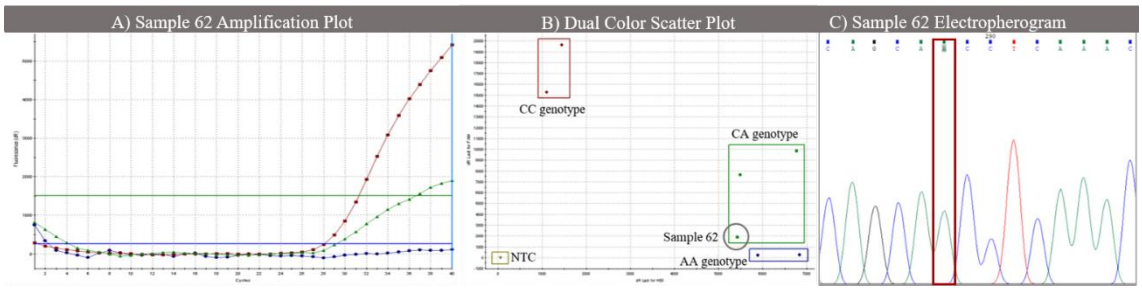


Figure 18 – Representation of real-time PCR result (A and B) and sequencing result to sample 62 (C). A) real time amplification plot of sample 62 indicating a heterozygotic. The figure B) reveals the dual scatter plot from reaction which includes the sample 62. C) sequencing result for sample 62 (indicated in red).

Analyzing these results, the real-time PCR identify the sample 62 as an heterozygotic genotype (CA) but the sequencing result does not confirm the real-time PCR genotyping. According to this technique, the individual from sample 62 is homozygotic (AA). Thus, when the genotyping result by real-time PCR provides this type of curves, in this study, the individuals cannot be considered heterozygotic. This type of curve was observed only for this sample.

3 – Immunohistochemistry

To investigate the possible influence of tumor infiltrating cells in tumor, specifically macrophages and T lymphocytes, immunohistochemistry protocols were applied in FFPE tissues using the antibodies CD68 to macrophages and CD3 to T lymphocytes. After performing the immunohistochemistry protocol, the tissue characteristics were evaluated, and ten hot-spots were selected to determine the number of macrophages and T lymphocytes. The total number of cases analyzed by immunohistochemistry was 34, all cases that were genotyping by real-time PCR.

The samples under study usually had inflammatory cells in the neoplastic stroma and rarely inside the tumor, showing a higher concentration in surrounded stroma. The hot spot selection was made in stroma adjacent to tumor, avoiding the normal mucosa. There were two samples with only normal mucosa, without tumor. It was possible to identify cases with ulcer and necrotic tissue, debris, exhibited bacteria, high mucus production and lymphoid nodules in submucosa and in the adjacent peritoneum; in other cases, neoplastic cells invading muscular layer and are also see metastasis in the peritoneum.

Relative to macrophages, T lymphocytes were the more present cells inside these tumors, with a median of 1215 cells per 724 mm^2 (minimum cell value: 299 per 724 mm^2; maximum cell value: 5077 per 724 mm^2). The macrophages were in smaller amount, with only 559 per 724 mm^2 as median (minimum cell value: 137 per 724 mm^2; maximum cell value: 1456 per 724 mm^2). Figure 19 represents a CR case with ulcer staining with H-E (19.A), 19. B and C with IHC for T cell and macrophages respectively (counterstained with Gill's hematoxylin).

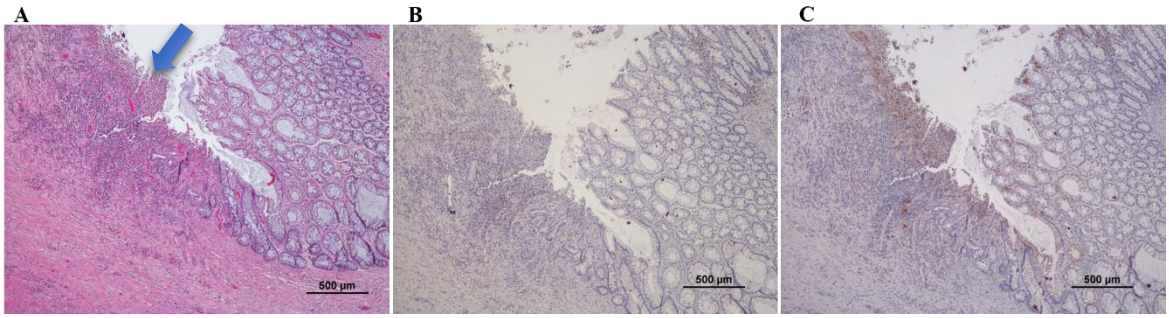


Figure 19 – Colorectal adenocarcinoma with ulcer. A) adenocarcinoma with ulcer (arrow) and normal mucosa. B) IHC representing the infiltration of T cells and macrophages (C). A) H-E. B and C) IHC contrasting with Gill's Hematoxylin.

Figures 20 and 21 represent some features of CR cases analyzed in this study.

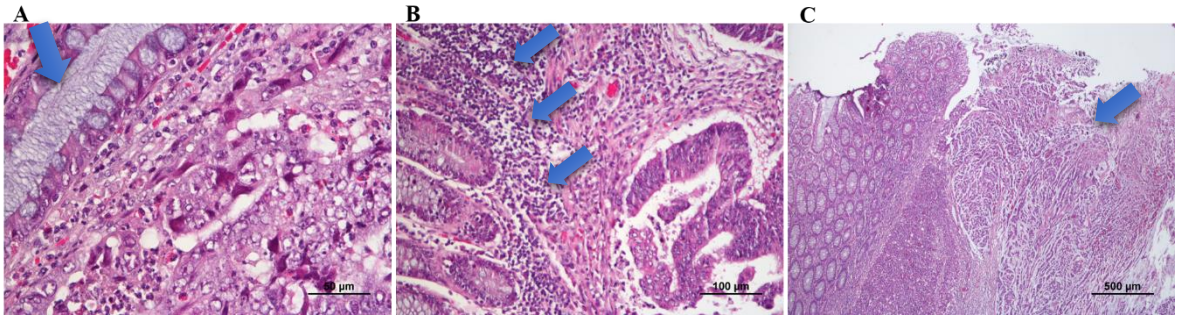


Figure 20 - Histological aspects of CR. A) note the neoplastic cells near the normal mucosa (arrow), with high content of inflammatory cells namely eosinophils. B) The inflammatory cells (arrows) surrounded the normal epithelial cells. C) A case of CR adenocarcinoma (arrow), near a normal mucosa. H-E.

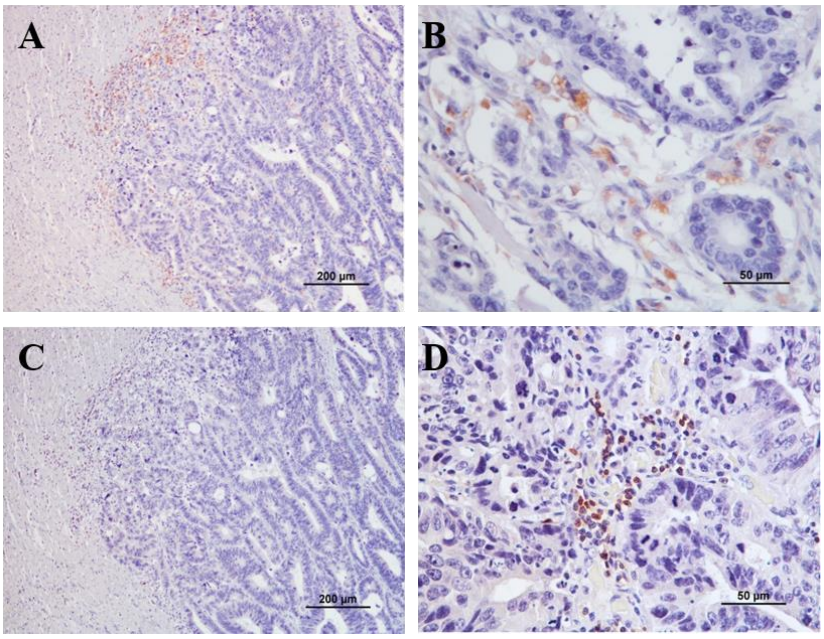


Figure 21 – Immunohistochemical expression of macrophages and T lymphocytes in colorectal adenomas. A) Macrophages identified by CD68 antibody, in the stroma, near the tumor; B) Macrophages surrounded the tumor; C) A few T lymphocytes were identified in the stroma near the tumor cells; D) T lymphocytes in the tumoral stroma. IHC counterstained with Gill's Hematoxylin.

4 – Statistical correlation

In this study, all patients had colorectal adenocarcinoma, without identification of the morphological specificity.

A descriptive analysis of the population under study is shown in table 6. The statistical analysis had a final population of 166 patients from the 172, since some individuals died due to other diseases, they were not included in the ultimate study.

The mean age at diagnosis was 65.2 years, the body mass index was 26.3, the lymphocyte count was 1434.1 and the neutrophils/lymphocytes ratio was 5.8.

Other clinicopathological aspects as gender, tumor localization and side, clinical stage, if the patient did radiotherapy after surgery, the presence of tumor in surgical margins and adjuvant or palliative chemotherapy were described in table 6.

Table 6 - Descriptive data of colorectal cancer patients included in the study (n=166).

	N (freq.)	**Median (IQR)**	**Mean ± SD**
Age, years	163	65.9 (57.5-74.3)	65.2 ± 11.6
BMI, kg.m^{-2}	104	26.2 (22.9-29.4)	26.3 ± 4.5
Lymphocyte count	160	1395.0 (835.0-1905.0)	1434.1 ± 696.6
Neutrophils/Lymphocytes ratio	160	2.9 (1.9-5.5)	5.8 ± 9.2
Gender			
Female	62 (0.37)	-	-
Male	104 (0.63)		
Localization			
Colon	96 (0.58)	-	-
Rectum	70 (0.42)		
Tumor side			
Right	40 (0.24)	-	-
Left	123 (0.75)		
Synchronous	1 (0.01)		
Clinical stage			
Localized	54 (0.33)		
Locally advanced	77 (0.47)	-	-
Metastatic	32 (0.20)		
Surgery/Radiotherapy			
No	9 (0.06)	-	-
Yes	156 (0.94)		
Surgical margins			
Negative	130 (0.87)	-	-
Positive	19 (0.13)		
Adjuvant chemotherapy			
No	54 (0.33)	-	-
Yes	112 (0.67)		

Palliative chemotherapy			
No	75 (0.45)	-	-
Yes	90 (0.55)		

N (freq.), number of cases and relative frequency; BMI, body mass index; IQR, inter-quartile range; SD, standard deviation.

Table 7 presents the empirical univariate and multivariate analysis by models Kaplan-Meier and Cox regression, respectively. The influence of clinicopathological and genetic variables in OS is described. This represents the time from initial diagnosis until the date of death or last clinical visit.

Table 7 - Empirical univariate (Kaplan-Meier) and multivariate (Cox regression) analyses of the endpoint survival for clinicopathological and genetic variables.

	Univariate analysis (Kaplan-Meier)		**Multivariate analysis (Cox regression)**	
	Mean (95%CI)	P (Log-rank)	HR (95%CI)	P
Age, years				
< median	120.2 (91.8-148.6)		-	-
> median	96.1 (83.2-109.0)	0.560		
Gender				
Female	138.1 (111.2-165.0)		-	-
Male	105.3 (83.6-127.1)	0.296		
Lymphocyte %				
< median	78.4 (34.3-122.4)[a]		Reference	
> median	109.5 (78.4-140.6)[a]	0.029	0.4 (0.2-0.8)	0.017
Localization				
Colon	91.1 (82.0-100.2)		-	-
Rectum	134.9 (114.4-155.3)	0.854		
Tumor side				
Right	74.4 (63.4-85.5)		Reference	

Left	118.2 (97.0-139.3)	0.035*	0.7 (0.3-1.8)	0.502
Stage				
Localized	142.2 (122.3-162.1)		Reference	
Locally advanced	92.9 (81.1-104.7)		1.6 (0.6-4.3)	0.340
Metastasis	60.2 (47.2-73.3)	0.008	0.7 (0.1-6.5)	0.792
Surgical margins				
Negative	116.5 (87.7-135.6)		Reference	
Positive	59.4 (41.2-77.6)	0.013	3.1 (0.3-28.5)	0.317
Adjuvant chemotherapy				
No	116.0 (91.5-140.6)		Reference	
Yes	119.8 (95.6-144.0)	0.010	0.4 (0.1-1.2)	0.106
Additive model				
CC	107.2 (82.6-131.9)			
CA	128.5 (94.3-162.7)		-	-
AA	61.4 (52.4-70.4)	0.548		
Recessive model				
CC/CA	116.1 (95.7-136.6)		-	-
AA	61.4 (52.4-70.4)	0.699		
Dominant model				
CC	107.2 (82.6-131.9)		-	-
CA/AA	125.9 (93.3-158.6)	0.374		

[a] median with 95 %CI* Breslow test. HR, hazard ratio. 95%CI, 95% confidence interval.

Kaplan-Meier analysis indicated the variables that were associated with CRC overall survival were: the blood lymphocytes percentage (P = 0.029 the tumor side (P = 0.035), stage (P = 0.008), surgical margins (P = 0.013), and adjuvant chemotherapy (P = 0.010) (table 7). Notably, *LTA* the rs1041981 was not associated with overall survival after analyses under the additive, recessive and dominant genetic models.

Cox regression analysis confirmed a shorter time to death in patients with lower peripheral blood lymphocyte percentage in at diagnosis (HR = 0.4; 95%CI = 0.2 - 0.8, P = 0.017).

Table 7 and 8 depict the empirical univariate Kaplan-Meier and the complementary multivariate analysis through Cox regression for OS and PFS, respectively. The influence of clinicopathological and genetic variables in PFS is presented in table 8.

Table 8 - Empirical univariate (Kaplan-Meier) and multivariate (Cox regression) analyses of the endpoint progression of disease for clinicopathological and genetic variables.

	Univariate analysis (Kaplan-Meier)		**Multivariate analysis (Cox regression)**	
	Mean (95%CI)	P (Log-rank)	HR (95%CI)	P
Age, years				
< median	71.6 (51.0-92.2)		-	-
> median	64.8 (52.6-76.9)	0.811		
Gender				
Female	80.9 (60.1-101.7)		-	-
Male	62.6 (52.4-72.8)	0.591		
Lymphocyte %				
< median	67.7 (48.4-87.1)		Referent	
> median	71.6 (60.8-82.5)	0.005	0.5 (0.3-0.9)	0.014

Localization				
Colon	58.0 (47.7-68.4)		-	-
Rectum	77.1 (54.0-100.2)	0.149		
Tumor side				
Right	35.3 (26.0-44.6)		Referent	
Left	82.4 (63.2-101.5)	0.002	0.4 (0.3-0.8)	0.003
Stage				
Localized	71.9 (58.7-85.1)		Referent	
Locally advanced	61.6 (50.5-72.6)		1.4 (0.8-2.5)	0.278
Metastasis	29.6 (21.2-38.0)	<0.001	2.0 (0.7-5.9)	0.215
Surgical margins				
Negative	86.7 (67.9-105.4)		Referent	
Positive	30.3 (19.1-41.6)	0.001	1.3 (0.4-3.8)	0.278
Adjuvant chemotherapy				
No	45.6 (33.2-58.1)		Referent	
Yes	93.4 (74.1-112.7)	0.001	0.7 (0.3-1.4)	0.278
Additive model				
CC	55.4 (44.4-66.5)		Referent	
CA	103.8 (83.4-124.2)		0.6 (0.4-1.1)	0.091
AA	35.4 (22.8-48.1)	0.046	1.2 (0.5-2.5)	0.722
Recessive model				

CC/CA	78.0 (60.2-95.7)		--- [a]	-
AA	35.4 (22.8-48.1)	0.087	--- [a]	
Dominant model				
CC	55.4 (44.4-66.5)		-	-
CA/AA	94.8 (76.2-113.4)	0.197		

[a] removed due to collinearity. HR, hazard ratio. 95%CI, 95% confidence interval.

Kaplan-Meier analysis indicated an association of blood lymphocyte percentage (P = 0.005), tumor side (P = 0.002), stage of the tumor (P < 0.001), surgical margins (P = 0.001), adjuvant chemotherapy (P = 0.001), and *LTA* additive (P = 0.046), and recessive models (P = 0.087).

Cox regression analysis confirmed a shorter time to disease progression in patients with lower % lymphocytes (HR = 0.5; 95%CI = 0.3 - 0.9, P = 0.014) and those with tumors on the right colon (HR = 0.4; 95%CI = 0.3 – 0.8, P = 0.003).

The evaluation of the association between clinicopathological and genetic variables in overall survival of patients with versus without lymphopenia is shown in table 9. Lymphopenia was considered with lymphocyte count <1000.

Table 9 - Empirical univariate (Kaplan-Meier) and multivariate (Cox regression) analyses of the endpoint survival for clinicopathological and genetic variables stratified by lymphopenia cutoff.

	Lymphopenia (lymphocyte count < 1x10^3/µL) (n=47)				**Normal lymphocytes (lymphocyte count > 1x10^3/µL) (n=110)**			
	Univariate		**Multivariate**		**Univariate**		**Multivariate**	
	Mean (95%CI)	P	HR (95%CI)	P	Mean (95%CI)	P	HR (95%CI)	P
Age, years								
< median	137.0 (99.3-174.7)		-	-	90.8 (79.7-102.0)		-	-
> median	61.1 (53.1-69.1)	0.438			91.6 (80.1-103.2)	0.770		
Gender								
Female	122.9 (47.9-197.9)		-	-	94.9 (81.7-108.0)		-	-
Male	75.1 (65.2-85.0)	0.898			87.8 (76.7-98.9)	0.306		
Localization								
Colon	64.9 (51.6-78.2)		Referent		96.3 (87.2-105.5)		Referent	
Rectum	156.5 (125.6-187.3)	0.058	0.2 (0.05-0.9)	0.038	67.5 (57.1-78.0)	0.044	2.2 (0.9-5.4)	0.084
Tumor side								
Right	75.0 (58.3-91.7)		-	-	75.8 (63.8-87.8)		-	-
Left	134.8 (102.9-166.6)	0.842			92.5 (83.5-101.5)	0.218		
Stage								
Localized	68.9 (50.8-87.0)		-	-	85.5 (78.3-92.7)		Referent	
Locally advanced	79.1 (68.8-82.3)				86.9 (72.9-100.9)		2.2 (0.7-7.2)	0.203
Metastasis	50.6 (42.0-59.2)	0.245			60.7 (45.5-75.9)	0.019	3.0 (0.8-11.0)	0.101

Adjuvant chemotherapy								
No	71.0 (54.6-87.4)		-	-	62.4 (51.3-73.5)		Referent	
Yes	127.5 (84.3-170.6)	0.643			96.7 (88.0-105.4)	0.003	0.3 (0.1-1.2)	0.092
Additive model								
CC	86.3 (79.9-92.6)				81.0 (68.5-93.5)		--- [a]	
CA	109.5 (61.1-157.8)		-	-	101.5 (91.3-117.1)		--- [a]	-
AA	62.6 (53.7-71.4)	0.131			55.1 (42.1-68.0)	0.060	--- [a]	
Recessive model								
CC/CA	131.3 (96.1-166.5)		-	-	90.5 (82.1-98.9)		-	-
AA	62.6 (53.7-71.4)	0.713			55.1 (42.1-68.0)	0.930		
Dominant model								
CC	86.3 (79.9-92.6)		Referent		81.0 (68.5-93.5)		Referent	
CA/AA	113.2 (74.9-151.5)	0.050	8.7 (1.0-77.3)	0.052	100.5 (90.4-110.6)	0.019	0.4 (0.1-0.9)	0.036

[a] removed due to collinearity. HR, hazard ratio. 95%CI, 95% confidence interval.

The empirical univariate analysis was used to evaluate clinicopathological and genetic factors that were associated with overall survival in lymphopenia and non-lymphopenia groups separately. The variables included in multivariate model for lymphopenic patients were: tumor localization (P = 0.058), and *LTA* SNP dominant model (0.050), The multivariate analysis confirmed that the time-to-death was shorter in colon cancer patients compared to rectum cancer (HR = 0.2; 95%CI = 0.05 - 0.9, P = 0.038).

For patients without lymphopenia the tumor localization (P = 0.044), stage (P = 0.019), adjuvant chemotherapy (P = 0.003), and *LTA* additive (P = 0.060), and dominant model (P = 0.019).

Multivariate analysis confirmed the worst prognosis of *LTA* SNP C homozygous carriers, showing a shorter time to death (HR = 0.4; 95%CI = 0.1 - 0.9, P = 0.036).

The clinicopathological and genetic variables with progression free survival was estimated using univariate and multivariate analysis (table 10).

Table 10 - Empirical univariate (Kaplan-Meier) and multivariate (Cox regression) analyses of the endpoint progression of disease for clinicopathological and genetic variables stratified by lymphopenia cutoff

	Lymphopenia (lymphocyte count < 1x10^3/µL) (n=47)				**Normal lymphocytes (lymphocyte count > 1x10^3/µL) (n=110)**			
	Univariate		**Multivariate**		**Univariate**		**Multivariate**	
	Mean (95%CI)	P	HR (95%CI)	P	Mean (95%CI)	P	HR (95%CI)	P
Age, years								
< median	84.8 (50.5-119.1)		-	-	51.0 (42.2-59.9)		-	-
> median	45.1 (32.7-57.5)	0.950			64.9 (50.7-79.0)	0.979		
Gender								
Female	94.7 (45.4-144.0)		-	-	58.4 (44.8-72.1)		-	-
Male	52.7 (39.8-65.5)	0.936			59.9 (47.9-72.0)	0.624		
Localization								
Colon	28.2 (18.3-38.0)		Referent		62.7 (51.2-74.2)		-	-
Rectum	111.7 (79.1-144.3)	0.002	0.3 (0.1-1.1)	0.063	48.9 (37.2-60.5)	0.633		

Tumor side								
Right	22.2 (12.4-32.0)		Referent		39.5 (28.5-50.6)		Referent	
Left	103.5 (74.9-132.0)	0.002	0.4 (0.1-1.3)	0.128	64.6 (53.6-75.5)	0.092	0.5 (0.3-0.9)	0.029
Stage								
Localized	44.6 (29.8-59.5)		Referent		59.5 (50.1-69.0)		Referent	
Locally advanced	60.5 (46.0-75.1)		0.6 (0.2-1.6)	0.273	56.0 (41.9-70.0)		1.9 (1.0-3.7)	0.068
Metastasis	25.4 (13.6-37.3)	0.055	3.2 (1.0-10.7)	0.060	31.3 (20.8-41.8)	0.002	1.7 (0.7-4.0)	0.204
Adjuvant chemotherapy								
No	46.6 (31.6-61.7)		-	-	33.1 (24.5-41.7)		Referent	
Yes	96.1 (66.7-25.6)	0.651			71.5 (60.0-83.1)	<0.001	0.4 (0.2-0.9)	0.023
Additive model								
CC	45.8 (33.6-57.9)		-	-	50.2 (38.4-61.9)		--- [a]	
CA	102.7 (65.6-139.8)				75.0 (60.9-89.1)		--- [a]	-
AA	39.6 (21.8-57.4)	0.791			28.6 (13.0-44.2)	0.016	--- [a]	
Recessive model								
CC/CA	92.9 (63.4-122.5)		-	-	63.0 (53.2-72.8)		Referent	
AA	39.6 (21.8-57.4)	0.496			28.6 (13.0-44.2)	0.070	2.5 (0.9-7.2)	0.080
Dominant model								
CC	45.8 (33.6-57.9)		-	-	50.2 (38.4-61.9)		Referent	
CA/AA	90.7 (58.8-122.6)	0.868			70.1 (56.7-83.5)	0.067	0.5 (0.3-0.9)	0.031

[a] removed due to collinearity. HR, hazard ratio. 95%CI, 95% confidence interval.

No definitive association was observed for patients under lymphopenia. Nevertheless, in group of CRC patients not lymphopenic there was association with tumor side (P = 0.092), tumor stage (P = 0.002); adjuvant chemotherapy (P < 0.001), and *LTA* gene analyzed by additive (P = 0.016), recessive (P = 0.070), and dominant (P = 0.067).

Multivariate analysis confirmed the association of tumor side (P = 0.029), adjuvant chemotherapy (P = 0.023) and *LTA* SNP rs1041981 dominant model to progression-free survival in CRC patients (P = 0.031).

In order to analyze the genotype-to-phenotype association between *LTA* and TAMs and TILs in tumor tissues we conducted a comparison between central tendency measures for each *LTA* genotype. No differences were observed between *LTA* genotypes and the count of TAMs and TILs infiltrated in tumors (Figure 22).

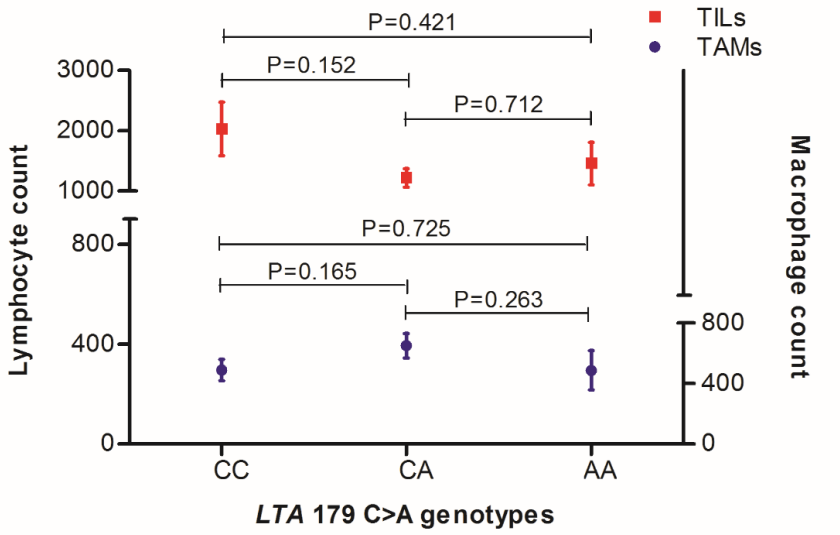


Figure 22 - Genotype-phenotype analyses. Comparison of tumor macrophages and lymphocytes between *LTA* 179 C>A genotypes. Data is presented as mean±SEM; TILs, tumor infiltrating lymphocytes; TAMs, tumor associated macrophages. Kruskal-Wallis followed by Mann-Whitney tests were used to compare differences between genotypes.

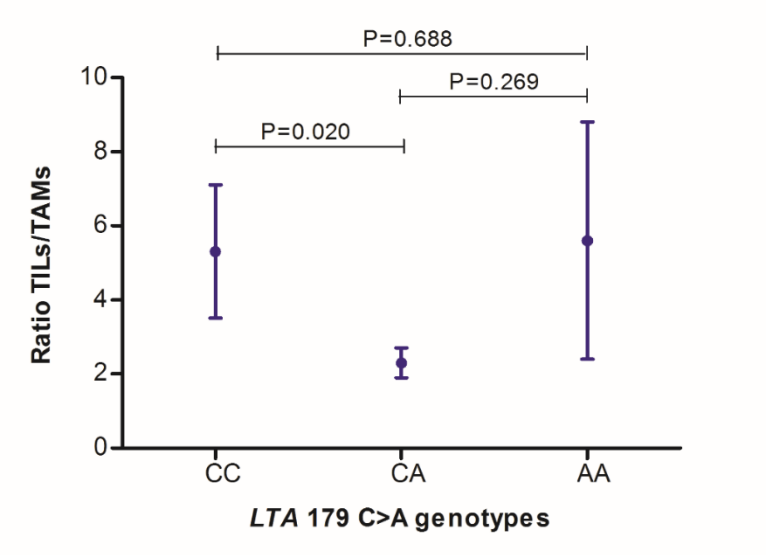


Figure 23 - Comparison of the ratio of TILs/TAMs across *LTA* 179 C>A genotypes. Data is presented as mean±SEM; TILs, tumor infiltrating lymphocytes; TAMs, tumor associated macrophages. Kruskal-Wallis followed by Mann-Whitney testes were used to compare differences between genotypes.

Interestingly, differences were found when we used a calculated TILs-to-TAMs ratio as dependent variable, between the genotypes CC and CA (P = 0.020) (Figure 23).

A significantly higher macrophage count was found in tumors located in colon (633.9±55.8) compared to rectum (450.9±100.1) (P=0.012), whereas no differences between anatomical location were found for infiltrating lymphocyte count (P=0.911) (data not show).

V- Discussion

The colorectal cancer is one of most prevalent cancer on the world. This cancer has one of highest mortality ratio, in part due to absence of symptoms manifestation at beginning of the disease (Bardhan and Liu, 2013 cit. Mahasneh *et al.,* 2017). CRC involves several risk factors as age, lifestyle (diet and sedentarism), inflammatory bowel diseases and hereditary factors. These elements lead to proliferation of colon and/or rectum mucosal cells, originating a polyp as initial lesion that progress to a malignant tumor (Grady and Markowitz, 2015). The polyp and its evolution are result of epigenetic alterations and mutations in important genes for cell viability such as tumor suppressor genes, cycle regulation and proto-oncogenes (Das *et al.,* 2017). The risk of cancer, response to treatment and other factors as disease relapse and inflammatory response can be influenced by variants in genes, genetic and epigenetic alterations (Barnett *et al.,* 2009 cit. Huang *et al.,* 2013).

Based on the premise that underlying germline genetic variants in genes coding for molecules involved in the regulation of immunoregulatory tumor microenvironment, we sought to analyze the association between the putatively functional rs1041981 SNP in *LTA* gene with survival and PFS in colorectal cancer patients. LTA protein and its effect on colorectal cancer remains controversial (Korneev *et al.,* 2017). Some studies described that LTA possess anti-tumor effects (Ito *et al.,* 1999, Zhang *et al.,* 2013), while others indicate a pro-tumor action (Lau *et al.,* 2014). Lymphotoxin alpha is expressed by active lymphocytes and dendritic cells, as extracellular homotrimer or a membrane bound complex heterotrimer. This protein is recognized as a pro-inflammatory cytokine and, after binding to its receptors, mediates several functions as inflammation, immunostimulation and apoptosis (Koni *et al.,* 1997 cit. Morishige *et al.,* 2013).

To perform the study and identify the possible association between *LTA* and colorectal cancer, we used blood samples from colorectal cancer survivor patients. To extract DNA from white blood cells, we tested three protocols: Isolate II Genomic DNA Kit, ExtractME® DNA blood Kit and Invisorb® Spin Blood Mini Kit to determine which was the best kit concerning to DNA quantity, purity and quality. Good purity DNA was in the range of 1.8-2.0 (Chacon-Cortes and Griffiths, 2014). We concluded that DNA with best performance regarding purity and yield was obtained with ExtractME® DNA blood Kit and Invisorb® Spin Blood Mini Kits, following the manufacture proceedings. The quality was determined by amplification of DNA with histone H4 primers as the histone genes are considered one of the most conserved genes in eukaryotes (Thatcher and Gorovsky 1994). All different DNAs were amplified using the same PCR reagents and thermal conditions and these products were analyzed after running on

electrophoresis in a gel of agarose. After analysis we concluded that ExtractME® DNA blood Kit yielded the best results of concentration, purity and integrity. It was then used to extract the remaining samples.

In this study, we used the real-time PCR technique using Taqman probes to allow the allele discrimination regarding rs1041981 SNP with fluorochromes FAM in the case of the C allele and VIC in the case of A allele.

After assessing genotype, based on real time PCR findings, approximately 5 % of all samples were randomly selected to confirm preliminary results using a different technique. Specific *LTA* exon 3 primers were selected, and a PCR protocol was optimized.

After purification of the PCR products with Illustra™ExoProstar™ 1-Step these samples were sent to STAB Vida for sequencing. The sequencing confirmed eight-out-of-nine real-time PCR results, except one sample result. This sample was identified, by real-time PCR, as heterozygotic (CA). However, when analyzing the sequencing result it was considered as homozygotic AA. This sample exhibit a different heterozygotic amplification plot profile comparatively with other plots. In this sample, both probes bind to DNA sequence, resulting in VIC fluorochrome with higher fluorescence detection, while the other heterozygotic samples had a higher FAM fluorescence detection. Furthermore, when analyzing the dual color scatter plot, it was possible to visualize that sample was in the heterozygotic edge between heterozygotic (CA) and homozygotic AA. Similarly, in homozygotic amplification plot of wild type (CC) it was detected a small fluorescence from VIC, but in this case, this outstanding fluorescence was below the fluorescence baseline gave by ROX fluorochrome.

According to Livak (1999), this background fluorescence can be explained by the existence of cross-reactivity between C-specific probe and A allele (explaining the detection of FAM background fluorescence), happening the same cross-reactivity in wild-type homozygotic, resulting in an interaction between A-specific probe and C allele. Furthermore, Oliver *et al.* (2000) suggest that higher amounts of DNA could be responsible for non-specific binding probe and detection of background fluorescence.

The retrospective cohort study allowed evaluating the role of *LTA* in primary and secondary end points, respectively overall survival and progression-free survival. Herein, the mean of age of participants was 65.2 which agrees similar studies from developed countries (Brenner *et al.*, 2014). The majority of participants in our study was males (63 %) and had a highest incidence of colon (0.58) than rectal cancer (0.42). Despite this, in our series, more patients presented with rectal cancer than usually described in literature (van der Sijp *et al.*, 2016).

We evaluated the possible influence of clinicopathological and genetic variables in overall survival and in progression-free survival. Using uni- followed by multivariate analysis. The analysis was also stratified intogroups of lymphopenic (blood lymphocyte counts < $1x10^3$ lymphocytes/µL) versus non-lymphopenic patients.

Blood lymphocyte percentage was shown to predict both PFS and OS. A better prognosis was observed for patients with a count of blood lymphocytes > 1395 lymphocytes/µL. This evidence is in accordance to Milasiene *et al.* (2005) and Iseki *et al.* (2016). Both studies revealed a good prognosis for colorectal cancer patients when the peripheral lymphocytes counts were higher than 1200 and 1100 lymphocytes/µL, respectively. The patients with blood lymphocytes < $1.0x10^3$ had a better overall survival when tumor was localized at the rectum, compared with colon. The van der Sjip *et al.* (2016) study revealed the principal differences between rectal and colon cancer. According to this study, and confirming our data, the patients with colon cancer had a worse overall survival. The patients with colon cancer had a higher incidence of complications after surgery (McArdle *et al*., 2005; Law *et al*., 2007) which may be associated with decreased mobility. If it last for a prolonged period, lower physical activity can influence the immune system (Wolin *et al.,* 2009), resulting in death.

The tumor side revealed an influence in progression-free survival. The left side tumor was associated with PFS for all subjects or if stratified as non-lymphopenic. This might suggest that, in our study, the patients with left side tumor had a better prognosis than patients with right tumor side. Concordantly, previous studies also revealed a better survival for patients with tumor localization on left side (Loupakis *et al.*, 2015; Petrelli *et al.,* 2016; Arnold *et al.,* 2017), which was also an influence in tumor treatment (Zhang *et al.,* 2017).

Adjuvant chemotherapy in patients with normal blood lymphocyte count was associated with a better progression-free survival.

The analysis of rs1041981 *LTA* dominant model showed association with clinical endpoints. A carriers compared with C-homozygous had a better overall survival and progression-free survival when the count of lymphocytes was > $1x10^3$ lymphocytes/µL. This finding suggests that the A allele of SNP rs1041981 from *LTA* gene is protective for the most relevant clinical outcomes in colorectal cancer. Park *et al.* (1998) found that the A allele was associated with increased risk for developing colorectal cancer and could have an important role in initial steps of tumorigenesis or in tumor relapse by reactivation of latent tumor cells. However, the A allele was associated with good prognosis in other cancer types. Niwa *et al.* (2007) described that the presence of CA and AA genotypes were associated with lower risk of endometrial cancer, while

Takei *et al.* (2008) revealed an association with lung cancer. The study of Ito *et al.* (1999) demonstrated the importance of LTA protein to the maturation and recruitment of NK cells and rapid tumor growth in LTA deficient mice. The A allele has been described as high bioactive allele. The A allele from *LTA* variant rs1041981 (Thr26Asn) was associated, by Ozaki *et al.* (2002), with increased protein activity in inducing, in smooth-muscle, a higher expression of VCAM1 and SELE mRNA when the amino acid change from threonine to asparagine. Furthermore, this allele was several times described as in almost complete linkage disequilibrium with intron 1 *LTA* variant (A252G) (Messer *et al*., 1991). In this variant, the G allele was associated with the rs1041981 A-allele and higher level of LTA transcription (Ozaki *et al*., 2002). Thus, patients with *LTA* A-allele might have increased transcriptional activity, which could correspond to higher capacity to activate NK cells, killing tumor cells, thus resulting in better prognosis (Fridman *et al.,* 2017). This may represent an explanation for association between A allele and better overall and progression-free survival observed in population under study.

To acquire new characteristics as resistance to apoptosis, angiogenesis and invasion of new tissues, it is necessary that the tumor and its neighbor environment have conditions to support the neoplasia progression (Hanahan and Weinberg, 2011). For that, are essential the cellular and noncellular components. Some cells in tumor microenvironment promote cancer progression while others are responsible by their elimination. Fibroblasts, immune, endothelial and adipose cells interact and are involved in the process of tumor promotion and elimination (Wang *et al.*, 2017).

TAMs and TILs have been associated with good prognosis in colorectal cancer in initial and advanced phases of cancer (Forssell *et al.*, 2007; Zhou *et al.*, 2010; Jochems and Schlom, 2011). In our study, after evaluating tissue characteristics, these cells were counted in ten hot-spots. Statistical differences in ratio TILs/TAMs was observed between *LTA* CC and CA genotypes. This analysis revealed a higher ratio TILs/TAMs for patients under study with CC genotype than patients with AA genotype, supporting that patients with CC genotype in this population had a higher number of tumor infiltrated lymphocytes in tumor at surgery.

The present study integrates different approaches including a detailed clinical characterization and follow up, allele and genotypic characterization of a functional SNP with potential impact on CRC and finally an immunohistochemistry characterization of the tumor and surrounding cells. These different points of view on the same subject represent a huge advantage of this project, since they imply inherent potentialities.

The patients' clinicopathological data was collected in detail, including a survey related with the specific treatment used in each case. This is of major importance once it represents the starting point for the search of correlations with the genetic data. In the future we intend to increase the number of patients under study aiming to increase the robustness of results. It will be interesting to investigate other genes with potential impact in CRC namely by a possible influence in inflammation and tumor microenvironment. Furthermore, the inclusion of a healthy control group will be of major importance to allow us to implement a case/control study in order to compare the variables in the two populations.

The real-time PCR technique and its results confirmation, by sequencing, allowed us to obtain the individuals' genotyping for LTA gene and ensuing the characterization of the allelic and genotypic frequencies, contributing to some important findings to be included in the molecular epidemiology of colorectal cancer. Furthermore, the IHC optimized protocol allowed the characterization of CRC tissues and will be extended to all the samples under study.

VI- Conclusion

We presented an innovative study with the association amongst clinicopathological data, genetic studies and immunohistochemistry characterization in colorectal cancer.

The optimization of the real-time PCR technique and its confirmation, by sequencing, allowed us to assess the individuals' genotype for the LTA genetic variant rs1041981 and ensuing characterization of the allelic and genotypic frequencies in 172 CRC patients. The C and A alleles presented, respectively, allelic frequency of 70 % 30 %. CC, CA and AA genotypic frequencies were 49%, 42% and 9%, respectively.

The present study showed a protective effect of CA and AA genotypes compared with C-homozygous regarding the association with disease progression for patients with normal lymphocytes count. In this group of patients, a significant protective effect for all-cause mortality was observed in A-carriers.

Furthermore, the IHC optimized protocol allowed the characterization of CRC tissues in 34 cases, using the anti-human CD68 against macrophages and the anti-human CD3 against the T cell. The statistical analysis for TAMs and TILs counts revealed significant differences between the ratio TILs/TAMs and the genotype of the LTA variant under study. It is essential to extend this preliminary approach to all the CRC patients.

The understanding of the impact of the LTA genetic variant rs1041981 on the evolution of the disease can bring new information on tumor microenvironment regulation. The investigation of other genes potentially important for CRC and its influence in inflammation and microenvironment and the inclusion of a healthy control group to case/control study will be an interesting future approach.

Thus, in the future it will be important to increase the number of patients and extend the study to other oncological centers and CRC populations in order to confirm the possible association of this variant in colorectal cancer and its relationship with the number of tumor infiltrating cells.

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VIII- Annexes

Annex I – Informed consent

Centro Hospitalar de Trás-os-Montes e Alto Douro, E.P.E.	Serviço de Oncologia Médica	
	SOM/PI/2016.01	A imprimir em triplicado

CONSENTIMENTO INFORMADO

Procedimento:

Colheita de Amostra de Sangue, Utilizaçao de Amostras Arquivadas e Consulta de Informação Clínica para investigação biomédica.

Finalidade:

Permitir a utilização de amostras arquivadas, colheita de sangue e consulta de dados clínicos para investigação biomédica para o estudo **SOM/PI/2016.01**:

MICROAMBIENTE DE IMUNOREGULAÇÃO NO CANCRO COLORECTAL E RESPOSTA CLÍNICA À QUIMIOTERAPIA E PROGNÓSTICO

Pressupostos:

- A colheita de amostras é feita para investigação biomédica para melhor conhecimento do cancro;
- A autorização dada pelo doente é voluntária e altruísta;
- Será respeitado o principio da confidencialidade e do anonimato.
- Não será efetuada divulgação/comunicação individual de resultados obtidos com esse material.
- As amostras serão preservadas até à sua utilização, sem tempo limitado de armazenamento.
- Não haverá comercialização de amostras.
- O consentimento pode ser retirado em qualquer momento pela própria pessoa ou, depois da sua morte ou incapacidade, pelos seus familiares, devendo nesse caso as amostras biológicas e seus derivados ser destruídos.

Efeitos Secundários:

A utilização destas amostras não implica risco nenhum para o doente, nem altera as decisões relativas a qualquer acto médico. A eventual recusa do doente a este consentimento não colocará em causa a assistência e acompanhamento médico que lhe são devidos como pessoa.

Declaração do Doador:

- Fui informado dos objectivos do procedimento acima descrito e dou o consentimento para a colheita de sangue, utilização de amostras arquivadas e consulta do meu registo clínico para fins de investigação.
- Declaro que compreendi a informação recebida e pude formular as perguntas que julguei oportunas.

Nº Processo: _____

Nome: _____ Assinatura _____

Declaração do familiar/representante legal, em caso de incapacidade do doente:

- Fui informado dos objectivos do procedimento acima descrito e dou o consentimento para a participação do meu familiar _____, para fins de investigação.
- Declaro que compreendi a informação recebida e pude formular as perguntas que julguei oportunas.

Nº Processo: _____

Nome: _____ Assinatura _____

INVESTIGADOR RESPONSÁVEL	MÉDICO ASSISTENTE
Declaro que me responsabilizo pela informação constada neste documento e pelo cumprimento dos pressupostos do estudo.	*Declaro que informei adequadamente o doente sobre o procedimento acima descrito.*
Nome, Nº Mecanográfico, Data e Carimbo	Nome, Nº Mecanográfico, Data e Carimbo

Centro Hospitalar de Trás-os-Montes e Alto Douro, E.P.E.	Serviço de Oncologia Médica	
	SOM/PI/2016.01	A imprimir em triplicado

CONSENTIMENTO INFORMADO – INFORMAÇÃO

1) O que é este documento?
Este documento consiste num convite para participar no estudo intitulado SOM/PI/2016.01 "Microambiente de Imunoregulação no Cancro Colorectal e Resposta Clínica à Quimioterapia e Prognóstico". Este documento irá também fornecer-lhe informação sobre a opção de participação.

2) Qual o objectivo deste estudo?
O objectivo deste estudo é avaliar fatores genéticos, histológicos e clínicos associados à regulação do microambiente tumoral no cancro coloretal.

3) Quem está a promover este estudo?
O Serviço de Oncologia Médica do CHTMAD será o responsável pelo estudo no CHTMAD, no entanto devido à sua envolvência multidisciplinar, poderão colaborar no seu desenvolvimento outros laboratórios/instituições.

4) Porque fui convidado para este estudo?
Vimos desta forma convidá-lo a participar neste estudo, dado estar a ser tratado no Serviço de Oncologia Médica por cancro coloretal. A sua participação neste estudo é voluntária. Se decidir não participar ou desistir deste estudo, não perderá quaisquer cuidados médicos.

5) Que procedimentos serão efectuados?
Solicita-se a sua autorização para a recolha de amostra de sangue, da qual serão estudados ácidos nucleicos e proteínas. Será utilizada uma pequena amostra do tumor que se encontra arquivado, bem como serão utilizadas informações clínicas recolhidas diretamente do processo clínico.

6) Quantas pessoas irão participar neste estudo?
O número exacto de indivíduos a participar neste estudo é desconhecido, mas estima-se que sejam necessários entre 200-250 indivíduos. No entanto quanto maior for o número de participantes neste estudo maior é a validade do mesmo.

7) Quanto tempo dura o estudo?
O estudo decorrerá até se ter um número suficiente de amostras capazes de permitir chegar a uma conclusão viável, estando dependentes do número de doentes que aceitem participar neste estudo.

8) Irei eu, ou terceiros, beneficiar com a minha participação neste estudo?
Poderá não receber benefícios directos pela sua participação neste estudo. Contudo, espera-se que este estudo possa adicionar informação aos factores genéticos e biológicos com valor preditivo e prognóstico da doença.

9) O que irá custar este estudo?
A sua participação neste estudo não irá acarretar qualquer custo adicional para si.

10) Como será mantida a confidencialidade dos meus registos?
Este estudo foi aprovado pela Comissão de Ética do CHTMAD. É garantida pela instituição e responsáveis do projecto a rigorosa confidencialidade de todos os dados registados, reservando-lhe o direito, caso assim o entenda, de pedir, em qualquer momento, a sua remoção, assim como, de todos os dados recolhidos, deste estudo.

11) Quem terá acesso à minha informação médica, caso eu assine este consentimento informado?
O acesso directo aos seus registos será efetuado por médicos da instituição, a pedido do promotor do estudo para verificar a informação obtida no decorrer do estudo. Ao assinar este Consentimento Informado, autoriza (ou o seu representante legal) o acesso a esta informação confidencial.
A confidencialidade dos seus registos médicos será mantida dentro dos limites permitidos pela legislação aplicável. Se os resultados deste estudo forem publicados, a sua identidade permanecerá confidencial.

12) Se eu concordar em participar neste estudo, que certezas posso ter?
- Que a sua participação é voluntária e que é inteiramente livre de desistir em qualquer altura, sem que isso ponha em risco a sua assistência médica futura.
- Que o promotor do estudo pode escolher retirá-lo deste estudo de investigação em qualquer altura.
- Que pode colocar questões acerca deste estudo em qualquer altura.

NOTAS FINAIS:
Se tiver questões acerca do consentimento informado ou dos seus direitos como doente a participar num estudo de investigação, pode contactar o Investigador Responsável no CHTMAD.

Annex II - DNA extraction protocols

The followed DNA extraction protocols are referent to other two protocol tested.

2.1 - Isolate II Genomic DNA Kit (Ref.: BIO-52065, Bioline).

1. Transfer 200 µL of buffy coat sample to sterile 1.5 mL microtube and 25 µL of the Proteinase K.
2. Add 200 µL of the Lysis Buffer G3. Vortex vigorously for 10-20 secs.
3. Incubate at 70 ºC for 15 min.
4. Add 210 µL of ethanol (96 – 100 %) and vortex.
5. Transfer the lysate onto ISOLATE II Genomic DNA spin Column placed in a collection tube. Centrifuge for 1 min at 10.200 rpm.
6. Transfer the ISOLATE II Genomic DNA spin Column to a new 2 mL collection tube.
7. Add 500 µL BW1 Buffer and centrifuge for 1 min at 10.200 rpm. Transfer the ISOLATE II Genomic DNA spin Column to a new 2 mL collection tube.
8. Add 600 µL BW2 Buffer and centrifuge for 1 min at 10.200 rpm. Discard the filtrate and reuse the collection tube.
9. Centrifuge for 1 min at 10.200 rpm to remove the residual ethanol.
10. Discard the collection tube and carefully transfer the ISOLATE II Genomic DNA spin Column to a sterile 1.5 mL microcentrifuge tube.
11. Add 100 µL Elution Buffer G, pre-heated to 70 ºC directly onto the purification minicolumn membrane.
12. Incubate at room temperature for 2 min.
13. Centrifuge at 10.200 rpm for 1 min.
14. Remove the minicolumn. Storage DNA for a short-time period at 4 ºC or long-term period at -20 ºC.

2.2 - Invisorb® Spin Blood Mini Kit (Ref.: 1031100200, STRATEC Molecular).

1. Transfer 30 µL of buffy coat sample to sterile 1.5 mL microtube.
2. Add 200 µL of the Lysis Buffer HL.
3. Vortex thoroughly 15 sec and incubate for 3 min at 56 ºC, with continuously shaking.

4. Add 20 µL Proteinase S and vortex.

5. Incubate the reaction tube at 56 ºC for 5 min with shaking the tube every 2-3 minutes.

9. Add 200 µL Binding Buffer HL and vortex thoroughly by 15 sec.

10. Transfer the mixture into RTA Spin Filter placed in a RTA Receiver Tube. Incubate for 1 min.

11. Centrifuge for 2 min at 10.200 rpm. Discard the filtrate and place the RTA Spin Filter in a new 2.0 mL RTA Receiver Tube.

12. Add 500 µL Pre-Wash Buffer and centrifuge for 1 min at 10.200 rpm. Discard the filtrate and place the RTA Spin Filter in a new 2.0 mL RTA Receiver Tube.

13. Add 700 µL Wash Buffer and centrifuge for 1 min at 10.200 rpm. Discard the filtrate and place the RTA Spin Filter in a new 2.0 mL RTA Receiver Tube.

14. Add 700 µL Wash Buffer and centrifuge for 1 min at 10.200 rpm. Discard the filtrate and reuse the 2.0 mL RTA Receiver Tube.

15. Centrifuge for 4 min at 12.300 rpm to remove the ethanol completely.

16. Discard the RTA Receiver Tube and transfer the RTA Spin Filter to 1.5 mL RTA Receiver Tube.

17. Add 200 µL Elution Buffer, which was pre-heated to 56 ºC.

18. Incubate at room temperature for 1 min.

19. Centrifuge at 10.200 rpm for 1 min.

20. Remove the RTA Spin Tube. Storage DNA for a short-time period at 4 ºC or long-term period at -20 ºC.

2.2.1 - Invisorb® Spin Blood Mini Kit protocol optimization.

1. Transfer 50 µL of buffy coat sample to sterile 1.5 mL microtube and add 150 µL of water.

2. Add 200 µL of the Lysis Buffer HL.

3. Vortex thoroughly 15 sec and incubate for 5 min at 56 ºC, with continuously shaking.

4. Add 20 µL Proteinase S and vortex.

5. Incubate the reaction tube at 56 ºC for 5 min with shaking the tube every 2-3 minutes.

9. Add 200 µL Binding Buffer HL and vortex thoroughly by 15 sec.

10. Transfer the mixture into RTA Spin Filter placed in a RTA Receiver Tube. Incubate for 2 min.

11. Centrifuge for 2 min at 10.200 rpm. Discard the filtrate and place the RTA Spin Filter in a new 2.0 mL RTA Receiver Tube.

12. Add 500 µL Pre-Wash Buffer and centrifuge for 1 min at 10.200 rpm. Discard the filtrate and place the RTA Spin Filter in a new 2.0 mL RTA Receiver Tube.

13. Add 700 µL Wash Buffer and centrifuge for 1 min at 10.200 rpm. Discard the filtrate and place the RTA Spin Filter in a new 2.0 mL RTA Receiver Tube.

14. Add 700 µL Wash Buffer and centrifuge for 1 min at 10.200 rpm. Discard the filtrate and reuse the 2.0 mL RTA Receiver Tube.

15. Centrifuge for 4 min at 12.300 rpm to remove the ethanol completely.

16. Discard the RTA Receiver Tube and transfer the RTA Spin Filter to 1.5 mL RTA Receiver Tube.

17. Add 100 µL Elution Buffer, which was pre-heated to 56 ºC.

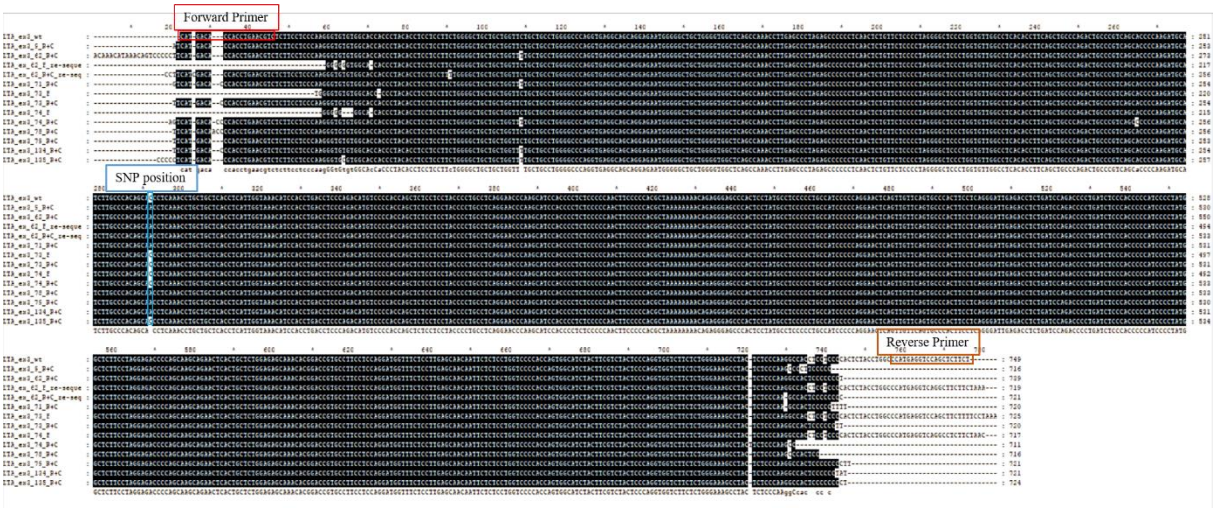
18. Incubate at room temperature for 5 min.

19. Centrifuge at 10.200 rpm for 1 min.

20. Remove the RTA Spin Tube. Storage DNA for short-time period at 4 ºC or for long-term period at -20 ºC.

Annex IIII – Sequences alignment

Alignment of corrected sequences and exon 3 fragment sequence selected from the human *LTA* gene. The sequence of primer Forward used for the amplification of exon 3 fragment of the *LTA* gene is marked in red and the reverse primer sequence is marked in brown. Signed in blue color is the polymorphism rs1041981 (C>A, Thr60Asn).



Annex IV - Summary of DNA quantification. With number of sample, kit name, buffy coat and elution buffer volumes. In this table, the yield and DNA purity (assessed by the ratio of absorbances at 260 and 280 nanometers (nm)) are also shown.

Sample	Extraction DNA Kit	Buffy Volume (µL)	Elution volume (µL)	Yield (ng/ µL)	Yield (ng)	$\frac{A260}{A280}$
CCR 5	Invisorb	50	200	17.81	3562	1.62
CCR 8	Extract Me	350	100	42.24	4224	1.87
CCR 18	Bioline	200	100	33.69	3369	1.58
CCR 19	Bioline	200	100	23.94	2394	2.11
CCR 24	Invisorb	30	200	15.87	3174	1.81
CCR 23	Invisorb	50	100	15.77	1577	2.12
CCR 24	Invisorb	50	100	8.5	850	3.69

Invisorb, Invisorb® Spin Blood Mini Kit; ExtractMe, ExtractME® DNA blood Kit; Bioline, Isolate II Genomic DNA Kit.