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Tesis doctoral

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"Acción de los compuestos bioactivos del café durante la carcinogénesis de colon en modelos *in vitro* e *in vivo*"

Memoria presentada por **Dña. Ariane Rocha Bartolomeu** para optar a la mención de Doctor Internacional por la Universidad de Granada

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“Imposible es solo una palabra lanzada por hombres pequeños a quienes les resulta más fácil vivir en el mundo que se les ha dado que explorar el poder que tienen para cambiarlo. Imposible no es un hecho. Es una opinión. Imposible no es una declaración. Imposible es potencial. Imposible es temporal. Imposible es nada.”

- Muhammad Ali -

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Summary

Resumen

El cáncer colorrectal (CCR) ocupa la tercera posición en el ranking de las neoplasias más mortales en todo el mundo. La incidencia está aumentando constantemente especialmente en países en desarrollo debido a la forma de vida "occidental". Según el informe estadístico más reciente, más de 1.9 millones de nuevos casos y 935 mil muertes fueran identificados en el año 2020 en todo el mundo. Actualmente, las opciones antitumorales y/o citotóxicas disponibles en la terapia del cáncer de colon poseen una eficacia alrededor del 50% y un modesto impacto en la supervivencia, debido a la resistencia primaria o adquirida a los fármacos convencionales, por o que casi la mitad de los pacientes recaen con metástasis. La dieta es un factor importante en el desarrollo de CCR, en las últimas décadas amplios estudios epidemiológicos y experimentales han vinculado los hábitos alimenticios a la prevención y baja tasa de riesgo. El café está incluido en la dieta diaria en casi todo el mundo cuyos beneficios fueron evidenciados a través de estudios epidemiológicos y experimentales. La actividad antitumoral relacionada al consumo de café cursa con varios mecanismos moleculares que están siendo elucidados gradualmente. Los tres principales compuestos bioactivos del café son los alcaloides cafeína y trigonelina, y el polifenol ácido clorogénico. Nuestro trabajo pretende aportar información que aun no ha sido explorada acerca de los compuestos mayoritarios del café tanto aislados como combinados frente al CCR. Por lo tanto, el objetivo principal de ese trabajo ha sido demostrar la acción de los compuestos mayoritarios del café frente a modelos experimentales del CCR *in vitro* e *in vivo*, además de evaluar si eses efectos actúan sobre la subpoblación quiescente de células madre cancerígenas. Para ello hemos usado *in vitro* líneas celulares tumorales procedentes de adenocarcinoma de colon humano (HCT-116 y HT-29) crecidas en monocapa y crecidas en colonoesferas, así como modelos experimentales *in vivo* de inducción química de carcinogénesis en ratones Swiss Webster e injertos xenográficos en ratones NSG. Tras el tratamiento con los compuestos del café hemos evaluado su citotoxicidad, efectos antitumorales y sus mecanismos moleculares, a través de estudios histológicos y de la modulación de algunas dianas involucradas en los "*hallmarks*" del CCR. Nuestros resultados muestran propriedades quimiopreventivas de los compuestos del café (cafeína y ácido clorogénico) frente al modelo de carcinogénesis de colon inducido por DMH/DCA en ratones, a través de la modulación de importantes citocinas (IL-6, TNF-α e IL-17), además de tener acción antiproliferativa por la disminución de la expresión de la proteína ki-67 y regulación negativa de un biomarcador crucial en el CCR, el oncomir miR-21. Del mismo modo fueron observados efectos antiproliferativos en células cancerígenas de colon (HCT-116 y HT-29)

cultivadas en monocapa, por parte de los compuestos del café cafeína y ácido clorogénico, mientras el compuesto trigonelina no produjo ningún efecto significativo. Además, las colonosferas secundarias enriquecidas en células madre cancerígenas (CMC) formadas con las mismas líneas celulares presentaron sensibilidad significativa a los compuestos aislados o combinados durante su exposición con 72 horas de manera dosis-dependiente, con excepción de la trigonelina que no presentó ningún efecto sobre dichas subpoblaciones. Diferentes interacciones antitumorales fueron observadas entre los dos compuestos cafeína y ácido clorogénico; dicha mezcla presentó un fuerte efecto antagónico sobre colonoesferas enriquecidas en CMC procedentes de la línea HCT-116, mientras que dicho efecto fue diferente en las colonoesferas enriquecidas en CMC procedentes de la línea HT-29, que dependió de la concentración elegida, observándose con la IC_{90} un fuerte efecto antagónico, con la IC_{50} un ligero efecto aditivo y con la IC_{10} un fuerte efecto sinérgico. Los marcadores de células madre cancerígenas fueran drásticamente reducidos (CD44 y CD326), así como la actividad de la enzima ALDH tras el tratamiento. Hubo un aumento en la retención del Hoescht 33342 que indicó también la reducción de las subpoblaciones de CMC tras la exposición a los compuestos del café. Además, hemos realizado dos protocolos de inoculación de colonoesferas en ratones, uno de pre-tratamiento oral con los compuestos del café más efectivos aislados o combinados y otra aproximación pos-tratamiento de la misma forma, teniendo en cuenta. que las dosis se basaron en el la concentración del consumo humano del café extrapolado a ratones. Los tumores procedentes de las CMC mostraron una significativa disminución en el grupo de ratones que recibió el pre-tratamiento en comparación con el grupo control al igual que ocurrió en el grupo de pos-tratamiento. Nuestros resultados demuestran, por tanto, que las proporciones de los mencionados compuestos del café correspondiente a una cantidad media del alto consumo humano pueden presentar propiedades preventivas o incluso terapéuticas respecto al desarrollo del CCR, abriendo una nueva aproximación para futuras investigaciones.

Palabras clave: cáncer colorrectal, compuestos bioactivos del café, miRNAs

Resumo

O câncer colorretal (CCR) ocupa a terceira posição no ranking das neoplasias mais mortíferas do mundo. A incidência está aumentando constantemente, especialmente nos países em desenvolvimento, devido ao modo de vida "ocidental". De acordo com o relatório estatístico mais recente, mais de 1,9 milhão de novos casos e 935 mil mortes foram identificados no ano de 2020 em todo o mundo. Atualmente, as opções antitumorais e/ou citotóxicas disponíveis na terapia do câncer de cólon são cerca de 50% eficazes e têm um impacto modesto na sobrevida, devido à resistência primária ou adquirida aos medicamentos convencionais, para os quais quase metade dos pacientes recai com metástases. A dieta é um fator importante no desenvolvimento do CCR, nas últimas décadas extensos estudos epidemiológicos e experimentais associaram hábitos alimentares à prevenção e baixo índice de risco. O café está incluído na dieta diária de quase todas as pessoas cujos benefícios foram evidenciados na literatura. A atividade antitumoral relacionada ao consumo de café segue vários mecanismos moleculares que estão sendo gradualmente elucidados. Os três principais compostos bioativos do café são os alcalóides cafeína e trigonelina, e o polifenol ácido clorogênico. Nosso trabalho visa fornecer informações ainda não exploradas sobre estes compostos, isolados ou combinados, contra o CCR. Portanto, o objetivo principal deste trabalho foi demonstrar a ação dos principais compostos do café contra modelos experimentais de CCR *in vitro* e *in vivo*, além de avaliar se esses efeitos atuam na subpopulação quiescente de células-tronco cancerígenas. Para isso foram utilizadas linhagens de células tumorais de adenocarcinoma de cólon humano (HCT-116 e HT-29) cultivadas em monocamada e em colonoesferas, bem como modelos experimentais *in vivo* de indução química de carcinogênese em camundongos Swiss Webster e enxertos xenográficos com camundongos NSG. Após o tratamento com compostos de café, avaliamos sua citotoxicidade e seus mecanismos moleculares, por meio de estudos histológicos e da modulação de alguns alvos envolvidos em marcadores de CRC. Nossos resultados mostram propriedades quimiopreventivas de compostos de café (cafeína e ácido clorogênico) contra o modelo de carcinogênese de cólon induzida por DMH/DCA em camundongos, através da modulação de importantes citocinas (IL-6, TNF-α e IL-17). Também foi observada a ação antiproliferativa com a diminuição da expressão da proteína ki-67 e regulação negativa de um biomarcador crucial no CCR, oncomir miR-21. Da mesma forma, efeitos antiproliferativos foram observados em células de câncer de cólon (HCT-116 e HT-29) cultivadas em monocamada, enquanto o composto trigonelina não produziu nenhum efeito significativo. As

colonosferas secundárias enriquecidas em células-tronco cancerígenas (CSC) formadas com as mesmas linhagens celulares mostraram sensibilidade significativa aos compostos isolados ou combinados durante sua exposição com 72 horas de forma dose-dependente. Diferentes interações antitumorais foram observadas entre os dois compostos cafeína e ácido clorogênico; esta mistura apresentou um forte efeito antagônico nas colonosferas enriquecidas em CSCs da linha HCT-116, enquanto tal efeito foi diferente nas colonosferas enriquecidas em CSCs da linha HT-29, que dependia da concentração escolhida, observado com o IC_{90} um forte efeito antagônico, com IC_{50} um leve efeito aditivo e com IC_{10} um forte efeito sinérgico. Os marcadores de células-tronco do câncer foram drasticamente reduzidos (CD44 e CD326), assim como a atividade da enzima ALDH após o tratamento. Houve um aumento na retenção de Hoescht 33342 que também indicou a redução das subpopulações de CSCs após exposição aos compostos do café. Realizamos dois protocolos de inoculação de colonosfera em camundongos, um de pré-tratamento oral com os compostos de café mais eficazes isolados ou combinados e outro de abordagem de pós-tratamento da mesma forma, levando em consideração que as doses foram baseadas no consumo médio humano de café. Os tumores de CSCs mostraram uma diminuição significativa no grupo de camundongos que recebeu o pré-tratamento em comparação ao grupo controle, bem como no grupo pós-tratamento. Nossos resultados, portanto, mostram que as proporções dos compostos de café acima mencionados baseado no consumo humano médio alto podem ter propriedades preventivas ou mesmo terapêuticas quanto ao desenvolvimento do CCR, abrindo uma nova abordagem para pesquisas futuras.

Palavras chave: câncer colorretal, compostos bioativos do café, miRNAs

1. INTRODUCTION

1.1. Cancer: definition and meaning

Cancer is a complex malignance inserted in a large group of disease, which may affect any organ or part of the body. Usually, this disease is termed as tumor and/or neoplasm. The main characteristic of the cancer is the uncontrollably cells grown, acquiring capacity to invade adjacent tissues of the body and go beyond the distant organs, which the attributed term is metastasis (Bray *et al.*, 2018). The cancer is occasioned mainly by aspects of environment/ style life and most of cancer may be prevented through avoiding exposure to the common risk factors, such as, smoking, sedentarism, carcinogens exposition etc. Likewise, most of cancers can be avoided with the right prevention through the early detection, quality diagnosis and specific treatment (McGuire, 2016).

The cancer is equipped of hallmarks, which comprises biological abilities gained during the multistep carcinogenesis. These hallmarks display an organizing principle for understanding the complexities of this malignance. Consisting of sustaining proliferative signalling, resisting cell death, enabling replicative immortality, evading growth suppressors, genome instability, tumor promoting inflammation, inducing angiogenesis and activation of invasion causing metastasis, reprogramming of energy metabolism and evading immune destruction **(Figure 1)** (Hanahan and Weinberg, 2011).

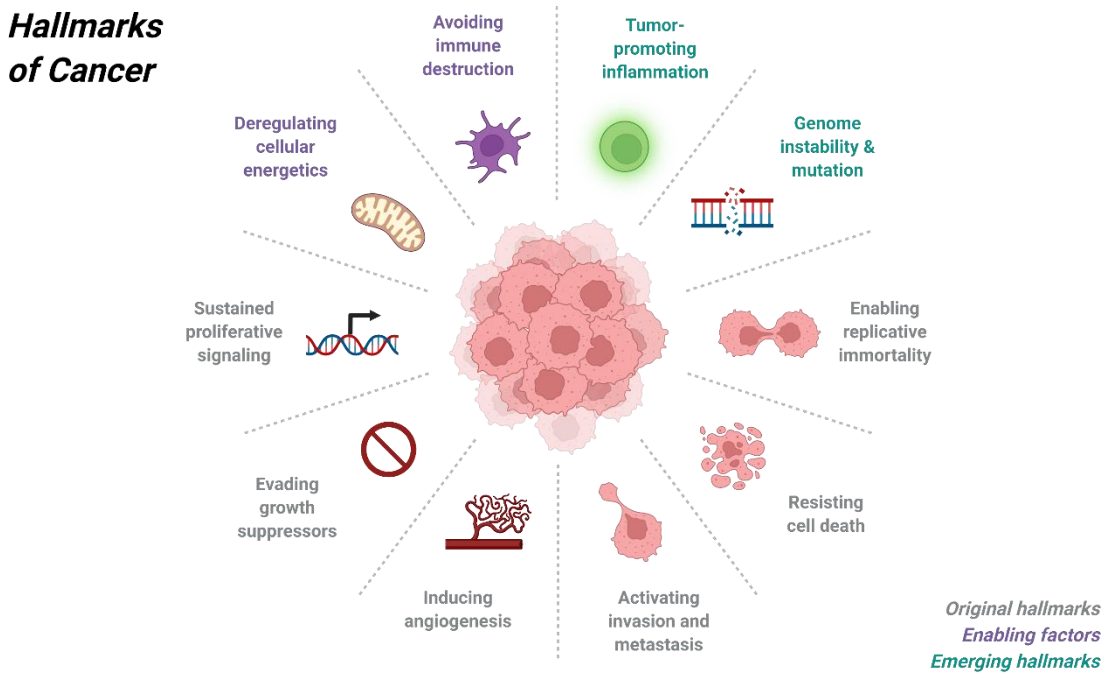

Figure 1. The cancer hallmarks. Source: Hanahan and Weinberg, 2011.

The cancer development is caused mainly by genetic alteration (mutations) whose can be inherited from our family in the germline cells or acquired during the lifetime as result of errors in the cell division or from carcinogenic exposition. Most of cancer harbour multiple genetic modifications in oncogenes as well as tumor suppressor genes, the first one induces aberrant growth and the second one contributes to the oncogenesis by a loss of function (Vogt, 1993). The most known oncogenes in human cancer are part of the *ras* family, involved in approximately 20% of tumor tissue, including about 50% of colon and 25% in lung carcinomas (Cooper, 2000). The mutations that convert *ras* proto-oncogenes to oncogenes result in constitutive *Ras* activity triggering the MAPK pathway (Molina and Adjei, 2006). Another kind of genetic aberration are the translocation of proto-oncogenes resulting in rearrangement of coding sequences, leading to the construction of abnormal gene materials. The most common aberrations by translocation accepted occur with *c-myc, bcl-2* and *abl* (Chial, 2008). Actually, more than 40 distinct human proto-oncogene are known and the oncogenes arise from mutations as nucleotide deletions or insertions that promote specific regions, gene amplification and chromosomal translocation. In addition, epigenetic disruption can lead the altered gene function and malignant cell transformation. Taken all together, the initiation and progression of cancer originally is seemed as genetic disease, with epigenetic abnormalities.

The neoplasm is not a simple arrange of malignant cells, but is like a complex of heterogeneous collection of infiltrating cells mixed with adjacent host cells, secreting molecules and surrounded of extracellular matrix (ECM). This phenomenon is named tumor microenvironment (TME), composed by immune cells, stromal cells, blood vessels and extracellular matrix which role is promotion of cancer progression (Truffi *et al.,* 2020). The TME variate depending of tumor type and it is constructed in different steps of tumorigenesis. Most of TME display responsibility by the tumor resistance to the therapies (radio- and chemoresistance). Nonetheless, the interaction of the cancer cells with stromal and inflammatory cells act differing the total of tumor mass protecting the cells (Hanahan and Weinberg, 2011; Marie-Egyptienne *et al*., 2013). In the beginning of tumor growth there are reciprocal interaction between cancer cells and components of TME that display key role in the survival of these cells, local invasion and metastatic dissemination. The TME is able to overcome a hypoxic situation and acidity coordinating a program that promotes angiogenesis restoring oxygen and nutrient supply removing metabolic waste (Anderson and Simon, 2020). Normal cells and tissues establish homeostasis of architecture also controlling the production and releasing of growth-promotion signals. Cancer cells deregulate those signals originating

uncontrolled cell growth and proliferation (Hanahan and Weingerg, 2011). Immune cells display important role on TME, depending on context, the interaction of immune cells with tumor cells can generate even tumor suppression as tumor promotion. Continual inflammation due to the chronic infection is a normal mechanism underlying tumor formation in many types of cancer, including colorectal cancer (CRC) (Anderson and Simon, 2020). Stromal cells support the cells recruitment from nearby endogenous tissue stroma helping important steps in tumor formation, constituting an important component of TME. This component variates according to the tumor types, the stromal part is constituted with vascular endothelial cells, fibroblasts, adipocytes and stellate cells. Stromal cells secrete many factors that influence angiogenesis, proliferation, invasion and metastasis (Denton *et al*., 2018). Besides the cellular components, there are the non-cellular components, such as the ECM and exosomes. The ECM is composed collagen, fibronectin, laminin and elastin within provide physical scaffold for cells and promote tumor cells dissemination. Solid tumor mass is constituted around 60% by ECM responsible by large collagen deposit (desmoplasia) which is strongly associated with poor prognosis. The MMPs are responsible by the break down the ECM proteins remodelling it to promoting the tumor metastasis and releasing cytokines together with growth factors. The ECM is like deposit for proangiogenic factors, like VEGF, FGF, PDGF and TGF-β (Eugundi *et al*., 2020). The exosomes are microvesicles and their contents vary depending on the cells from which they are derived along with protein, DNA, RNA and lipids. The exosomes display role on the crosstalk between the stromal cells and the cancer cells promoting inflammation, tumor progression, angiogenesis and metastasis. In hypoxia condition, it occurs an increase the exosomes release by the cancer cells beneficiating the transition of stromal cells into cancer-associated fibroblasts (Tung *et al*., 2019). Thus, the TME is extremely important in the tumor dissemination, treatment failure and tumor relapse.

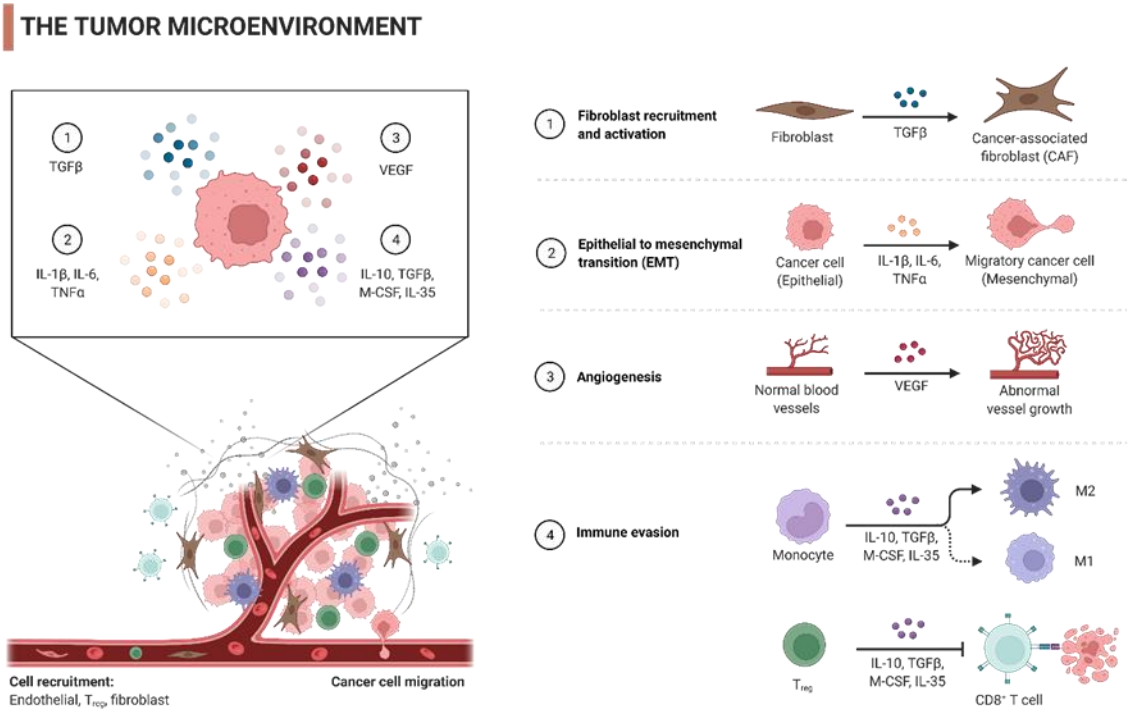

Figure 2. The tumor microenvironment is constituted by cellular components adjacent to the tumor mass, immune cells, epithelial cells and acellular components including the ECM and blood vessels. The cancer cells produce molecules that contribute to the tumor growth by immune evasion, distant metastatic niche formation, neoangiogenesis and among others functions that compute malignance to the neoplasm.

1.2. Colorectal cancer – origin and epidemiology

1.2.1. Epidemiology

Colorectal cancer (CRC) is one of most frequent human neoplasm occupying the second place in the top ranking of cancer mortality in both men and women and all ages. Over than 1.9 million new cases and 935 thousand deaths were estimated worldwide couple of years ago (Sung *et al*., 2021). CRC was third most common diagnosed cancer worldwide in 2020, comprising 10% of new cases **(Figure 3)** (Sung *et al*., 2021). The incidence rates are higher in developed countries than in developing countries, however the mortality rates are more common in this last one. CRC has been frequently associated with the socioeconomic status and the Human Development Index (HDI) with highest incidence in Eastern Europe, South Eastern Asia and Northern America. In Hungary and Norway, the CRC is ranking the top one in men and women, noteworthy its incidence rates tend to be low in most of regions of Africa and South-Central Asia **(Figure 4)** (Bray, 2015). The relation between the HDI and CRC

incidence suggest that this malignancy has it as a socioeconomic development marker, also it might to reflect in changes on lifestyle habits like increasing intake of animal-source foods and sedentarism, which are independently associated with CRC risk (Siegel *et al*., 2020). Furthermore, heavy alcohol consumption, cigarette smoking, increasing intake of red and processed meat represents additional factor meanwhile adequate intake of whole grains, fibber and adequate diet seems to reduce the risk for CRC (Berlau, Glei and Pool-Zobel, 2002; Paulmer, 1985). Nonetheless, epidemiological data show slightly higher prevalence in men, although this pattern is changing due to rising incidence in women, and strong association with increasing age. Both environmental risk factors and hereditary display an important part on CRC development (Henricson *et al*., 2015). These epidemiological data evoke the importance of CRC as a global health problem and explicitly the necessity for novel preventive and therapeutic strategies.

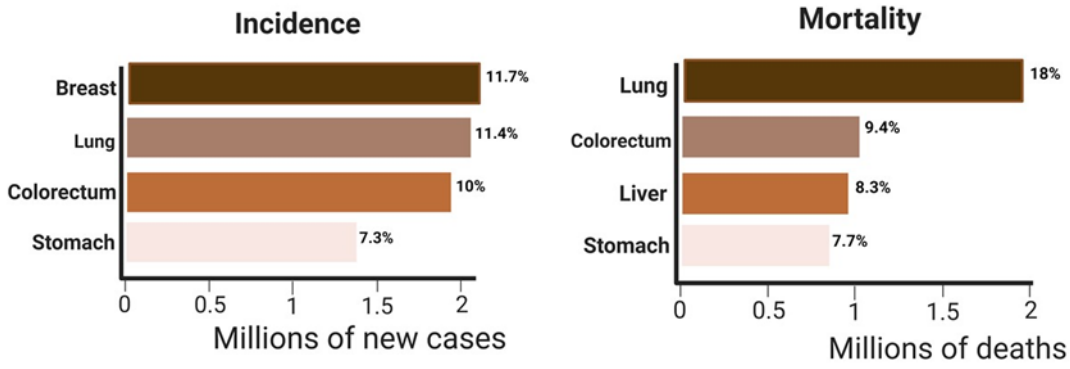

Figure 3. Infographics of epidemiological data on main cancers obtained in 2020. The CRC was responsible about 9.4% of 9 958 133 of deaths and 18% of 19 292 789 new cases of all cancers types last year. Source: Globocan, 2020.

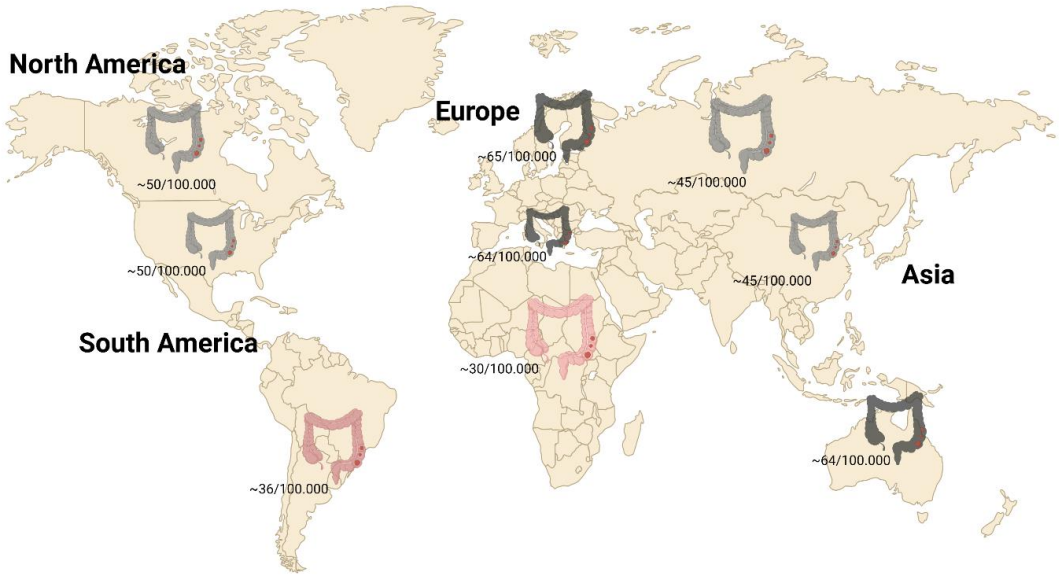

Figure 4. Global CRC age-standardized distribution of incidence by 100.000 habitants in both women and men. Source: Globocan, 2020.

1.2.2. Colorectal anatomy

In order to understand the mechanism of CRC in different segments of the large bowel is important to mention some aspects of anatomy, embryology and physiology. The colorectum is divided in segments: cecum, appendix, ascending colon, transverse colon, splenic flexure, descending colon, sigmoid colon and rectum **(Figure 5)** (Carmichael and Mills, 2016). The colon is an expansive tube that practically surround the small intestine like an arch, it is variable in length, averaging around 150 cm which correlate one quarter of the small intestine length. Its diameter may be considerably distended increasing the intestinal lumen (Carmichael and Mills, 2016). The major function of colon and rectum is nutrient/water absorption, and faecal storing. The small intestine differentiates anatomically of large intestine in some aspects as position, calibrecaliber and degree of fixation into the abdomen wall. The colon presents three specific characteristics: the taeniae coli (longitudinal muscle), the haustra sacculation (outpouchings of the bowel) and the appendices epiploicae (small appendages of fat) (Fraser *et al*., 1981). The cecum is the sacculated segment that is blind pouch located exactly below the entrance of the ileum, situated into the iliac fossa usually invested with peritoneum and its motility is limited by the mesocecum. There are two ligaments responsible for the angulation

of the cecum: ileocecal inferior and superior. These structures are surrounded with a circular sphincter which relax from response to the food entrance in the stomach and composed with an ileocecal valve that regulates its emptying (Kumar and Phillips, 1987). The roles of the cecum are prevention of reflux from the colon to the ileum, absorption of sodium and water (Li and Lai, 2009). The appendix is a vermiform and elongated diverticulum that emerges from the cecum around 3.0 cm below the ileocecal junction. The assemblage of the three taeniae is a useful to locate the appendix base (Oaklay, 1933). During years, the appendix was accredited with very little physiological role, however, some evidences indicated that this little structure has a significant role in young and adult's human. This vermiform structure is enriched in lymphoid tissue and it contributes to intestinal immunity, due to its position and shape, the appendix is a reservoir of microbiota (Girard-Madoux *et al.*, 2018). The ascending colon is most involved in absorptive process of remaining water, electrolytes and other key nutrients from indigestible material that consequently led to faeces formation, its ventral side is covered by the peritoneum whereas the posterior surface is retroperitoneally attached to the iliac fascia (Mazzuccelli and Maurer, 2004). This structure is approximately 15 cm long, which ascends from the level of the ileocecal to the psoas muscle and iliacus, the quadratus lumborum and the lower pole of the right kidney. In the lateral peritoneal reflection, is visible the white line of Toldt, whereas it is more evident in the descending colon (Carmichael and Mills, 2016). Transverse colon is about 45 cm, the longest segment of the large bowel, crossing the abdomen it extends from the right flexure into the spleen flexure. This part of large bowel is completely invested with peritoneum, but with bigger omentum make the fusion on its anterosuperior aspect. Further, its function is mainly moving waste material forward and perfused by the mesenteric artery, which supply the colon with arterial blood (Azzouz and Sharma, 2021). The descending colon extends downward from the splenic flexure; it is retroperitoneal in majority of animals located anteriorly to the left kidney, over its lateral border. This structure stores faeces that occasionally is emptied into the rectum (Gworys, Domagala and Markocka-Maczka, 2004). From the sigmoid flexure downside of descending part, begin the sigmoid colon which is 35-40 cm long, mobile, omega-shape loop totally invested by the peritoneum. The mesosigmoid is adhered to pelvic walls around the third sacral vertebrae level with an inverted shape, binding into the intersigmoid fossa (Carmichael and Mills, 2016). The sigmoid colon contracts to make the pressure inside this organ and the rectosigmoid junction stool to move into the rectum. This terminal portion is responsible to holding chamber of faecal material until its release, however, the sigmoid retains water, extract vitamins and minerals (Shafik, 1996). The rectum is known by its wide, distensibility lumen, and absence of taeniae, haustra, epicloic

appendices and well-defined mesentery. Measuring 12-15 cm this portion has three lateral curves: the upper and lower are convex to the right and the middle is convex to the left, these curves is named folds or valves of Houston. The mesorectum is commonly removed during the surgery for rectal cancer, due its capacity to became metastatic area (Cawthorn *et al*., 1990; Heald, Husband and Ryall, 1982).

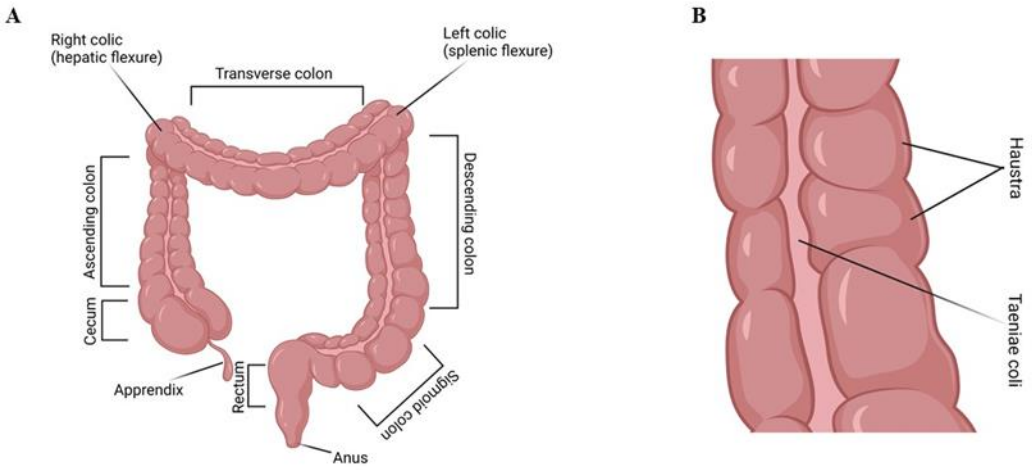

Figure 5. Segments of colorectum: **(A)** the right side originating the proximal to the splenic flexure (cecum, ascending colon and transverse colon) and left side known as distal part (descending colon, sigmoid and rectum). **(B)** Macroscopic features of large intestine the longitudinal muscle (Taeniae coli) and outpouchings (Haustra). Source: Feng and Mao, 2009.

1.2.3. Histological and molecular features of CRC

The natural CRC development is a multistep process that occur across four main stages: initiation, promotion, progression and metastasis **(Figure 6)**. The initiation stage concern in genetic damage that (such as DNA adducts) induce affected cells to subsequent neoplastic transformation. Most CRCs arise from a polyp, which process begins by an aberrant crypt (AC) or aberrant crypt foci (ACF). The ACF is considered the earliest neoplastic lesion, its evolution to adenoma/adenocarcinoma depends of genetic factors that deregulate the differentiation and uncontrol the cell proliferation (Orlando *et al.*, 2008). The ACF can be classified regarding the histology and topographic features. The topographic observations when compared with adjacent normal mucosa show the ACF as elevated structure, deeply stained, with larger crypts and shape polymorphism (rounded, serrated and elongated). Rarely the AC are isolated, it is most common to identify with two or more AC, the luminal of these structures are elliptical (Kirstt, Bryan and Gal, 2000). Histologically, the ACF is classified as hyperplasic, mild/moderate or severe dysplastic lesions. This classification depends of several factors: crypt

shape, serration, mucin depletion, maturation, nucleic shape, polymorphism, hyperchromatism and crypt compartments. The hyperplasic ACF commonly have histopathologic features similar to hyperplasic polyps. Nonetheless, the hyperplasic ACF are larger and longer than normal crypts with apical branching. The dysplastic ACF presents characteristic of adenomatous epithelium with hypercellularity, abnormal nuclear features and reduced cytoplasmatic mucin content and disordered maturation of upper region in the crypt epithelium (Nucci *et al*., 1998).

The two major CRC precursor are Adenomatous polyp (adenomas) and Serrated polyps (Conteduca *et al*., 2013). A colon polyp is a small cluster of cells that forms into the lumen of the colon epithelium. Polyps are divided in non-neoplastic and neoplastic; these two subtypes are classified histologically due to its appearance, inherited familiar factors, dysplasia degree and structural configuration **(Table 1)** (Rubio *et al*., 2002).

Table 1. Classification of non-neoplastic/neoplastic polyps and polyposis (Rubio *et al*., 2002).

<table>
<tr><th colspan="2">Non-neoplastic</th><th colspan="2">Neoplastic</th></tr>
<tr><th>Sporadic</th><th>Hereditary</th><th colspan="2">Sporadic</th><th>Hereditary</th></tr>
<tr><td>Hyperplastic polyps</td><td>Hyperplastic polyposis</td><td rowspan="3">Begins adenomas:</td><td>Tubular</td><td rowspan="3">FAP</td></tr>
<tr><td></td><td></td><td>Villous</td></tr>
<tr><td></td><td></td><td>Tubovillous</td></tr>
<tr><td>Inflammatory polyps</td><td>Juvenile polyposis</td><td rowspan="2">Serrated adenomas:</td><td>Sessile Serrated</td><td rowspan="2">HNPCC</td></tr>
<tr><td></td><td></td><td>Traditional serrated</td></tr>
<tr><td>Juvenile polyps</td><td rowspan="3">Peutz-Jeghers Syndrome</td><td rowspan="3">Peutz-Jeghers Syndrome:</td><td>Carcinoma in situ</td><td rowspan="3">MUTYH associated polyposis</td></tr>
<tr><td>Lymphoid polyps</td><td>Intramucosal CRC</td></tr>
<tr><td></td><td>Invasive CRC</td></tr>
</table>

The colonic epithelium is a continually renewing tissue and it is composed by four distinct cell lineages: enterocytes, goblet cells, endocrine cells and stem cells Paneth cells (this last one into the small intestine). The ancient cells on the top of the villus are released into the lumen and replaced by new cells raised from colonic crypts. In the bottom of colonic crypt there are stem cells whose role is proliferate and differentiate into cellular compartment of colon mucosa (Cernat *et al*., 2014). The stem cells possess the capacity to divide into identical daughter cells, which proliferate and differentiate to enterocytes, goblet cells and endocrine cells meanwhile their upward movement through the crypt (Ricci-Vitiani *et al*., 2009). High canonical WNT activity and nuclear β-catenin in colonocytes arise from aberrant crypts and evolve into a tubular or tubule-villous polyp. The over proliferation of polyps can lead to the early adenoma development with low degree of dysplasia. The early adenoma extends into advanced adenoma with high degree of dysplasia, increasing the accumulation of mutation in daughter cells progressing lately into carcinoma; this phenomenon is called transition of adenoma to carcinoma (Cernat *et al*., 2014). One of the most relevant factors in this transition is the size of the adenoma, once the malignant neoplastic develops in 1% of adenomas <1 cm, in 10% of adenomas >2 cm and 50% of adenomas >2 cm (Conteduca *et al*., 2013). Sessile serrated adenomas, traditional serrated adenomas and conventional adenomas differ among each other in their malignant potential, which reflects differences in the molecular pathways of carcinogenesis. However, there are some similarities between sporadic CRC and colitis-associated CRC.

1.2.4. Molecular hallmarks

CRC neoplasms may be broadly characterized as hypermutated (over than 12 mutations per 106 bases) or non-hypermutated (less than 8.24 mutation per 106 bases) (Cancer Genome Atlas, 2012). The hallmarks of CRC are genetic and epigenetic aberrations that gives aggressiveness to the epithelial cells which include the chromosomal instability (CIN), CpG island methylator phenotype (CIMP) alterations length of microsatellite (MSI) and mismatch repair (MMR) (Niguyen, Goel and Chung, 2019). The CIN (~80% of CRCs) is characterized by anomaly in chromosomal copy number (such as aneuploidy and polyploidy) and structure, which supposed to have origin from errors during mitosis typically occasioned by an APC mutation (APC 11307K, for example), followed by RAS activation or loss of TP53 and alteration in SMAD family member 4 **(Figure 6)** (Bakhoum *et al*., 2014; Pino and Chung,

2010). MSI occur in around 15% of sporadic CRCs, assuming the inactivation of genes involved in repair of mismatches bases in DNA, known as MMR genes (hMSH2, hMLH1, hPMS1 or hMSH6). The loss of MMR function affects directly the repair of slippage within repetitive DNA sequence elements into the cells. Consequently, this phenomenon develops microsatellites (mono- or dinucleotides) that once spreading throughout the genome may reaching regions which contains sequences that encodes important tumor suppressor genes, genes, such as, encoding transforming growth factor-β (TGF- β) receptor type II and Bcl-2-associated X protein (BAX). In HNPCC the microsatellites are inherited in most of the cases (¬95%) involving the genes hMSH2 or hMLH1, although some cases are acquired as observed in tumors with methylation-associated silencing a gene responsible by a MMR protein, for example, biallelic silencing the promoter region of the MLH1 trough methylation (Jasperson *et al*., 2010; Hewish *et al*., 2010). Nonetheless, germline deletions in the EpCAM (epithelial cell adhesion molecule) gene, also named as TACSTDI, were found in a subset family with Lynch syndrome and MMR gene mutation absent, it was an uncommon hypermethylation of the hMSH2 promoter (Ligtenberg *et al*., 2008). The MSI status is classified due to the level of its instability as MSI-low (<30% of instability) and MSI-high (>40% of loci instability). MSI-high occur in over than 90% of individuals with inherited predisposition for CRC, but only 15% of those with sporadic cases (Ligtenberg *et al*., 2008). The sporadic MSI tumors are associated with the serrated adenomas and usually carry BRAF V600E mutations, which are absent in individuals with germline mutation in MMR genes. Thus, the positive cases for BRAF mutation in a MSI tumor, are golden standard to exclude the Lynch syndrome (Pritchard and Grady, 2010). There are two MAP-associated mutation in MYH identified: Y179C and G396D, individuals with this mutation presents germline inactivation of a base excision repair gene, the mutY homologue (MUTYH-associated polyposis). The MYH protein excises from DNA the 8-oxoguanine product of oxidative damage to guanine (Lubbe *et al*., 2009). CIMP is a form of epigenetic modification concerned in hypermetilation of repetitive CG nucleotides (known as CpG islands), located in promoter regions enriched with tumor suppressor genes (as MLH1, MINT1, MINT2 and MINT3), usually these genes are silenced post hypermetilation. The CIMP is observed in about 50% of premalignant colonic adenomas and around 50% of CRCs, suggesting strong marker for an early stage of colorectal tumorigenesis. MSI is found in 45% of CIMP-positive and in 100% of CIMP-positive cases with methylate hMLH1 (Weisenberger *et al*., 2006). However, the understanding about CIMP remains elusive; its definition is wide without any criteria to classify CIMP subtypes (Nazemalhosseini *et al*., 2013; Jia *et al*., 2016). Additionally, one or more whole cellular pathways become deregulated, the most studied

pathways are: Wnt-β-catetin, TGF-β, epidermal growth factor receptor (EGFR), Ras/Raf/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (P13K) (Conteduca *et al*., 2013; Dekker *et al*., 2019).

Colon Cancer Progression

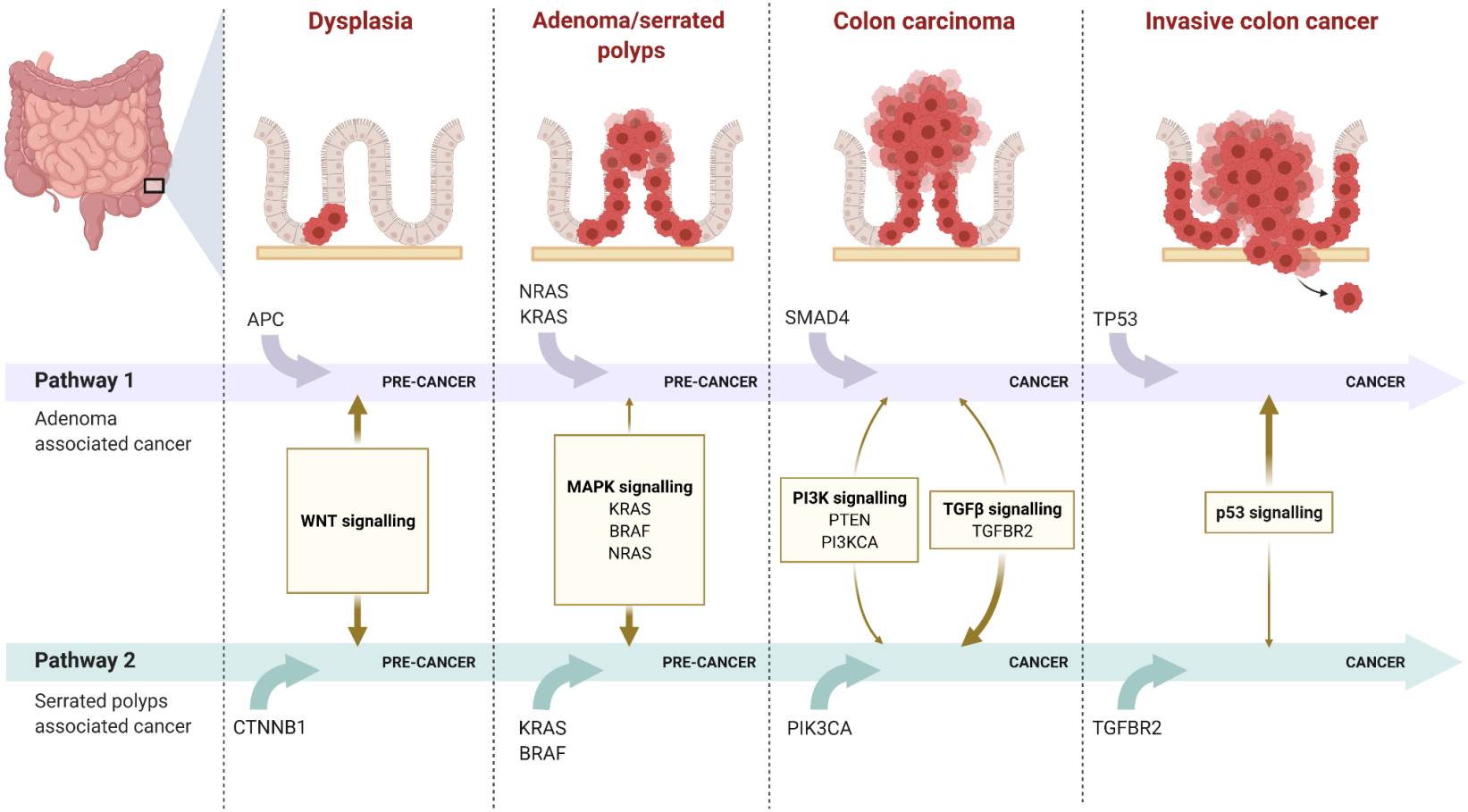

Figure 6. Colon cancer progression pathways. At first occur low degree of dysplasia most due to mutation in APC (~95% of cases) or in CTNNB1 (83% of cases) genes resulting in increasing inappropriately the activity of Wnt-signaling pathway in both adenomas and serrated associated pre-neoplastic lesions. Subsequently, both cancer subtypes present alteration in the Ras family members, however, the MAPK-signalling pathway is most deregulated in serrated polyps associated cancer. When the adenoma evolves to carcinoma different phenomenon occur depending of the cancer subtype, the P13k may be present in both subtypes meanwhile TGF-β is stronger altered in serrated cancers. At the end, acquiring metastatic characteristics through the loss of wild-type TP53 in adenomas and also mutation in the TGFBR2 in serrated associated cancers. Source: Cancer Genome Atlas and Dekker *et al.*, 2019.

Aberrant miRNA expression in CRC has been postulated and its role is gaining relevance. The microRNAS (miRNAs) are a small non-coding RNAs that regulates genes expression through control of RNA expression. These small molecules may act as oncogenes or tumor-suppressor genes and their differential expression is considered also a hallmark of CRC (O´brien *et al.*, 2018). Silencing or triggering different pathways, the miRNAs contribute for the transition from normal colonocytes to adenoma or carcinoma (Liu and Lee, 2019). Danese *et al.,* (2017) identified suitable reference of miRNAs from tissue and exosomes plasma of CRC patients trough high-throughput screening. The authors described 7 miRNAs (miR-27a, let-7g, miR-25, miR-152, miR-132, and miR-103a) targeting at least two oncogene or tumor suppressor gene, specially the miR-16 showed interaction with BCL1, KRAS, BMI-7, MYB, RECK, TP53 genes (Danese *et al*., 2017). The miR-143 and miR-145 are tumor suppressors with reduced expression since the early stage of adenomas contributing for its tumorigenesis, which may suggest both miRNAs as biomarker for early steps of CRC (Akao *et al*., 2010). On the other hand, the miR-21 is upregulated in both adenomas and carcinomas, as much as increasing the expression of this miRNA more advanced is the tumor stage, indicating a potential role of mir-21 in the CRC development (Schetter *et al*., 2008). In addition, the miR-21 expression was observed highly increased in situ hybridization during the transition from adenoma to carcinoma (Yamamishi *et al*., 2009). The oncomiR-21 is well stablished by literature and its elevated expression leads to increase in cell proliferation, reduction in apoptosis and favors tumor cell migration, intravasation and metastasis. This miRNA was validated experimentally targeting several genes involved on colon carcinogenesis, such as PDCD4, PTEN, RECK, NFIB, TPM1, SPRY2, RHOB, TIMP3, maspin, CDC25a, TIAM1 and MSH2 genes (Lu *et al*., 2008; Asangani *et al*., 2007; Meng *et al*., 2007). Moreover, the miR-

21 is predicted to target others diverse genes, such as, MAPK1 emphasizing the needy for novel experiments for well validation once MAPK1 is the key gene in MAPK-pathway (Liu *et al*., 2013).

CRC diagnosed in the wall of the intestine (stages I and II) is potentially curable when occur the early detection followed of surgical and therapeutic interventions. However, most of countries have no containment protocols for this cancer. Otherwise, the rate for survival in individuals with regional-stage (nodal stage III) or distant-stage (metastatic phase, stage IV) is very low 50-70% and 10%-14% respectively (Venook *et al*., 2016). The problematic in the therapy success is the tumor breaching the intestinal wall and reaching another organ through lymphatic or bloodstream system. The CRC is aetiological heterogeneous depending on anatomical location, for example, features of right-side (proximal) are different compared with left-side (distal) or rectal cancers (Loree *et al*., 2018). According to demographic factors proximal colon cancer is more incident in women, older individuals, meanwhile distal cancers is most prevalent in men and younger individuals, and rectal cancer although rare, is most diagnosed in people before 50 years old (Wolf *et al*., 2018). Hence the molecular features by side, proximal colon is enriched with subtypes characterized by (MSI)-high, CpG island methylator, phenotype (CIMP)-high or BRAF mutation whereas distal colon cancer by the (CIN)-positive subtype. Those hereditary cancers syndrome, HNPCC-associated occurs mainly in proximal colon, while (FAP)-associated CRC occurs in distal part **(Figure 7)**. (Keum and Giovanucci, 2019).

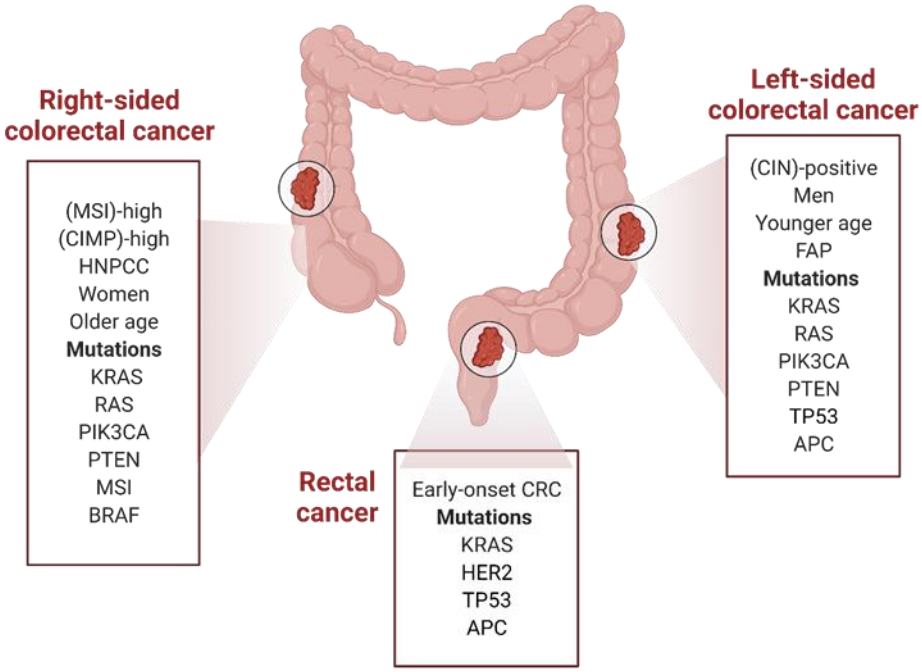

Figure 7. Right vs left sided features of CRC. As mentioned before the colorectum is basically partitioned into three parts (proximal, distal and rectum), each segment is heterogeneous across anatomical location of tumor. Source: Keum and Giovannucci, 2019.

1.2.5. Inflammation on CRC development

Inflammatory bowel disease (IBD), especially ulcerative colitis, are important risk factor on CRC development. The incidence of CRC in patients with IBD was reported to be up 60% higher than general population (Long, Lundsmith and Hamilton, 2017). The sporadic cancer may exhibit prominent inflammatory response, named as tumor-induced inflammation (Long, Lundsmith and Hamilton, 2017). Although the role of inflammation is well stablished in CAC, in the CRC is nuanced. Epidemiologic and experimental studies suggest that adjuvant treatment with anti-inflammatory may prevent or delay the CRC development (Friis *et al*., 2015). Inflammation is a hallmark of CRC and may arise from several factors and display dual role in cancer context, targeting specific cancer cells by cytotoxicity T-lymphocytes or dampening of unspecific inflammation trough regulatory T cells (T-regs) (Galoon *et al*., 2006). Several cell types cross-talk among each other predominantly by network of cytokines, chemokines and other growth factors. Current knowledge of translational research into inflammatory and cancer context is obtained mainly by three major murine models. The induction of inflammation in rodent with dextran sulphate sodium (DSS), AOM or DMH has

been widely applied (Long, Lundsmith and Hamilton, 2017; Tanaka *et al*., 2003). For instance, the interleukin-6 (IL-6) has been identified as overexpressed in the serum of CRC patients and its levels is correlated with tumor size (Becker *et al*., 2005). Nonetheless, this cytokine promotes the growth of stablished colon cancer cells *in vitro* (Becker *et al*., 2005). This pleiotropic cytokine has both pro- and anti-inflammatory action besides to promoting haematopoiesis, plasma cell development and also regulates the proliferation of intestinal epithelial cells (Tebbut *et al*., 2002). Tebbut *et al*., (2002), investigated a possible function of IL-6 in inducing growth of dysplastic lesions *in vivo*. In order to block IL-6 signaling, dnTGFβRII transgenic and wild-type mice submitted to AOM/DSS treatment received a weekly dose of a neutralizing antibody against IL-6 receptor α chain. Interestingly, those mice that received the neutralizing antibody were protected contrary to the control wild-type group. Thus, this study suggests that IL-6 receptor signaling promotes colon carcinogenesis in mice and elevated IL-6 levels contribute to the tumor progression. According to Simone *et al*., (2015), the phenomenon that tumor-infiltrating leukocytes (TIL increase CRC cells proliferation is through the activation of transducer and activator of transcription 3 (STAT3) and nuclear factor-kappa B (NF-κB). Thus, compounds that are able to inhibit simultaneously STAT3 and NF-κB, may contribute positively against CRC development while epidemiological and experimental studies associated the overexpression of TNF-α with CRC development (Pakdermirli and Kocal, 2020; Wuang *et al*., 2017; Lasry, Zinger and Ben-Neriah, 2016). Zhao and Zang (2018), demonstrated that TNF-α is the key member on inflammatory niche into the tumor microenvironment suggesting that this protein promote colon cancer invasion by upregulating TROP-2 expression via ERK1/2 signaling pathway. The tumor promoting role for IL-17 cytokine has been identified for both inflammation-associated and sporadic gastrointestinal cancers, this cytokine is elevated at firsts stages of tumor formation in mouse models and high levels in patients' serum is associated with poor prognosis (Junjie *et al*., 2020; Simone *et al*., 2015). Downregulation of IL-17 has been shown to suppress metastasis and increase the action of radio- and chemotherapy. In the gut, IL-17 interacts inclusive with bacterial microbiota expanding and recruiting neutrophils for control and early invasive extracellular bacteria or fungi (Sparber *et al*., 2018; Cho *et al*., 2010). Furthermore, IL-17 is useful in maintain the tight junctions between intestinal epithelial cells or promoting their proliferation in order to wounding (Zepp *et al*., 2017). However, depending of colonization type (pathogenic microbiota), the IL-17 acquire tumorigenic function through the sustained tissue repair instigating the proliferation of pre-malignant cells (Chung *et al*., 2018). Thus, chronic IL-17 activity leads to a protumor microenvironment due to its production of

inflammatory mediators, mobilizing myeloid cells and changing the phenotype of stromal cells (Junjie *et al*., 2020). Summarizing the role of these cytokines in the CRC development, the therapeutic agents that downregulate these proteins may be potentially employed as chemopreventive or adjuvant treatment.

1.3. Cancer Stem cells (CSCs)

The hypothesis that tumor growth is driven by cancer stem cells (CSCs), a small subpopulation of cells that displays embryonic stem cell characteristics and exert influence in tumorigenesis (Tsunekuni *et al.*, 2019). Likewise, dividing asymmetrically in order to produce differentiated identical daughter cancer cells, these CSCs exhibit diverse capacity of cancer promoting such as self-renewal (O´brien *et al*., 2007), chemoresistance (Ricci-Vitiani *et al*., 2007), and metastatic potential (Todaro *et al*., 2007). These cells are quiescent and emerge by gene mutations or deregulation of genetic programs in normal stem/progenitor cells within derived from differentiated intestinal cells or intestinal stem-cells (ISCs) (Lau, Ho and Lee, 2017). In a homeostatic situation, the stem cells reside in a "Stem cell" niche, a specific environment that plays a key role in regulation these cell maintenance and self-renewal. A similar concept occurs with CSCs, however, with a tumor specific microenvironment within comprise stromal cells, immune cells, networks cytokines, growth factors, hypoxic regions and the extracellular matrix (ECM) (Borah *et al*., 2015). Between the solid tumors, the CRC share the major intrinsic factors into the microenvironment that maintain the stemness of CSCs such as (A) abnormal activation of proliferating signaling pathways as Wnt, Notch and Hedgehog, (B) high tumorigenicity, (C) strong drug or radiation resistance and (D) interruption of the master transcriptional regulators that sustain embryonic stem cell self-renewal like NANOG, OCT-4 and SOX-2 (Borah *et al*., 2015; Kim and Orkin, 2011). When given these specific environmental stimuli, those cells that exhibit plasticity enable the CSCs to convert reversibly into transition of epithelial-to-mesenchymal (ECM) (Cabrera, Hollingsworth and Hurt, 2015). The colorectal CSCs are surrounded by surface markers, between these surface markers utilized to identify the colorectal CSCs are CD44, CD133, CD326, ALDH1 and EpCAM (Dalerba *et al*., 2007). The CD44 is a hyaluronic acid encoded by CD44 gene. This molecule is a transmembrane protein that balance the cell-cell interaction, cell adhesion and cell migration. Those CSCs that present CD44+ display more aggressiveness characteristics, high colony formation, resistance to chemo- and radiotherapies (Spring *et al*., 1988). The CD133 is expressed in hematopoietic cells, endothelial cells and neuroepithelial cells (Li *et al*., 2012). This transmembrane glycoprotein when positive in colorectal CSCs gives it characteristic of

self-renewal and multi-directional differentiation potential (Kazama *et al.*, 2018). This glycoprotein is considered a specific marker of primary colorectal CSCs and associated with cell differentiation and tumor size (Todaro *et al.*, 2010). The EpCAM (CD326) is one of the first tumor-associated antigens identified. The extracellular domain is believed to certain two epidermal growth factor (EGF)-like domains, its common biological function is that they can potentially inhibit cathepsins, a family of cysteine proteins frequently produced by tumor cells and known to be involved in metastasis (Baeuerle and Gires, 2007). The aldehyde dehydrogenase isoform 1 (ALDH1) catalyses the changeover of aldehyde to carboxylic acid. This molecule is often used as a surrogate marker of CSCs and non-CSCs in different cancers (Rassouli, Matin and Saeinasab, 2016). In CRC the overexpression of ALDH1 is associated with poor prognosis, poor differentiation and high metastatic potential. Different biological function has been attributed to the ALDH1, such as, protection of CSCs against high levels of reactive oxygen species, tumorigenic and metastatic promoting role (Rodriguez-Torres and Allan, 2015). Altogether, evidences suggest that CSCs are the root of cancer metastasis, relapse and recurrence. The state of survival of CSCs depends of several factors derived mainly from the microenvironment of the cells niche. These cells must to be eradicated to prevent the cancer recurrence or relapse.

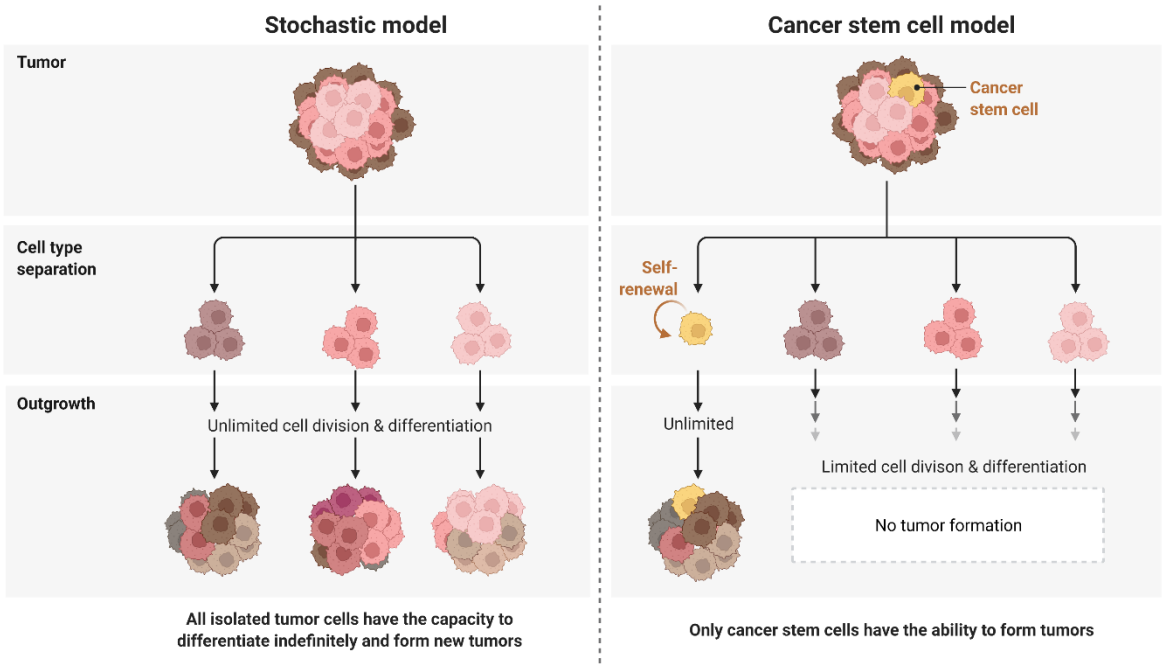

Figure 8. The cancer stem cell is quiescent and its capacity to make division is unlimited promoting the cancer recurrence.

1.4. Murine CRC models

The understanding the mechanisms of CRC development, human biopsy, tumor xenotransplants and *in vitro* cell culture models have providing important tools for developing and testing hypotheses regarding this malignance. However, establishing tumors tissues or colon cancer cell lines is at least challenging due to the mutational complexity, absence of normal tumor microenvironment and limitation concerned in ethical aspects. The rat and mouse intestine resembles the human intestine in what concern development, structure and function (Kobaek-Larsen *et al*., 2000). Despite histological similarities, rats and mice have few differences in intestines compared to the humans, such as, the lack of adipose tissue in the submucosa (Oliveira *et al*., 2020). Between the translational models for the colon carcinogenesis, 1,2-dimethylhidrazine hydrochloride (DMH) and its metabolites azoxymethane (AOM) associated with a promoting stimulus such as dietary deoxycholic acid (DCA) is a reliable chemically-induced model applied o induce colonic preneoplastic (i.e., aberrant crypt foci) and neoplastic (i.e., adenomas and adenocarcinomas) lesions with similar e morphological and molecular features in relation to the human disease (Gurley, Moser and Kemp, 2015). DMH is an alkylating agent dependent of the liver biotransformation to become an active carcinogen. Thus, DMH is oxidized into the liver converting itself to AOM, then it suffers hydroxylation to form methylaxymethanol (MAM). The MAM form is converted mainly in methyldiazonium ions that make alkylation on DNA, RNA and proteins (Kobaek-Larsen *et al*., 2000; Hawks and Magee, 1974). The colon carcinogenesis induction using this carcinogen was described for the first time in 1967 by Druckrey and colleagues, with subcutaneous injections at dose of 21 mg/kg. They observed that DMH induced cancer in the distal portion of the colon resembling histopathological features to the humans CRC. This colon carcinogen may be administered in different routes, subcutaneously, intraperitoneal, oral and intrarectal (Newmark *et al*., 2009). The time of bioassay depends of study goals, for example, administrating subcutaneously the dose of 20 mg/kg once a week during twenty weeks induces colonic adenomas in about 60% of male F344 rats (Reddy, 1998). Post 7 weekly subcutaneous injection of DMH at dose of 196 mg/kg in Swiss Webster mice revealed several adenomas and adenocarcinomas (Temple and Basu, 1987; Nascimento-Gonsalves *et al*., 2021).

In addition to the chemically-induced models, CRC cells or grafts of tumor tissue can be exerted into the animals providing an excellent tool to evaluate tumor development or to analyze the effects of chemotherapy with new drugs or natural compounds. In this model, human tumor cells or tumor fragments are implanted in immunocompromised animals in

several ways, such as, subcutaneously (most utilized), intrasplenically or directly in the colon/rectum (orthoptic models). For example, the adenocarcinoma cells HCT-116 is widely utilized in xenografts model, Tao *et al*., (2015) in order to evaluate the effects of an herbal medicine against CRC injected HCT-116 in BALB/C mice axilla, and after tumor growth they were replaced into de cecum. Morata-Tarifa *et al*., (2015) demonstrated that CSCs cells obtained with cultured spheroids of colon adenocarcinoma cells HT-29 and HCT-116, may approximate the microenvironment of stem-cell niche and induce metastasis. Both chemically-induced or xenografts models have advantages and disadvantages, for example, chemical induction of carcinogenesis can be a slowly process leading a long-term, meanwhile, exerting into immunocompromised mice require special care and housing.

1.5. A cup of coffee against the hallmarks of CRC

Considering that environmental factors display one of major role in pathogenesis of CRC and extensive evidences suggest that nutrition exerts strong influence as much as protective or causal, we decided to explore the idea of coffee may present preventive effects against CRC. Coffee is the second most popular beverage worldwide, being party of dietary habits in any nation with a consumption of approximately two billion of cup per day. Due to it cheap cost and easy preparation it is consumed in almost all social classes and different cultures with many different modes of preparation (Gaascht, Dicato and Diedrich, 2015). Despite its simple appearance, a cup of coffee contains hundreds of compounds that vary widely depending of the origin of coffee tree or its metabolism. Due to its complexity and its rich mixture, coffee is considered a pharmacopeia presenting antiproliferative, antineoplastic, anti-inflammatory and others properties against the hallmarks of cancer (Gaascht, Dicato and Diedrich, 2015; Mestdalgh *et al*., 2014). The coffee diversified compounds include chemical elements, amino acids, melanoidins, lipids, sugar, diterpenes and polyphenols (Kitzberger, Scholz and Benassi, 2014). The roasting and brewing process change the coffee beverage composition as result of many volatile compounds. Thus, many factors may influence the content from the harvest of green beans to the consumption of serving cup of coffee (Caporaso *et al*., 2014). The main alkaloid found in coffee beverages is the caffeine (CAF), however the brewing techniques affect the quantity of this compound in coffee, for example, a cup of espresso (25 ml) contains around 60.25 mg of CAF meanwhile a mug of filtered coffee (125 ml) with identical coffee contains 173.25 mg (Wei *et al*., 2012). The second most abundant alkaloid is the trigonelline (TRI) (1-methylpyridinium-3-carboxylate) with phytoestrogen which concentration increase as much as the coffee fruit develops and also suffer partial

pyrolyzed into nicotinic acid during the roasting process (Wei *et al*., 2012). The major group of phenolic compounds identified in coffee have origin from chlorogenic acids (CGA) (Wei *et al*., 2012). Recently, Romualdo *et al*., (2019) showed through epidemiological and experimental data that the coffee beverages and their predominant bioavailable compounds individually offer protective effects against gastrointestinal and liver cancer development. Some meta-analyses of prospective, case-control and cohort studies highlight the beneficial effects of coffee. Generally, these studies consider relative risk (RR) or overall survivor (OS) comparing the low (≤ 1 cup of coffee/day) versus high (≥3 cups of coffee/day) (Machintosh *et al*., 2020). Mackintosh *et al*., (2020) found that increase coffee intake is associated with lower risk of CRC progression and death. Regarding *in vitro* context, Ekbatan *et al*., 2018 showed antiproliferative and cytotoxic effects of CGA towards the human colon cancer Caco-2 cells by cell membrane damage and the cell cycle arrest at S-phase. Another study showed that the coffee extract reduces the Caco-2 viability and decreases KRAS expression through modulation of the miRNAs miR-30c and miR-96, which target the KRAS gene, while CGA isolated in the same proportions of its coffee extracts did not exhibit any effect (Sadeghi *et al*., 2018). These findings showed that the effects of coffee extract under these conditions was stronger than CGA isolated suggesting that the bioactive properties of these compounds might suppress the cell's expansion by a sort of interaction between each other, such as synergism. Moreover, CGA induces reactive oxygen species (ROS) in human colon cancer HCT-116 and HT-29 cells, inhibiting its viability as well as arresting S-phase and extracellular signal-related kinase (ERK) inactivation (Hou *et al*., 2017). CAF showed significant pro-apoptotic effect against different gastric cancer cells by the upregulation of caspases-3/caspase-9 and Cyt-c levels (Liu, Zhou and Tang, 2017). This alkaloid is able to prevent HIF-α, VEGF and IL-8 accumulation induced by the activation of adenosine receptor in human HT29 cells. The vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) display essential role in tumor angiogenesis, and the hypoxia-inducible factor-1 (HIF-1) is well known to regulate this phenomenon (Merighi *et al*., 2007). The most prominent mechanisms on *in vivo* findings come from hepatocarcinogenesis models what highlight the exclusivity of this thesis. Moreover, Soares *et al*., (2018) showed that caffeinated coffee intake, but not decaffeinated coffee or caffeine, counteracted the development of dysplastic ACF during the initial stages of MNNG-induced colon carcinogenesis. In addition, rather than decaffeinated coffee, caffeinated coffee-treated rats also displayed fewer ACF positive for metallothionein, which is proposed as a stem cell mutation marker. In addition, each treatments reduced DNA damage (phosphorylate H2A histone family/member X, cH2AX) and only caffeinated coffee and caffeine diminished

proinflammatory cyclooxygenase 2 (COX-2) expression in colonic mucosa. The authors suggested that the anti-inflammatory and antigenotoxic effects was exerted by caffeinated coffee (Soares *et al*., 2018). Keeping this context, Silva *et al*., (2014) administrated common organic/commercial caffeinated coffee brews diminished DMH-induced mutagenicity (micronuclei) and toxicity (apoptotic cells) in colonocytes of male Swiss Webster mice. Curiously, the most prominent results were observed in infusions prepared with organic powder. A xenograft model CT-26 exerted with CRC cells was exposed to both decaffeinated coffee and CGA isolated, showing similar reduction of potential in lung metastasis in a dose dependent-manner by targeting key molecules of CRC (Kang *et al*., 2011). Pre-clinical bioassays do not usually utilize the dosage found in coffee beverages considering the human intake and supraphysiological approaches are frequent. For this, the well stablished Human Equivalent Dose (HED) calculation take in consideration the body surface area (BSA) in order to normalize for dose translation from the human to the rodents (Reagan-Shaw, Nihal and Ahmed, 2008). Moreover, the most *in vitro* and *in vivo* bioassays are, basically, the exposition of tumor cell lines to the coffee compounds individually, missing the complexity of interaction as observed in filtered coffee.

Thus, taken all together, considering this comprehensive literature overview our focus in this study was explore the effects of coffee major bioavailable compounds against the colon carcinogenesis *in vitro* and *in vivo*. Offering for the first-time insights of pre-clinical bioassays and translational significance for further mechanistic investigations.

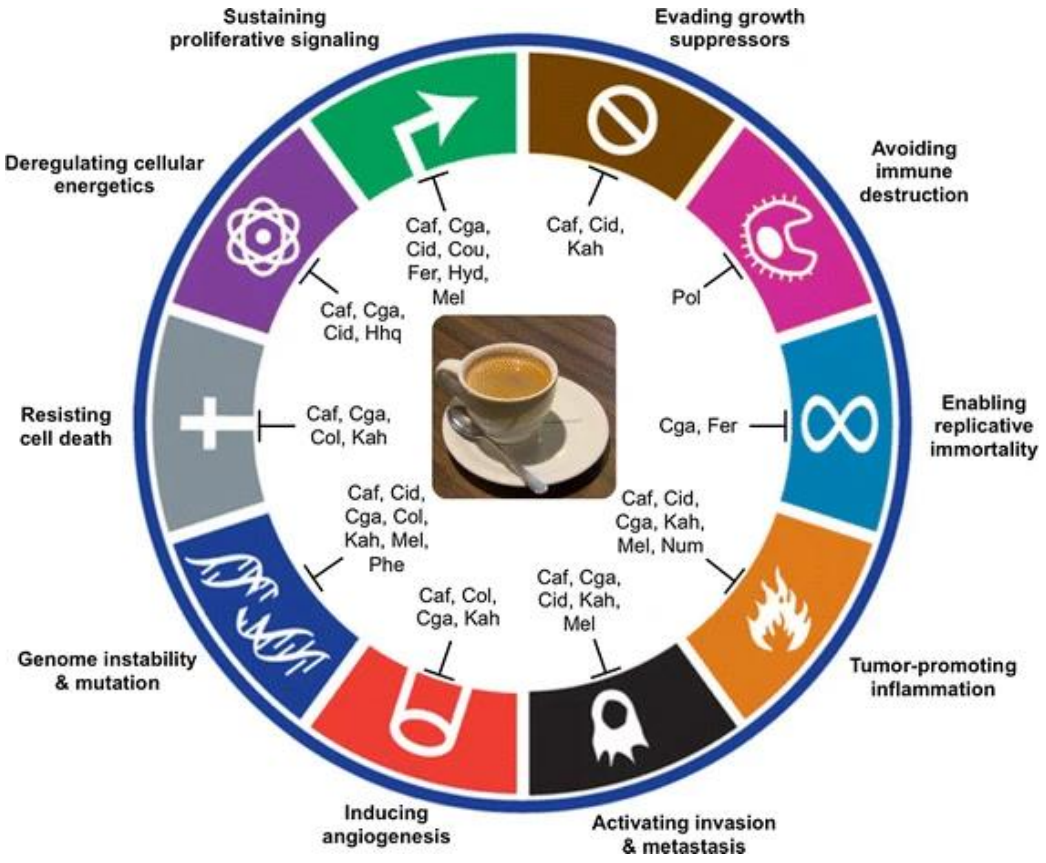

Figure 9. Bioavailable compounds of coffee targeting the hallmarks of cancer, based on Hanahan and Weinberg, (2011) and Gaascht, Dicato and Diedrich, (2015). *Caf* = caffeine, *Col* = cafestol, *Cid* = caffeic acid, *Cga* = chlorogenic acids, *Cou* = coumaric acid, *Fer* = feluric acid, *Cid* = caffeic acid, *Hhq* = hydroxyhydroquinone, *Hyd* = 5-hydroxymethylfurfural, *Kah* = kahweol, *Mel* = melanoidins, *Num* = N – methylpyridinium, *Phe* = phenolic compounds, *Pol* = polysaccharide

2. HYPOTHESIS

The CRC is the 3rd most common cancer worldwide and it implicate in a public health issue. Considering the toxicity of therapies, the lack of specificity and selectivity, the resistance against radio- and chemotherapy, the identification of new strategies with therapies targeting molecules of early or late stages of carcinogenesis is extremely necessary. The quiescent subpopulation of colon cancer stem cells (CSCs) is the major responsible by the tumor recurrence and metastasis, the treatment failure is attributed to the incomplete eradication of this particular group of cancer cells. An efficient therapy must to act against low proliferating quiescent cells to improve the overall survival. Furthermore, it has been stablished that miRNA play a crucial role in the tumor development, its differential expression may promote greater tumorigenic potential. The sensibility of these miRNAs signatures is important to the treatment efficacy. The etiology of CRC is multifactorial, with both environmental and genetic as risk factors. Several systematic studies have summarized evidence for associations between dietary factors and the incidence of CRC. Epidemiological and experimental data correlates coffee intake with improving CRC patients' survival and chemopreventive effects were observed in bioassays.

Thus, our hypothesis is based on the following premises:

- Coffee bioavailable compounds based on the proportions of human high intake (≥3 cups a day) would present effects against the CRC carcinogenesis in bioassays.
- Caffeine, chlorogenic acid and trigonelline are the major coffee compounds that may act against CRC by interacting between each other.
- miRNAs are key players in the steps of the colon carcinogenesis.
- The coffee main bioactive compounds will be able to target the subpopulation of CSCs eliminating them, thus avoiding the recurrence of cancer, among others.

3. OBJECTIVES

The main goals of this project were:

1. To evaluate cytotoxicity of coffee main compounds (caffeine, chlorogenic acid and trigonelline) isolated or combined against the adenocarcinoma colorectal cells line

(HCT-116 and HT-29) cultured on monolayer as well as colonospheres enriched in CSC subpopulation.

2. To analyze combined effects of the bioactive compounds (synergy, additive or antagonistic action).
3. To study the modulation of miRNAs profile trough the evaluation of miRNA global expression.
4. To assess whether the expression of specific proteins evolved on the cell's proliferations: Apoptosis induction, cell cycle arrest, cytokines expression and modulation of CSCs markers.
5. To investigate the caffeine acute toxicity in bioassays.
6. To evaluate effects of oral intake during the CRC development *in vivo* in a well stablished chemically colon carcinogenesis induction and xenograft model enriched with CSCs subpopulation in two approaches: pre-treatment and pos-treatment.

4. MATERIALS AND METHODS

4.1. Tumor cell line characteristics

Human colon cancer cells (mentioned below) were obtained from the American Type Culture (ATCC) and gently maintained in the cell bank of Centro de Instrumentación Científica – CIC, Universidad de Granada – UGR, Spain.

Cell line	Status MSI	Panel CIMP 1	Panel CIMP 2	CIN	KRAS	BRAF	P13CA	PTEN	P53
HCT-116	MSI	+	+	-	G13D	WT	H1047R	WT	WT
HT-29	MSS	+	+	+	WT	V600E	P449T	WT	R273H

Table 2. Molecular features of the CRC cells line utilized for the training. Abbreviations – microsatellite instability (MSI); CpG island methylator phenotype (CIMP); instability pathway (CIN) and wild type (WT) (Ahmed *et al.*, 2013).

4.2. Tumor cells maintenance

Human colorectal cancer cells HCT-116 and HT-29 were maintained in DMEM (Dulbecco´s Modified Eagles Medium; Sigma Chemical Co, St Luis, MO, EEUU) supplemented with 10% Fetal Bovine Serum (FBS) (BioWhittaker, Lonza, Basel, Switzerland), and 1% penicillin/streptomycin solution (10.000 U/ml penicillin G and 10 mg/ml de streptomycin; Sigma Chemical Co, St Luis, MO, EEUU) in flasks of 25 or 75 cm^3 (BD Falcon, Franklin Lakes, NJ). All cells were grow incubated at 37 ºC with 5% CO_2 in a humidified incubator (Steri-Cult CO_2 Incubator, Thermo Electron Corporation, Waltham, MA, USA). The cells dissociation was done with the trypsin replacement TrypLE reagent (Life Technologies) as soon as it reaches 80-90% of confluence.

In order to preserve the cells characteristics during long periods, the detached cells from flasks with trypsin were centrifuged at 1500 rpm during 5 minutes in medium with FBS forming a pellet, the supernatant was discarded. Pellets were suspended in specific storage medium (0.5 x 10^6 cells/vial) and frosted at -80 ºC. Storage medium: inactivated FBS and dimethyl sulfoxide (DMSO). The cells thawing occur in 37 ºC with immediate resuspension in medium, followed by centrifugation (1500 rpm/5min) and discarding the supernatant to eliminate the DMSO residue.

The cells manipulation was proceeding onto sterile conditions. Assays of quality control were routinely processed to preserve the cells authenticity and maintaining mycoplasma free.

4.3. Viability assay by MTT and choice of compounds concentration

MTT assay was performed to assess cell viability according to the manufacturer. The monolayer cultured cells were exposed during 24, 48 and 72 hours, respectively, to a range of concentration starting from 0 to 10 mM (caffeine, chlorogenic acid and trigonelline solved on PBS) obtaining the IC_{50} of each cell line. After, 100 µl of MTT solution (1 mg/ml) (Thiazolyl Blue Tetrazolium Bromide, Sigma) were added to each well and cells were incubated for 4 h. The formazan product was dissolved in DMSO (100 µl) and absorbances were measured using an automated plate reader (BioTek Instruments, USA) at ~ 540 nm. Absorbance from untreated cells was considered as 100% cell viability, and percentage (%) of cell viability was calculated according to the formula: % = [mean experimental absorbance/mean control absorbance]x100%.

4.4. Pharmacologic interaction assay

The doses-interaction evaluation was carried out as described by Chou and Talalay, 2010. The Combination Index (CI) was quantified by a software "CompuSyn" based on "mass action law". The combination ratio was 1:2 of IC_{50} obtained previously in each mixed compound. The CI was classified as synergism (CI < 1), additive effect (CI = 1) or antagonism (CI > 1). The Dose Reduction Index (DRI) indicates no dose-reduction, favorable dose-reduction and not favorable dose-reduction when DRI=1, > 1 and < 1, respectively.

4.5. Colonospheres forming assay

After trypsinization and centrifugation (1500 rpm/5 min), the tumor cells were submitted to three washes with phosphate buffer saline (PBS) (Lonza, Basel, Switzerland) in order to eliminate the FBS. Posteriorly, the cells were seeded into specific medium for spheroids: DMEM/F12 (Sigma Chemical Co, St Luis, MO, EEUU) supplemented with 1x of B27 (Gibco, Big Cavin, OK, EEUU); hydrocortisone (1 µg /ml) (Sigma-Aldrich); heparin (4ng/ml) (Sigma-Aldrich); 10 µg/ml of insulin (Gibco, Big Cavin, OK, EEUU); EGF(10 ng/ml) (Sigma-Aldrich); FGF 20 (ng/ml) (Sigma-Aldrich); 1% of penicillin/streptomycin solution (10.000 U/ml and 10 mg/ml, respectively); HGC and IL-6 (10 ng/µl) (Miltenyi Biotec).

The cell density used was $3.5x10^4$ cells/well in 6/wells ultra-low adherence plates (Corning Inc., Corning, NY, EEUU) with final volume at 2 ml. Post 72 h, the primary spheres were suspended in 2 ml microtubes ("eppendorfs"), which are centrifuged at 1500 rpm for five minutes. The supernatant was discarded resting a few microliters with the pellet. The pellet disaggregating was mechanically undone trough soft movements with syringe with insulin needle (JET BIOFIL, FPE204030). The counting cells was realized in trypan blue on Neubauer chamber. After the cell count, the cells were seeded onto low adherence 96/well plates (Corning Inc., Corning, NY, EEUU) with around 190 µl/well at density of $1x10^4$ cell/well in order to receive the concentration of bioactive compounds.

4.6. Cytotoxicity assay with CCK8

The inhibitory effects on secondary spheroids were obtained due to its exposition towards the two major coffee bioactive compounds. Summarily, spheres (4×10^3 cells/well) were seeded onto 96-well plates and incubated for 24 h, then treated with the doble of different IC_{50} concentration previously obtained from MTT assay on monolayer culture during 72 h at 37 ºC. Thereafter, cells are processed as follow, 15 µL of CCK8 reagent (Dojindo, CK04-13) added to each well and incubated at 37 °C for 3 hours. Absorbance is recorded at 450 nm with the reader Titertek Multiscan apparatus (Flow, Irvine, CA, USA). Controls used were untreated cells, with maximum and minimum absorbance (only medium). Nonetheless, the plate edges were not filled with cells in order to eliminate the border effect. Usually, the experiment lasts 6 days to form the secondary's spheres.

4.7. CSCs characterization by ALDH activity

The assay was handed by ALDEFLUOR marker assays (Stem Cell Technologies) to detect ALDH1 activity according to manufacturer's instructions. Cell CSCs surface levels were determined with anti-human antibody CD44-APC and CD326-APC (Miltenyi Biotec). All samples were analysed on a FACS CANTO II (BD Biosciences) using the FACS DIVA software. ALDH1, CD44 and CD326 markers allowed identifying the CSCs isolated. The CSCs growled in specific enriched spheres medium (above-mentioned).

4.8. Side population assay

Analysis of cells overexpressing ABC transporters with capacity to efflux drugs was performed using Hoechst 33342 (Sigma-Aldrich) dye. Hoechst was added to $1x10^6$ cells/ml, resuspended in DMEM with 10% of FBS, to a final concentration of 5 µg/ml, and incubated at

37ºC for 90 min in the dark. For negative controls, 5 µM verapamil (Sigma-Aldrich) was used for maintaning the capacity to efflux channel closed inhibiting the capacity to efflux Hoechst by cells. After incubation, cells were spin down at 4ºC and resuspended in cold PBS. The brightly fluorescent Hoechst positive cells were mesured by Flow cytometry in Hoechst blue (440/40) and Hoechst red (695/40) channels using a FACS Aria III flow cytometer (Becton Dickinson, BD Biosciences, Franklin Lakes, NJ, USA) from the Scientific Instrumental Center (University of Granada).

4.9. Cell cycle analysis

To determine whether the compounds would induce cell cycle arrest. The secondary spheres formed with HCT-116 and HT-29 cells were seeded onto 6-wells plates at density of $3x10^6$ cells/well and exposed during 48 h to the double of IC_{50} obtained on monolayer culture assay. The cells were disaggregated mechanically with a needle and washed with PBS and the pellet was resuspended and fixed in an ice-cold solution of 70% ethanol in PBS (v/v) at –20 °C for 1 h. Fixed cells were stained with 1 µl of PI (1 mg/ml) and 3 µl of RNase A (100 mg/ml) in PBS) at 37°C for 1 h. Samples were analysed acquired in a Flow Cytometer FACSCantoTM II using FACSDiva (BD Biosciences) software. The percentages of cells in G_0/G_1, S and G_2/M phases of the cell cycle were determined using FlowJo software vX 10.6 (Tree Stars Inc).

4.10. Apoptosis analysis

The induction of apoptosis by the variables was assessed by annexin V-FITC/PI apoptosis kit (Becton Dickinson, USA) in both cell lines. The secondary spheres were seeded (3×10^6 cells/mL) in 6-well plates and treated with the double of the IC_{50} previously obtained during 48 h. Untreated spheres were used as a MOCK and control. Cells were centrifuged (200 g/10 min) and washed twice with PBS. Staining was performed according to the manufacturer instructions- Samples were acquired in a FACSCantoTM II (BD Biosciences, USA) Flow Cytometer with emission filters of 515-545 nm for FITC (green) and 600 nm for PI (red) using FACSDiva (BD Biosciences) software and analysed using FlowJo software vX 10.6 (Tree Stars Inc). The percentages of early apoptotic (AV+, PI-), late apoptosis or necrotic (AV+, PI+) and live cells (AV-, PI-) were determined.

4.11. Dose determination in vivo

High coffee consumption, as observed worldwide, leads to an estimated caffeine intake range of 200-300 mg/day (~2.8 to 4.0 mg/kg, using a 70 kg adult as example). The suggested

caffeine dose (50 mg/kg) is based on allometric translation of Human Equivalent Dose (HED) in order to reproduce the human high intake of filtered coffee (≥ 3 cups/day) (Elmeliegy *et al*., 2021; Reagan-Shaw, Nihal and Ahmad, 2008). The dose of CGA concentration display a ratio of 1:2 as determined was based on a previous study by our research group (CAF 1.0 and CGA 0.41 mg/ml of coffee) (Efsa, 2015). The rodents age, around seventh weeks old (sexual maturity), is explained by human exposure to coffee, which starts from puberty and extends over the adult age (Romualdo *et al*., 2019).

4.12. Caffeine acute toxicity

Acute toxicity was determined in eight weeks old male CD.1 mice (n=6 per group "G") during 5 days. Caffeine dissolved in distillate water was administered in a single oral dose (gavage administration) at 25, 50, 75 and 100 mg/kg once a day during five days/week **(Figure 10)** Control mice were administrated with the maximum volume of distillate water that a mouse is able to consume (0.2 ml). Mice were maintained under standard conditions and for each treatment schedule, were weighed and assessed daily for systemic toxicity (listlessness, weight loss) and local toxicity (alopecia, skin reaction, and leg motility). After one week the last treatment, mice were sacrificed and macroscopic examination of the organs was done.

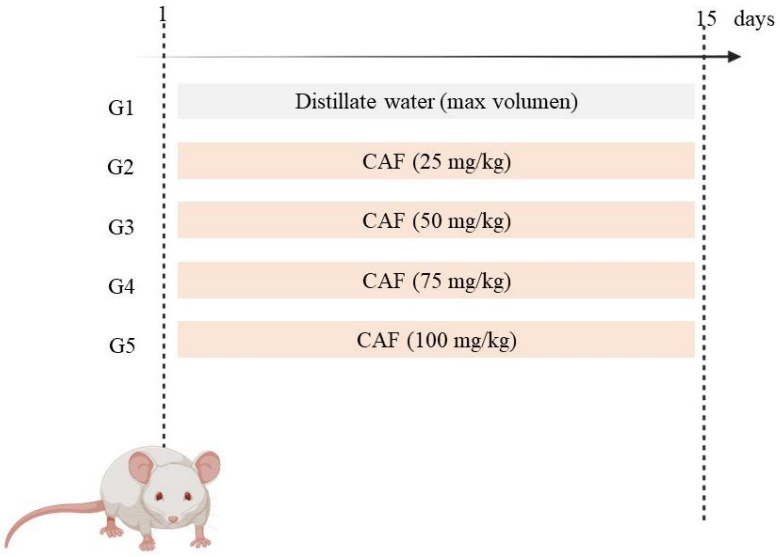

Figure 10. Caffeine acute toxicity was realized by intragastric treatments. CD-1 male mice. CAF = caffeine.

4.13. Experimental design in vivo (chemical colon carcinogenesis induction)

Seven-week-old male Swiss Webster mice were randomly distributed into five experimental groups (n = 10/group) and submitted to a colon carcinogenesis model. In brief, mice were initiated for colon carcinogenesis by receiving one intraperitoneal (i.p) injection of DMH per week [40 mg/kg body weight (b.wt) in EDTA 0.0001 M; Merck KGaA, Darmstadt, Germany] for two weeks (weeks 1 and 2) (Gurley, Moser and Kemp, 2015). To promote colon carcinogenesis after DMH initiation, the animals received a balanced diet supplemented with deoxycholic acid (DCA) at 0.02% (Merck KGaA, Darmstadt, Germany) for 10 weeks (weeks 3 to 13). Control mice received a DMH vehicle and non-supplemented balanced diet. Concomitantly to DCA intervention, mice received CAF (50 mg/kg b.wt./day), CGA (25 mg/kg b.wt./day), CAF+CGA (50 and 25 mg/kg b.wt./day, respectively) or just distilled water as a vehicle (intragastrically, five times a week) (8–10 am) for 10 weeks **(Figure 11)**. The bioactive coffee compounds were diluted daily in distilled water. All mice were euthanized by exsanguination under ketamine/xylazine anesthesia (100/16 mg/kg b.wt. i.p) at week 13. At necropsy, the liver was removed, weighted in order to evaluate absolute (g) relative (%) liver weights. The large intestine was removed and opened longitudinally and after a rapid macroscopic analysis, proximal, medial and distal parts were fixed in 10% formalin solution during 24 h for posterior histological and immunohistochemistry assays. A sample of the distal part (2 cm) was aseptically processed for molecular analysis, snapped frozen in liquid nitrogen, and stored at −80 ◦C.

All animals were obtained from ANILAB—Laboratory of Animals, Paulínia, São Paulo State, Brazil. Mice were kept in a room with continuous ventilation (16-18 air changes/h), relative humidity (45-65%), controlled temperature (20-24 °C) and light/dark cycle of 12:12 h and were given water and a balanced diet (Nuvilab, Quimtia, Colombo, Brazil) *ad libitium*. Body weight and food consumption were recorded once a week during the whole experiment. These protocols were approved by the Botucatu Medical School/UNESP Ethics Committee on the Use of Animals (CEUA) (Protocol number 1254/2017) and all animals received human care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” (National Research Consul (US), 2011).

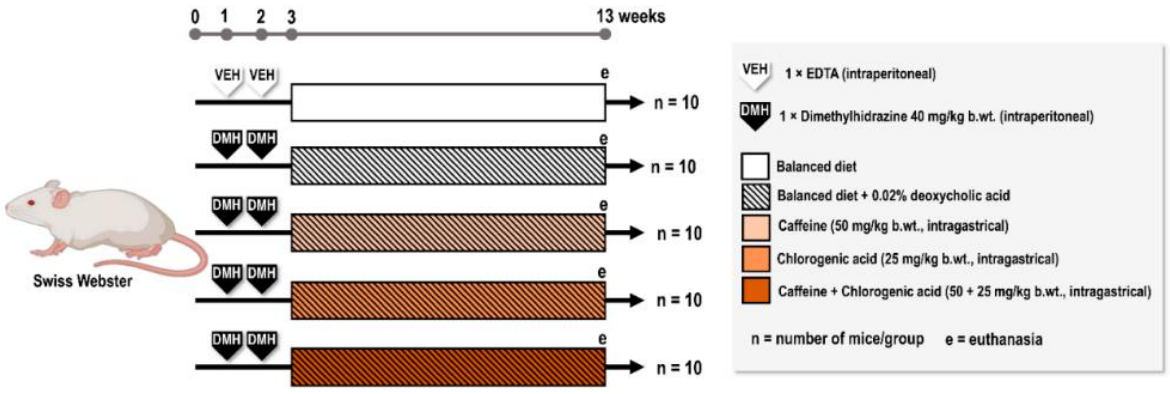

Figure 11. Experimental design of chemically colon carcinogenesis induction

4.14. Topographic identification of early pre-neoplastic lesions (ACF)

For ACF screening development (n = 10 animals/group), a classical preneoplastic colonic lesion [30] was made, the colon was removed, opened longitudinally, washed with distilled water, and measured (length, in cm). Samples of colon were fixed flat in 10% phosphate-buffered formalin solution for 24 h, then stored in ethanol 70%. Each colon sample was stained with 2% of methylene blue for 2 min, placed onto histological slides and then observed under conventional bright-field microscopy (Axiostar Plus, Zeiss, Oberkochen, Germany) at 20× magnification. For an ACF analysis, samples of proximal, medial and distal colonic mucosa were evaluated using well-stablished criteria (Bird, 1987). Total number of ACF (mean per mice/group) and aberrant crypt (AC) (mean per mice/group) were calculated for each group. Moreover, considering those ACF that were ≥2 AC, the mean number of ACF or AC per colon length analyzed was calculated.5.4. Colonic Proliferation and Apoptosis Indexes

After ACF screening, colon samples were swiss-rolled and embedded in paraffin, sections were obtained and stained with haematoxylin-eosin (HE).

Immunostaining for Ki-67 (ab16667, 1:100, Abcam, UK) and cleaved caspase-3 (ab179817, 1:200, Abcam, Cambridge, UK) were conducted using specific primary antibodies. Five-micrometer sections of colon samples were deparaffinized and hydrated through xylene-alcohol-water graded series. Slides were submitted to antigen retrieval in 0.1 M citrate buffer in a pressure chamber (Dako Cytomation, Glostrup, Denmark), incubated with 3% hydrogen peroxide solution (10 min), and treated with skimmed milk (1 h). Slides were incubated with primary antibodies 4 °C overnight. The sections were washed with phosphate buffer saline (PBS) solution and incubated with biotinylated universal polymer (Erviegas, Indaiatuba, SP,

Brazil) for 20 min. In order to stain the immunocomplexes, slides were incubated with 3′3-diaminobenzidine (DAB) chromogen solution (Sigma-Aldrich, St. Louis, MO, USA). Finally, the sections were counterstained with Harris' hematoxylin. Immunostained sections were evaluated in conventional light microscopy (Olympus BX53, Tokyo, Japan). The proliferation and apoptosis indexes (PI% and AI%) were calculated in 20 randomly selected colonic crypts by dividing the number of Ki-67 or caspase-3 positive cells per total number of cells analyzed (n = 10 animals/group) (Caetano *et al*., 2020).

4.15. Enzyme-linked Immunosorbent Assay (ELISA)

Around 100 mg of colon samples (n = 7 animals/group) was homogenized in RIPA buffer (Cell Signaling, Danvers, MA, USA) containing 1% protease inhibitor cocktail (Sigma-Aldrich, USA), the proportion was 100 µL buffer for each 30 mg tissue, then maintained at 4 °C for 2 h. The homogenate content (tissue/buffer) was centrifuged (10,000× g, 4 °C, 30 min) and the supernatant was collected for the protein quantification using the Bradford method. Levels of tumor necrosis alpha (TNF-α), interleukin-17 (IL-17) and -6 (IL-6) were obtained by the Luminex multiple analyte profiling (xMAP) methodology using a 96-well plate with specific magnetic beads for each cytokine, based on the manufacturer's instructions (MCYTOMAG-70 K, Millipore, Burlington, MA, USA).

4.16. RNA isolation and miRNA profiling

About 30 mg of colon samples (n = 5 animals/selected groups) were homogenized in 1 mL of QIAzol (Qiagen, Manchester, UK). Total RNA was isolated in each one using a QIAGEN RNeasy column-based system according to the manufacturer's instructions (Qiagen, Hilden, Germany). The RNA quantification was assessed in a Qubit 2.0 fluorometer and its integrity was observed in Agilent 2100 Bioanalyzer platform (Agilent Technologies, Santa Clara, USA). Only samples with RNA integrity number (RIN) ≥ 7.0 were considered for the miRNA expression. The RNA samples were stored until the analysis at −80 °C.

A total of 100 ng of whole RNA was utilized for nCounter Mouse v1.5 miRNA global expression assay (efficient in detection of 600 murine and murine-related viral miRNAs, Supplementary Data S1) in an automated system (NanoString Technologies, Seattle, WA, USA). These analyses were carried out at the Molecular Oncology Research Center in the Barretos Cancer Hospital (Barretos, Brazil). The NanoString methodology consists in the incubation of total RNA samples with specific probes that bind 3′ ends of each mature miRNA normalizing the miRNA melting temperature. The tag excess was removed, the complexes that

formed RNA-tags were incubated with 10 µL and 5 µL of reporter and capture probes, at 64 °C during 18 h. There were specific fluorescent signals for each complex in miRNA at the 5′end, and capture probes are biotinylated at the 3′end. Post purification, the mixture was pipetted in a streptavidin-covered cartridge by nCounter Prep Station. Finally, cartridges were analyzed in nCounter Digital Analyzer, acquiring 280 fields of view per sample and the miRNA-reporter probe complexes were counted. miRNA expression was analyzed by raw counting the miRNA-reporter probe complexes that had been normalized using the median of the top 100 miRNAs and that presented the lowest coefficient of variation (low CV values) using the NanoString package. A pair of comparisons were made considering $p \leq 0.05$, a fold change (FC) > 1.5 and presented as log2 (FC). Commonly/differentially expressed miRNAs were evaluated using a Venn Diagram.

4.17. In silico analysis of miRNA targets and pathways

After the analysis of differentially modulated miRNAs, each miRNA was identified and classified on microT-CDS (v5.0), DIANA tools and mirPath v.3 (http://snf-515788.vm.okeanos.grnet.gr/; (accessed on 10 July 2019)). The output list of validated targets was submitted to the analysis of the functional enrichment of biological processes (BP) in the DAVID Bioinformatics Resource 6.8 online Platform (https://david.ncifcrf.gov/; (accessed on 10 July 2019)) [68]. Principal BP observations were organized by the high significance and lowest adjusted p values, considering $p < 0.05$. A network confidence analysis of the miRNA targets was carried out using the STRING database (https://string-db.org/; (accessed on 10 July 2019).

4.18. In vivo tumorigenicity assay

In order to evaluate the *in vivo* anti-tumor ability of caffeine and CGA, isolated or in association, on CSCs HCT-116 spheroids, tumors were generated by subcutaneous injections of 5×10^3 cells/mouse using 26-gauge needles (in the back of legs) in NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, NSG). Animals (n = 6 per group) were housed and maintained at 20° C to 24 ° C, 50% humidity a light-dark cycle (14 p.m to 10 p.m) and with food and water ad libitum. The tumor growth was observed twice a week. Tumor growth was assessed using a digital caliper and the tumor volume was calculated by the formula as follow:

$V = (\text{length})2 \times \text{width} \times \pi/6$.

Animal experimentation was performed according to the protocols reviewed and approved by the Institutional Animal Care and Use Committee of the University of Granada.

4.18.1. Pre-treatment approach

Male NSG mice were randomly distributed (n=6) and receiving intragastric treatment five times a week with CAF (50 mg/kg), CGA (25 mg/kg) and CAF+CGA during 15 days before the subcutaneous injections of CSCs and following oral treatments until the euthanasia **(Figure 12)**. The negative control received only the vehicle (distillate water).

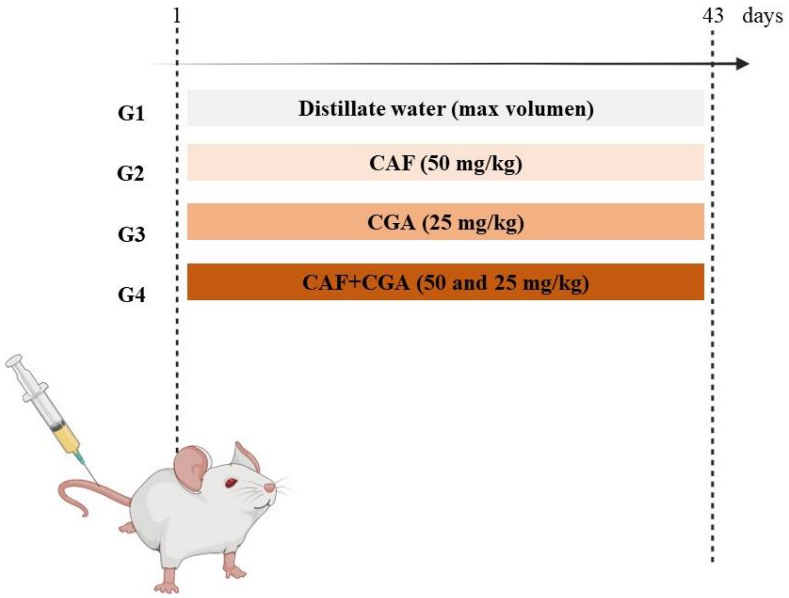

Figure 12. The treatments were realized 15 days before the inoculation of CSCs. Five times a week NSG male mice received intagrastric coffee compounds.

4.18.2. Post-treatment approach

Post inoculation when appearing the first tumors (sand grains length felt by touching), these mice were randomly distributed (n=6) and started to receive the intragastric treatments isolated or associated as follow CAF (50 mg/kg), CGA (25 mg/kg) and CAF+CGA (50 and 25 mg/kg).

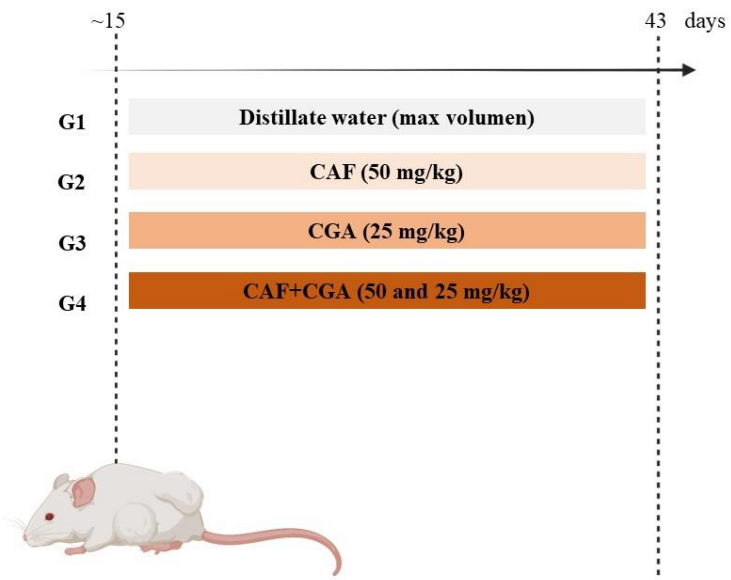

Figure 13. Post inoculation of CSCs treatments. Intragastric coffee compounds in male NSG mice.

4.19. Statistical analysis

Data were analyzed using one-way ANOVA or Kruskal–Wallis and *post hoc* Tukey's test and presented as mean ± standard deviation (SD). The number of samples per group for each analysis is represented by n. Statistical analysis was performed using GraphPad Prism Software 8.0 (GraphPad, San Diego, CA, USA), and differences were considered significant when $p \leq 0.05$.

5. RESULTS

5.1.Antitumor effect of coffee compounds: The exclusion of trigonelline (TRI)

We realized the "*cut off*" in order to decide which compound act better against the colon carcinogenesis alone or combined. Firstly, we tested all combination possible, CAF, CGA, TRI, CAF+CGA, CAF+TRI, CGA+TRI, TRI+CAF and CAF+CGA+TRI. The general findings showed not differences between all these groups (data not shown), however in the analysis of pre-neoplastic lesions the exposition of the mice to every isolated compound and its combination in pairs presented drastic reduction in the quantification of AC and ACF, in counterpart, the combination with all compounds (CAF+CGA+TRI) presented not difference compared with the positive group (DMH/DCA) and slightly tendence to increase the quantification of both. Moreover, apparently the compound with most prominent effect was the CGA followed by CAF. The *in vitro* monolayer viability assay, the TRI showed not effectivity against the colon cancer cell lines (HCT-116 and HT-29). Thus, we decided to eliminated the TRI and its associations in further experiments.

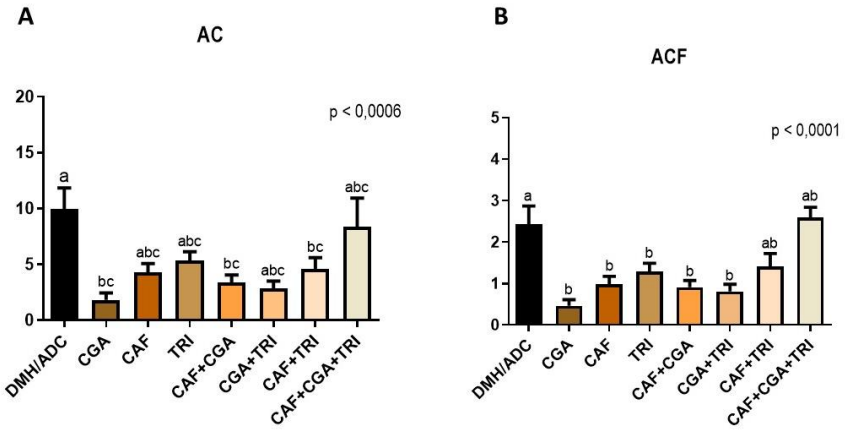

Figure 14. Effects of three major bioavailable coffee compounds in the pre-neoplastic development. **(A)** Quantification of Aberrant Crypts (AC) and **(B)** quantification of Aberrant Crypt Foci (ACF).

Table 3. Antiproliferative effects of coffee major compounds on colon cancer cells obtained 72h post exposition

Monolayer MTT assay	CGA (IC_{50}/mM)	CAF (IC_{50}/mM)	TRI (IC_{50}/mM)
HT-29	3,96	7,75	-
HCT-116	~ 1,19	4,43	-

5.2. Caffeine acute toxicity in mice

We determined the HED based on high human coffee consumption (≥ 3 cups/day), thus the caffeine dissolved in water was given to the mice in several proportions (range from 25 to 100 mg/kg). After one week the last treatment, the mice were healthy, without weight variation or sign of toxicity. After all, the organs were macroscopically evaluated presenting no systemic or local toxicity **(Figure 15)**. Thus, the proportion of caffeine founded in coffee common beverages (50 mg/kg) was considered safety to the male mice.

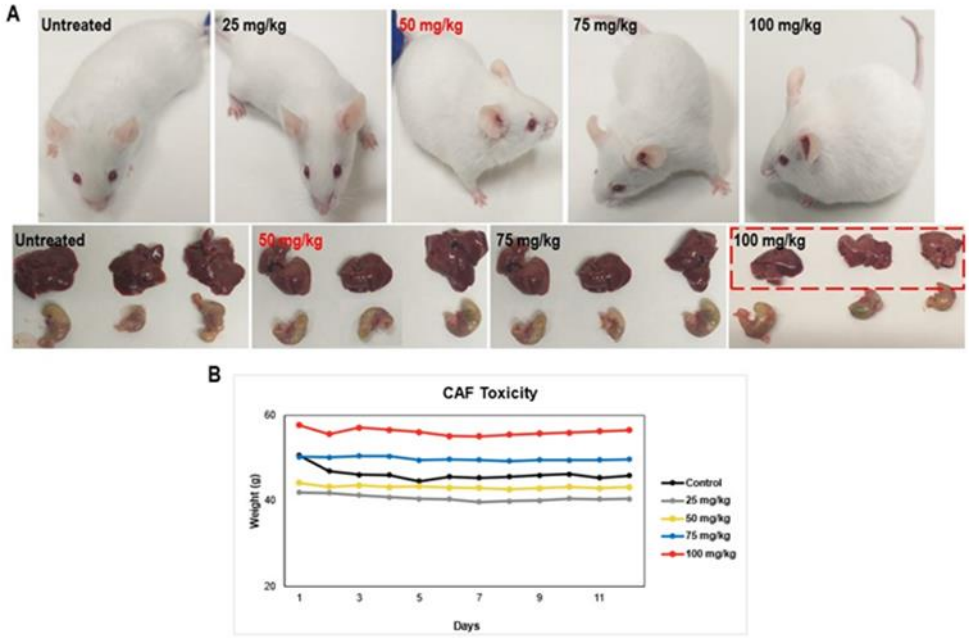

Figure 15. Caffeine acute toxicity assay. Eight weeks old male CD.1 mice orally treated with caffeine **(A)**. Body mass variation during the weeks of treatments **(B)**.

5.3.Experimental model of chemically induction of colon carcinogenesis

5.3.1. General findings during bioactive coffee compounds interventions

Firstly, the effect of coffee compounds on food consumption were analyzed. Body and liver weights from mice submitted to the DMH/DCA-induced colon carcinogenesis were taken. The animals exhibited similar initial body weights **(Table 4)**. The DMH/DCA protocol and different interventions with bioactive coffee compounds did not alter food consumption, body weight gain or final body weight. Although interventions did not modify absolute liver weight, all groups submitted to the DMH/DCA protocol demonstrated a significant increase in relative liver weight (%) at the end of week 13 ($p < 0.05$), and coffee compounds did not modify this DMH/DCA-induced effect **(Table 4)**.

Table 4. Effects of coffee compounds on food consumption, body weight, relative and absolute liver weights in DMH/DCA-induced colon carcinogenesis.

Groups/Treatments	Food Intake (g/mice/day)	Body Weight (g)			Absolute Liver Weight (g)	Relative Liver Weight (%)
		Initial	Final	Gain		
Untreated	6.19 ± 1.52	29.8 ± 2.5	40.3 ± 3.2	7.3 ± 3.6	2.0 ± 0.2	4.8 ± 0.4
DMH/DCA	5.69 ± 1.50	28.4 ± 1.6	37.1 ± 3.5	6.7 ± 4.2	2.4 ± 0.4	6.3 ± 1.0 *
DMH/DCA+CAF	5.57 ± 1.48	28.1 ± 2.8	36.8 ± 3.3	6.1 ± 4.4	2.8 ± 0.5	7.1 ± 1.5 *
DMH/DCA+CGA	5.72 ± 1.84	30.5 ± 2.8	37.0 ± 3.3	6.8 ± 4.3	2.5 ± 0.7	6.5 ± 0.8 *
DMH/DCA+CAF+CGA	5.61 ± 1.78	30.0 ± 1.6	38.4 ± 2.7	7. 7 ± 3.9	2.3 ± 0.3	6.8 ± 0.6 *

n = 10 animals/group. Data are expressed as mean ± S.D. Untreated: EDTA vehicle (i.p)/balanced diet. DMH/DCA = 1,2-dimethylhydrazine (2 × 40 mg/kg b.wt., i.p)/deoxycholic acid supplemented diet (0.02% w/w, 10 weeks). CAF = caffeine (50 mg/kg b.wt. intragastrical), CGA = chlorogenic acid (25 mg/kg b.wt. intragastrical) and CAF+CGA = caffeine + chlorogenic acid (50 and 25 mg/kg b.wt. intragastrical) for 10 weeks. * Statistical differences compared to the untreated group using ANOVA and Tukey's test ($p < 0.05$).

5.3.2. Analysis of colonic preneoplastic AC and ACF

After a whole-mount colonic mucosa analysis, all groups that received coffee compound interventions similarly demonstrated a significant reduction in the mean number of AC and ACF ($p < 0.0001$ and $p = 0.004$, respectively) per area of colon mucosa analyzed when compared to the DMH/DCA counterpart **(Figure 16)**. Representative photomicrographs of ACF stained by methylene blue, as shown in **Figure 16**. The protocol utilized here is based on firsts steps of colon carcinogenesis, however, some animals developed dysplastic ACF together with tumors without significant or representative differences **(Figure 17)**.

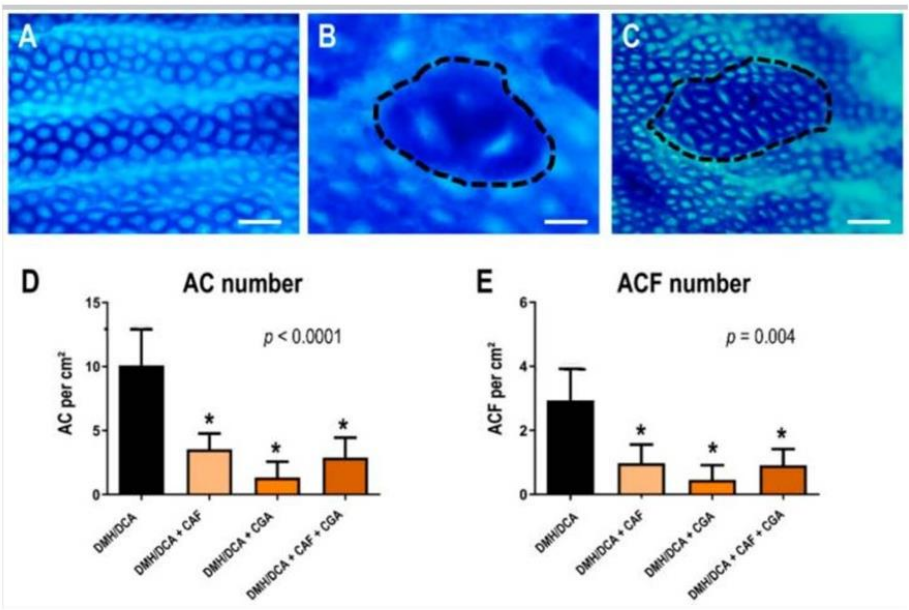

Figure 16. Representative methylene blue-stained colonic epithelium, showing **(A)** normal colon epithelium (scale bar: 100 µm, untreated group), **(B)** aberrant crypt foci with three crypts (scale bar: 50 µm, dotted line) and **(C)** aberrant crypt foci with >20 aberrant crypts (scale bar: 100 µm, dotted line). Effects of coffee compounds on the development of **(D)** AC or **(E)** ACF during DMH/DCA-induced colon carcinogenesis. n = 10 animals/group. Data are expressed as mean ± S.D. Untreated: 2×EDTA vehicle (i.p.)/balanced diet. DMH/DCA = 1,2-dimethylhydrazine (2 × 40 mg/kg b.wt., i.p.)/deoxycholic acid supplemented diet (0.02% w/w, 10 weeks). CAF = caffeine (50 mg/kg b.wt. intragastrical), CGA = chlorogenic acid (25 mg/kg b.wt. intragastrical) and CAF+CGA = caffeine + chlorogenic acid (50 and 25 mg/kg b.wt. intragastrical) for 10 weeks. * Statistical differences compared to the untreated group using ANOVA and Tukey's test ($p < 0.05$).

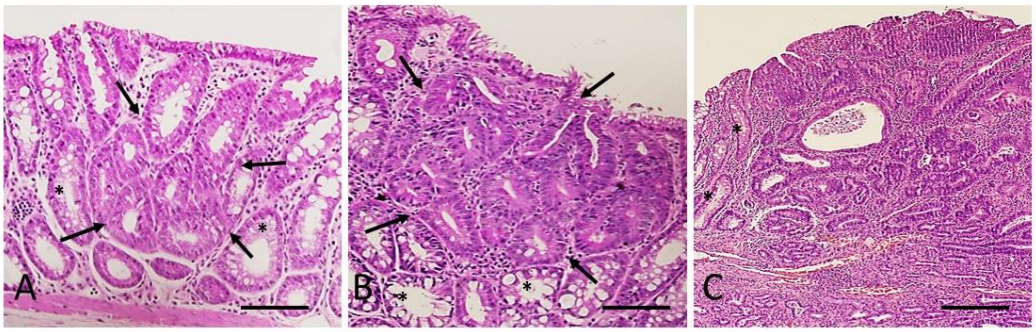

Figure 17. Representative imagens of colon sections stained by HE and showing one ACF conventional **(A, arrows)**, ACF dysplastic **(B, arrows)** and one tumor **(C)** in mice submitted to the DMH/CDA protocol. * Normal colonic crypts; Bar= 100 µm **(A, B)** or 200 µm **(C)**.

5.3.3. Proliferation and apoptosis index in colonic crypts

After a colonic epithelium analysis, all groups submitted to the DMH/DCA-induced mouse model demonstrated an increase in cell proliferation in colonocytes compared with the untreated counterpart ($p < 0.0001$) measured by immunoreactivity for Ki-67. CAF or CGA interventions individually did not change this DMH/DCA-mediated effect on colonocyte proliferation. However, only the CAF+CGA intervention significantly reduced colonocyte proliferation in normal-appearing crypts compared to the DMH/DCA counterpart ($p < 0.0001$) **(Figure 18)**. Moreover, only the CAF+CGA intervention significantly increased ($p < 0.0001$) the percentage of apoptotic colonocytes in the crypts, measured by immunoreactivity for cleaved-caspase 3 **(Figure 18)**.

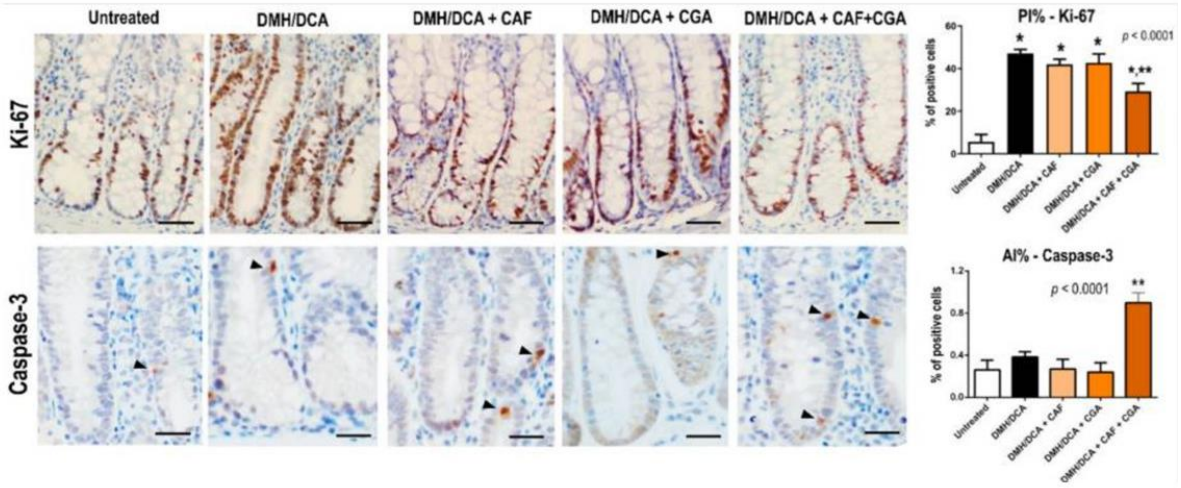

Figure 18. Effects of coffee compounds on colonic proliferation (PI%) and apoptosis indexes (AI%) during DMH/DCA-induced colon carcinogenesis. n = 10 animals/group. Data are expressed as mean ± S.D. Representative photomicrographs of Ki-67-positive colonocytes (scale bar: 50 µm) and cleaved caspase-3-positive apoptotic bodies (scale bar: 25 µm, arrowheads) are also displayed. Untreated: 2 × EDTA vehicle (i.p.)/balanced diet. DMH/DCA = 2 × 1,2-dimethylhydrazine (40 mg/kg b.wt., i.p.)/deoxycholic acid supplemented diet (0.02% w/w, 10 weeks). CAF = caffeine (50 mg/kg b.wt. intragastrical), CGA = chlorogenic acid (25 mg/kg b.wt. intragastrical) and CAF+CGA = caffeine + chlorogenic acid (50 and 25 mg/kg

b.wt. intragastrical) for 10 weeks. Asterisks correspond to statistical differences compared to the untreated (*) or DMH/DCA (**) counterparts using ANOVA and Tukey test ($p < 0.05$).

5.3.4. Proinflammatory cytokine analysis

The histological analysis showed that the DMH/DCA group presented more inflammatory infiltrate of lymphocytes, plasma cells and neutrophils in the lamina propria compared to the untreated counterpart. In addition, this colonic low-grade inflammation induced by DCA treatment was reduced by treatments with isolated or combined bioactive coffee compounds in comparison with the DMH/DCA counterpart **(Figure 19)**. In concordance with histological findings, the colonic cytokines analysis demonstrated that the DMH/DCA group notably increased colonic levels of IL-6 ($p < 0.0001$), IL-17 ($p < 0.0001$) and TNF-α ($p = 0.0003$) compared to the untreated counterpart, showing the activation of pro-inflammatory axis **(Figure 19)**. In contrast, treatments with isolated or combined bioactive coffee compounds similarly reduced the expression of IL-6, IL-17 and TNF-α in comparison with the DMH/DCA counterpart **(Figure 19)**.

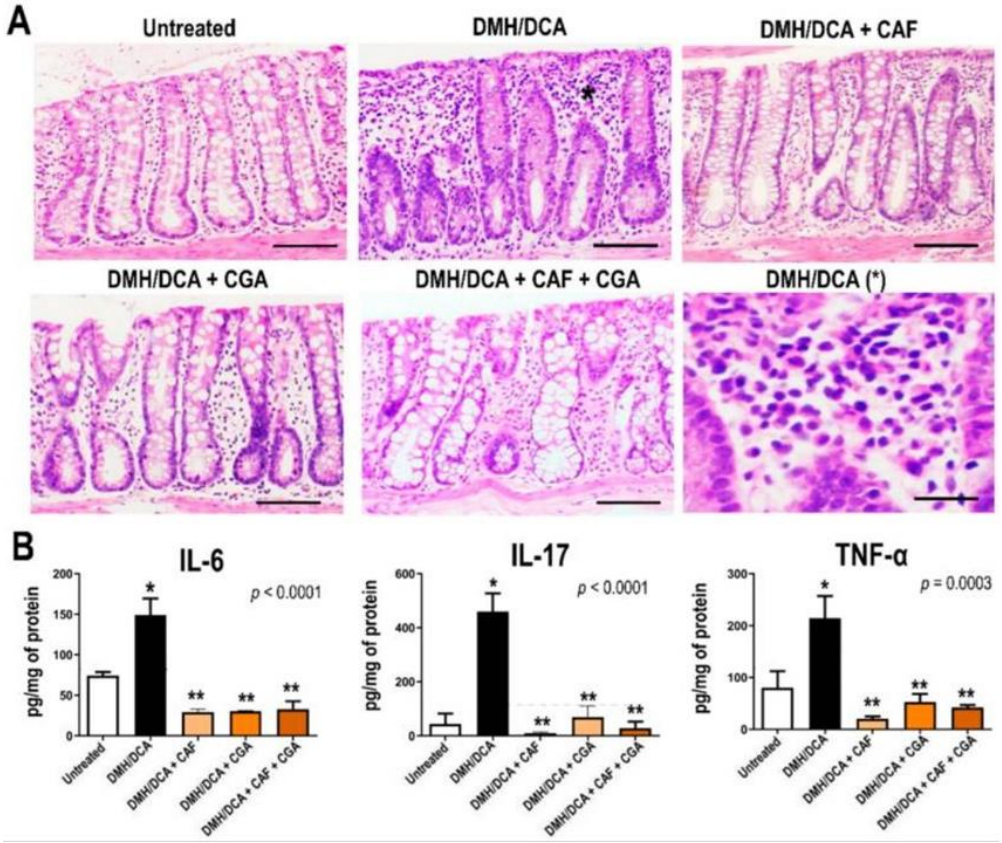

Figure 19. (A) Representative photomicrographs of HE-stained sections of colonic crypts (scale bar: 100 µm). * Detail on the inflammatory infiltrate present in the lamina propria of

DMH/DCA group (scale bar: 20 µm). **(B)** Effects of coffee compounds on colonic levels of interleukins 6 (IL-6), 17 (IL-17) and the tumor necrosis factor alpha (TNF-α) during DMH/DCA-induced colon carcinogenesis. n = 7 animals/group. Data are expressed as mean ± S.D. Untreated: 2×EDTA vehicle (i.p.)/balanced diet. DMH/DCA = 2 × 1,2-dimethylhydrazine (40 mg/kg b.wt., i.p.)/deoxycholic acid supplemented diet (0.02% w/w, 10 weeks). CAF = caffeine (50 mg/kg b.wt. intragastrical), CGA = chlorogenic acid (25 mg/kg b.wt. intragastrical) and CAF+CGA = caffeine + chlorogenic acid (50 and 25 mg/kg b.wt. intragastrical) for 10 weeks. Asterisks correspond to statistical differences compared to the untreated (*) or DMH/DCA (**) counterparts using ANOVA and Tukey's test ($p < 0.05$).

5.3.5. Global miRNA expression

The DMH/DCA model showed the upregulation of six miRNAs and the downregulation of four miRNAs in the colon **(Table 5, Figure 20A)**, including the upregulation of the oncomiR miR-21a-5p, that featured the highest FC compared to the untreated counterparts. As CAF+CGA intervention demonstrated the most pronounced effects on reducing ACF development and colonic cytokine levels (as observed similarly in CAF and CGA treatments), while increasing crypt apoptosis, and decreasing crypt proliferation (exclusive to this group); it was evaluated whether this treatment modulated DMH/DCA-induced effect on colonic miRNA expression. CAF+CGA intervention significantly increased the expression of two miRNAs, while it downregulated four miRNAs compared to the DMH/DCA group **(Table 5, Figure 20A)**. Of those miRNAs differentially expressed in our model, CAF+CGA decreased the expression of the oncomiR miR-21a-5p, as depicted in the Venn diagram **(Figure 20A)**.

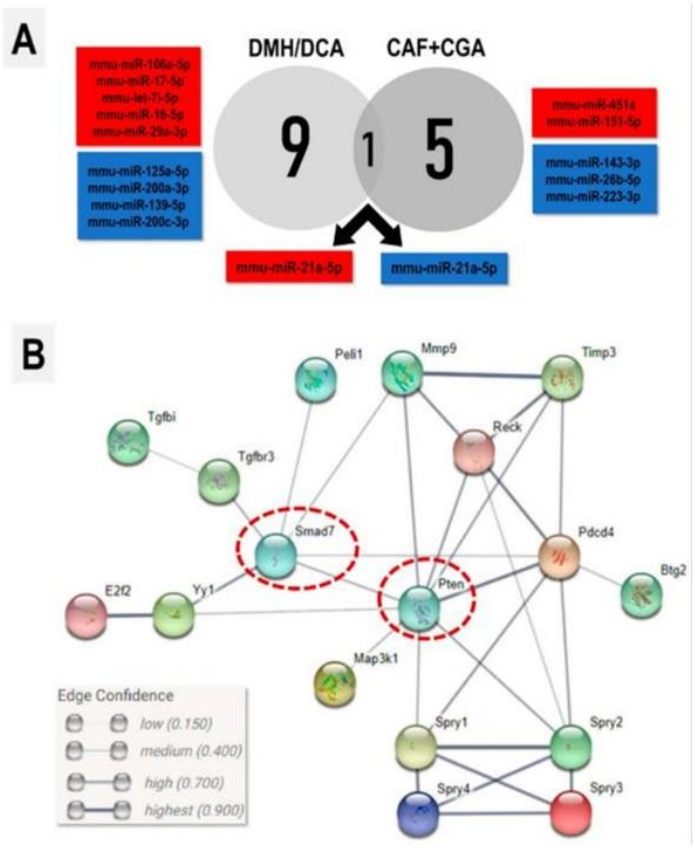

Figure 20. The Venn diagram depicting the differentially expressed miRNAs (red: upregulated; blue: downregulated) in DMH/DCA (vs. untreated group) and CAF+CGA (vs. DMH/DCA). **(B)** STRING confidence network analysis of miR-21a-5p validated targets. Nodes in the correlated proteins are shown (with 3D structure inside). Edges correspond to the confidence of functional correlation (caption). DMH/DCA and CAF+CGA shared the differential expression of oncomiR miR-21-5p, which was upregulated in the DMH/DCA group and downregulated in CAF+CGA intervention. DMH/DCA = 2 × 1,2-dimethylhydrazine (40 mg/kg b.wt., i.p.)/deoxycholic acid supplemented diet (0.02% w/w, 10 weeks). CAF = caffeine (50 mg/kg b.wt. intragastrical), CGA = chlorogenic acid (25 mg/kg b.wt. intragastrical) and CAF+CGA = caffeine + chlorogenic acid (50 and 25 mg/kg b.wt. intragastrical) for 10 weeks.

Table 5. Considering Fold Change (FC) (>1.5) and $p \leq 0.05$; Untreated: 2 × EDTA vehicle (i.p.)/balanced diet. DMH/DCA = 2 × 1,2-dimethylhydrazine (40 mg/kg b.wt., i.p.)/deoxycholic acid supplemented diet (0.02% w/w, 10 weeks). CAF = caffeine (50 mg/kg b.wt. intragastrical), CGA = chlorogenic acid (25 mg/kg b.wt. intragastrical) and CAF+CGA = caffeine + chlorogenic acid (50 and 25 mg/kg b.wt. intragastrical) for 10 weeks.

DMH/DCA vs. Untreated			DMH/DCA+CAF+CGA vs. DMH/DCA		
miRNA	**Log2 (FC)**	***p* Value**	**miRNA**	**Log2 (FC)**	***p* Value**
mmu-miR-21a-5p	2.32	0.03	mmu-miR-451a	2.09	0.001
mmu-miR-106a-5p	1.73	0.001	mmu-miR-151-5p	1.17	0.03
mmu-miR-17-5p	1.73	0.001	mmu-miR-21a-5p	−2.06	0.03
mmu-let-7i-5p	1.53	0.02	mmu-miR-143-3p	−1.58	0.04
mmu-miR-16-5p	1.42	0.009	mmu-miR-26b-5p	−1.51	0.04
mmu-miR-29a-3p	1.34	0.04	mmu-miR-223-3p	−1.45	0.02
mmu-miR-125a-5p	−1.65	0.01			
mmu-miR-200a-3p	−1.39	0.01			
mmu-miR-139-5p	−1.36	0.02			
mmu-miR-200c-3p	−1.29	0.01			

5.3.6. Analysis miR-21a-5p target genes and network/functional correlation

Considering that miR-21a-5p was downregulated by CAF+CGA treatment, target analysis of this miRNA revealed 35 experimentally validated proteins **(Table 6)**. Pathway analysis showed that most of these targets are involved with the "negative regulation of ERK1 and ERK2 cascade", "negative regulation of TGF-β receptor signaling pathway", and "positive regulation of apoptotic process" **(Table 7)**. In keeping with these findings, Phosphatase and tensin homolog (Pten) and mothers against decapentaplegic homolog 7 (Smad7) were as central nodes in the target network analysis **(Figure 20B)**, as proteins directly involved with the modulated biological processes.

Table 6. Experimentally validated targets genes of miR-21a-5p

Target gene	Database	Confidence
Fasl	miRTarbase, miRDB	Reporter assay and Western blot
Peli1	miRTarbase, miRDB, TargetScan	qPCR and Western blot
Pdcd4	miRTarbase, miRDB, TargetScan	Reporter assay, pPCR and Western blot
Spry2	miRTarbase, miRDB, TargetScan	Reporter assay and Western blot
Pten	miRTarbase	Reporter assay, pPCR and Western blot
Reck	miRTarbase, miRDB, TargetScan	Reporter assay, pPCR and Western blot
Spry1	miRTarbase, miRDB, TargetScan	Reporter assay and Western blot
Tgfbi	miRTarbase, miRDB, TargetScan	Reporter assay, pPCR and Western blot
Pdcd4	miRTarbase, miRDB, TargetScan	Others
Btg2	miRTarbase, miRDB, TargetScan	Others
Spry4	miRTarbase,	qPCR
Spry3	miRTarbase	Western blot
Elavl4	miRTarbase	Others
Pias3	miRTarbase	Western blot
Tgfbr3	miRTarbase	Reporter assay, pPCR and Western blot
Tnfaip8l2	miRTarbase	Reporter assay, pPCR and Western blot
Smad7	miRTarbase, miRDB, TargetScan	qPCR and Reporter assay
Gt(ROSA)26Sor	miRTarbase	qPCR and Reporter assay
Yy1	miRTarbase	qPCR and Reporter assay
Eif4e3	miRTarbase	qPCR and Reporter assay
Pdcd10	miRTarbase	qPCR and Reporter assay
Timp3	miRTarbase, miRDB, TargetScan	qPCR
YOD1	miRTarbase, miRDB, TargetScan	Reporter assay, pPCR and Western blot
PDCD4	miRTarbase, miRDB, TargetScan	qPCR and Western blot
Mmp9	miRTarbase	qPCR and Western blot
Kcnk6	miRTarbase	NGS
Map3k1	miRTarbase, miRDB, TargetScan	NGS
Cyfip1	miRTarbase	NGS
Rmnd5a	miRTarbase, miRDB, TargetScan	NGS
Tns1	miRTarbase, TargetScan	NGS
Gid4	miRTarbase, miRDB, TargetScan	NGS
E2f2	miRTarbase	NGS
Rpp40	miRTarbase	NGS
Moap1	miRTarbase	NGS
AK010878	miRTarbase	NGS

Table 7. Biological processes correlated to the validated targets of miR-21a-5p

Groups/Treatments	Food Intake (g/mice/day)	Body Weight (g)			Absolute Liver Weight (g)	Relative Liver Weight (%)
		Initial	Final	Gain		
Untreated	6.19 ± 1.52	29.8 ± 2.5	40.3 ± 3.2	7.3 ± 3.6	2.0 ± 0.2	4.8 ± 0.4
DMH/DCA	5.69 ± 1.50	28.4 ± 1.6	37.1 ± 3.5	6.7 ± 4.2	2.4 ± 0.4	6.3 ± 1.0 *
DMH/DCA+CAF	5.57 ± 1.48	28.1 ± 2.8	36.8 ± 3.3	6.1 ± 4.4	2.8 ± 0.5	7.1 ± 1.5 *
DMH/DCA+CGA	5.72 ± 1.84	30.5 ± 2.8	37.0 ± 3.3	6.8 ± 4.3	2.5 ± 0.7	6.5 ± 0.8 *
DMH/DCA+CAF+CGA	5.61 ± 1.78	30.0 ± 1.6	38.4 ± 2.7	7. 7 ± 3.9	2.3 ± 0.3	6.8 ± 0.6 *

5.4. CSCs experimental bioassays

5.4.1. Suppression of colon cancer cells in conventional culture

We determined whether these compounds could suppress colon cancer cells proliferation. The monolayer HCT-116 and HT-29 cultured cells were exposed to the coffee compounds isolated and/or in association (CAF, CGA and TRI) at the range from 0 to 70 mM obtaining the best inhibitory effect at 72 h of incubation time. The determined IC_{50} were respectively 7.75 and 3.96 mM of CAF and CGA on HT-29, meanwhile the IC_{50} over HCT-116 were ~1.19 and 4.43 mM to the CAF and CGA exposition, respectively **(Table 8)**. The trigonelline showed no effects against these colon cancer cell lines (as explained above). The association between CAF+CGA showed significant effects decreasing the IC_{50} to 2.60 and 1.33 mM in HT-29 and HCT-116 cells, respectively. These results indicate that the coffee major compounds exert a significant anti-proliferative effect in concentration and time-dependent manner on colon cancer cells.

5.4.2. Inhibitory effects of coffee compounds on colonospheroids enriched with CSCs

The spheres mimic the tumour architecture and are also enriched in cancer stem cell-like (CSCs). We evaluated the colonospheres viability trough the colorimetric cell counting obtaining the best inhibitory effects at 72 h of exposition starting the range with at least the double of IC_{50} previously identified on monolayer culture. The spheres formed with HT-29 cell line where less sensitive showing the IC_{50} at 7.1, 53.1 and 49.2 mM front the CAF, CGA and CAF+CGA treatments respectively. In counterpart, HC-T116-derived colonospheres showed higher sensitivity than HT-29 colonospheres with the IC_{50} 27, 23 and 17 mM after CAF, CGA and CA+CGA exposition. The colonospheres were not exposed to the TRI once it was not effective on the monolayer assays. Considering microscopy images from spheres, the shape of non-exposed HC-T116-derived colonospheres was indeed more spherical, regular and continuous which could be described as compact "tumor" packaging. On the other hand, the HT-29 (~200 µm) derived colonospheres were smaller with less regular outline and lower / fewer cell aggregates. The upraising concentration of the bioactive compounds were altering the colonospheroids shapes, in the CAF exposition the shape was reduced but not deregulated, meanwhile, with the presence of CGA the colonospheroids got darker, with irregular shape and presence of debris **(Figure 21)**. The monolayer model suggest that each cell is capable of initiation and propagation, however, increasing of heterogenicity with microenvironmental

influence highlight the possibility of high proliferative and migrative cells development with CSCs characteristics. Caffeine presented more effectiveness against HT-29-derived colonospheroids.

Monolayer MTT assay	**CGA (IC_{50}/mM)**	**CAF (IC_{50}/mM)**	**CGA+CAF (IC_{50}/mM)**
HT-29	3.96	7.75	2.60
HCT-116	~ 1.19	4.43	1.33
Colonospheres CCK8	**CGA (mM)**	**CAF (mM)**	**CGA+CAF (mM)**
HT-29	7.1	53.9	49.2
HCT-116	23	27	17

Table 8. The IC_{50} values obtained by a curve response, represents the concentration of each compound that inhibits the cells viability by 50%.

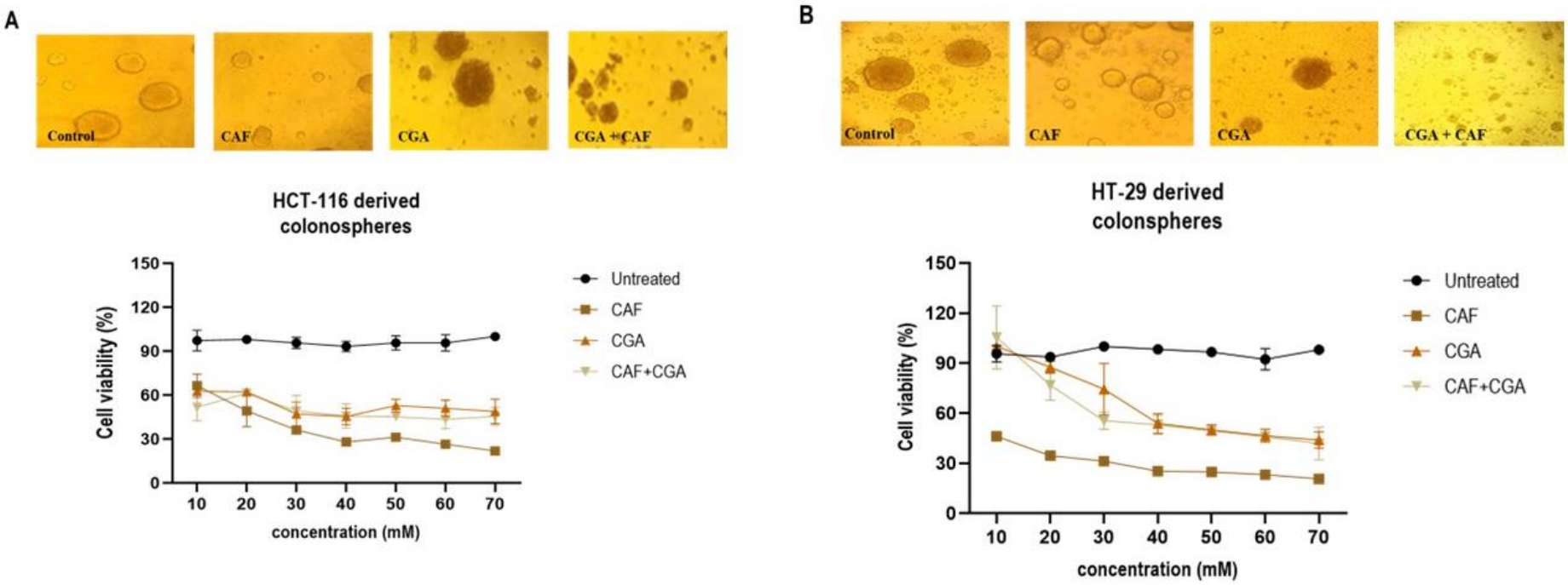

Figure 21. Anti-proliferative effects of bioactive coffee compounds. **(A)** Light microscopy-derived image, morphology of colonospheres derived from HCT-116 and **(B)** from HT-29 culture exposed to the higher concentration of compounds during 72 h (scale bar 100 µM) with the curve of concentration-response of 7 concentrations range. Decreasing spheroids viability compared with the untreated spheres were evaluated statistically with performing differences among the groups by ANOVA and *post hoc* Tukey test ($p < 0.001$).

5.4.3. Combination index (CI) from the effects of the combination between CAF and CGA over CSCs

The CAF+CGA presented strong antagonistic effects, with followed CI = 2.2 in the colonospheroids formed from HCT-116 cells, meanwhile the spheroids of HT-29 showed different response according to the dose average as follow, very strong antagonism at IC_{90} (CI = 12.2), nearly additive effects at IC_{50} (CI = 1.1) and a very strong synergism at IC_{10} (CI = 0.14). The DRI obtained at the IC_{50} in the HCT-116 cell line, indicates no dose-reduction for CGA (DRI = 0.64) and favorable rise of CAF concentration (DRI = 1.4), whereas in the other cell line the DRI collected emphasize the necessity to increase the concentration in both compounds (CAF DRI = 1.3 and CGA DRI = 2.2) in order to accurate the synergy in the combination **(Table 9)**. These findings suggest that the proportion 1:2 of these compounds founded in common coffee beverages might interact and reduce the synergy that exerts anti-proliferative effects.

Table 9. Parameters of methods for the drugs combination obtained from the software "Compusyn". The median-effect plot linearizes all dose-effect curves and provide m, Dm and r values. The Dm defines potency, "m" is the slope means shape of curve (m = 1, > 1 and < 1 indicates hyperbolic, sigmoidal and flat sigmoidal respectively) and the "r" value is related to the conformity of the data (r = 0.7 to 0.9 is good or acceptable). *CI is the Combination Index where (CI < 1) indicates synergy, (CI = 1) additive effect and (CI > 1) is antagonism. The Dose Reduction Index (DRI) is related with the needy of rise or decrease the compounds in a combination.

HCT116	IC_{10}		IC_{50}		IC_{90}		Dm	m	r
	CI	DRI	CI	DRI	CI	DRI			
CAF	-	1.90	-	1.49	-	1.16	29.6913	2.41528	0.88233
CGA	-	0.83	-	0.64	-	0.50	25.7433	2.40312	0.96909
CAF+CGA	1.7	-	2.2	-	2.8	-	59.7802	1.89260	0.78336
HT29	IC_{10}		IC_{50}		IC_{90}		Dm	m	r
	CI	DRI	CI	DRI	CI	DRI			
CAF	-	8.17	-	1.33	-	0.21	6.08838	0.87409	0.99108
CGA	-	40.3	-	2.29	-	0.13	21.0145	1.50126	0.96818
CAF+CGA	0.14	-	1.187	-	12.25	-	13.7268	0.50758	0.75301

5.4.4. Apoptosis induction and cell cycle arrest following CAF and CGA treatments over CSCs.

In order to corroborate these findings even over the resistant CSCs subpopulations, we evaluated the cell cycle arrest and apoptosis induction by flow cytometry analysis exposing the colonospheres to the IC_{50} previously obtained. The CAF, CGA and its association significantly exerts proapoptotic effects on HCT-116-derived colonospheroids, being the percentage of positive Annexin V cells 59% CAF, 54% CGA and 64% CAF+CGA during 48 h of exposition. Otherwise, the HT-29-derived colonospheroids showed significant apoptosis induction only with CGA treatment. The inhibition of cell cycle progression occurred drastically with the CGA and the CAF+CGA treatments over HCT-116 spheroids, arresting most of cells in the S phase. Furthermore, the HT-29 had significantly cell cycle arrest at S phase with CGA and G_1 in the combination exposition. These results support the potential proapoptotic effects of these bioactive compounds and also the inhibition of the cell cycle progression, consistently with the antiproliferative effect **(see Figure 22 and 23)**.

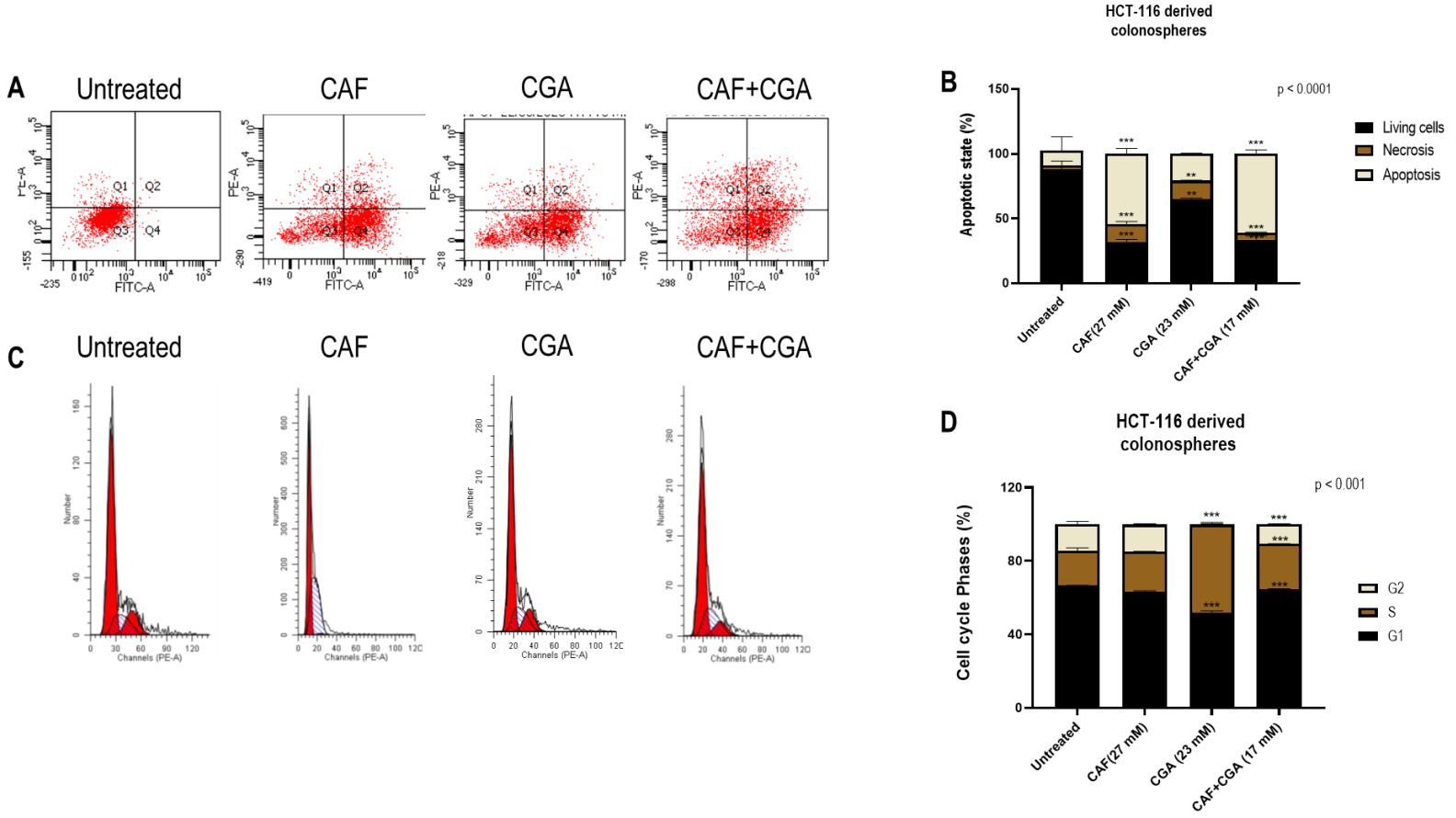

Figure 22. Effects of bioactive coffee compounds on HCT-116 derived colonospheres survival. **(A and C)** dot plots showing the respective quadrants of PE-A and FITC-A channels. (B and D) representative image of statistics differences defined as ** $p < 0.001$ and *** $p < 0.0001$ performed among the groups by ANOVA and *post hoc* Tukey test.

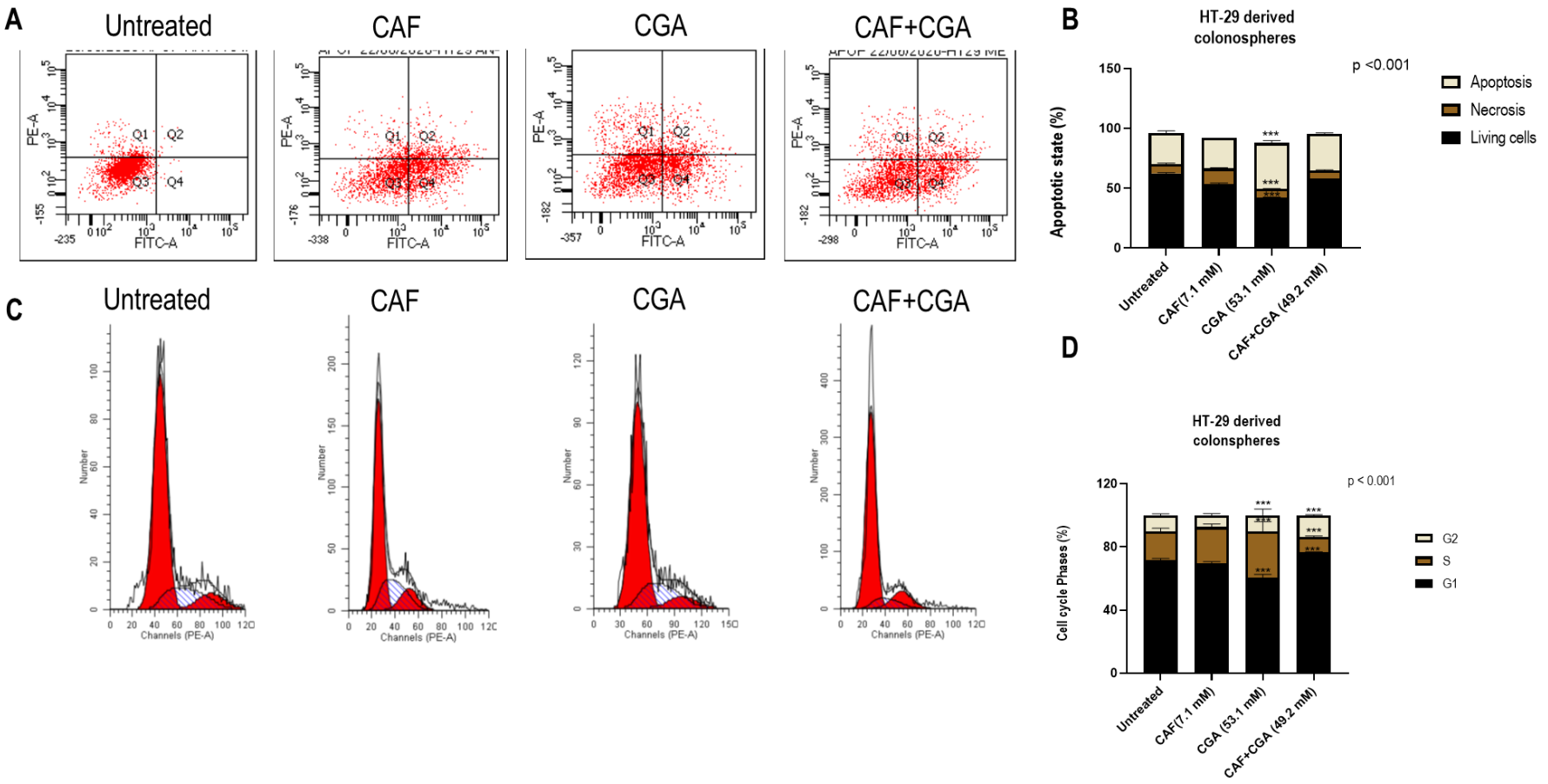

Figure 23. Effects of bioactive coffee compounds on HT-29 derived colonospheres survival. **(A and C)** dot plots showing the respective quadrants of PE-A and FITC-A channels. **(B and D)** representative image of statistics differences defined as *** $p < 0.001$ performed among the groups by ANOVA and *post hoc* Tukey test.

5.4.5. Coffee major compounds against CRC with stemness properties based on ALDH activity and specific surface markers

We quantified the ALDH activity, the differential expression of CD44 and CD326 by flow cytometry in spheroids enriched with CSCs. We observed significant decreasing in the ALDH activity in HCT-116 derived colonospheroids after all treatments. On the other hand, this phenomenon was observed in HT-29 derived colonospheroids only in CAF and the CAF+CGA treatments during the 48 h of exposition. Thereafter, the surface CSCs CD44 and CD326 markers were quantified. The CD44 expression was drastically reduced in both cell lines at 36 hours post- all treatments. The CD326 expression decreased significantly in the HCT-116 line in all treatments, meanwhile, the HT-29 spheroids showed significant reduction in the expression of CD326 only in the treatments with CAF and the CAF+CGA. These findings indicate potential effects of coffee compounds towards a cell subpopulation enriched with CSCs **(Figure 24)**.

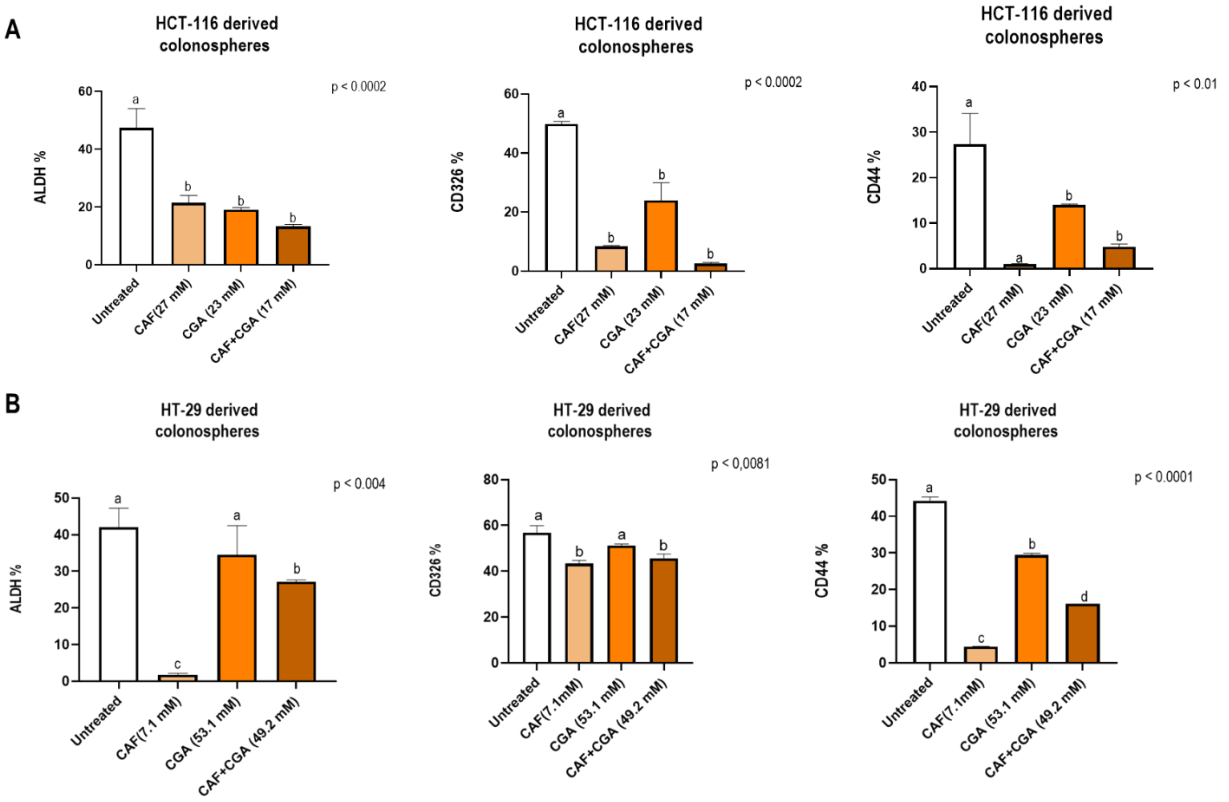

Figure 24. Effects of coffee major compounds on CSCs markers post 36 h of exposition **(A)** HCT-116 derived colonospheres and **(B)** HT-29 derived colonospheres. Statistical differences (expressed by upper letters) were performed among the groups by ANOVA and *post hoc* Tukey test.

5.4.6. Bioactive major coffee compounds reduce the side population of CSCs

The method of staining with hoescht 33342 enables the identification of side population based on the dye efflux properties onto the ATP-biding cassette (ABC-transporters). This population is enriched in CSCs and characterized by their capacity to efflux the fluorescence DNA-binding dye Hoescht 33342 through their ABC transporters (25). Both cell lines of colonoespheroids presented significant reduction of side population with CSCs characteristics post 48 h of incubation with caffeine **(figure 25)** but not with the others treatments. This data supports the findings previously demonstrated, reinforcing its potential against CSCs.

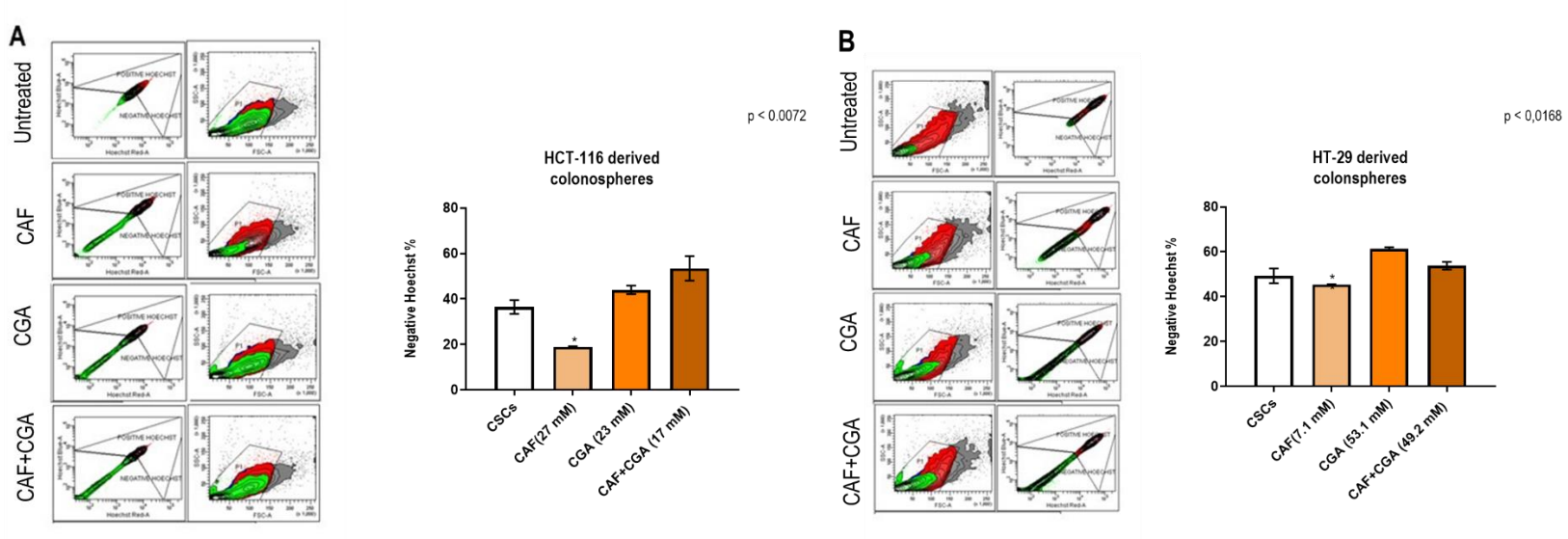

Figure 25. Identification of Side Population through Hoesch 33342 staining. Plots of flow cytometry are showed in **(A)** and **(B)**. The difference between the treatments is exposed in **(A)** and **(B)** post 48 h of incubation. Statistical differences (expressed by upper *) were performed among the groups by ANOVA and *post hoc* Tukey test.

5.4.7. Overall subcutaneous xenograft tumour formation with HCT116 - derived spheroids enriched with CSCs

According to greater proapoptotics effects and to significant CSCs subpopulation reduction, we decided to start the xenograft assays inserting HCT-116 derived colonospheroids on Matrigel-based. Due to the HCT-116 colon cancer cell line responded better to the treatments than HT-29, with higher sensitivity, we chosen this cell line to proceed with xenograft experimentation. Thus, the effects of these compounds were evaluated in two periods, firstly we carried out the 15 days of pre-treatment obtaining drastic tumour growth inhibition with both CAF and CGA isolated treatments, however, the combination showed no significant effects corroborating with the antagonistic effects showed previously. However, the post-treatments showed no antitumor effects in these conditions, suggesting that these bioactive compounds work mainly as cancer prevention agent and that we need to carry out more experiments in order to conclude the preventive and/or therapeutic effect of these compounds alone or in combination with conventional chemotherapy. **(Figure 26)**.

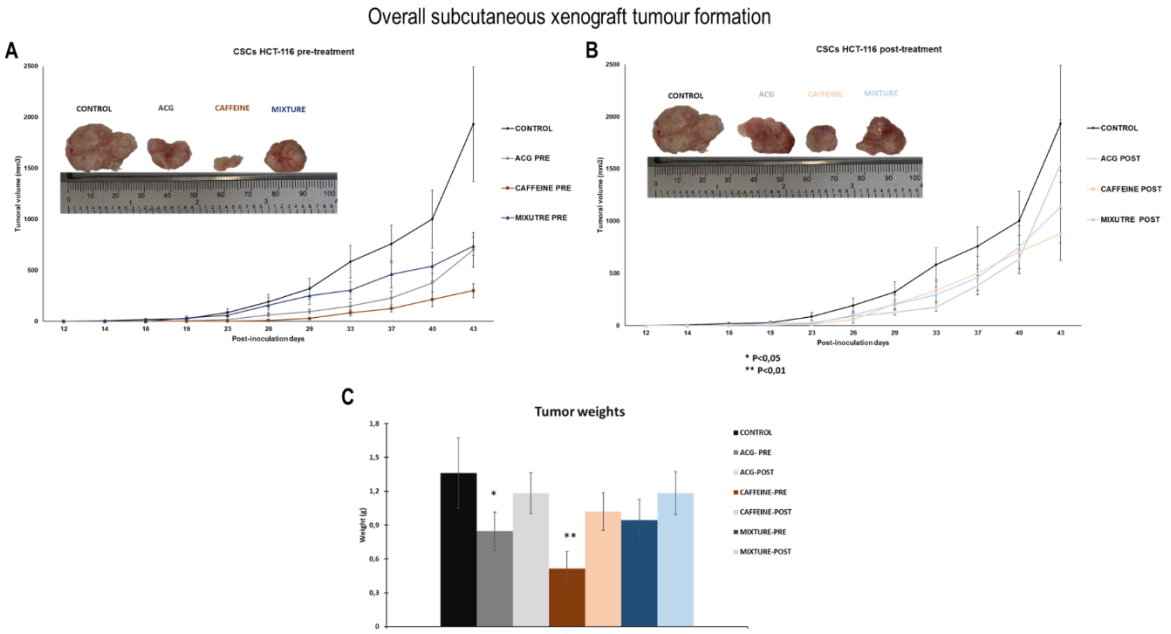

Figure 26. Antitumoral effects of coffee major compounds on NSG male mice. Tumor growth enriched with CSCs of HCT-116 derived colonspheroids in pre-treatment with CGA, CAF and its CAF+CGA **(A)** and post-treatment **(B)**, tumor weigh S-phase through the pre- and post-treatments periods **(C)**. Statistical differences (expressed by upper *) were performed among the groups by ANOVA and *post hoc* Tukey test ($p < 0.01$ and $p < 0.05$).

6. DISCUSSION

This study aimed evaluating the beneficial effects of the main coffee alkaloids and polyphenols, CAF and CGA, individually or in association, showing that the TRI was not such efficient. As elicited in a recent prospective study demonstrating that high coffee consumption (≥3 cups a day) improved the overall survival of CRC patient (Mackintosh *et al*., 2020) further mechanistic studies are needed in order to discriminate which bioactive compounds are involved in this protective effect, and whether these bioactive molecules interact with each other. Our findings may contribute to this gap in the literature, as CAF+CGA intervention had the most pronounced effects on decreasing epithelial cell proliferation (Ki-67) and increasing apoptosis in colonic crypts. This treatment also decreased the levels of proinflammatory cytokines IL-6, IL-17 and TNF-α, and downregulated the oncomiR miR-21a-5p in the colon of well established chemically induced carcinogenesis bioassay (Bird, 1987). Ultimately, CAF+CGA attenuated preneoplastic ACF development. Note that our coffee compound intervention followed a human equivalent dose (HED) translational approach, equivalent to the CAF and ACG contained in three cups of coffee (high coffee consumption). Actually, we also demonstrated the potential effects of CAF individually or combined with CGA against highly aggressive CSCs enriched colon cancer cell subpopulations and its growth grafts on Matrigel into well-stablished xenograft mice model. The CRC is a disease, which contains heterogenous populations of tumor epithelial cells, exhibiting multiple differentiation due to randomly successive mutations.

Diet displays important role on CRC development, and coffee bean-derived beverages, as the widely consumed espresso and “common”, are important and safe as part of the human dietary habit worldwide (Romualdo *et al*., 2019). Previous studies reported that direct contact of colonic mucosa with bioactive coffee compounds, mainly CGA, generate metabolites due to the microbiota metabolization, harboring, *in situ*, several microbial metabolites in amounts higher or similar to those utilized in the *in vitro* assays, also reaching extremely low peak plasm concentrations (Stalmach *et al*., 2009; Stalmach *et al*., 2010). CAF is quickly and practically absorbed entirely in the gastrointestinal tract within 45 min, the smallest part in the upper (~20%) and the largest part (~80%) in the lower gastrointestinal tract, where it is sufficiently hydrophilic to cross biological membranes, reaching the liver, where a biotransformation occurs through the cytochrome P450 (CYP) enzymes, generating metabolites (paraxanthine, dimethylxanthine and theobromine). These metabolites are immediately bioavailable, reaching a plasma peak of ~33 μM within 60–80 min after the higher consumption, equivalent to the

intake of three cups/day (~350 mL) of common filtered coffee (Kot and Daniel, 2008; Lang *et al*., 2013). According to Christopher *et al*. (2021), the increase in coffee consumption (≥3 cups/day) is inversely proportional to the progression of cancer in patients with advanced or metastatic CRC. Interestingly, this same study observed that when caffeinated and decaffeinated coffee were considered separately, both improved the overall survival; however, the caffeinated coffee presented lower effectiveness on progression-free survival, reinforcing the need for further approaches to identify how these bioactive molecules interact (Christopher *et al*., 2021). Given the importance to explore the complexity of interaction between several bioactive compounds found in a traditional beverage, this study evaluated the effects of combination between the major coffee compounds. According with Makino *et al*. (2021) the kahweol and cafestol, both diterpenes founded in non-filtered coffee beverages affects synergistically the prostate cancer cell viability. Further, interestingly our findings show different interactions in CAF combined with CGA presenting strong antagonistic effects in colonospheres formed with HCT-116 (CI = 2.2), meanwhile, the other line respond different depending the proportion of both compounds. The antiproliferative effects of CAF and CGA isolated was greater with lower IC_{50} on monolayer culture, otherwise, the colonospheres showed less sensitivity to the treatments. However, the difference of sensibility in both models is due to the quantity of CSCs, spheroids are enriched of this subpopulation that are more resistant to each / every stimulus (Visvader *et al*., 2008). Even if the colonospheres presented lower IC_{50} post incubation with the compound's association compared with isolated them isolated, an antagonistic mechanism was observed at the ratio 1:2. Nonetheless, depending of DRI is possible to obtain synergic effects in the right proportions of each compound. The principle of mass-action law is helpful to avoid pitfalls in drug combination studies. CAF showed synergistic effects with cisplatin against two sarcoma cell lines and primary sarcoma cells culture isolated from clonogenic assay. Moreover, an enhancement in the efficacy of others chemotherapy by CAF was observed in the combination with adriamicyn, mitomycin, cyclosphosphamide and doxorubicin (Tomita and Tsuchiya, 1989; Kakuyama and Sadzuka, 2001). The combined therapy might reveal a great strategy enhancing the antitumor activity more than a single-agent therapy. Most of studies report the synergy, but rarely report the antagonism as the main topic, anyhow, it should be noted that antagonism can also be important (Chou, 2010). The most *in vitro* assays involving coffee compounds are based on exposing tumoral cells to bioactive compounds individually, losing the complexity of compound combination in whole coffee beverages.

In our chemically induced mouse model, DMH is a procarcinogen that is biotransformed in the liver into highly reactive ions that alkylate specific genomic DNA bases, resulting in specific DNA adducts, such as O^6-methylguanine (O^6-mG) and N^7-methylguanine (N^7-mG) in colonic epithelial cells. These DNA adducts can lead to genomic instability and mutation in cancer, a hallmark that significantly contributes to the initiation of rodent colon carcinogenesis (Rosenberg, Giardina and Tanaka, 2009). In addition to DMH administration, dietary DCA was used to promote the early-stage of colon carcinogenesis. Secondary biliary acids are known to contribute to the inflammatory milieu that promotes (pre)neoplastic lesions by different mechanisms, including NLRP3 inflammasome activation that leads to cytokine production (Zhao *et al*., 2016). As such, a clear inflammatory colonic context was found in a DMH/DCA model, as IL-6, IL-17 and TNF-α were substantially increased in this group. Although the mechanisms are not fully understood, DCA is also proposed to induce colonic tumors in mice, as a 0.2% DCA in a diet for 8–10 months, without carcinogen initiation, and that led to development of colonic neoplasia (Bernstein *et al*., 2011). The appearance of ACF in both humans and rodents are thought to be the earliest identifiable tumor precursor lesions. However, only a small fraction of ACF evolve into an adenoma–carcinoma sequence. Early screening of these lesions is well accepted in short/medium term rodent bioassays as an early marker for CRC prevention (Orlando *et al*., 2008; Rosenberg, Giardina and Tanaka, 2009; Venkatachalam *et al*., 2020). Indeed, it was found that coffee compounds individually or in combination similarly decreased ACF development. Our findings are in keeping previous bioassays demonstrating that caffeinated coffee (Soares *et al*., 2018), CAF (Carter *et al*., 2007) and CGA (Morishita *et al*., 1997) individually attenuated the development of ACF or dysplastic crypts during the initial stages of different chemically induced colon carcinogenesis models in rodents. Nonetheless, the landscape of potential molecular mechanisms involved was not investigated, in detail, in these studies.

Hence early apoptosis induction and cell cycle arrest were observed mainly in HCT-116 derived colonospheroids, indicating that CAF and CGA displays potential properties in important keys of cancer hallmarks. Apoptosis is a fundamental tumor suppressor, in which genes products as p53 among others play key roles in eliminate damaged cells (Makin and Dive, 2001) and cell cycle is critical to control the progression of the DNA-damaged cells. Simultaneously, we observed apoptosis induction in colonic crypts in mice exposed to the DMG/DCA carcinogen by increasing expression of caspase-3 in the combined compounds treatment. Kurata *et al*., (2007) showed that CGA inhibited tumor cell proliferation in a dose-

dependent manner, through the activity of DNA-ladder and caspase-3, as well as increasing the expression of c-Jun, reinforcing its cytotoxic effect. Also, our findings indicate that most of tumor cells were arrested in the S-phase, which is the cellular defense against DNA damage in replicating cells. When DNA polymerases encounter errors like mutations and lesions, occur the inhibition of DNA-synthesis and the progression of cycle is stopped, therefore, most of chemotherapeutic antimetabolites drugs display roles in the S-phase (Mills, Kolb and Sampson, 2018).

As mentioned before, the cancer stem cells (CSCs) is a subpopulation responsible by the drug resistance, tumor recurrence and metastasis, being main problem on the therapeutic failure (Tirino *et al.*, 2013). The drug resistance and recurrence presented by this small cell population are mainly explained by the overexpression of multidrug resistance (MDR) membrane proteins and high activity of aldehyde dehydrogenase (ALDH). The ALDH is a CSCs marker and protects the drug-tolerant subpopulation from the potential effects of elevated levels of ROS and pharmacologic disruption of its activity lead the accumulation of ROS and consequently apoptosis (Raha *et al*., 2014). In this study, we showed significative reduction on ALDH activity after cells exposure to the bioavailable coffee bioactive compounds. The indirect or direct ALDH activity inhibition, has shown the loss of stem cell traits, reduction of cell proliferation, invasion and enhanced the drug sensitization (Toledo-Guzmán *et al*., 2019). We suggest that combining these compounds with other trivial treatment for CRC could open a new field of investigation.

The relevance of this subpopulation has yield to develop methodologies for their identification and isolation. Several reports show evidences that CSCs can be characterized based on either the use of hoescht 33342 dye exclusion technique and/or cell surface markers (Feng, Wu and Yi, 2015). Colon cancer stem-cells may be identified through the expression of cell surface proteins such as, CD133, CD44 and CD326 (Olejniczak, Szaryńska and Kmieć, 2018). The CAF and CGA combined or individually decreased significantly CD44 and CD326 cell surface proteins expressions, reinforcing the capacity of these compounds against CSCs. CAF reduced significantly the efflux of hoescht 33342 in both colonoespheres cell lines. The xanthines can dramatically reduce the activity of drug transporter ATP-binding-cassette (ABC), the caffeine xanthine treatment significantly increases the retention of a stablished ABC substrate in MCF/MX100 cells. Moreover, the combination with caffeine and mitoxantrone decreased significantly the IC_{50} of this chemotherapeutic drug front resistant breast cancer cells (Ding, Allman and Salvi, 2012). Exceptionally, CAF and CGA metabolism

is relatively comparable in human and rodents, which simplify the establishment of translational approaches (Walton, Dorne and Renwick, 2001).

Although caffeine is an authentic stimulator of gastric acid secretion and GI motility, it may cause also disturb on stomach mucosa. Some cases are related to caffeine overdoses; however, it is extremely rare (Evans, Richards and Battisti, 2021). The lethal doses of caffeine have been reported at peak plasma of ~100 µg, which can be reached by the ingestion of ~10 mg or greater (Cappelletti *et al.*, 2018). The recommended amount of caffeine is up to 400 mg/day for healthy adults and the most accurate estimate of the DL50 in rats of caffeine orally ingestion is 367 mg/kg (Adamson, 2016). We showed that the orally ingestion of caffeine concentration found in the high coffee consumption (≥3 cups/day HED ~50 mg/kg) presents no toxicity up to 100 mg in CD-1 mice. There is no evidence about the toxicity of CGA, this compound is absorbed in small intestine around 30% then most of part reaches the colon. The CGA is almost indetectable after 1 h of ingestion (~0.035 µM in the plasma) (Lang *et al.*, 2013). The *in vitro* assays generally apply coffee compounds in supraphysiological concentration ranging from high micromolar (µM) or millimolar (mM) levels, trying to mimic the amount observed in coffee and not considering the metabolic approach (Gaascht, 2015; Wilson, 2018). Nonetheless, the *in vitro* findings can provide meaningful insight to the potential target and mechanisms of action for proposed active compound for the next step that will therefore be to develop compounds that mimic the structure of the bioavailable compound without the negative effects even in supraphysiological concentrations.

It was found that a CAF+CGA combination counteracted the DMH/DCA-induced upregulation of miR-21a-5p, reducing the expression of this oncomiR in the colon. In humans, miR-21-5p is also overexpressed in a colon tumor compared with normal adjacent tissue, and its expression is positively correlated to CRC staging (Kanaan *et al.*, 2012; Schee *et al.*, 2012). Our DMH/DCA-induced model reflected this marked molecular hallmark of colon carcinogenesis. Increased expression of miR-21-5p in CRC is also associated to a poor prognosis, including poor differentiation, lymph node metastasis and advanced TNM (Schee *et al.*, 2012). This miRNA negatively regulates the target gene Pcdc4, resulting in increased invasion, migration and cell proliferation of different human CRC cell lines (HT-29, Colo206f, LIM 1863, SW480 and DLD1), contrasting with a knockdown of miR-21-5p cells (Cottonham *et al.*, 2010; Deng *et al.*, 2014). In addition, it is recognized that tumor suppressor PTEN is inversely associated with miR-21-5p levels in CRC tissues and the HCT-116 colon cancer cell line. When these cells were transfected with miR-21-5p inhibitor, proliferation and migration

were suppressed while PTEN protein levels were increased (Carter *et al*., 2007). These findings elicit that miR-21-5p targets tumor suppressor PTEN at the post-transcriptional level, attenuating the PTEN/PI3K/Akt signaling pathway, which is involved in the negative regulation of proliferation and the positive regulation of apoptosis (Xiong et a., 2013).

Our target analysis of this miRNA also revealed that Pten is a validated target in mice. Moreover, it was found that ~13-20% of miR-21a-5p targets are involved in the (A) negative regulation of ERK1 and ERK2 cascade—closely involved with cell proliferation—and (B) positive regulation of apoptosis. Note that both BP annotations included Pten, which was also a central node in the network analysis, demonstrating the importance of miR-21a-5p/Pten axis on the regulation of colon proliferation/apoptosis. In accordance with the downregulation of miR-21a-5p, CAF+CGA was the only treatment displaying a reduced Ki-67 labeling indexes on colonocytes and increased the percentage of colonocytes in apoptosis. Cell proliferation is closely related to (pre)neoplastic lesion development, as its increase may promote a clonal expansion of DMH-initiated epithelial cells, and ultimately promote ACF development (Ma, Williamson and Rowlands, 2002; Roncucci *et al*., 2000). Furthermore, the ability to induce apoptosis in DMH-initiated epithelial cells may also prevent the emergence of (pre)neoplastic lesions (Schee, 2012). As such, the modulation of crypt proliferation/apoptosis by coffee compounds may be involved with miR-21a-5p decrease, resulting in a decreased ACF burden.

DCA is a naturally occurring secondary bile acid that presents potential pro-carcinogenic and pro-inflammatory actions (Bernstein, 2011; Liu *et al*., 2018). Some animal studies have demonstrated that mice receiving a DCA-supplemented diet developed gut dysbiosis and intestinal inflammation (Liu *et al*., 2018; Xu *et al*., 2021). Our findings indicate that CAF+CGA intervention reduced the expression of pro-inflammatory cytokines IL-6, IL-17 and TNF-α in the colon. Note that Smad7, a negative regulator of pro-inflammatory TGF-β signaling, is a target of miR-21a-5p. When active, TGF-β signaling drives the pro-inflammatory shift of many stromal cells involved in the (pre)neoplastic lesion microenvironment (Itatani, Kawada and Sakai, 2019). The connection between inflammation and colon carcinogenesis is well stablished. The infiltration of CD8+ and CD3+ cells and other immune cell subsets in CRC have been associated with clinical prognoses and outcomes (Terzic *et al*., 2010; Mezheyeuski *et al*., 2021). IL-6 is overexpressed in CRC patients and is correlated with a larger tumor size, the occurrence of liver metastasis and reduced survival rates, as this interleukin is also a potent stimulator of colon cancer cell proliferation (Yu, Pardoll and Jove, 2009; Waldner, Foersch and Neurath, 2012; Heichler *et al*., 2020). The TNF-

α is expressed initially in the first steps of inflammation, this cytokine is responsible for triggering many reactions, including the production of other cytokines, chemokines and endothelial adhesion molecules, besides increasing vascular permeability and recruiting immune cells to the site of infection (Mager *et al.*, 2016; Popivanova *et al.*, 2008; Kruglov *et al.*, 2008). Finally, the IL-17 is also expressed significantly higher in CRC tissues, and its upregulation begins in the adenoma stage and is at a higher level in the malignant stage. IL-17 promotes tumorigenesis through the production of myeloid-derived suppressor cells (MDSCs) and stimulates IL-6 secretion from stromal tumor cells activating the STAT3 pathway (Parker, Beury and Ostrand, 2015). TNF- α and IL-17 presented a synergistic effect on the proliferation of HT-29 cells through stimulating the extracellular receptors of Kinase (ERK1/2) and increasing IL-17 downstream genes, such as MMP-9, MMP-7 and MMP-2 (Xie *et al.*, 205). As such, it was suggested that the negative regulation of colonic mucosa inflammation by coffee compounds may be involved with a decrease in miR-21a-5p, also contributing to the decreased ACF burden.

Here was evaluated the oral administration of CAF, CGA and its combination on NSG mice, in two approaches: 1) pre-treatment – before the spheroids inoculation and 2) post-treatment – after first tumor appearance. Whereas post-treatment did not show tumor reduction, a preventive effect was observed of both compounds isolated in the pre-treatment xenograft assay. The combination of both compounds showed no significant tumor inhibition corroborating with the antagonistic interaction of compounds. Epidemiological evidences point the coffee as chemopreventive factor on CRC. According to Wang *et al.*, (2013) comparing 816 cases of CRC versus 815 controls, there is a significant inverse association between the consumption of coffee or its polyphenols, especially in distal colon cancer. Similarly, an analysis of two case-control studies in Japan reported a 22% of reduction in the risk of CRC in high coffee consumption (≥ 3 cups/day), especially in distal and rectal colon cancer, suggesting the heterogeneous effect depending of anatomic subside (Nakagawa-Senda *et al.*, 2017). In addition, clinical findings on coffee consumption and CRC are missing; Kang *et al.*, (2011) reported that any intake of caffeinated or decaffeinated coffee beverages showed downregulation of extracellular signal regulated-kinase (ERK) phosphorylation in CRC tissue compared to non-consumers (Kang *et al.*, 2011).

In general, the modulation of key regulators on colon tumorigenesis underscore the importance of more deep investigation *in vitro* and *in vivo* approaches. We showed for the first

time the potential antitumor effects of two major coffee compounds against CSCs cells *in vitro* and *in vivo*.

7. CONCLUSIONS

1. The caffeine together with the chlorogenic acid in the concentrations founded in the coffee beverages are able to suppress the colon carcinogenesis in preventive way or in early stages.

2. Both compounds display antiproliferative and proapoptotic effects against highly aggressive colon cancer cells subpopulation lowering the CSCs main markers

3. Nonetheless, the concentration in combination of both compounds must to be administrated in the right proportion to maximize the interaction and obtain synergic effects

4. All compounds were able to immunomodulate pro-inflammatory cytokines that display key role in the CRC hallmarks

5. The combined oral treatment downregulated an extremely important oncomiR, the miR-21a-5p

6. Finally, our preclinical results suggest that the main coffee bioavailable compounds attenuate early- and late-stage of colon carcinogenesis. These beneficial effects are probably mediated by the downregulation of an important oncomiR, thus modulating proliferation, apoptosis and inflammation. Our findings provide insights into which coffee compounds are involved on the recent reported protective effects of coffee intake on CRC outcomes in humans and may inspire future clinical studies.

8. CONCLUSIONES

1. La cafeína junto con el ácido clorogénico en las concentraciones que se encuentran en las bebidas de café son capaces de suprimir la carcinogénesis de colon en forma preventiva o en etapas tempranas.

2. La cafeína junto con el ácido clorogénico muestran efectos antiproliferativos y proapoptóticos frente a la subpoblación de células madre cancerígenas de colon altamente agresivas como se demuestra por la reducción de la expresión de los principales marcadores de CMC.

3. La concentración en combinación de la cafeína junto con el ácido clorogénico debe administrarse en la proporción adecuada para maximizar la interacción y obtener efectos sinérgicos.

4. Todos los compuestos fueron capaces de inmunomodular citocinas proinflamatorias que desempeñan un papel clave en las características distintivas del CCR.

5. El tratamiento oral combinado reguló a la baja un oncomiR extremadamente importante en cáncer, el miR-21a-5p.

6. Finalmente, nuestros resultados preclínicos sugieren que los principales compuestos biodisponibles del café atenúan las etapas temprana y tardía de la carcinogénesis de colon. Estos efectos beneficiosos probablemente estén mediados por la regulación baja de un oncomiR importante, modulando así la proliferación, la apoptosis y la inflamación, así como por la disminución de las subpoblaciones CSCs. Nuestros hallazgos brindan información sobre qué compuestos del café están involucrados en los efectos protectores mostrados recientemente en la ingesta de café frente a CRC en humanos y pueden inspirar futuros estudios clínicos.

9. CONCLUSÕES

1. A cafeína juntamente com o ácido clorogênico nas concentrações proporcionais ao consumo humano médio/alto de café são capazes de suprimir a carcinogênese do cólon de forma preventiva ou em estágios iniciais.

2. Ambos os compostos exibem efeitos antiproliferativos e pró-apoptóticos contra a subpopulação de células de câncer de cólon altamente agressivas, diminuindo os principais marcadores de CSCs

3. No entanto, a concentração em combinação de ambos os compostos deve ser administrada na proporção certa para maximizar a interação e obter efeitos sinérgicos

4. Todos os compostos foram capazes de imunomodular citocinas pró-inflamatórias que apresentam papel fundamental nas características do CRC

5. O tratamento oral combinado regulou negativamente um oncomiR extremamente importante, o miR-21a-5p

6. Finalmente, nossos resultados pré-clínicos sugerem que os principais compostos biodisponíveis do café atenuam os estágios inicial e tardio da carcinogênese do cólon. Esses efeitos benéficos são provavelmente mediados pela regulação negativa de um importante oncomiR, influenciando a proliferação, apoptose e inflamação. Nossas descobertas fornecem informações sobre quais compostos de café estão envolvidos nos efeitos protetores relatados recentemente da ingestão de café nos resultados do CCR em humanos e podem inspirar futuros estudos clínicos.

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