UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE – UFCSPA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Gabriela Gonçalves Roliano

Participação do sistema imune-inflamatório na progressão do câncer colorretal: análise do impacto da obesidade e da sinalização purinérgica.

Porto Alegre
2021

Gabriela Gonçalves Roliano

Participação do sistema imune-inflamatório na progressão do câncer colorretal: análise do impacto da obesidade e da sinalização purinérgica.

Dissertação submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre

Orientadora: Profa. Dra Elizandra Braganhol
Coorientador: Prof. Dr. Antônio Nocchi Kalil

Porto Alegre
2021

Catalogação na Publicação

Roliano, Gabriela Gonçalves
Participação do sistema imune- inflamatório na progressão do câncer colorretal: análise do impacto da obesidade e da sinalização purinérgica / Gabriela Gonçalves Roliano. -- 2021.
143 p. : il., graf., tab. ; 30 cm.

Dissertação (mestrado) -- Universidade Federal de Ciências da Saúde de Porto Alegre, Programa de Pós-Graduação em Ciências da Saúde, 2021.

Orientador(a): Elizandra Braganhol ; coorientador(a): Antônio Nocchi Kalil.

1. Câncer colorretal. 2. Obesidade. 3. Macrófagos associados ao tumor. 4. Sistema purinérgico. I. Título.

Sistema de Geração de Ficha Catalográfica da UFCSPA com os dados fornecidos pelo(a) autor(a).

AGRADECIMENTOS

Agradeço à UFCSPA, por disponibilizar o espaço físico e o fornecimento de muitos insumos, possibilitando a realização do presente trabalho.

Às fontes fornecedoras de fomento, CAPES, CNPq e FAPERGS, espero retribuir o investimento em mim empregado com a publicação dos artigos contidos neste manuscrito.

À minha orientadora, professora doutora Elizandra Braganhol, por todo o empenho que fez com que esse trabalho fosse realizado, por acreditar em mim e me dar forças para continuar nas piores horas.

Ao meu co-orientador, professor doutor Antônio Kalil, por nos proporcionar uma visão clínica do processo do câncer.

À minha família, aos meus irmãos pelo incentivo, e em especial aos meus pais, por todo o apoio para que eu nunca parasse de estudar.

Às colegas de grupo, cada experimento realizado nesses 3 anos foi sempre com ajuda delas. Na pesquisa, não consegue-se realizar nada sozinho, e um grupo parceiro como esse foi primordial, as “Elizetes” tem meu grande reconhecimento.

À professora doutora Márcia Wink e aos colegas, alunos da professora Márcia, que foram acolhedores comigo e com meu grupo, tornando-nos todos uma grande família científica.

Meu agradecimento especial a duas colegas e amigas, doutora Juliana Azambuja e doutoranda Thaís Paim, por dividirem muito mais do que pesquisa, pessoas que fizeram total diferença na minha caminhada e que vou ser eternamente grata. E aos meus amigos, que ficam repetindo incansavelmente o quanto sou capaz e que vou conseguir alcançar os meus objetivos, e que sabem o quanto o apoio emocional importa para mim.

E por fim, agradeço a todos os professores da UFCSPA que passaram na minha vida e me fizeram acreditar no meu potencial.

RESUMO

O câncer colorretal (CCR) é um problema de saúde mundial, com alta taxa de mortalidade quando alcança o grau metastático. Quando associado à obesidade o risco é aumentado, devido a alteração imunológica sofrida pelo organismo. Estudos indicam que a participação de um microambiente inflamatório, composto por células imunes e seus produtos de secreção, é essencial para a progressão tumoral. Dando ênfase à imunidade inata, macrófagos associados ao tumor (TAM) frequentemente apresentam uma polarização do tipo-M2 (predominantemente imunossupressora), a qual pode desempenhar importantes funções de promoção tumoral. Umas das vias alteradas nas células imunes durante a modificação gerada pelo microambiente tumoral é o sistema de sinalização purinérgico. O objetivo do presente trabalho foi investigar a participação do sistema imune/inflamatório na progressão do câncer colorretal. Para tanto, primeiro foi realizada uma revisão da literatura buscando concentrar os dados sobre sinalização purinérgica no câncer colorretal publicados até o momento. Segundo, em experimentos *in vitro*, as linhagens Caco-2 e HCT116, foram cultivadas com o soro dos grupos: obesos e não-obeso e parametros de viabilidade, migração, proliferação e adesão celular. Além disso, a secreção das citocinas IL-6, IL-10 e TNF-α foram analisadas no soro e no sobrenadantes das linhagens celulares. Em terceiro, foram realizadas culturas primárias de macrófagos humanos obtidos a partir da diferenciação de monócitos circulantes. Tais culturas foram co-cultivadas diretamente com células de linhagem de CCR humano (HCT116) ou tratadas com o meio condicionado. A avaliação dos fenótipos M1/M2 dos macrófagos foi realizada por citometria de fluxo para os marcadores CD204, CD163 e HLA-DR ou por CBA para TNF, IL-1β, IL-6, IL-10 e IL-8; espécies reativas de oxigênio e a produção de óxido nítrico, assim como morte celular por necrose, proliferação e potencial de membrana também foram avaliados. Em suma, os principais resultados foram: o aumento da expressão da enzima CD73 e dos receptores P1 (A1, A2A, A2B) leva a progressão tumoral no CCR; os receptores da família P2 (P2X e P2Y) e os níveis de ATP apresentaram resultados contraditórios, com efeitos tanto pró- quanto anti-tumoral. Citocinas pró-inflamatórias (TNF-$\boldsymbol{\alpha}$ e IL-6) foram mais elevadas no grupo obeso, no soro e no sobrenadante das linhagens celulares de CCR. Os macrófagos que foram co-cultivados com células tumorais apresentaram perfil de liberação de citocinas mesclado, aumento de proliferação, diminuição de ROS e aumento da expressão de

CD206 e CD163, sinais característicos de um fenótipo pró-tumoral. Como conclusão, a participação do sistema purinérgico, o estado de inflamação causado pela obesidade e a adaptação dos macrófagos a células tumorais foram elementos auxiliares para o desenvolvimento/progressão tumoral.

PALAVRAS-CHAVES: Câncer colorretal, Obesidade, Macrófagos associados ao tumor e Sistema purinérgico.

ABSTRACT

Colorectal cancer is a worldwide health problem, with a high mortality rate when it reaches the metastatic level. When associated with obesity, the risk is increased, due to the immunological alteration suffered by the body. Studies indicate that the participation of an inflammatory microenvironment, composed of immune cells and their secretion products, is essential for tumor progression. Emphasizing innate immunity, tumor-associated macrophages (TAM) often display an M2-type polarization (predominantly immunosuppressive), which can play important tumor-promoting functions. One of the altered pathways in immune cells during the modification generated by the tumor microenvironment is the purinergic signaling system. The present work aimed to investigate the participation of the immune/inflammatory system in the progression of colorectal cancer. To do so, first, a literature review was carried out, seeking to concentrate the data on purinergic signaling in colorectal cancer published so far. Second, in a block of experiments, the Caco-2 and HCT116 strains were cultivated with the serum of the groups: obese and non-obese, evaluated on viability, migration, proliferation, and adhesion; the cytokines IL-6, IL-10, and TNF-α were analyzed in serum and cell culture supernatants after exposure to serum. Third, in another block of experiments, primary cultures of human macrophages obtained from the differentiation of circulating monocytes were performed. Such cultures were co-cultured with human CCR lineage cells (HCT116) or cultured with a conditioned medium of the same lineage. The evaluation of M1/M2 phenotypes of macrophages was performed by flow cytometry (FACS) for the markers CD204, CD163, and HLA-DR or by CBA for the markers TNF, IL-1β, IL-6, IL-10, and IL- 8; reactive oxygen species and nitric oxide production, as well as cell death by necrosis, proliferation and membrane potential were also evaluated. In summary, the main results were: increased expression of the CD73 enzyme and P1 receptors (A1, A2A, A2B) leads to tumor progression in RCC; P2 family receptors (P2X and P2Y) and ATP levels presented contradictory results, with both pro- and anti-tumor effects. Pro-inflammatory cytokines (TNF-**α** and IL-6) were higher in the obese group, in serum, and the supernatant of CCR cell lines, after cell exposure, as well as in the group with the highest cell adhesion. Macrophages that were co-cultured with tumor cells showed a mixed cytokine release profile, increased proliferation, decreased ROS (promotes angiogenesis), and increased labeling for CD206 and CD163, characteristic signs of

TAM. In conclusion, the participation of the purinergic system, the state of inflammation caused by obesity and the adaptation of macrophages to tumor cells were auxiliary elements for tumor development/progression.

KEYWORDS: Colorectal cancer (CRC). Obesity. Tumor cell lines. Tumor-associated macrophages (TAM). Purinergic signaling.

LISTA DE ABREVIATURAS

5-FU 5-Fluorouracil

A1 Receptor de adenosina 1

A2A Receptor de adenosina 2A

A2B Receptor de adenosina 2B

A3 Receptor de adenosina 3

ADP Adenosina di-fosfato

AMP Adenosina monofosfato

ATP Adenosina tri-fosfato

BRAF Gene humano que produz a proteína B-Raf (Proto-oncogene)

CBA Cytometric Bead Array

CCL1 Ligante 1 de quimiocina (motivo C-C)

CCL16 Ligante 16 de quimiocina (motivo C-C)

CCL18 Ligante 18 de quimiocina (motivo C-C)

CCR Câncer Colorretal

CCR3 Receptor de quimiocina C-C tipo 3 (CD193)

CCR4 Receptor de quimiocina C-C tipo 4 (CD194)

CCR8 Receptor de quimiocina C-C tipo 8 (CDw198)

CD163 Receptor do complexo hemoglobina-haptoglobina

CD206 Receptor de manose

CD39 Enzima trifosfato de ecto nucleosido difosfo-hidrolase-1

CD40L Ligante da proteína CD40

CD73 Enzima 5'-nucleotidase (ou ecto-5'-nucleotidase)

CSF1 Fator estimulador de colônia 1

DAMP Padrões moleculares associados a danos

DNA Ácido Desoxirribonucleico

EGFR Receptor do fator de crescimento epidérmico

HLA-DR Receptor de superfície celular MHC classe II

IL-1 Interleucina 1

IL-10 Interleucina 10

IL-12 Interleucina 12

IL-1β Interleucina 1 beta

IL-2 Interleucina 2

IL-4 Interleucina 4

IL-6 Interleucina 6

IL-8 Interleucina 8

IMC Índice de Massa Corporal

IFNγ Interferon gama

LPS Lipopolissacarídeo

M1 Macrófagos 1, com perfil pró-inflamatório

M2 Macrófago 2, com perfil anti-inflamatório

Mφ Macrófago

NK Natural Killer

NO Óxido Nítrico

OMS Organização Mundial de Saúde

P1 Família do receptor purinérgico 1

P2 Família do receptor purinérgico 2

P2X Receptor purinérgico 2X

P2Y Receptor purinérgico 2Y

PAMP Padrões moleculares associados a patógenos

PCR Proteína C reativa

RAS RAt Sarcoma virus, ou vírus do sarcoma de rato (Oncogene)

RNA Ácido Ribonucleico

ROS Espécies reativas de oxigênio

TAM Tumor-associated macrophages

Tc1 Célula tipo 1 T citotóxica

TGF-β Fator de crescimento transformante-β

TGF-β1 Fator de crescimento transformante-β 1

Th1 Célula tipo 1 T helper

Th2 Célula tipo 2 T auxiliar

TME Microambiente tumoral

TNF Fator de necrose tumoral

TNF-**α** Fator de necrose tumoral alfa

Treg Linfócito T regulatório

UTP Trifosfato de uridina

VEGF Fator de crescimento endotelial vascular

VEGF-A Fator de crescimento endotelial vascular A

SUMÁRIO

1 INTRODUÇÃO

1.1 Câncer colorretal (CCR)

O câncer colorretal (CCR) é caracterizado por tumores que se desenvolvem no cólon, segmento do intestino grosso, e no reto. Geralmente tem início com pólipos, lesões benignas que se desenvolvem na parede interna do intestino ou reto, e com o passar do tempo vão adquirindo características de malignidade (Figura 1) (INCA, 2018). Na maioria das vezes, quando detectado em estágio precoce é curável, entretanto quando não tratado, pode espalhar-se para outros órgãos e aumentar o risco de letalidade (MILLER *et al.*, 2019). A metástase é a agravante no CCR, cerca de 20% dos pacientes possuem metástase no fígado no momento do diagnóstico, sendo esta, a principal causa de morte para os pacientes (BRASIL. MINISTÉRIO DA SAÚDE., 2014). Os sintomas geralmente não são notados até a fase avançada da doença. No entanto, as lesões pré-cancerosas podem ser identificadas pela pesquisa de sangue oculto nas fezes e colonoscopia (HABR-GAMA, 2005). Entre o aparecimento do pólipo adenomatoso e a transformação em tumor estima-se um período superior a 10 anos (BYE *et al.*, 2017; HABR-GAMA, 2005).

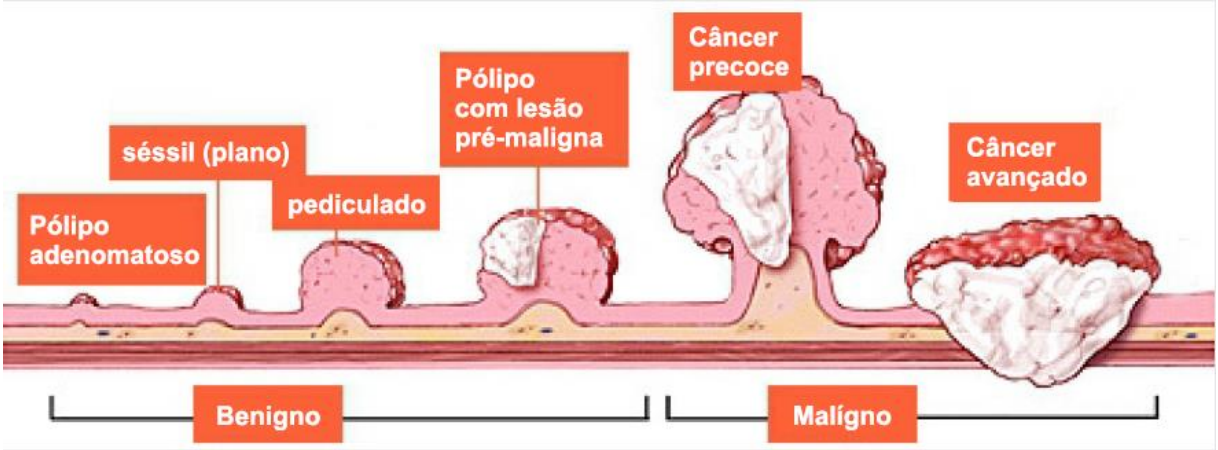


Figura 1 - Progressão das alterações no Câncer Colorretal. Fonte: Jacob & Paresoto. Disponível em: http://jacobeparesoto.com.br/antigo/cancer-de-colon-e-reto/. Acesso em 21/09/2021.

Dentre os fatores de risco para o desenvolvimento do CCR estão a infecção bacteriana, principalmente para indivíduos acima de 60 anos (MUGHINI-GRAS *et al.*,

2018); a obesidade (BARDOU; BARKUN; MARTEL, 2013); modificações epigenéticas relacionadas ao tabagismo (LIMSUI *et al.*, 2010) e ingestão de álcool (FEDIRKO *et al.*, 2011). Dados da literatura mostram que o sistema imunológico participa da patogênese da doença. Doenças inflamatórias intestinais , como a colite ulcerativa , e a Doença de Crohn, apresentam maior risco do desenvolvimento do tumor (BALKWILL, F. R.; MANTOVANI, 2012; GUPTA; MASSAGUÉ, 2006; ITZKOWITZ; HARPAZ, 2004; ITZKOWITZ; YIO, 2004; JESS; RUNGOE; PEYRIN-BIROULET, 2012; RUTTER *et al.*, 2004; TRIANTAFILLIDIS; NASIOULAS; KOSMIDIS, 2009)

A carcinogênese colorretal é uma progressão da mucosa normal ao adenoma, depois ao carcinoma e, finalmente, à metástase (Figura 2); essa cascata inclui a proliferação de tumor primário, a invasão e a sobrevivência das células tumorais durante a migração no sangue periférico, a transferência para os órgãos remotos e, proliferação tumoral, angiogênese e adaptabilidade ao novo ambiente (KRIJGER *et al.*, 2011).

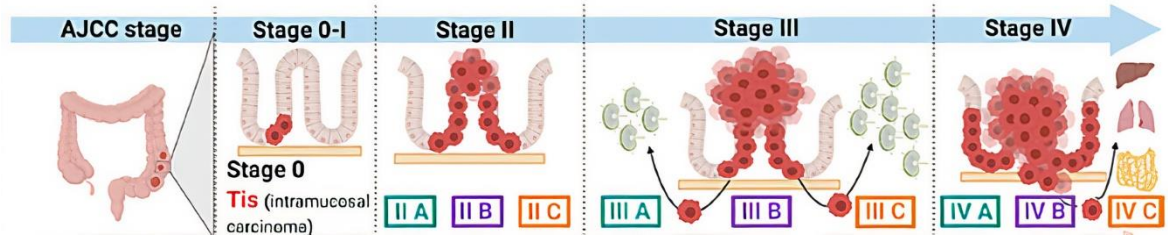


Figura 2 - Estágios de desenvolvimento do câncer colorretal. Fonte: adaptado de Shek et al (2021).

Ao longo da última década o resultado clínico para pacientes com CCR metastático melhorou, atualmente a sobrevivência global média para pacientes com metástase é de aproximadamente 30 meses quando tratados, mais do que o dobro de 20 anos atrás (VAN CUTSEM *et al.*, 2016). Entretanto, 50% dos pacientes que são inicialmente diagnosticados com CCR primário, acabam apresentando doença metastática (ATREYA; YAEGER; CHU, 2017).

A classificação normalmente é feita pelo estadiamento TNM para carcinomas do cólon e do reto, essa sigla corresponde à profundidade da invasão do tumor para o interior ou extrapolação das paredes do cólon (T), invasão ou aderência a órgãos ou estruturas adjacentes (T), números de linfonodos regionais envolvidos (N) e presença ou ausência de metástases (M). Esse sistema de estadiamento clínico/patológico se

aplica a todos os carcinomas que surgem no cólon e no reto, a maioria desses cânceres é estagiada após o exame patológico de um espécime (EDGE; COMPTON, 2010).

Conforme a sétima edição do Manual de estadiamento do câncer da American Joint Committee on Cancer (AJCC), os cânceres do cólon e do reto são classificados por grupos de estágios, sendo eles 0, I, II, III, IVA e IVB, onde em cada um destes estágios se inserem as características de estadiamento TNM (EDGE; COMPTON, 2010), como mostra a Tabela 1.

Tabela 1 - Classificação TNM.

Estágio	T	N	M
0	T *in situ*	N0	M0
I	T1	N0	M0
	T2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-T2	N1/N1c	M0
	T1	N2a	M0
IIIB	T3-T4a	N1/N1c	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
IIIC	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N1	M0

IVA	Qualquer T	Qualquer N	M1a
IVB	Qualquer T	Qualquer N	M1b

Fonte: adaptado de Edge & Compton (2010).

A definição de TNM está apresentada na Tabela 2.

Tabela 2 - Definição TNM.

Tumor primário (T)	
TX	Tumor primário não pode ser avaliado
T0	Nenhuma evidência de tumor primário
T *in situ*	Carcinoma in situ: itraepitelial ou invasão da lâmina própria
T1	Tumor invade a submucosa
T2	Tumor invade muscular própria
T3	Tumor invade através da muscular própria em tecidos pericolorretais
T4a	Tumor penetra na superfície do peritônio visceral
T4b	Tumor invade diretamente ou é aderente a outros órgãos ou estruturas
Linfonodo Regional (N)	
NX	Linfonodos regionais não podem ser avaliados
N0	Sem metástase linfonodal regional
N1	Metástase em 1-3 gânglios linfáticos regionais
N1a	Metástase em um linfonodo regional
N1b	Metástase em 2-3 linfonodos regionais

N1c	Depósito de tumor na subserosa, mesentério ou tecidos pericólicos ou perirretais não peritoneais sem metástase nodal regional
N2	Metástase em quatro ou mais linfonodos regionais
N2a	Metástase em 4-6 linfonodos regionais
N2b	Metástase em sete ou mais linfonodos regionais
Metástase distante (M)	
M0	Nenhuma metástase distante
M1	Metástase à distância
M1a	Metástase confinada a um órgão ou local
M1b	Metástases em mais de um órgão / local ou peritônio

Fonte: adaptado de Edge & Compton (2010).

A ressecção cirúrgica é a terapia primária para CCR não metastático (BRASIL. MINISTÉRIO DA SAÚDE., 2014). Já o tratamento utilizado para o CCR metastático tem sido a quimioterapia sistêmica com o 5-fluorouracil (5-FU), um integrante da família das fluoropirimidinas. Seu mecanismo de ação consiste na inserção errônea de fluoronucleotídeos no DNA e RNA, inibindo a biossíntese dos ácidos nucleicos (CREMOLINI *et al.*, 2015; WEINBERG *et al.*, 2016). O tratamento atual de primeira linha é a quimioterapia citotóxica combinada usando fluoropirimidina com oxaliplatina (FOLFOX ou XELOX) ou irinotecano (FOLFIRI ou XELIRI) em combinação com o agente anti-VEGF (bevacizumab) ou agentes anti-EGFR (cetuximab ou panitumumab) para pacientes com os genes RAS e BRAF do tipo selvagem (ATREYA; YAEGER; CHU, 2017).

1.2 Obesidade

A obesidade é definida pela OMS como índice de massa corporal (Kg/m^2) acima de 30.0 Kg/m^2 (OBESITY, [*s. d.*]). Adotada pela Assembleia Mundial da Saúde em 2004 e reconhecida novamente em uma declaração política de 2011, A Estratégia Global de Dieta, Atividade Física e Saúde, foi elaborada pela OMS com o propósito de direcionar mudanças nas políticas públicas que forneçam acesso a alimentos saudáveis e ambientes seguros para atividade física em escolas, locais de trabalho e comunidades (GLOBAL STRATEGY ON DIET, PHYSICAL ACTIVITY AND HEALTH, [*s. d.*]; OBESITY AND OVERWEIGHT, [*s. d.*]). Entretanto, em 2016, mais de 1,9 bilhão de adultos tinham excesso de peso, correspondendo a 39% da população mundial, entre esses, mais de 650 milhões eram obesos (13%) (WHO, Obesity, 2021)(OBESITY, [*s. d.*]), o que mostra que a iniciativa da OMS não foi executada suficientemente para eliminar essa doença que é prevenível.

Níveis mais elevados de IMC estão relacionados com o aumento do risco de desenvolver CCR, com chances até 60% maiores, em comparação com indivíduos com peso normal; entretanto, os mecanismos que podem estar envolvidos nesta associação entre excesso de peso e CCR permanecem pouco esclarecidos (BIANCHINI; KAAKS; VAINIO, 2002; MA *et al.*, 2013) . Uma das possíveis vias seriam os adipócitos e pré-adipócitos, presentes na camada interna do intestino, que com o acúmulo de ácidos graxos acabam perdendo a função fisiológica de equilíbrio energético e metabolismo dos lipídios. Essa desordem leva resistência à insulina, responsável pela diabetes tipo 2, e também por prejudicar a resposta imune adaptativa, gerando um maior risco de desenvolver diversos tipos de câncer (CALLE *et al.*, 2003; KUSHI *et al.*, 2006; ROCK *et al.*, 2020).

Entre as alterações causadas no organismo estão o aumento de hormônios peptídicos, como leptina, resistina e insulina, fator de crescimento tumoral alfa (TNF-α) e dos marcadores de inflamação, como proteína C reativa (PCR), ligante da proteína CD40 (CD40L), e interleucina 1 (IL-1). Além disso ocorre a diminuição de adipocinas (BIANCHINI; KAAKS; VAINIO, 2002; MA *et al.*, 2013; TESAURO *et al.*, 2007).

A leptina tem seu nível sérico intimamente ligado à quantidade de tecido adiposo, e pode ser responsável pelo aumento dos níveis de fatores de crescimento em células de CCR, estimulando a proliferação destas (AMEMORI *et al.*, 2007).

A adiponectina, secretada pelas células adiposas, possui propriedades anti-inflamatórias e anti-aterogênicas e, em níveis baixos, pode contribuir para resistência à insulina e um estado pró-inflamatório (TESAURO *et al.*, 2007).

A disfunção metabólica gerada pela obesidade promove o aumento de citocinas pró-inflamatórias que fazem o recrutamento de células mieloides e linfoides para o tecido adiposo, gerando uma leucocitose no tecido (ANDERSEN; MURPHY; FERNANDEZ, 2016; GRANT, R. *et al.*, 2014; GRANT, R. W.; DIXIT, 2015). Evidências sugerem que há um mecanismo bidirecional, imunológico e metabólico, responsável por controlar o consumo de energia e gerar respostas imune inata e adaptativa de sucesso. Ao que parece, os receptores de reconhecimento de padrões que acreditava-se ser exclusivos para defesa do hospedeiro também respondem ao estresse metabólico e contribuem para a patogênese da doença na obesidade (KANNEGANTI; DIXIT, 2012) (Figura 3).

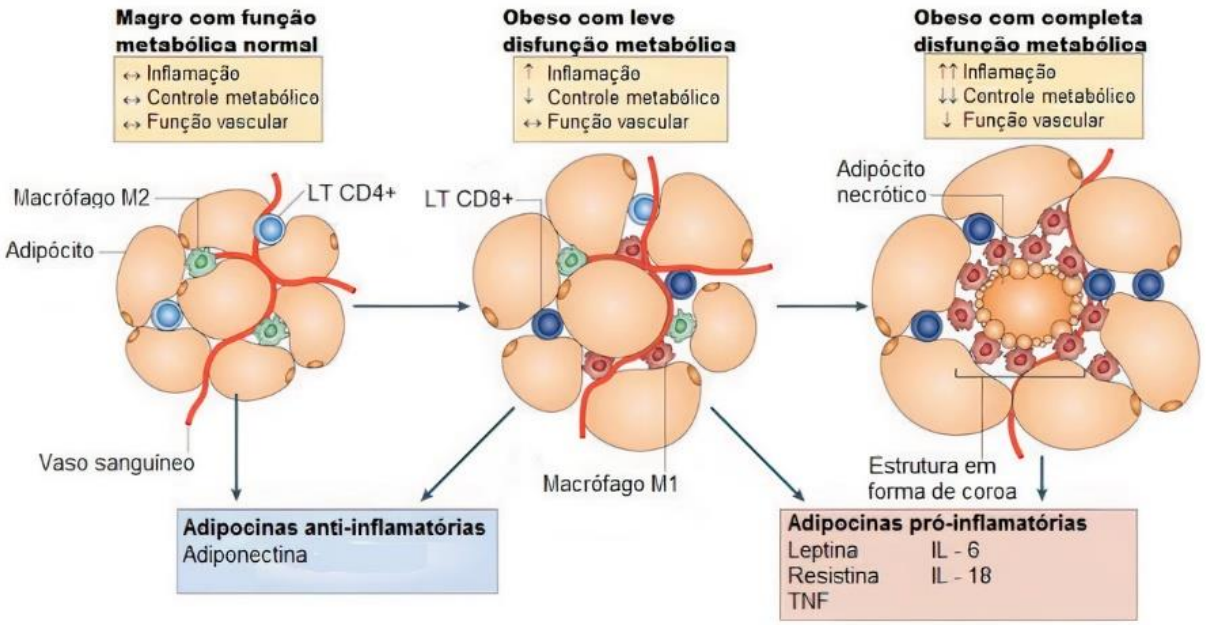


Figura 3 - Aumento do infiltrado imune no tecido adiposo de obeso. Fonte: ASSUMPÇÃO (2016).

Então, a relação entre a obesidade e o CCR se daria por uma soma de fatores, alterações no metabolismo hormonal (insulina), proteínas segregadas pelo tecido

adiposo (adipocinas) que contribuem para o regulamento de resposta imune (leptina), e resposta inflamatória (TNFα, IL-6), além de outros fatores como vasculatura, interações estromal, angiogênese (fator de crescimento endotelial vascular 1) e componentes da matriz extracelular (colágeno tipo VI) (RAJALA; SCHERER, 2003) (Figura 4). Nesse contexto, mais estudos sobre a associação da obesidade com CCR, levariam a maior apropriação do tema e maiores chances de sucesso nas intervenções.

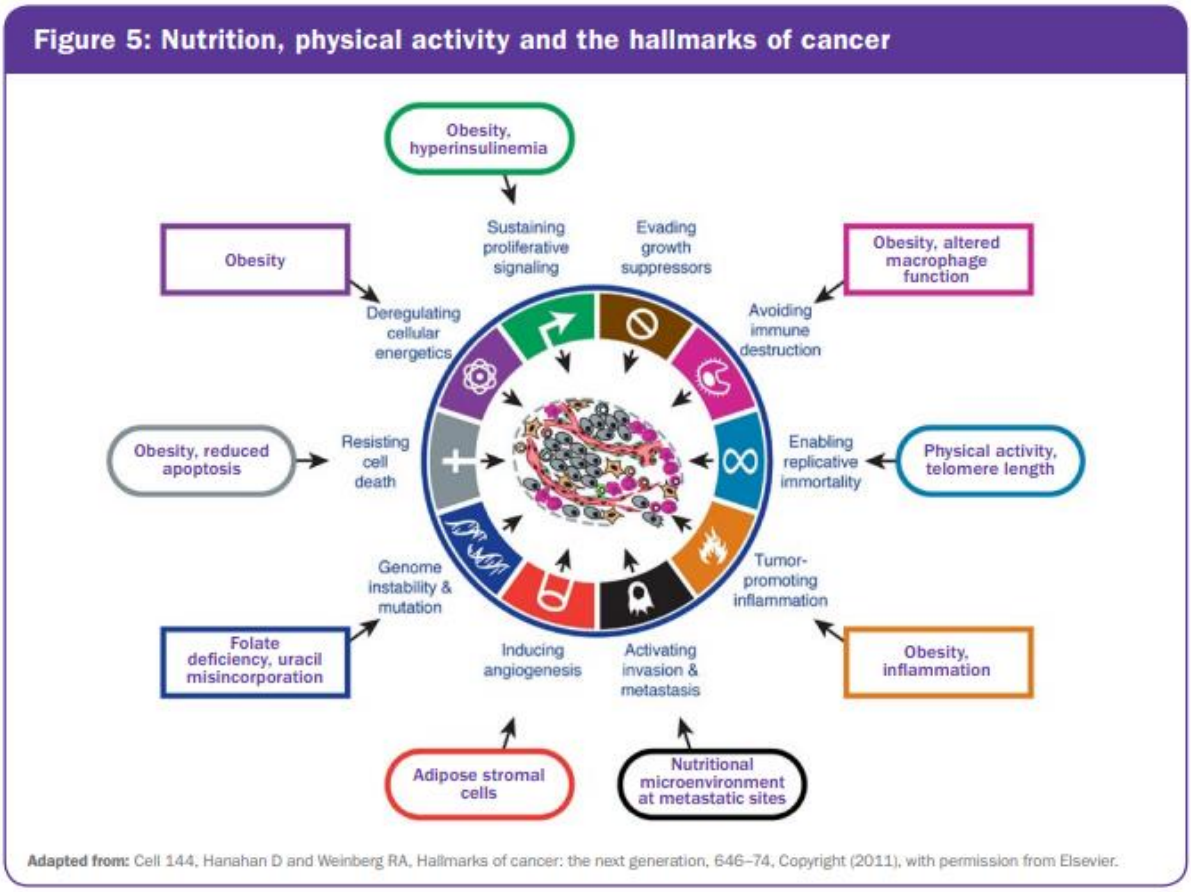


Figura 4 - Relação da obesidade como as vias de progressão do câncer. Fonte: World Cancer Research Fund / American Institute for Cancer Research. Diet, Nutrition, Physical Activity and Cancer: a Global Perspective, A summary of the Third Expert Report. 2018. Disponível em: Diet, Nutrition, Physical Activity and Cancer: a Global Perpective (wcrf.org). Acesso em: 24/09/2021.

1.3 O processo inflamatório e os macrófagos

As principais funções dos macrófagos, fisiologicamente, são fagocitose, endocitose, secreção de moléculas imunomoduladoras e proteção contra patógenos invasores. Os macrófagos podem ser células residentes no tecido ou serem recrutados dos monócitos da circulação sanguínea durante a inflamação (MANTOVANI *et al.*, 2004; MARTINEZ; GORDON, 2014). Quando ativados, possuem papéis de quimiotaxia, adesão, funções tróficas, interagem com linfócitos T e B na imunidade adaptativa e geram uma resposta mais aprimorada (MARTINEZ; GORDON, 2014).

Os macrófagos são orquestrados por um espectro de ativação, onde a polarização M1/clássica (pró-inflamatória) pode ser induzida por citocinas como o interferon-γ (IFN-γ) ou fator de necrose tumoral (TNF), sozinhos ou em conjunto com produtos microbianos, como lipopolissacarídeos (LPS) (MANTOVANI *et al.*, 2004). Tal população de células é caracterizada por alta capacidade de apresentação de antígeno; produção elevada de citocinas pró-inflamatórias, como IL-12, IL-6, IL-8, TNF, IL-1β e óxido nítrico (NO) e espécies reativas de oxigênio (ROS) (MANTOVANI *et al.*, 2004; MARTINEZ; GORDON, 2014; WADWA *et al.*, 2006). Quando polarizados, os macrófagos M1 participam do recrutamento de células Th1, Tc1 e NK, coordenando uma resposta imune tipo I ou clássica, que tem função de ataque a patógenos intracelulares e resistência tumoral (LOCATI; MANTOVANI; SICA, 2013).

Por outro lado, a polarização M2/alternativa (anti-inflamatória) é induzida pela IL-4, que é produzida pelas células Th2, eosinófilos, basófilos ou os próprios macrófagos (MARTINEZ; GORDON, 2014). Esse fenótipo é associado com diminuição da fagocitose, aumento da atividade da arginase, aumento da expressão de receptores de manose (CD204), aumento da liberação de citocinas anti-inflamatórias como IL-10, inibição da expressão de citocinas pró-inflamatórias levando a regeneração tecidual e resolução da inflamação, além disso, promove o recrutamento de células imunes como eosinófilos, basófilos, células Th2, Tregs e T virgens, e imunorregulação, pela produção de agonista de CCR3, CCR4, CCR8 e das citocinas CCL1, CCL16 e CCL18 (FIORENTINO; BOND; MOSMANN, 1989; MANTOVANI *et al.*, 2004) (Figuras 5 e 6).

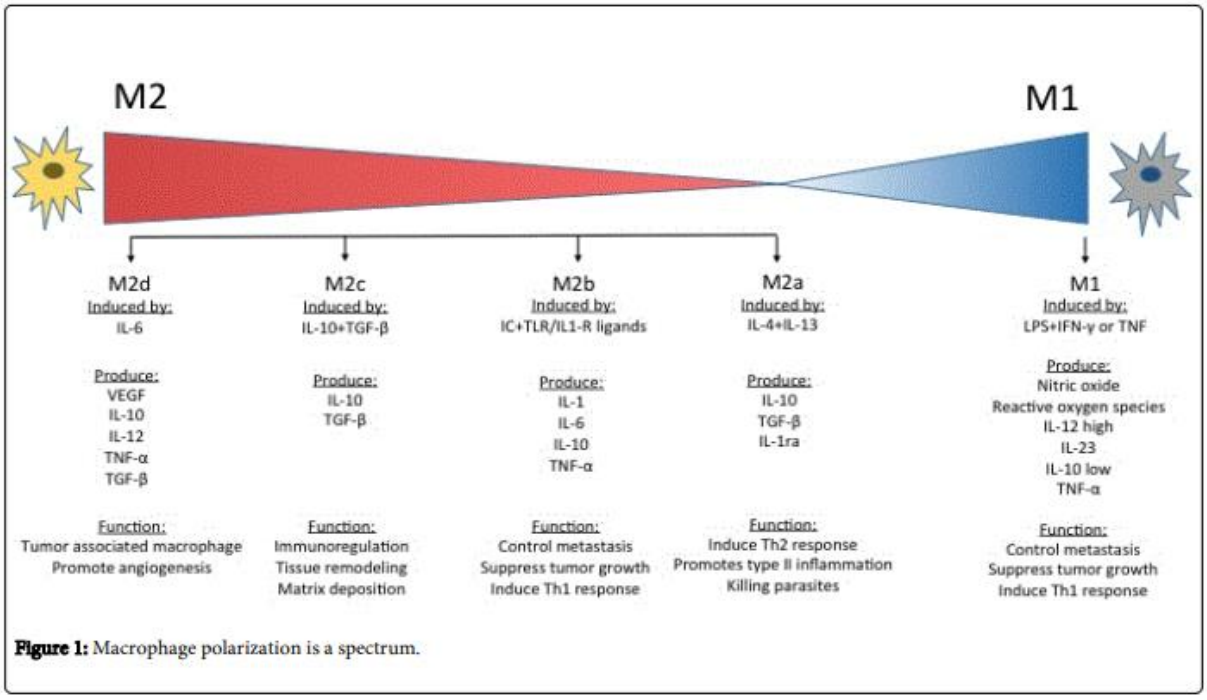


Figure 1: Macrophage polarization is a spectrum.

Figura 5 - Espectro de polarização dos macrófagos. Fonte: Weagel et al (2015).

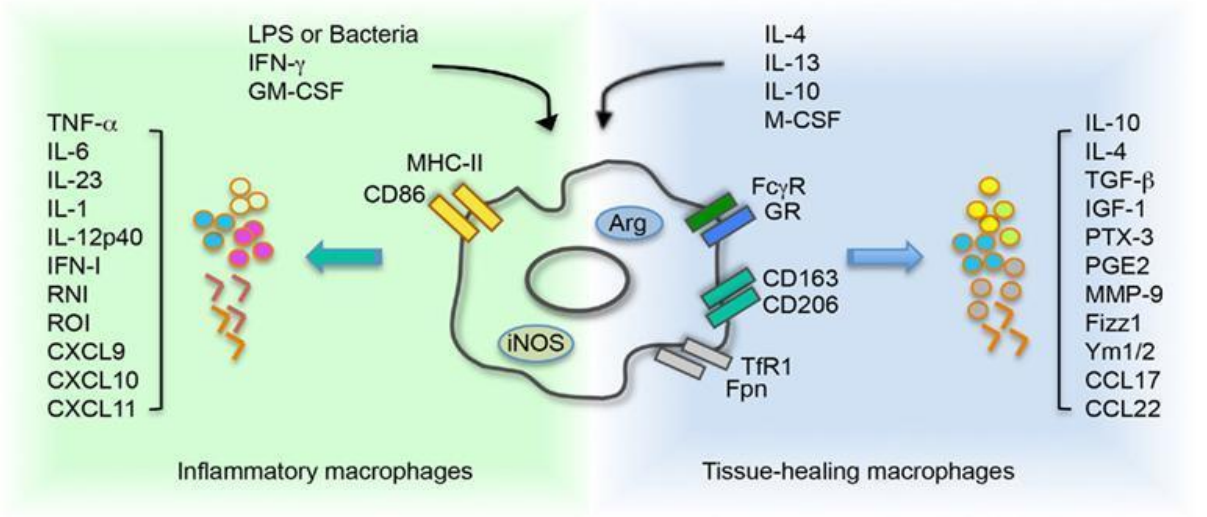


Figura 6 - Representação esquemática das características apresentadas pelos fenótipos M1 e M2 dos macrófagos. Fonte: Bosurgi et al (2011).

1.4 Macrófagos associados ao tumor (TAM)

No início do processo tumoral, ocorre o recrutamento dos macrófagos, buscando extinguir as células tumorais, entretanto, quando o tumor se estabelece,

esses macrófagos são “educados” para terem um comportamento pró-tumoral, passando a atuar como macrófagos-associados ao tumor (TAM) (BALKWILL, F. R.; MANTOVANI, 2012; POLLARD, 2004; QIAN; POLLARD, 2010). Os TAM podem apresentar características mistas de polarização M1 e M2, no entanto, com comportamento predominantemente imunossupressor (QIAN; POLLARD, 2010). A acidose local no tumor é responsável por inibir a produção de citocinas pró-inflamatórias, como interleucina 2 (IL-2) e interferon gama (INF-γ), produzidas pelas células T, e ainda gerar a morte dessas células a longo prazo, para gerar uma região anti-inflamatória, tipo Th2 (FISCHER *et al.*, 2007; NOY; POLLARD, 2014).

Atualmente, é evidente que a resposta inflamatória persistente gera um ambiente com potencial de iniciar o tumor, como no caso da doença de Crohn, que aumenta relativamente o risco do CCR (BALKWILL, F.; CHARLES; MANTOVANI, 2005; GRIVENNIKOV; GRETEN; KARIN, 2010). Essas células também produzem fatores de crescimento e/ou citocinas que favorecem o crescimento de células epiteliais, que acabam adquirindo mutações associadas à progressão do tumor. Por consequência, o recrutamento de mais células inflamatórias, resultando em um ciclo vicioso e aumentando a progressão do câncer (BALKWILL, F. R.; MANTOVANI, 2012; QIAN; POLLARD, 2010).

O ambiente Th2 é caracterizado pela presença do fator de crescimento transformador-β1 (TGF-β1) e Arginase 1, assim como um aumento do número de células T CD4 + (DENARDO *et al.*, 2009). Pode-se dizer, portanto, que para o tumor conseguir se desenvolver é preciso que modificações no microambiente favoreçam a polarização dos macrófagos residentes, ou dos monócitos recrutados, para o fenótipo pró-tumoral (NOY; POLLARD, 2014).

Acredita-se que os TAMs sejam originados dos monócitos recrutados da medula óssea. O fator estimulador de colônia 1 (CSF1) é o principal regulador da diferenciação da maioria das populações de macrófagos, e além disso, é um fator quimiotático (CHITU; STANLEY, 2006; SHAND *et al.*, 2014). Concentrações elevadas de CSF1 nos tumores estão associadas a um pior prognóstico, em contraste, o bloqueio de CSF1 leva a perda de características pró-tumorais associadas aos TAMs (MANTOVANI *et al.*, 2017). O VEGF-A também recruta progenitores de macrófagos que se diferenciam em TAMs sob influência de IL-4, provando que este também é um estimulador de TAMs (MANTOVANI *et al.*, 2017).

Os mecanismos pró-tumorais no microambiente ofertado pelos TAM são a criação de vasos sanguíneos que permitam a passagem de oxigênio e nutrientes ao tumor e a promoção de invasão. Tal passo é importante, uma vez que aumenta o número de células tumorais circulantes e, portanto, beneficia a formação de metástases (HANAHAN; WEINBERG, 2011; WYCKOFF *et al.*, 2007). Na sequência, ocorre imunossupressão que é pelo menos em parte mediada por macrófagos ou seus progenitores, e que também envolve células Treg, bem como a evasão imune mediada por células tumorais (COUSSENS; POLLARD, 2011; GAJEWSKI; SCHREIBER; FU, 2013; MOVAHEDI *et al.*, 2010). Os TAM expressam IL-10 e TGF-β, citocinas envolvidas na modulação das funções das células T, onde o TGF-β inibe as funções citotóxicas, enquanto a IL-10 inibe as funções auxiliares (BROWN; TROWSDALE; ALLEN, 2004; NG *et al.*, 2013; OH; LI, 2013) (Figura 7).

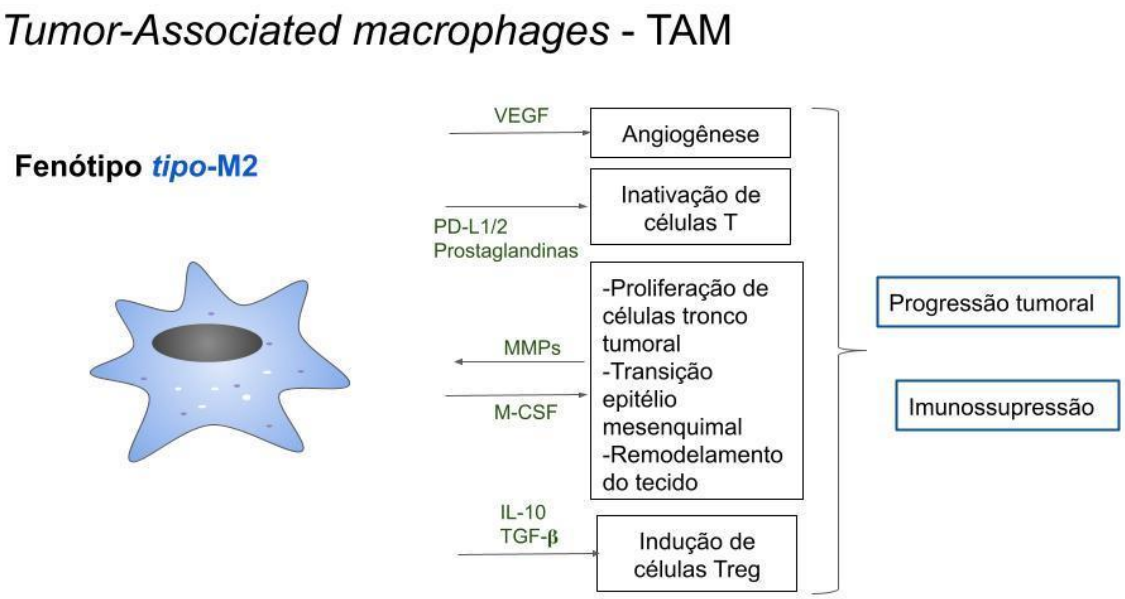


Figura 7 - Representação esquemática das características dos macrófagos associados ao tumor (TAM), conforme informações extraídas de Gonzalez et al, 2018. Fonte: adaptado de Gonzalez H, Hagerling C, Werb Z (2018).

Porém, no CCR este processo ainda é controverso. Zhang et al demonstra que os TAM favorecem a resistência ao quimioterápico 5-FU (ZHANG *et al.*, 2016), já Malesci et al sugere que a alta densidade dos TAM melhora a resposta à quimioterapia no estágio III da doença (MALESCI *et al.*, 2017). Dados que indicam a necessidade da continuidade da pesquisa com macrófagos e o microambiente no CCR.

Conjuntamente às funções das células imunes no microambiente tumoral (TME) existem alterações no sistema purinérgico. Os nucleotídeos e nucleosídeos são liberados para o TME e modulam o comportamento das células imunes. Neste contexto, moléculas como ATP e adenosina, em níveis patológicos, favorecem a anergia do sistema imune no combate a essa patologia levando a progressão tumoral (BURNSTOCK, Geoffrey; DI VIRGILIO, 2013).

1.5 Sistema Purinérgico

O processo imune/inflamatório é regulado por uma série de mecanismos, entre os quais está incluído o sistema purinérgico. Acreditava-se anteriormente que o 5'-trifosfato de adenosina (ATP) liberado pelas células era somente consequência de danos sofridos por essas, entretanto, agora há um conceito bem estabelecido de que muitos tipos de células liberam ATP fisiologicamente e que o mesmo atua como sinalizador via sensibilização de purinoreceptores (BODIN; BURNSTOCK, 2001; BURNSTOCK, Geoffrey; DI VIRGILIO, 2013). De fato, os nucleotídeos e nucleosídeos extracelulares desempenham funções importantes relacionadas à regulação do sistema imune/inflamação, neurotransmissão, proliferação/diferenciação/morte celular, motilidade, regeneração, envelhecimento e câncer (ABBRACCHIO; BURNSTOCK, 1998; BRAGANHOL *et al.*, 2015).

Os nucleotídeos responsáveis pela sinalização purinérgica, como o ATP e o 5'-trifosfato de uridina (UTP), podem ser liberados nos fluidos extracelulares através do dano celular e morte, onde as células necróticas e apoptóticas liberam ATP representando "sinais de perigo" ou padrão molecular associado ao dano (DAMP) (DI VIRGILIO; FERRARI; ADINOLFI, 2009; ELLIOTT *et al.*, 2009; IDZKO *et al.*, 2007). Além disso, esses nucleotídeos também podem ser liberados de maneira ativa em resposta a vários tipos de estresse, como estimulação mecânica, hipóxia ou em resposta ao padrão molecular associado a patógenos (PAMP) (HOMOLYA; STEINBERG; BOUCHER, 2000; LAZAROWSKI; BOUCHER; HARDEN, 2003; SÉROR *et al.*, 2011).

Quando se encontra no meio extracelular, o ATP é hidrolisado por uma família de enzimas chamadas de ectonucleotidases. As ectonucleosídeo trifosfato difosfoidrolases (ENTPDases), exemplo NTPDase1/CD39), as ecto-pirofosfato-fosfodiesterases (E-NPP) e fosfatases alcalinas (ALPs) são enzimas pertencente a via adenosinérgica canônica, que degradam o ATP em adenosina 5'-difosfato (ADP) e posteriormente em adenosina monofosfato (AMP). Enquanto que a CD73/ecto-5'-nucleotidase converte o AMP em adenosina, e por fim a adenosina desaminase (ADA) gera inosina (BURNSTOCK, G., 2007). No microambiente intracelular, a adenosina é fosforilada por ADO quinase (AdoK) e adenilato quinases em ADP (AZAMBUJA *et al.*, 2019). A adenosina também pode ser gerada pela via adenosinérgica não canônica por nicotinamida adenina dinucleotídeo (NAD +) - glicohidrolase / CD38 que é subsequentemente metabolizado por CD73 em adenosina, a CD73 representa ligação comum entre as duas vias adenosinérgicas (BAGHERI; SABOURY; HAERTLÉ, 2019; FERRETTI *et al.*, 2019).

Os receptores para nucleotídeos extracelulares e seus produtos de degradação, como a adenosina, são caracterizados da seguinte maneira: uma divisão dos receptores purinérgicos entre P1 (adenosina) e P2 (nucleosídeos di- e trifosfato). Os subtipos de receptores para purinas e pirimidinas são diversos, constituindo atualmente 4 subtipos do receptor P1 (adenosina) (A1, A2A, A2B e A3), 7 subtipos de receptores de canais de íons P2X (P2X1-P2X7) e 8 subtipos de receptores acoplados a proteína G P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$) (BURNSTOCK, G., 2007) (Figura 8).

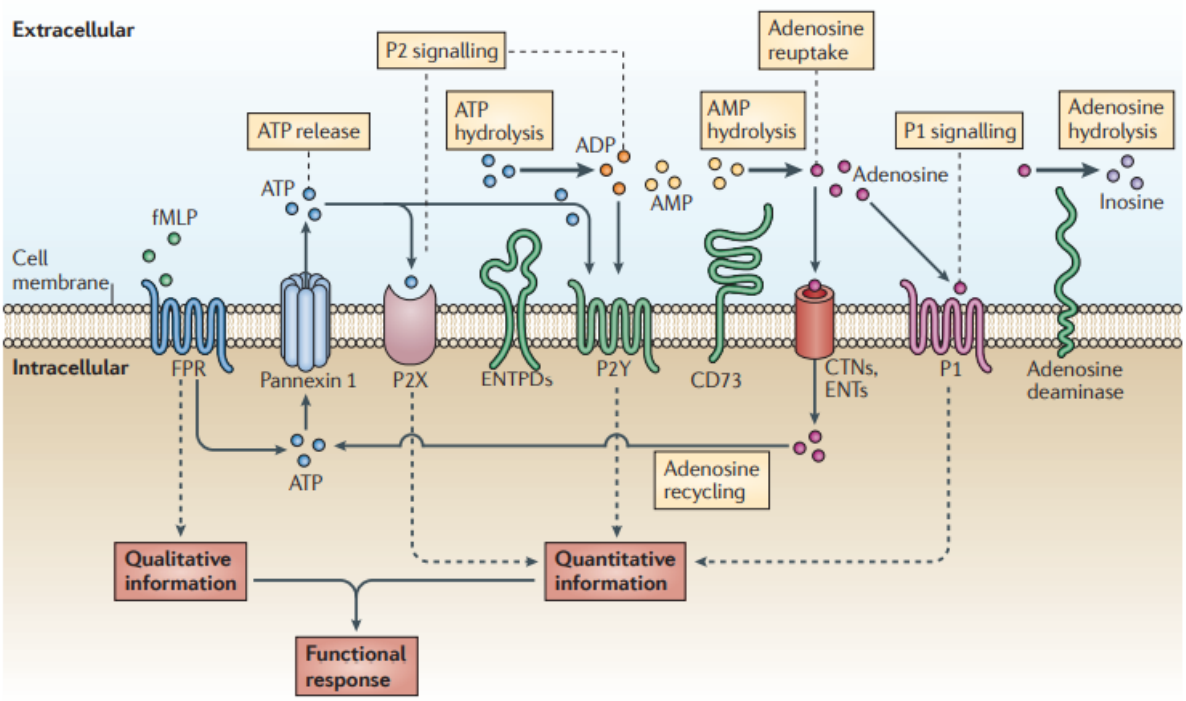


Figura 8 - Componentes da sinalização purinérgica, via adenosinérgica canônica. Fonte: Junger W (2011).

1.6 Sistema Purinérgico no Câncer

No câncer, novas evidências demonstram um papel crucial do sistema purinérgico alterado, é notável a expressão aumentada de CD73, enzima que catabolisa AMP a adenosina. A adenosina auxilia no início e na progressão do câncer por gerar um microambiente imunossupressor e angiogênico. Além disso também participa da formação de metástases (ALLARD *et al.*, 2014; ALLARD; ALLARD; STAGG, 2016; ANTONIOLI *et al.*, 2013a, 2013b; YEGUTKIN, 2014). Já o ATP está relacionado ao recrutamento de monócitos aos sítios alvos e a estimulação dos macrófagos a produzir mediadores inflamatórios (VENTURA; THOMOPOULOS, 1995) (Figura 8). Os aspectos do sistema purinérgico relacionados à promoção do CCR serão melhores apresentados no capítulo 3 desta dissertação.

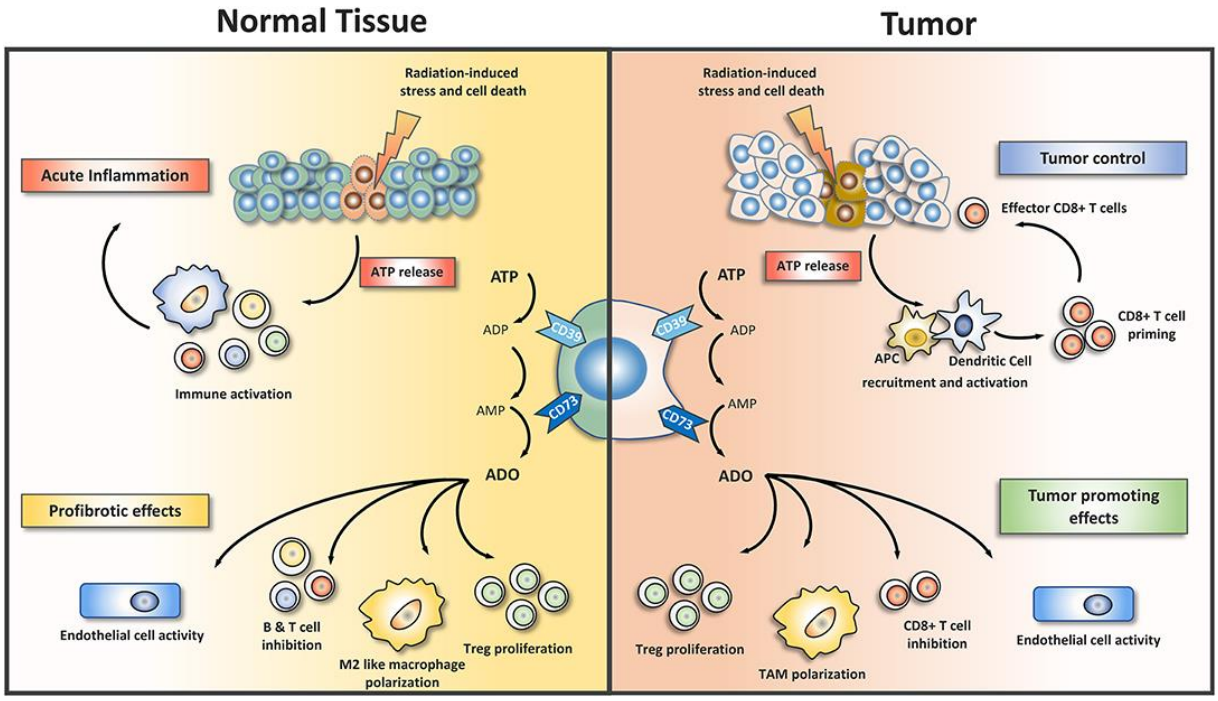


Figura 8 - Sinalização purinérgica no contexto fisiológico e tumoral. Fonte: de Leve S, Wirsdörfer F, Jendrossek V (2019).

Fica claro que, desvendar os mecanismos celulares e moleculares envolvidos nesse processo complexo que é o câncer e as alterações que o cercam, é de extrema importância, pois permitirá uma intervenção terapêutica mais eficiente, objetivando melhorar o prognóstico dos pacientes.

2 HIPÓTESE DO ESTUDO

Muitas evidências conectam CCR, obesidade e inflamação, por sua vez, o sistema de sinalização purinérgica tem sido visto como um elemento essencial na resposta imune/inflamatória e na progressão tumoral. Apesar disso, a participação das células do sistema imune inato, mais especificamente dos macrófagos, e da sinalização purinérgica no processo de malignidade do CCR ainda é pouco conhecida. Considerando que as células tumorais moldam o comportamento dos macrófagos a contribuir para o crescimento tumoral, e que o estado de inflamação local possui impacto sobre a disfunção imune/inflamatória, nossa hipótese é que a comunicação entre células tumorais e os macrófagos no microambiente tumoral poderiam ter como principais consequências: 1) a fuga do sistema imune de vigilância, devido a ativação crônica gerada pelo excesso de tecido adiposo, levando ao início do estabelecimento do tumor, (2) a produção de adenosina que neutraliza o ataque ao tumor pelos linfócitos T citotóxicos ou pelas NK (natural killer), resultando em sobrevivência tumoral e (3) a liberação desses fatores para a circulação modula o fenótipo das células imunes circulantes, as quais favorecem a formação do nicho pré-metastático no tecido-alvo. Existem mecanismos ainda não conhecidos que geram esse ambiente enriquecido em fatores imunossupressores. A elucidação dessas vias pode ser importante para melhor compreender os mecanismos de progressão do CCR e, assim, criar estratégias terapêuticas para contornar o manejo clínico desses pacientes. TME dos obesos no CCR

3 JUSTIFICATIVA

O presente estudo se justifica pelo grande número de casos de câncer de intestino, no Brasil e no mundo nas últimas décadas. Os fatores de risco para esse câncer têm aumentado em muitos países em desenvolvimento devido ao estilo de vida pouco saudável predominantemente adquirido. A obesidade também apresenta números crescentes, e é preocupante por estar associada a muitas doenças. O estudo da relação entre obesidade com o câncer colorretal e as alterações causadas no organismo ganharam importância no cenário da saúde pública. O sistema imunológico possui os componentes da sinalização purinérgica, mecanismo capaz de diminuir a resposta imune ao tumor, aumentando o potencial de malignidade e controlar diretamente a progressão tumoral. Nossa motivação partiu de trabalhos anteriores realizados pelo nosso grupo de pesquisa, que avaliaram a liberação de citocinas pró e anti-inflamatórias pelos macrófagos e a expressão de enzimas e receptores purinérgicos no glioblastoma. Acreditamos que as células imunes estão sendo direcionadas para o fenótipo pró-tumoral, entre outros fatores, pelo sistema purinérgico. A pesquisa procurou associação entre as citocinas pró- e anti-inflamatórias no estado de obesidade e a alteração imune local; a ativação dos macrófagos e a expressão de enzimas e receptores purinérgicos com o CCR. Esperamos contribuir para a descoberta de novas hipóteses de intervenções. Além disso, nossas conclusões podem ser usadas como bases para outros estudos.

4 OBJETIVOS

4.1 Objetivo geral

- Investigar a participação do sistema imune/inflamatório na progressão do câncer colorretal.

4.2 Objetivos específicos:

- Revisar os estudos sobre sinalização purinérgica e câncer colorretal.

- Investigar o efeito de fatores séricos liberados por indivíduos obesos sobre a viabilidade, proliferação e migração de células de câncer colorretal (Caco-2 e HCT116) *in vitro*.

- Avaliar a polarização de cultura primária de macrófagos humanos co-cultivados com células tumorais de CCR (linhagem HCT116).

- Efetuar um rastreamento de citocinas e mediadores inflamatórios alterados após o cultivo de macrófagos humanos com linhagens de CCR.

5 REFERÊNCIAS

ABBRACCHIO, M P; BURNSTOCK, G. Purinergic signalling: pathophysiological roles. **Japanese journal of pharmacology**, [*s. l.*], v. 78, n. 2, p. 113–145, 1998. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/9829617. Acesso em: 4 dez. 2018.

ALLARD, Bertrand *et al.* Anti-CD73 therapy impairs tumor angiogenesis. **International Journal of Cancer**, [*s. l.*], v. 134, n. 6, p. 1466–1473, 2014. Disponível em: https://doi.org/10.1002/ijc.28456. Acesso em: 4 dez. 2018.

ALLARD, Bertrand; ALLARD, David; STAGG, John. Methods to Evaluate the Antitumor Activity of Immune Checkpoint Inhibitors in Preclinical Studies. *In*: METHODS IN MOLECULAR BIOLOGY (CLIFTON, N.J.). [*S. l.: s. n.*], 2016. v. 1458, p. 159–177. Disponível em: https://doi.org/10.1007/978-1-4939-3801-8_12. Acesso em: 4 dez. 2018.

AMEMORI, S *et al.* Adipocytes and preadipocytes promote the proliferation of colon cancer cells in vitro. **American journal of physiology. Gastrointestinal and liver physiology**, [*s. l.*], v. 292, n. 3, 2007. Disponível em: https://doi.org/10.1152/AJPGI.00145.2006. Acesso em: 25 set. 2021.

ANDERSEN, Catherine J; MURPHY, Kelsey E; FERNANDEZ, Maria Luz. Impact of Obesity and Metabolic Syndrome on Immunity. **Advances in Nutrition**, [*s. l.*], v. 7, n. 1, p. 66, 2016. Disponível em: https://doi.org/10.3945/AN.115.010207. Acesso em: 25 set. 2021.

ANTONIOLI, Luca *et al.* CD39 and CD73 in immunity and inflammation. **Trends in Molecular Medicine**, [*s. l.*], v. 19, n. 6, p. 355–367, 2013a. Disponível em: https://doi.org/10.1016/j.molmed.2013.03.005. Acesso em: 4 dez. 2018.

ANTONIOLI, Luca *et al.* Immunity, inflammation and cancer: a leading role for adenosine. **Nature Reviews Cancer**, [*s. l.*], v. 13, n. 12, p. 842–857, 2013b. Disponível em: https://doi.org/10.1038/nrc3613. Acesso em: 4 dez. 2018.

ATREYA, Chloe E.; YAEGER, Rona; CHU, Edward. Systemic Therapy for Metastatic Colorectal Cancer: From Current Standards to Future Molecular Targeted Approaches. **American Society of Clinical Oncology Educational Book**, [*s. l.*], v. 37, p. 246–256, 2017. Disponível em: https://doi.org/10.14694/edbk_175679

AZAMBUJA, Juliana Hofstätter *et al.* Inhibition of the Adenosinergic Pathway in Cancer Rejuvenates Innate and Adaptive Immunity. **International Journal of Molecular Sciences**, [*s. l.*], v. 20, n. 22, p. 5698, 2019. Disponível em: https://doi.org/10.3390/ijms20225698. Acesso em: 21 nov. 2019.

BAGHERI, S; SABOURY, AA; HAERTLÉ, T. Adenosine deaminase inhibition. **International journal of biological macromolecules**, [*s. l.*], v. 141, p. 1246–1257, 2019. Disponível em: https://doi.org/10.1016/J.IJBIOMAC.2019.09.078. Acesso em: 6 out. 2021.

BALKWILL, Frances; CHARLES, Kellie A.; MANTOVANI, Alberto. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. **Cancer Cell**, [*s. l.*], v. 7, n. 3, p. 211–217, 2005. Disponível em: https://doi.org/10.1016/j.ccr.2005.02.013. Acesso em: 4 dez. 2018.

BALKWILL, Frances R.; MANTOVANI, Alberto. Cancer-related inflammation: Common themes and therapeutic opportunities. **Seminars in Cancer Biology**, [*s. l.*], v. 22, n. 1, p. 33–40, 2012. Disponível em: https://doi.org/10.1016/j.semcancer.2011.12.005

BARDOU, Marc; BARKUN, Alan N; MARTEL, Myriam. Obesity and colorectal cancer. **Gut**, [*s. l.*], v. 62, n. 6, p. 933–947, 2013. Disponível em: https://doi.org/10.1136/GUTJNL-2013-304701. Acesso em: 24 set. 2021.

BIANCHINI, France; KAAKS, Rudolf; VAINIO, Harri. Overweight, obesity, and cancer risk. **The Lancet. Oncology**, [*s. l.*], v. 3, n. 9, p. 565–574, 2002. Disponível em: https://doi.org/10.1016/S1470-2045(02)00849-5. Acesso em: 8 out. 2021.

BODIN, P; BURNSTOCK, G. Purinergic signalling: ATP release. **Neurochemical research**, [*s. l.*], v. 26, n. 8–9, p. 959–969, 2001. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/11699948. Acesso em: 4 dez. 2018.

BRAGANHOL, Elizandra *et al.* Nucleotide receptors control IL-8/CXCL8 and MCP-1/CCL2 secretions as well as proliferation in human glioma cells. **Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease**, [*s. l.*], v. 1852, n. 1, p. 120–130, 2015. Disponível em: https://doi.org/10.1016/j.bbadis.2014.10.014. Acesso em: 4 dez. 2018.

BRASIL. MINISTÉRIO DA SAÚDE. Diretrizes diagnósticas e terapêuticas em oncologia - carcinoma colorretal. [*s. l.*], 2014.

BROWN, D.; TROWSDALE, J.; ALLEN, R. The LILR family: modulators of innate and adaptive immune pathways in health and disease. **Tissue Antigens**, [*s. l.*], v. 64, n. 3, p. 215–225, 2004. Disponível em: https://doi.org/10.1111/j.0001-2815.2004.00290.x. Acesso em: 4 dez. 2018.

BURNSTOCK, G. Purine and pyrimidine receptors. **Cellular and Molecular Life Sciences 2007 64:12**, [*s. l.*], v. 64, n. 12, p. 1471–1483, 2007. Disponível em: https://doi.org/10.1007/S00018-007-6497-0. Acesso em: 26 set. 2021.

BURNSTOCK, Geoffrey; DI VIRGILIO, Francesco. Purinergic signalling and cancer. **Purinergic Signalling**, [*s. l.*], v. 9, n. 4, p. 491–540, 2013. Disponível em: https://doi.org/10.1007/s11302-013-9372-5. Acesso em: 4 dez. 2018.

BYE, William A *et al.* Strategies for detecting colon cancer in patients with inflammatory bowel disease. **The Cochrane Database of Systematic Reviews**, [*s. l.*], v. 2017, n. 9, 2017. Disponível em: https://doi.org/10.1002/14651858.CD000279.PUB4. Acesso em: 24 set. 2021.

CALLE, Eugenia E *et al.* Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. **The New England journal of medicine**, [*s. l.*], v. 348, n. 17, p. 1625–1638, 2003. Disponível em: https://doi.org/10.1056/NEJMOA021423. Acesso em: 29 set. 2021.

CHITU, Violeta; STANLEY, E Richard. Colony-stimulating factor-1 in immunity and inflammation. **Current Opinion in Immunology**, [*s. l.*], v. 18, n. 1, p. 39–48, 2006. Disponível em: https://doi.org/10.1016/j.coi.2005.11.006. Acesso em: 4 dez. 2018.

COUSSENS, L. M.; POLLARD, J. W. Leukocytes in Mammary Development and Cancer. **Cold Spring Harbor Perspectives in Biology**, [*s. l.*], v. 3, n. 3, p. a003285–

a003285, 2011. Disponível em: https://doi.org/10.1101/cshperspect.a003285. Acesso em: 4 dez. 2018.

CREMOLINI, Chiara *et al.* FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment of patients with metastatic colorectal cancer: updated overall survival and molecular subgroup analyses of the open-label, phase 3 TRIBE study. **The Lancet Oncology**, [*s. l.*], v. 16, n. 13, p. 1306–1315, 2015. Disponível em: https://doi.org/10.1016/S1470-2045(15)00122-9. Acesso em: 25 set. 2021.

DENARDO, David G. *et al.* CD4+ T Cells Regulate Pulmonary Metastasis of Mammary Carcinomas by Enhancing Protumor Properties of Macrophages. **Cancer Cell**, [*s. l.*], v. 16, n. 2, p. 91–102, 2009. Disponível em: https://doi.org/10.1016/j.ccr.2009.06.018. Acesso em: 4 dez. 2018.

DI VIRGILIO, Francesco; FERRARI, Davide; ADINOLFI, Elena. P2X7: A growth-promoting receptor - Implications for cancer. **Purinergic Signalling**, [*s. l.*], v. 5, n. 2, p. 251–256, 2009. Disponível em: https://doi.org/10.1007/s11302-009-9145-3

EDGE, Stephen B.; COMPTON, Carolyn C. The american joint committee on cancer: The 7th edition of the AJCC cancer staging manual and the future of TNM. **Annals of Surgical Oncology**, [*s. l.*], v. 17, n. 6, p. 1471–1474, 2010. Disponível em: https://doi.org/10.1245/s10434-010-0985-4

ELLIOTT, Michael R. *et al.* Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. **Nature**, [*s. l.*], v. 461, n. 7261, p. 282–286, 2009. Disponível em: https://doi.org/10.1038/nature08296. Acesso em: 4 dez. 2018.

FEDIRKO, V. *et al.* Alcohol drinking and colorectal cancer risk: an overall and dose-response meta-analysis of published studies. **Annals of Oncology**, [*s. l.*], v. 22, n. 9, p. 1958–1972, 2011. Disponível em: https://doi.org/10.1093/annonc/mdq653. Acesso em: 8 nov. 2018.

FERRETTI, E *et al.* Canonical and non-canonical adenosinergic pathways. **Immunology letters**, [*s. l.*], v. 205, p. 25–30, 2019. Disponível em: https://doi.org/10.1016/J.IMLET.2018.03.007. Acesso em: 10 out. 2021.

FIORENTINO, David F.; BOND, Martha W.; MOSMANN, Tim R. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. **The Journal of Experimental Medicine**, [*s. l.*], v. 170, n. 6, p. 2081, 1989. Disponível em: https://doi.org/10.1084/JEM.170.6.2081. Acesso em: 25 set. 2021.

FISCHER, Karin *et al.* Inhibitory effect of tumor cell-derived lactic acid on human T cells. **Blood**, [*s. l.*], v. 109, n. 9, p. 3812–3819, 2007. Disponível em: https://doi.org/10.1182/blood-2006-07-035972

GAJEWSKI, Thomas F; SCHREIBER, Hans; FU, Yang-Xin. Innate and adaptive immune cells in the tumor microenvironment. **Nature Immunology**, [*s. l.*], v. 14, n. 10, p. 1014–1022, 2013. Disponível em: https://doi.org/10.1038/ni.2703. Acesso em: 4 dez. 2018.

GLOBAL STRATEGY ON DIET, PHYSICAL ACTIVITY AND HEALTH. [*s. l.*],

GRANT, Ryan *et al.* Inactivation of C/ebp homologous protein-driven immune-metabolic interactions exacerbate obesity and adipose tissue leukocytosis. **The**

Journal of biological chemistry, [*s. l.*], v. 289, n. 20, p. 14045–14055, 2014. Disponível em: https://doi.org/10.1074/JBC.M113.545921. Acesso em: 25 set. 2021.

GRANT, Ryan W.; DIXIT, Vishwa Deep. Adipose tissue as an immunological organ. **Obesity (Silver Spring, Md.)**, [*s. l.*], v. 23, n. 3, p. 512, 2015. Disponível em: https://doi.org/10.1002/OBY.21003. Acesso em: 25 set. 2021.

GRIVENNIKOV, Sergei I.; GRETEN, Florian R.; KARIN, Michael. Immunity, Inflammation, and Cancer. **Cell**, [*s. l.*], v. 140, n. 6, p. 883–899, 2010. Disponível em: https://doi.org/10.1016/j.cell.2010.01.025. Acesso em: 4 dez. 2018.

GUPTA, Gaorav P.; MASSAGUÉ, Joan. Cancer Metastasis: Building a Framework. **Cell**, [*s. l.*], v. 127, n. 4, p. 679–695, 2006. Disponível em: https://doi.org/10.1016/J.CELL.2006.11.001

HABR-GAMA, Angelita. Câncer coloretal: a importância de sua prevenção. **Arquivos de Gastroenterologia**, [*s. l.*], v. 42, n. 1, p. 2–3, 2005. Disponível em: https://doi.org/10.1590/S0004-28032005000100002. Acesso em: 24 set. 2021.

HANAHAN, Douglas; WEINBERG, Robert A. Hallmarks of Cancer: The Next Generation. **Cell**, [*s. l.*], v. 144, n. 5, p. 646–674, 2011. Disponível em: https://doi.org/10.1016/j.cell.2011.02.013. Acesso em: 4 dez. 2018.

HOMOLYA, L; STEINBERG, T H; BOUCHER, R C. Cell to cell communication in response to mechanical stress via bilateral release of ATP and UTP in polarized epithelia. **The Journal of cell biology**, [*s. l.*], v. 150, n. 6, p. 1349–1360, 2000. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/10995440. Acesso em: 4 dez. 2018.

IDZKO, Marco *et al.* Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. **Nature Medicine**, [*s. l.*], v. 13, n. 8, p. 913–919, 2007. Disponível em: https://doi.org/10.1038/nm1617. Acesso em: 4 dez. 2018.

INCA. **Colorretal**. [*S. l.*], 2018. Disponível em: http://www2.inca.gov.br/wps/wcm/connect/tiposdecancer/site/home/colorretal. Acesso em: 6 nov. 2018.

ITZKOWITZ, Steven H.; HARPAZ, Noam. Diagnosis and management of dysplasia in patients with inflammatory bowel diseases. **Gastroenterology**, [*s. l.*], v. 126, n. 6, p. 1634–1648, 2004. Disponível em: https://doi.org/10.1053/j.gastro.2004.03.025

ITZKOWITZ, Steven H.; YIO, X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. **American journal of physiology. Gastrointestinal and liver physiology**, [*s. l.*], v. 287, n. 1, 2004. Disponível em: https://doi.org/10.1152/AJPGI.00079.2004. Acesso em: 25 set. 2021.

JESS, Tine; RUNGOE, Christine; PEYRIN-BIROULET, Laurent. Risk of Colorectal Cancer in Patients With Ulcerative Colitis: A Meta-analysis of Population-Based Cohort Studies BACKGROUND & AIMS: Patients with ulcerative colitis. [*s. l.*], 2012. Disponível em: https://doi.org/10.1016/j.cgh.2012.01.010. Acesso em: 8 nov. 2018.

KANNEGANTI, Thirumala-Devi; DIXIT, Vishwa Deep. Immunological complications of obesity. **Nature Immunology**, [*s. l.*], v. 13, n. 8, p. 707–712, 2012. Disponível em: http://dx.doi.org/10.1038/ni.2343

KRIJGER *et al.* MicroRNAs in colorectal cancer metastasis. **The Journal of pathology**, [*s. l.*], v. 224, n. 4, p. 438–447, 2011. Disponível em: https://doi.org/10.1002/PATH.2922. Acesso em: 25 set. 2021.

KUSHI, Lawrence H. *et al.* American Cancer Society Guidelines on Nutrition and Physical Activity for cancer prevention: reducing the risk of cancer with healthy food choices and physical activity. **CA: a cancer journal for clinicians**, [*s. l.*], v. 56, n. 5, p. 254–281, 2006. Disponível em: https://doi.org/10.3322/CANJCLIN.56.5.254. Acesso em: 25 set. 2021.

LAZAROWSKI, Eduardo R.; BOUCHER, Richard C.; HARDEN, T. Kendall. Mechanisms of Release of Nucleotides and Integration of Their Action as P2X- and P2Y-Receptor Activating Molecules. **Molecular Pharmacology**, [*s. l.*], v. 64, n. 4, p. 785–795, 2003. Disponível em: https://doi.org/10.1124/MOL.64.4.785. Acesso em: 26 set. 2021.

LIMSUI, David *et al.* Cigarette smoking and colorectal cancer risk by molecularly defined subtypes. **Journal of the National Cancer Institute**, [*s. l.*], 2010. Disponível em: https://doi.org/10.1093/jnci/djq201

LOCATI, Massimo; MANTOVANI, Alberto; SICA, Antonio. Macrophage Activation and Polarization as an Adaptive Component of Innate Immunity. **Advances in Immunology**, [*s. l.*], v. 120, p. 163–184, 2013. Disponível em: https://doi.org/10.1016/B978-0-12-417028-5.00006-5

MA, Yang *et al.* Dendritic cells in the cancer microenvironment. **Journal of Cancer**, [*s. l.*], v. 4, n. 1, p. 36–44, 2013. Disponível em: https://doi.org/10.7150/JCA.5046

MALESCI, A *et al.* Tumor-associated macrophages and response to 5-fluorouracil adjuvant therapy in stage III colorectal cancer. **Oncoimmunology**, [*s. l.*], v. 6, n. 12, 2017. Disponível em: https://doi.org/10.1080/2162402X.2017.1342918. Acesso em: 26 set. 2021.

MANTOVANI, A *et al.* The chemokine system in diverse forms of macrophage activation and polarization. **Trends in Immunology**, [*s. l.*], v. 25, n. 12, p. 677–686, 2004. Disponível em: https://doi.org/10.1016/j.it.2004.09.015. Acesso em: 4 dez. 2018.

MANTOVANI, Alberto *et al.* Tumour-associated macrophages as treatment targets in oncology. **Nature reviews. Clinical oncology**, [*s. l.*], v. 14, n. 7, p. 399–416, 2017. Disponível em: https://doi.org/10.1038/nrclinonc.2016.217. Acesso em: 4 dez. 2018.

MARTINEZ, Fernando O; GORDON, Siamon. The M1 and M2 paradigm of macrophage activation: time for reassessment. **F1000prime reports**, [*s. l.*], v. 6, p. 13, 2014. Disponível em: https://doi.org/10.12703/P6-13. Acesso em: 4 dez. 2018.

MILLER, Kimberly D *et al.* Cancer treatment and survivorship statistics, 2019. **CA: a cancer journal for clinicians**, [*s. l.*], v. 69, n. 5, p. 363–385, 2019. Disponível em: https://doi.org/10.3322/CAAC.21565. Acesso em: 8 out. 2021.

MOVAHEDI, Kiavash *et al.* Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. **Cancer Research**, [*s. l.*], v. 70, n. 14, p. 5728–5739, 2010. Disponível em: https://doi.org/10.1158/0008-5472.CAN-09-4672

MUGHINI-GRAS, Lapo *et al.* Increased colon cancer risk after severe Salmonella infection. **PloS one**, [*s. l.*], v. 13, n. 1, 2018. Disponível em: https://doi.org/10.1371/JOURNAL.PONE.0189721. Acesso em: 24 set. 2021.

NG, T. H. Sky *et al.* Regulation of Adaptive Immunity; The Role of Interleukin-10. **Frontiers in Immunology**, [*s. l.*], v. 4, p. 129, 2013. Disponível em: https://doi.org/10.3389/fimmu.2013.00129. Acesso em: 4 dez. 2018.

NOY, Roy; POLLARD, Jeffrey W. Tumor-Associated Macrophages: From Mechanisms to Therapy. **Immunity**, [*s. l.*], v. 41, n. 1, p. 49–61, 2014. Disponível em: https://doi.org/10.1016/j.immuni.2014.06.010. Acesso em: 4 dez. 2018.

OBESITY. [*S. l.*], [*s. d.*]. Disponível em: https://www.who.int/health-topics/obesity#tab=tab_1. Acesso em: 25 set. 2021.

OBESITY AND OVERWEIGHT. [*S. l.*], [*s. d.*]. Disponível em: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight. Acesso em: 25 set. 2021.

OH, S. A.; LI, M. O. TGF- : Guardian of T Cell Function. **The Journal of Immunology**, [*s. l.*], v. 191, n. 8, p. 3973–3979, 2013. Disponível em: https://doi.org/10.4049/jimmunol.1301843

POLLARD, Jeffrey W. Tumour-educated macrophages promote tumour progression and metastasis. **Nature Reviews Cancer 2004 4:1**, [*s. l.*], v. 4, n. 1, p. 71–78, 2004. Disponível em: https://doi.org/10.1038/nrc1256. Acesso em: 25 set. 2021.

QIAN, Binzhi; POLLARD, Jeffrey W. Macrophage Diversity Enhances Tumor Progression and Metastasis. **Cell**, [*s. l.*], v. 141, n. 1, p. 39, 2010. Disponível em: https://doi.org/10.1016/J.CELL.2010.03.014. Acesso em: 25 set. 2021.

RAJALA, Michael W; SCHERER, Philipp E. Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. **Endocrinology**, [*s. l.*], v. 144, n. 9, p. 3765–3773, 2003. Disponível em: https://doi.org/10.1210/EN.2003-0580. Acesso em: 25 set. 2021.

ROCK, Cheryl L. *et al.* American Cancer Society guideline for diet and physical activity for cancer prevention. **CA: A Cancer Journal for Clinicians**, [*s. l.*], v. 70, n. 4, p. 245–271, 2020. Disponível em: https://doi.org/10.3322/CAAC.21591. Acesso em: 25 set. 2021.

RUTTER, Matthew *et al.* Severity of Inflammation Is a Risk Factor for Colorectal Neoplasia in Ulcerative Colitis. **Gastroenterology**, [*s. l.*], v. 126, n. 2, p. 451–459, 2004. Disponível em: https://doi.org/10.1053/j.gastro.2003.11.010

SÉROR, Claire *et al.* Extracellular ATP acts on P2Y2 purinergic receptors to facilitate HIV-1 infection. **The Journal of Experimental Medicine**, [*s. l.*], v. 208, n. 9, p. 1823–1834, 2011. Disponível em: https://doi.org/10.1084/jem.20101805. Acesso em: 4 dez. 2018.

SHAND, Francis H W *et al.* Tracking of intertissue migration reveals the origins of tumor-infiltrating monocytes. **Proceedings of the National Academy of Sciences of the United States of America**, [*s. l.*], v. 111, n. 21, p. 7771–7776, 2014. Disponível em: https://doi.org/10.1073/pnas.1402914111. Acesso em: 4 dez. 2018.

TESAURO, Manfredi *et al.* Vascular, metabolic, and inflammatory abnormalities in

normoglycemic offspring of patients with type 2 diabetes mellitus. **Metabolism: clinical and experimental**, [*s. l.*], v. 56, n. 3, p. 413–419, 2007. Disponível em: https://doi.org/10.1016/J.METABOL.2006.10.026. Acesso em: 25 set. 2021.

TRIANTAFILLIDIS, John K; NASIOULAS, Georgios; KOSMIDIS, Paris A. Colorectal cancer and inflammatory bowel disease: epidemiology, risk factors, mechanisms of carcinogenesis and prevention strategies. **Anticancer research**, [*s. l.*], v. 29, n. 7, p. 2727–2737, 2009. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/19596953. Acesso em: 8 nov. 2018.

VAN CUTSEM, E. *et al.* ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. **Annals of Oncology**, [*s. l.*], v. 27, n. 8, p. 1386–1422, 2016. Disponível em: https://doi.org/10.1093/annonc/mdw235. Acesso em: 4 dez. 2018.

VENTURA, M A; THOMOPOULOS, P. ADP and ATP activate distinct signaling pathways in human promonocytic U-937 cells differentiated with 1,25-dihydroxy-vitamin D3. **Molecular pharmacology**, [*s. l.*], v. 47, n. 1, p. 104–114, 1995. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/7838119. Acesso em: 4 dez. 2018.

WADWA, R Paul *et al.* Soluble interleukin-2 receptor as a marker for progression of coronary artery calcification in type 1 diabetes. **The international journal of biochemistry & cell biology**, [*s. l.*], v. 38, n. 5–6, p. 996–1003, 2006. Disponível em: https://doi.org/10.1016/J.BIOCEL.2005.09.015. Acesso em: 25 set. 2021.

WEINBERG, Benjamin A. *et al.* A paradigm shift from one-size-fits-all to tailor-made therapy for metastatic colorectal cancer. **Clinical Advances in Hematology and Oncology**, [*s. l.*], v. 14, n. 2, p. 116–128, 2016.

WYCKOFF, Jeffrey B. *et al.* Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. **Cancer Research**, [*s. l.*], v. 67, n. 6, p. 2649–2656, 2007. Disponível em: https://doi.org/10.1158/0008-5472.CAN-06-1823

YEGUTKIN, Gennady G. Enzymes involved in metabolism of extracellular nucleotides and nucleosides: Functional implications and measurement of activities. **Critical Reviews in Biochemistry and Molecular Biology**, [*s. l.*], v. 49, n. 6, p. 473–497, 2014. Disponível em: https://doi.org/10.3109/10409238.2014.953627. Acesso em: 4 dez. 2018.

ZHANG, Xin *et al.* Macrophages induce resistance to 5-fluorouracil chemotherapy in colorectal cancer through the release of putrescine. **Cancer letters**, [*s. l.*], v. 381, n. 2, p. 305–313, 2016. Disponível em: https://doi.org/10.1016/J.CANLET.2016.08.004. Acesso em: 26 set. 2021.

CAPÍTULO I: Colorectal cancer and purinergic signalling: an overview

ROLIANO, G.G.[1], AZAMBUJA, J.H.[2], BRUNETTO, V.T.[4,5], KALIL, A.N.[1,3], BRAGANHOL, E.[1,4,5]*

[1] Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil.

[2]Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA.

[3]Serviço de Cirurgia Oncológica, Santa Casa de Misericórdia de Porto Alegre (ISCMPA), Porto Alegre, Rio Grande do Sul, Brazil.

[4]Programa de Pós-Graduação em Biociências, UFCSPA, Porto Alegre, RS, Brazil.

[5]Instituto de Cardiologia do Rio Grande do Sul/Fundação Universitária do Instituto de Cardiologia (IC-FUC), Porto Alegre, RS, Brazil.

*Corresponding Author

Elizandra Braganhol (E-mail: ebraganhol@ufcspa.edu.br)

Universidade Federal de Ciências da Saúde (UFCSPA)

Sarmento Leite St, 245 – Main Building – room 304

CEP: 90.050-170 Porto Alegre, RS, Brazil

Phone: +55 51 3303 8762

As normas da revista *Purinergic Signalling*, para a publicação do artigo de revisão estão disponíveis em: https://www.springer.com/journal/11302/submission-guidelines#Instructions%20for%20Authors_Types%20of%20papers

All authors contributed to the study conception. G.G.R, J.H.A., and V.T.B. selected, critically revised the articles, and drafted the manuscript. A.N.K. and E.B. discussed and critically revised the manuscript. E.B. supervised all stages of manuscript preparation. All authors agree with the content of the submitted paper.

Abstract

Colorectal cancer remains one of the most common cancers and has a high fatality rate when at an advanced stage. Gut inflammation is related to colorectal cancer, with immune cells losing physiological role. The cells of the innate and adaptive immune system, including macrophages, neutrophils, mast cells, and lymphocytes are present in most solid tumors. The purinergic signaling is a way of communication between immune cells and tumor microenvironment, and so far it has been seen that it can alter the immune response to tumor progression. This system is regulated by the availability of extracellular purines to sensitize purinoceptors (P1 and P2) and tightly controlled by nucleotidases/phosphatases (E-NPP, CD73/CD39, ADA) and kinases, which interact with nucleotides and nucleosides to exert their effects. In this review, the objective was to compile the results exposed on the relationship of the purinergic system with the progression of colorectal cancer so far. As results, we found that the increased expression of CD73 leads to blocking the response of the effector immune cells and tumor progression in CCR. In receptors, A1, A2A and A2B were related with tumor progression, but A2B also increased apoptosis; and A3 had no association. P2X5, P2X7, P2Y2, P2Y6, P2Y12 increased main progression factors. ATP at high levels had greater tumor progression.

Keywords

Colorectal cancer. Purinergic signaling. Immune system. Tumor microenvironment.

Highlights

CD73 leads to tumor progression in colorectal cancer.
CD39 related with immunosuppressive T cell and poor prognosis.
Receptors P1 and P2 were main associated with poor prognosis to colorectal cancer patients.
Purinergic enzymes and receptors can be key to stop tumor development.

1. Introduction

Colorectal cancer (CRC) is the third most common cause of cancer death worldwide [1]. The CRC is characterized by two main mechanisms of instability genetic: low mutation rate with a high frequency of DNA somatic copy number alterations that generate chromosomal instability (84%); and hypermutated CCR with defective mismatch repair (MMR) that generate microsatellite instability (MSI) (13%) [2]. MSI is a predictive marker for response to chemotherapy and to immunotherapy [3].

The CRC classification is performed by analyzing tissue biopsy obtained from surgery following the TNM staging - tumor invasion (T), lymph nodes commitment (N), and metastasis formation (M) [4]. In the CCR first stage, the treatment is surgical. However, after the tumor progresses to a more advanced stage (III and IV), association of chemo/radiotherapy is required [5, 6]. The first line chemotherapy is 5-fluorouracil, an agent intercalating of DNA [7, 8]. Some monoclonal antibodies have been tested (anti-VEGF, anti-EGFR), but the therapeutic decision is personalized for each patient [9, 10].

Despite many efforts to develop new therapeutic strategies, the 5-year relative survival rate for patients with metastasis is just 14% [11], characterizing the main cause of death of patients with CRC [12, 13]. Moreover, considering the high CRC heterogeneity, TNM stage exhibits limitations in the cancer staging, which can generate over- or undertreatment of patients [14]. Therefore, in addition to considering exclusively histological factors, it has been proposed to include new cellular and molecular markers to enable the early diagnosis/prognosis and the follow-up of patients during the treatment [15, 16].

Diseases related with chronic inflammation, such as Crohn's Disease (CD), ulcerative colitis (UC) are clearly associated with a higher risk of developing CRC [17, 18]. Immune cells participate of tumor initiation; non-transformed microenvironmental cells communicate in a dynamic form with the cancer cells, creating an immunosuppressive environment which favor cancer progression and metastasis [19–21]. In the beginning of tumor development, NK cells and $CD8^+$ lymphocytes perform the recognition of the immunogenic cancer cells and try to eliminate them. However, those cells less immunogenic and “cold” persist raising the first tumor site [22]. Therefore, the immune cell composition in the tumor microenvironment (TME) may be a predictor of prognosis

for patients, and the presence of T cells or macrophages is related to good and bad prognosis for solid tumors, respectively [22].

However, whether the microbiota regulates the functional state of MPs in the TME and the signaling pathways involved are unknown. We reveal that microbiota-derived signals are needed to program mononuclear phagocytes in the TME toward immunostimulatory monocytes and DCs; absence of these signals shifts the mononuclear phagocytes repertoire toward tumor-promoting macrophages. Mechanistically, we demonstrate that microbiota-derived stimulator of interferon genes (STING) agonists such as c-di-AMP induce type I interferon (IFN-I) production by intratumoral monocytes, which regulates their skewing and natural killer (NK) cell-DC crosstalk. This mechanism can be triggered by microbiota manipulation with a high-fiber diet (FD) to improve the antitumor response. We show that transplantation of fecal microbiota from responder but not from non-responder individuals is sufficient to trigger IFN-I and remodel the innate immune TME in melanoma patients [23].

In this scenario, purinergic signaling emerges as a key element of cancer progression, since it orchestrates the immune/inflammatory responses and tumor proliferation/migration events as well [24–26]. Extracellular nucleotides and nucleosides, as ATP and adenosine, via purinorreceptor sensitization regulate fundamental functions of immune cells, including clonal expansion, migration, polarization, cytokine production and so on [27, 28]. Purinergic system is characterized by a signaling cascade mediated by nucleotides and nucleosides in the extracellular space [29, 30]. One of the players, “the messengers”, are extracellular purines such as adenosine 5’-triphosphate (ATP), adenosine 5’ diphosphate (ADP), and adenosine or pyrimidines uridine-5’-triphosphate (UTP) and uridine diphosphate (UDP) which act as signaling molecules mediating cell communication in physiological or pathological conditions [31].

Considering that the immune cell dysfunction is related to CRC progression and probably with metastasis, we hypothesized that alteration in purinergic signaling may contribute to cancer-associated immune dysfunction. Therefore, here we revised the potential role of the immune cells in cancer-related inflammation, focusing in the available data about CRC. Further, purinergic signaling in CRC cells as well as in the tumor-associated immune cells is discussed, highlighting the main purinergic signaling

alterations with diagnostic, prognostic or therapeutic value. A better understanding of purinergic signaling participation on CRC progression and metastasis will open new avenues for future therapeutic interventions.

2. Methodology:

The electronic databases used were “Pubmed”, “Web of Science”, “ScienceDirect” and “Scopus”. The function “advanced” was used in the Pubmed and ScienceDirect, in the Web of Science, sometimes we used the term “in title” and to receptor terms we used “in topic”, and in the Scopus, quotation marks (“) at the beginning and the end of expression was used, and the field tag abstract was used. The descriptors used included: “colorectal cancer and CD73”; “colorectal cancer and CD39”; “colorectal cancer and NTPDase1”; “colorectal cancer and ecto-5’-nucleotidase”; “colorectal cancer and purinergic signaling”; “colorectal cancer and adenosine”; “colorectal cancer and adenosine triphosphate”; “colorectal cancer and purinergic receptors”; “colorectal cancer and inflammation and purinergic signaling”; “colorectal cancer and metastasis and purinergic signaling”; “colorectal cancer and A1 receptor”; “colorectal cancer and A2A receptor”; “colorectal cancer and A2B receptor”; “colorectal cancer and A3 receptor”; “colorectal cancer and P2X receptor”; “colorectal cancer and P2Y receptor”. A total of 932 papers were found. After copies were excluded, we had a number of 439. Therefore, the evaluation of the title and abstract of the papers was done, and 91 articles were selected. Thirty-two productions were excluded because they were abstract, book chapter, reviews, in other languages different from English or with access not available. Finally, 59 articles were included in this review. Two independent researchers made the choice of papers that has criteria of eligibility and when there was conflict, a third researcher made the tiebreaker. Only complete articles written in English published in the period from 2009 (January) to 2021 (November) were included. The variables extracted were about the material studied, sample size, target and main results briefly (see Table 1). A second reviewer then checked details. The data investigated were related to the purinergic receptors and enzymes, immune cells, tumor cells, over survival.

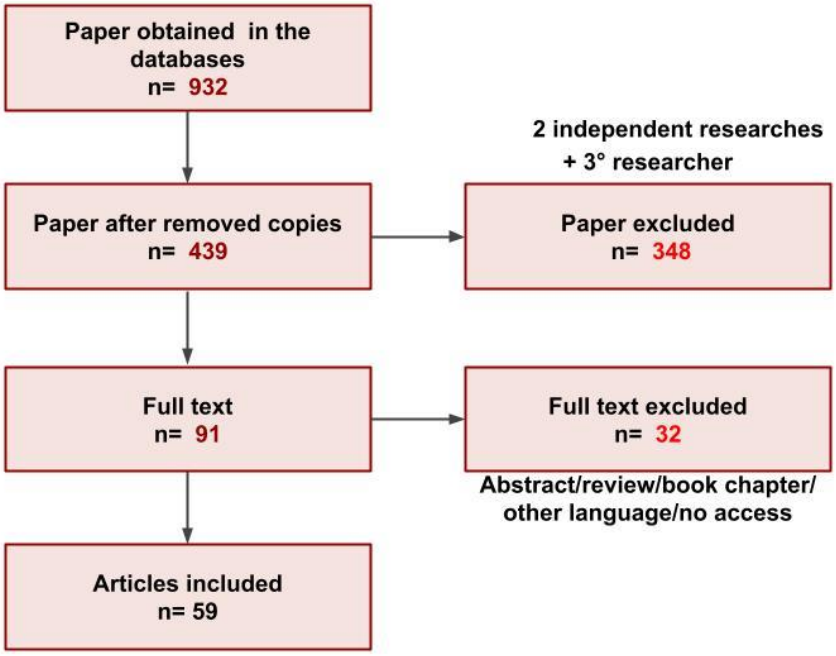


Figure 1. Overview of the paper selection for inclusion in the present review.

3. Immune cell dysfunction associated to cancer progression

The cells of the innate and adaptive immune system, including macrophages, neutrophils, mast cells, and lymphocytes are present in most solid tumors. These cells mediating inflammatory responses are necessary both for tumor progression and for its elimination. Several studies have broadened the concept that inflammation is a critical component of tumor progression [32]. [33].

First, scientists observed that the mass of solid tumors was composed in large number by immune cells [34, 35]. The first hypothesis was that these cells would be recruited into the TME to recognize the transformed cells and initiate an immune response to eliminate and the tumor helping to slowing down the tumor progression. However, as we began to understand more about the tumor-immune cells crosstalk, we realized that the hypothesizes was not accurate.

Immune cells are recruited in to the TME through the secretion of various soluble factors released by tumor cells, such as cytokines and chemokines. However, after arriving they are educated by tumor cells through mechanisms that induce anergy and an immunosuppressive phenotype leading to tumor growth.

3.1. Macrophages and neutrophils

Macrophages and neutrophils are said to be critical to cancer-related inflammation. In breast, skin, pancreatic cancer, the presence of extensive immune infiltrate is correlated with poor prognosis [36–38]. Neutrophils and macrophages are the first players in an inflammation situation, and are components of the immune innate response. Neutrophils' role is to promote death by phagocytosis, degranulation and neutrophil extracellular traps (NET) extravasation that promote immobility of pathogens to help cell phagocytes. They recruit the phagocytes to operate against pathogens and/or tissue injury [39].

It has been described that these two cells can be activated in different physiological phenotypes according to the environment signaling: classical (M1/N1) and alternative polarization (M2/N2), where M1/N1 profile does the antigen combat, eliminating altered/immunogenic cells, while M2/N2 profile reduce the inflammation, recomposing tissue homeostasis [40, 41]. In TME, studies are highlighting the capacity of tumor cells to induce and “corrupt” TAM and tumor associated-neutrophils (TAN) to a M2-*like*/N2-*like* phenotype, which contribute to tumor progression. TAM produce cytokines that promote angiogenesis, immunosuppression by T cells inactivation, Treg induction, dendritic cell activity inhibition, epithelial–mesenchymal transition and, thus, cancer cell proliferation and chemoresistance [42, 43].

TAMs promote metastasis initiation by tissue remodeling, downregulation of Natural killer (NK), recruitment of more immunosuppressive macrophages and increasing of NETs deposit. NETs apparently increase adhesion of tumor cells, concentrating groups of tumor cells and allowing the degradation of extracellular matrix for tumor spread [22, 44]. TAN induces angiogenesis in tumor sites by MMP9 and VEGF secretion [45]. Other possible tumor-promoting activities of TAN include tumor cell invasion and growth, T cell immunosuppression, induction of chronic inflammatory environment, favoring the establishment of pre-metastatic niche [46, 47]. Briefly, TAM and TAN favor the tumor cells on immune escape and tumor survival. On the other hand, there is evidence that they can also have an antitumor activity, TAM and TAN are associated with better response to chemotherapy in CRC patients [48, 49].

3.2. Myeloid-derived suppressor cells

The myeloid-derived suppressor cells (MDSCs) are a group of heterogeneous cells originating in the bone marrow that was recently described as potent immunosuppressive agents in cancer and other conditions such as chronic

inflammation and infections [50, 51]. The main mechanisms of these cells are induction of other immunosuppressive cells - M2 and regulatory T cells (Treg) - and blockade of immunoinflammatory response.

3.3. Lymphocytes

The NK cells = are the most cytotoxic cells of innate immune response, which are responsible for immune surveillance and operate mainly by recognition of major histocompatibility class I (MHC-I) expression [52, 53]. MHC-I expression is downregulated in tumor cells, which promotes the failure of NK cells through the lack of inhibitory signals. However, tumor cells can escape from the immune response via different mechanisms, such as (1) cleavage of costimulatory ligands and (2) secretion of immunoregulatory molecules such as adenosine (ADO), TGF-α and prostaglandin E2 [54–58]. In addition, low NK levels and activity were detected in various types of solid tumors, including CRC [59–61].

T cells are the main players of adaptive immunity response and are divided in subpopulations [62]. Effector T cells are divided in $CD8^+$, cells with cytotoxic activity against target cells, and T helper 1-$CD4^+$, which produce inflammatory cytokines that amplify antitumor response through macrophages and NK activation [63, 64]. Once finished the inflammatory process, the remaining cells turn into memory T cells [65]. The participation of effector T-cells have been extensively investigated in cancer-related inflammation and they are associated with better prognostic for cancer patients including CRC, depending on the immune composition of TME [63, 66]. However, similarly as observed for TAM and TAN, tumor cells and the TME cells reduce T-effector cells activity and promote the recruitment of T immunosuppressive T-$CD4^+$ regulatory cells [62]. Treg ($CD4^+CD25^+FOXP3^+$) are responsible for immunological self-tolerance and for tissue homeostasis during inflammation, decreasing the number and the activity of effector immune cells and stimulating tissue repair [67]. Treg suppress effector cells T-$CD8^+$, Th$CD4^+$, macrophages, NK cells and neutrophils [67, 68] through a variety of mechanisms: (1) via contact with other cells through inhibitory receptors CTLA4, PD-1, PDL-1 and LAG3 [69, 70] and (2) via production of immunosuppressive molecules, such as IL-10, TGFα and ADO [71].

Treg infiltration has been associated with tumor progression and patients' worst prognosis in various types of cancer [72]. However, the association between Treg

infiltration and prognosis in CRC is not yet understood, considering there are several studies that concluded both positive and negative correlations [49, 73–76]. The blockade of PD-1/PDL-1 and CTLA4 using monoclonal antibodies was the first strategy studied for cancer-based immunotherapy and, even though it has been beneficial to oncological patients [77–80], targeting immune checkpoints is still a challenge to CRC therapy. Since a variety of tumors types with different mutations profiles do not respond [81–83].

Another T cell subpopulation that has been extensively studied in inflammation, infection and cancer is the exhausted $CD8^+$ cells (Tex). After long term stimulation, the effector T cells fail in turning into memory cells and lose their ability to produce inflammatory cytokines and proliferate, enabling the immune response and the senescence of these cells [84–86]. The Tex cells are characterized by the high and persistent expression of inhibitory receptors such as PD-1 and the exhaustion can be reversed through the PD-1 blockade, which consists in an important strategy to cancer therapy [87, 88]. In CRC, Tex are abundant in the TME and it has been proposed as a potential biomarker and therapeutic target [89–91].

In summary, tumor cells induce escape immune by decrease antigens presentation human leukocyte antigens (HLA) and by immunosuppression promotion, with Treg, TAM and TAN recruitment, anti-inflammatory cytokines secretion (IL-10, TGFα), purinergic signalling alterations and immune modulatory receptor expression (PD-1, CTLA4). In this context, understanding the mechanisms involved in this complex relationship is of most importance to build an efficient strategy to beat cancer and re-educate immune cells. One of the important mechanisms that controls the immune response and the progression of cancer is the purinergic system.

Reference	Type of study	Lineage	Molecule	Results	Overcome
Wen et al, 2019	In vitro / In vivo	CT29 / Balb/c	ATP	Increase of ATP levels in the microenvironment	Decrease of tumor progression by ATP-mediated immune response
Yagushi et al, 2010	In vitro	Caco-2	ATP	ATP-mediated PKC inhibition via P2R stimulation	Decrease of tumor cell viability and proliferation
Vinette et al, 2015	In vitro	Caco-2	ATP	ATP-mediated MRP2 expression increase via P2R stimulation	Increase of tumor cell survival and chemoresistance
Kim et al, 2019	Tumor (N=136)	CRC (stage III)	ATP	ATP sensitivity of patients is directly correlated with better response to chemoterapy	Improvement of OS and PFS
Dillard et al, 2021	In vitro	HT29, HCT116, LS513 and LS174T	ATP and ADO	ATP induce cell death and have anti-proliferative effects in CRC cells	Worst tumor cell development
Tokunaga et al, 2019	Blood (N=451)	Liver (but not limite to) metastatic CRC	ADO	Adenosine SNP (CD73 rs2229523, A2BR rs2015353 and CD39 rs2226163) correlated with OS	Improvement of OS
Kunzli et al, 2011	In vivo	CD39 transgenic and CD39-/-	CD39	- High CD39 expression in endothelium, stromal and mononuclear infiltrating tumor cells - High P2Y2 expression in liver metastatic tumors	Increased tumor cell spread and liver-mestastasis formation
	Tumor (N=63) /adjacent tissue (N=13)	CRC stage		Lower expression of CD39 and P2Y2 in tumor tissue of inicial stages of CRC compared to metastatic tumors.	Author suggested that lower levels of CD39 and P2Y2 could be associated with longer survival and less invasive tumors

McCarthy et al, 2013	In vivo In situ	HCT-116 and HCT 15	CD39L 4	Lower levels of ATP in transfected mt-PCPH and myc-tagged PCPH cells. However, the NTPDase activity of mt-PCPH was undetectable.	Decreased levels of ATP intracelular
Parodi et al, 2013	Tumor (N=33, 2 CRC), blood donor (13)	Renal, Bladder, CRC stage (III)	CD39	-High CD39 expression in blood from tumor patients -High molecular density per cell CD8+ Treg intratumoral.	CD39 expression was correlated with supression activity mediated by CD8+ Treg
Zhang et al, 2013	Blood (N= 64) and tumor (N=5) and In vitro	CRC (I, II, III, IV) cells from blood	CD39	-Increased MDSCs positively correlated with tumor metastasis and TNM -MDSCs CD39high potential to inhibit CD3+ Tcells proliferation	Immunossuppressive activity of MDSCs cells from CRC patients
Scurr et al, 2014	Tumor/adjace nt tissue, blood/donor (N=40)	CRC stage I, II (majority), III	CD39	Increased expression of CD39 in boths FOXP3+ and FOXP3- in tumor tissue compared with blood from healthy donors or CRC patients	Possible regulatory role of CD4+ FOXP3- T cells intraturmoral
Sundstrom, 2016	Blood (N=45) and tumor (N=7)	-	CD39	- Higher expression of CD39 in Tregs in blood and tumor tissue. - Adenosine reduced migration of T cells through the reduction of monocyte capacity to activate the endothelium.	CD39 enzyme activity contributes to adenosine-mediated reduction of TEM in CRC patients.
Limagne et al, 2016	Blood mCRC (25) and donor (20)	CRC stage IV	CD39	- High gMDSC (high PD-L1, CD39 and CD73) leves associeted with poor progosis. - FOLFOX plus bevacizumad decreased gMDSC.	Immunosuppressive effect on T cells, demonstrated by reduced TNFa and Ki67 proliferation marker expression
Timperi et al, 2016	Tumor / adjancet tissue, blood (N=34)		CD39	-High Tregs CD39high, IL-17 and IL-1b increased in tumor site. - The genotypes at polymorphism (ENPD1	Phenotype compatible with a strong suppressive activity, a high proliferative potential

				SNP, rs10748643) were contibute to CD39 expression.	and a stable regulatory program.
Hu et al, 2017	In vitro	SW480	CD39	- A2A and A2B antagonists blocked effect of gama-delta-Treg. - CD39+ gama-delta-Treg inhibited CD3+ T cell proliferation.	Increased adenosine extracellular concentration
	Turmor / adjacent tissue (109), boold donors	-		- CD39high in gama-delta-Treg in tumor, correlated with high CTLA-4, PD-1, FOXP3 and IL-10, IL-17A, GM-CSF, TGF-b1, TNF-a production. - TGF-b1 induced gama-delta-Treg with higher ADO.	Immunosuppressive function on T cells, higher than conventional T reg CD4+. Correlated with advanced TNM stage.
Khaja et al, 2017	Tumor / adjacent tissue (N=12)	CRC stages (I,II,III and IV)	CD39	CD3+T cells in the TME were CD4+FoxP3+T with high co-expression of PD-1/CTLA-4 and PD-1/CD39. CD39 was higher in tumor. FoxP3+Helios+ and FoxP3+Helios− Treg had high co-espression of PD-1/CTLA-4 and PD-1/CD39.	Authors sugest that expression of PD-1, CTLA-4 and CD39 compatible with potent immunossuppresive subsets of Tregs
Zhulai et al, 2018	Blood (42) / donors (30), tumor (5) stage III	Initial (stages I and II) and advanced (stages III and IV) CRC groups	CD39	CRC advanced stages incresead CD4+CD39+ lymphocytes in blood and tumor tissue, negative correlation with CD3+CD4+ T helpers and CD3–CD19+ B cells and the immunoregulatory index (CD4+ to CD8+ ratio). Association between FOXP3 and CD39 in CD4+CD25high T cells.	Increased CD4+CD39+ T cells correlated to a imunossupression phenotype of the cells in blood and tumor tissue.
Simoni et al, 2018	Tumor / adjacent tissue (N=94)	Stage I, II, III and IV	CD39	Lower CD39 expression in TIL CD8+ bystander than TIL CD8+ tumor specific. CD39+ was correlated with proliferation and exhaustion asociated genes.	Possible TIL CD8+ maker in CRC

Strasser et al, 2019	Tumor /adjacent tissue, gene data (298)	-	CD39	Higher levels of CD39+Helios+ T cells and pro-inflammatory IFNγ -producing T cells in CRC tissue	Authors sugest that TME induces pro-inflamatory and imunossupressive activity from T cells and macrophages.
Gaibar et al, 2021	Tumor mCRC (N=57, paraffin)	Stage IV	CD39	Variant alele CD39 patients had better response to bevacizumab plus chemotherapy, but no changes to OS or PSF	SNPs as predictors to prognosis to tratament
Gallerano et al, 2020	Tumor / adjacent tissue, blood (N=60)	Stage I, II, III and IV	CD39	Lymphocytes T CD8+ CD39high more expressive in tumor, initial stage of CRC (I-II), with high PD-1 expression and lower INF-y production, correlated with T cells exhausted and suppressed CD4+ T cell proliferation.	CD39 marker to T cells supression
Park et al, 2021	In vivo / In vitro	CT26. balb/C subcutaneously injected into the flank. intraperitoneally injected POM-1 daily for 2 weeks.	CD39	CD39 inhibitor increased CD11b and Ly6C expression in M1 TAM and F4/80 in macrophages in vitro. CD39 inhibitor promote smaller tumor growth, Ly6C and MHC II in F4/80+, increased CD8 T cells in the spleen , CD4 in the blood, and Caspase-3 expression, comparated with control group in vivo.	CD39 inhibitor increased M1 TAM phenotype in colorectal cancer model
Rodin et al, 2021	Tumor / adjacent tissue (28 male, 19 female) 10 cm away from the tumor	colon tumors	CD39	MAIT (mucosal-associated invariant T cell) infltrating CRC have a terminally exhausted phenotype (PD-1highTim-3+CD39+) and increased proliferation. The MAIT cells have reduced polyfunctionality with decreaased of anti-tumor efector molecules, and blocking antibodies to PD-1 improved activation of tumor-infltrating MAIT cells in vitro.	MAIT cells express CD39 and is associeted with immune escape

Zhao et al, 2020	In vitro	MC38 and HT29	CD39	Expression level of CD39 in colorectal tumor tissues was higher than that in normal tissues. CD39 was also highly expressed in both human and murine colorectal cancer cell lines MC38 and HT29. CD39 inhibitor inhibited MC38 cell growth at 48 and 72 h. CD39 inhibitor inhibited cell proliferations in a dose-dependent manner.	High CD39 expression in CRC cells, CD39 inhibitor ihibits cells growth.
Zhan et al, 2021	Tumor / adjacent tissue (N=129) / In vivo / In vitro	TNM I, II, III and IV. In vitro CT26. In vivo: CT26-Vec/Pla2g4a cells transplanted into the caecal BALB/c.	CD39	Left-sided CRC had less CD39+γδ Tregs frequency that right-sided CRC. The RSCRC had adenosine level, IL-17A–producing incresed and IFN-γ–producing decreased.	Tumor in right side is most malignant that left side, by imunossupression mediated by CD39 +γδ Tregs
Matsuyama et al, 2010	In vitro	SW48 and SW48LM2	CD73	Reduced CD73 expression in highly liver metastasis cell line	Decrease in purine nucleosides, increase in purine nucleotides metabolism
Wu et al, 2012	Tumor (16 fress, 358 paraffin)	CRC 54 - I , 147 - II , 124 - III , 30 - IV	CD73	High CD73 expression in CRC tissue fresh or paraffined	Worst overall survival rate
Cushman et al, 2014	Tumor (N=103)	mCRC and respective primary tumor	CD73	Higher levels of CD73 expression predict for PFS benefit from cetuximab	Better response to chemoterapy
Zhang et al, 2015	Tumor / adjacent tissue (N=90)	CRC (11 - I , 38 - II , 40 - III , 1 - IV)	CD73	Higher xpression of CD73 in both tumor and stromal tissue compared to peritumor tissue.	CD73high worse outcomes in tumor. CD73high in stromal

					correlated with less advanced tumours and better outcome.
Wu et al, 2016	In vitro	RKO, SW480, HCT-15, LoVo and KM12	CD73	CD73 expressed in all five cells lines of CRC; overexpression of CD73 promoved β-catenin/cyclin D1 and EGFR expression.	Cell growth ad cell cycle
	In vivo	CRC human with/without CD73 interference		CD73 increased tumor size and weight	CD73 promoted tumor growth
Hatch et al, 2016	Blood (N=152), tumor (N=71)	-	CD73	High plasma levels of CD73 were predictive of shorter OS in all patients, although it also correlated to PFS benefit in wt KRAS group treated with cetuximab	Potential predictive marker of benefit from cetuximab in mCRC.
Xie et al, 2017	In vitro	HEK293T cells, SW480, HCT116, LoVo, CaCo2, HT29, RKO, DLD1, HCT8	CD73	miR-30a has a negative effect in regulating expression of CD73 mRNA and protein, with decreased proliferation and incresed apoptosis.	inverse correlations between the levels of miR-30a and CD73.
	In vivo	BALB/c nu/nu mice injection of SW480		Decreased in the mean weight of miR-30a gruop.	
	Tumor / adjacent tissue (N=27)	-		Lower expression levels of miR-30a and higher expression levels of CD73 than the corresponding adjacent control tissues.	
Sun et al, 2017	In vitro	CT26, RAW 264.7	CD73	CD73 knockdown and ADO receptors antagonist correlated to inhibbited M2 polarization and tumor cell proliferation	Fasting induced CRC cells autophagy, downregulated ADO by suppressing CD73, modulated TAM polarization

					and inhibits tumor growth.
	In vivo	WT BALB/c mice		Mice in dietary restriction had reduced tumor growth without body weight reduction, with reduced M2 polarization	
Wang et al, 2019	Boold (N=232) / donors (158)	TNM stage= I + II (110), III + IV (122)	CD73	Higher CD73 expression in CRC patients. Correlation between CD73 expression and several clinicopathological features. Shorter OS in higher CD73 expression.	Worst prognosis
Liu et al, 2020	In vivo	Colitis-associated tumorigenesis	CD73	CD73 inhibitor decresed body weight loss, N° tumors, longer colon, lower histopathological score and downregulated expression CRC tumorigenesis-associated genes. ADO antagonist was opposite, and increased TNF-α and IL-6	CD73 inhibitor inhibit colitis-associeted tumorigenesis
Yu et al, 2020	In vivo	CD73null / A2Bnull	CD73	CD73 promoted tumor progression; inactivation antitummor immunity and regression. ADO released in cell death binds to A2B (increases CD73), and A2A (immune suppression)	CD73 expression in CAFs promotes immussupression.
	In vitro	EG7.OVA and MC38			
	Tumor (N=25)	-			
Messaoudi et al, 2020	Microarray CRCm (N=251), blood (N=193)	CRC stage IV	CD73	CD73high associated with more aggressive CRCm liver, poorer reponse to pre-operative chemotherapy and mutated KRAS, shorter time to recurrence and disease-specific survival.	Both high CD73 expression in the stroma or in cancer cells were associated with poorer outcomes.
Kim et al, 2021	In vitro / In vivo	CT26 implantation in	CD73	Nt5e and Entpd1 expressions affect TCR diversity and transcriptional profiles of T cells;	CD73 inhibition is distinct from PD-1 inhibition and

		Balbc; colitis-associated cancer model mouse by injection of azoxymethane and Dextran Sulfate Sodium		CD73 inhibitor (AB680) improved the anticancer functions of immunosuppressed cells, including Treg and exhausted T cells, and caused increased activation of CD8+ T cells.	exhibits potential as anticancer immunotherapy for CRC, alone or combined with anti-PD-1 therapy.
Lai et al, 2021	In vitro / In vivo Tumor and blood	Male C57BL/6 and P14 TCR transgenic, CD28KO, P14CD28KO and CD73KO mice	CD73	CD28-/- upregulate CD73 expression in CD8+ T cells, without diference to CD39 expresssion, and with increse of adenosine level in culture supernatant. CRC tumor and PBL had CD73 upregulation in Cd28-/-Cd8+ T cells. Reduction of cytolytic activity of CD8+ T cells in CD28-/- supernatant tratment	CD28 costimulation inhibits the expression of CD73 in CD8+ T cells
Ploeg et al, 2021	In vitro	H292, OvCAR3, DLD1, PC-3M and CHO–K1	CD73	Extracellular vesicles cancer cells lines and patients derived are enriched in CD73. The CD73 inhibition in extracellular vesicles reactivate proliferative and cytotoxic capacity of T cells.	CD73 in extracellular vesicles promote immune supression by T cells attack.
Terp et al 2021	In vitro, human samples (dataset)	HCT116, SKBr3, CT26.CL25 (CT26), A549, PC9, MC38 and 4T1.2 (4T1)	CD73	CD73 expression was significantly lower in tumors of responders vs. nonresponders, lower PFS in CD73high vs. CD73low patients. High CD73 expression is associated with a poor response to anti-EGFR treatment.	High CD73 expression takes Immunesupression and poor outcome.
P1					

Lan et al, 2017	*In vitro*	HCT116 and SW480	A1	Increased A1 expression, suppressed the proliferation, growth inhibition and apoptosis induced by metformin in an AMPK-TOR pathway dependent manner.	Metformin have anti-tumor effects
Wu et al, 2019	Tumor / adjacent tissue (N=204)	CRC stage I/II (106) and III/IV (98)	A2A	Higer A2A expression in tumor than non tumor tissue, correlated with tumor size, depth of tumor invasion, TNM stage and PD-L1 expression.	Correlated with smaller OS
Kitsou et al, 2020	*In silico* (N=453)	RNA seq and clinicophatological data	A2A	A2A had lower expression in CRC compared to normal tissue and was not correlated to OS. In COAD, TIL load positevely correlated to A2A expression.	A2A not correlated to clinical outcome in CRC patients
Ma et al, 2010	*In vitro*	DLD1, SW480, HCT-15, LOVO, COLO205	A2B	Higher A2B expression than A1, A2A, and A3 in tissues samples and in cell lines that was increased in hypoxia coditions. Inhibition of A2B decreased cell growth.	A2B with role in proliferation of CRC
	Tumor (N=88)/ adjacent tissue (N=62)	-			
Long et al, 2013	*In vitro*	Saos-2, Pheonix Eco, U2OS, HCT116	A2B	A2B is upregulated directly by p53, activated by cellular stress, induce cell death by apoptosis. It is seen in hypoxic codition and in response to chemoterapy.	A2B promoted cancer cell death
Molck et al, 2016	*In vitro*	DLD1, SW480, CPP14, HEK293T	A2B	A2B antagonist increased mitochondrial oxygen consumption and intracellular ROS levels	A2B antagonist change cell metabolism, but it was independ of A2B activation
Balber et al, 2017	*In vitro*	HT-21 and CHO-K1	A3	High A3 expression in HT-29 cells	A3 high expressed in tumor cells and [18F]FE@SUPPY

	In vivo	Immunodeficient CB17-SCID		No difference between the CHO-K1 and HT-cells xenografts.	correctly marked in vitro and in human tissue.
	Tumor / adjacent tissue (N=2)	-		[18F]FE@SUPPY accumulation was higher in CRC than in healthy tissue and corresponded to high expression of A3	
Marucci et al, 2018	*In vitro*	Caco-2, PC3, HepG2, CHO	A3	Agonist inhibited Caco-2 cells growth and migration, promoted apoptosis and elevated ROS levels. However, the A3 knockdown did not prevent the effect from the agonist.	The agonist could act as a anticancer treatment, but indepedent from A3.
P2					
			P2X		
Gao et al, 2018	*In silico* (N=206)	-	P2X5	P2X5 expression related with worse prognosis and the expression was higher in the high risk group.	P2X5 like prognostic or preditive maker.
Janakiram et al, 2015	*In vivo*	Rag1-/-ApcMin/+	P2X7	Increased expression of P2X7R upon Treg transfer and NK depletion, increasing tumor cell proliferation, intestinal tumor formation and growth.	Promote tumor and less survival in mice.
Hofman et al, 2015	*In vivo*	Disrupted P2X7 gene	P2X7	P2X7 blockade stimulated Treg accumulation, reduced colonic inflammation and increased tumor proliferation, associated with elevated expression of TGFB1.	P2X7 blockade increased CAC proliferation and development, while promoted an immunossupressive microenviroment.
Quian et al, 2017	Tumor (N=12 fresh /116 paraffin)	TNM stage I (31), II (36), III (44) and IV (5)	P2X7	P2X7 up-regulated in tumor tissue and correlated with higher expression in high-TNM stage.	Preditive to worst prognosis in CRC patients
Zhang et al, 2019	*In vitro*	NCM460, HCT116,	P2X7	Higher expression of P2X7 in CRC cell lines, expression higher in the cell line derived from tumor metastasis.	Higher expression of P2X7 related to advanced disease and poorer prognosis.

		SW480, SW620			
	Tumor / adjacent tissue (N=97)	TNM stage I (16), II (30), III (41) and IV (10)		P2X7high (N=56) versus P2X7low (N=41). Higher expression of P2X7high in tumor tissue, associated advanced disease and shorter survival. P2X7 expression was higher in mCRC.	
Zhang et al, 2021	*In vitro, in vivo*	HT116, SW620	P2X7	P2X7 inhibitor (A438079) inhibits CRC cells line proliferation, invasion, migration and promotes apoptosis by Bcl-2/caspase9/caspase3 pathway. In vivo P2X7 inhibitor inhibits tumor growth.	Inhibitor promote apoptosis
Yang et al, 2020	*In vitro / In vivo*	Injected 10 μL of 2 × 106 CT26-Con or CT26-mP2X7R cells into the subserosa of the caecum	P2X7	P2X7R promoted proliferation, migrated, ability to invade, increased the number of tumorspheresof of CRC cells in vitro. P2X7 overexpression increase the growth and weight of tumors, infiltration of macrophages, TAM recruitment, stimulation of angiogenesis in vivo.	TAM stimulation and tumor growth
			P2Y		
Limami et al, 2012	*In vitro*	HT-29	P2Y2	Ursolic acid induced an increase in intracellular ATP and in P2Y2 mRNA. p38 activation was dependent on P2Y2 activation.	Resistence to apoptosis
Placet et al, 2018	*In vitro*	HT-29	P2Y6	P2Y6R agonist prevented apoptosis. Stimulation of P2Y6R prior to 5-FU treatment provided protection.	Resistence to apoptosis
	In vivo	P2Y6 -/- or P2Y6+/+ mice		Less number and volume of CRC tumors in P2ry6-/- mice P2Y6R increased expression of XIAP and	Worst outcome in mice

				correlated with AKT phosphorylation and resistance to 5-FU.	
Girard et al, 2020	*In vitro*	Caco-2	P2Y6	P2Y6 incresead cell migration, through PKCα that stabilizes the actin cytoskeleton	Promote migration
Wright et al, 2020	*In vitro*	HT-29	P2Y12	Reduced cells agregation and adhesion.	P2Y12 help tumor cell in agregation and adhesion
	Tumor (N=6) / Donors (N=22)	mCRC		Higher levels of spontaneous platelet aggregation and P-selectin expression in mCRC tissues	Promote platelet agregation

Table 1. CRC and Purinergic Signaling: Main results of 46 studies found on CCR and purinergic signaling, separated by focus molecule (ATP, ADO, CD39, CD73, P1 receptors and P2 receptors (P2X and P2Y).

4. Purinergic Signaling in Colorectal Cancer

4.1. Ectonucleotidases - General Aspects

Purinergic signaling is regulated by the availability of extracellular purines to sensitize purinoceptors and tightly controlled by nucleotidases/phosphatases and kinases [29]. The canonical adenosinergic pathway includes the ecto-nucleoside triphosphate-diphosphohydrolases (E-NTPDases), [ATP->ADP and ADP-> AMP], the ecto-pyrophosphate-phosphodiesterases (E-NPP), [ADP-> AMP and of AMP -> ADO, alkaline phosphatases (ALPs), [ATP-> ADP, ADP-> AMP, and AMP-> ADO] , the ecto-5'-nucleotidase/CD73 (CD73) [AMP -> ADO] and adenosine deaminase (ADA) [ADO -> inosine] [30, 92, 93]. Extracellular ADO levels are also regulated by nucleoside equilibrative transporters (ENTs) or concentrative nucleoside transporters (CNTs), expressed in the cell membrane and responsible for ADO transport into the cells [25, 94]. In the intracellular microenvironment, ADO is phosphorylated by ADO kinase (AdoK) and adenylate kinases into ADP [26].

However, ADO can also be generated by the non-canonical adenosinergic pathway by nicotinamide adenine dinucleotide (NAD^+)-glycohydrolase/CD38 (NAD^+ -> ADP-ribose (ADPR)) and NPP1/CD203a (PC-1) (ADPR -> AMP) that is subsequently metabolized by CD73 to ADO. CD73 represents the common link between the two adenosinergic pathways [95].

In the end of this cascade, we have the enzyme responsible for ADO remotion and INO production. ADA is present in virtually all human tissues, but the highest levels are found in the lymphoid system such as lymph nodes, spleen, and thymus [96]. Furthermore, ADA is reported to bind to the cell surface through an ADA binding protein (CD26) [97].

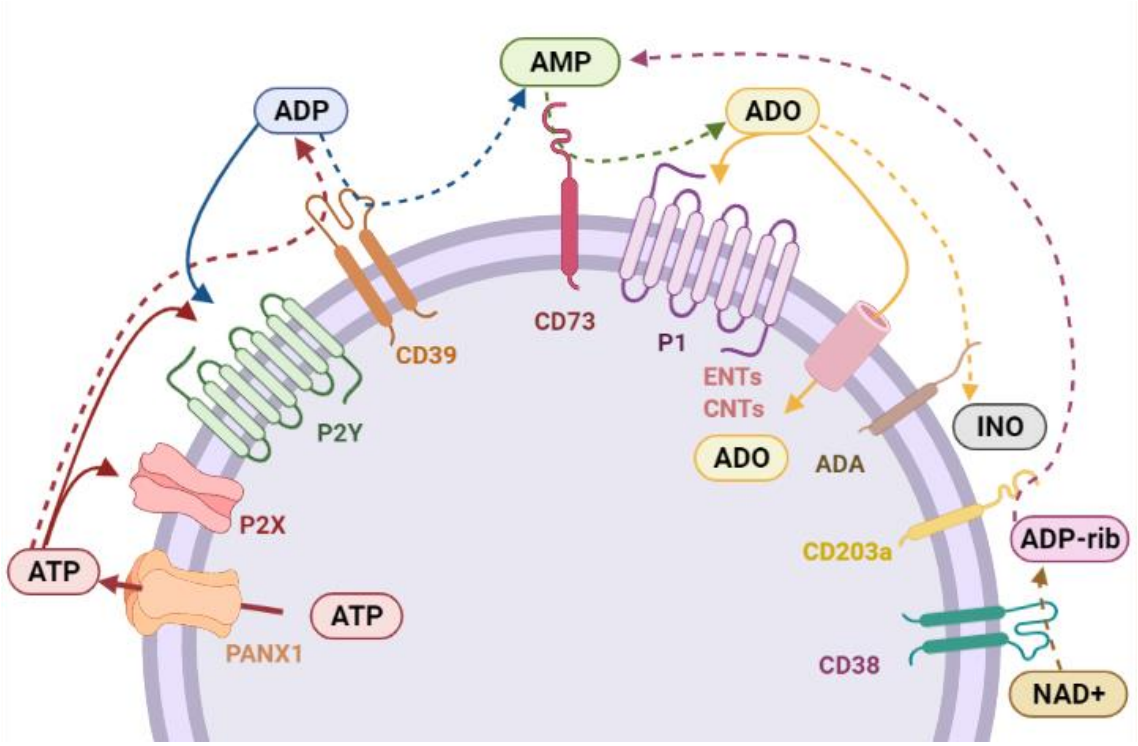


Figure 2. Purinergic signaling cascade: Intracellular ATP is externalized by PNX1 channel and sensitizes P2 receptors. P2R are divided in two major categories: P2X1-7 and $P2Y_{1,2,4,6,11–14}$. ATP is hydrolyzed by E-NTPDase1/CD39 to ADP, which also binds to P2YR and is further hydrolyzed by CD39 to AMP. AMP is also formed through an alternative pathway that involves the enzymes NAD^+-glycohydrolase/CD38, which hydrolyzes NAD^+ in ADP-ribose and NPP1/CD203a, which hydrolyzes ADP-ribose in AMP. Once AMP is formed by the canonical and/or the non-canonical pathway, it is hydrolyzed by ecto-5'-nucleotidase/CD73 to ADO, thus connecting both adenosinergic pathways. ADO binds to the P1 receptors, which are divided in A1, A2A, A2B and A3, each one with different affinities to its ligand. ADO is internalized in the cell through ENTs or CNTs and/or it is deaminated to INO by ADA.

Abbreviations: ATP (adenosine triphosphate); ADP (adenosine diphosphate); AMP (adenosine monophosphate); ADO (adenosine), INO (inosine); NAD+ (nicotinamide adenine dinucleotide); PNX1, P2X (ionotropic purinergic receptors), P2Y (metabotropic purinergic receptors), P1 (adenosine metabotropic receptors), E-NTPDase1/CD39 (ecto-nucleoside triphosphate diphosphohydrolase 1), ENTs (nucleoside equilibrative transporters); CNTs (concentrative nucleoside transporters); ADA (adenosine deaminase).

4.1.1. Ectonucleotidases - the CD39 role in CRC progression

Several studies have been investigating Treg in CRC TME, aiming to characterize its subpopulations and its potency of immunosuppression. Abundant evidence with different methodologies highlights $CD39^{+}FOXP3^{+/-}$ as the major regulatory T cells present in tumor tissue and also elevated in peripheral blood from patients compared to healthy donors [98–104]. Furthermore, the levels of $CD39^{+}$Tregs in peripheral blood from CRC patients correlated to TNM staging and clinicopathological features, since the expression of this protein gradually increase from the initial to the advanced stages [98, 101, 105]. Like FoxP3, Helios is a Treg marker that confers immunosuppression function [106] and Helios co-expression with CD39 was found in some studies [100, 104]. In addition, Treg co-expressed CD39, CTLA-4 and PD-1 in CRC tumor and peripheral blood, suggesting a potent immunosuppressive activity [98–100]. Khaja et al even highlight the simultaneous blockade of the two molecules - CD39 and PD-1 - as a new modality of treatment for CRC patients.

Besides the levels of these cells in CRC TME, the immunosuppressive activity of $CD39^{+}$ Treg was investigated in few studies. *In vitro*, $CD39^{+}$ γδTreg (a subtype of T cells, mainly present on gastrointestinal tract) from CRC tissues inhibited $CD3^{+}$ effector T cells proliferation and function with more potency than all other T cells subsets extracted from the same tissues [98]. Furthermore, the investigation of the mechanism by which $CD39^{+}$ γδTreg exhibits the immunosuppressive effect showed few influences of IL-10, TGF-β, CTLA-4 and PD-1, while the blockade of ADO receptors drastically reduced the function of these cells. Hu et al also suggest that tumor cells expression of TGF-β1 induces differentiation of tumor-infiltrating $CD39^{+}$ γδTreg in an ADO-dependent manner. Already Zhan et al, found that right side CRC had worst results compareted with left side CRC, about: more frequency of $CD39^{+}$ γδTreg, high production of IL-17A and low of IFN-γ, increase of adenosine level, higher inhibited both CD4+/CD8+ T cell proliferation, lower expression IFN-γ by CD4+ T cells. Furthermore, the results indicated that this CD39+γδ Treg are derived from intestinal tissue–resident γδ T cells. The PLA2G4A gene had induced CD39 expression in γδ-TILs *in vitro* and *in vivo*, and increased on growth of tumor, metastasis and invasion *in vivo*. In tumor sample, in the right side, the low expression was better outcome and left side the low expression had poor outcome [107].

Another investigation positively correlated the high frequency of $CD39^+$ Treg and their immunosuppressive activity, which was drastically reduced by its knockdown using siRNA [101].

In addition, the Treg $CD39^{high}$ expression derived from CRC patients were tested *in vitro* on their capacity to reduce T cells transendothelial migration (TEM). Treg from cancer patients reduced TEM compared to cells from healthy volunteers. The enzyme activity contributes to ADO-mediated reduction of T cell migration, since the addition of ADO reduced TEM while the CD39 and ADO receptors blockade enhanced it. The mechanism was then investigated, and results showed that ADO-induced reduction of TEM is mediated through monocytes, which decrease the capacity to activate the endothelium and promote ICAM-1 expression. In the same study, the CD73 expression on Treg was analyzed, but no difference was observed between tumor or healthy tissue samples [99]. Although more studies to investigate the effect of CD39 expression on regulatory T cells in CRC are needed, these data showed different perspectives of the immunosuppressive activity of these cells, indicating their majority in TME and potent ability to reduce T effector cell proliferation, function and migration into the tumor.

The CD39 expression was also observed in non-regulatory cells derived from CRC patients. Two different subtypes of $TCD8^+$ cells were identified in CRC tumors, one referred as bystander, which are specific for unrelated antigens in an antigen-specific response, and in tumor-specific tumor-infiltrating lymphocytes (TIL) $CD8^+$. Marked lack of CD39 was found $CD8^+$ bystanders, while high expression was found in TIL $CD8^+$. The TIL-$CD39^+$ had a high expression of genes related to proliferation and exhaustion, characteristic of chronic stimulation of T cells, referring to an exhausted cell population, while TIL-$CD39^-$ was referred as TIL-$CD8^+$ bystander. Furthermore, TIL$CD8^+CD39^+$ cells had expression genes related to pathways of antigen presentation and processing. The authors suggest CD39 as a biomarker for $TCD8^+$ cells, which could identify and isolate T cells with a bystander role [108]. Others authors find lymphocytes T CD8+ $CD39^{high}$ were more expressive in tumor than non-tumor sample, present in initial stage of CRC (I-II), and with high PD-1 expression and lower INF-y production, correlated with T cells exhausted and suppressed CD4+ T cell proliferation [109]. Already Rodin et al, studied an specific mucosal-associated invariant T cell (MAIT), that

recognizing microbial metabolites, with effector functions that are relevant for tumor immunity. Theses MAIT cells had a terminally exhausted phenotype (PD-1highTim-3+CD39+) and increased proliferation, the participation of CD39 activity as source of immunossupressive adenosine in the TME remains to be investigated in this scenario. The exhausted MAIT cells have reduced polyfunctionality with regard to production of important anti-tumor effector molecules, and blocking antibodies to PD-1 partly improved activation of tumor-infltrating MAIT cells *in vitro* [110].

The T cells are the most cells studied in TME and specially for the CD39 expression, considering it has been proposed as a marker for more suppressive Treg. However, other immune cells are present in the tumor as discussed before and importantly interact with the microenvironment. Accumulation of both Treg and MDSCs were found and the role and of MDSCs were analyzed through the course of treatment in peripheral blood from mCRC patients in advanced stage receiving FOLFOX plus bevacizumab. A higher level of these cells was observed in untreated mCRC, especially granulocytic MDSCs (gMDSCs) and it was associated with decreased overall survival (OS) and progression-free survival (PFS). Meanwhile, the FOLFOX plus bevacizumab reduced gMDSCs and increased OS and PFS. Then, the study investigated the expression of PDL-1, CD39 and CD73 in MDSCs and the gMDSCs of mCRC patients expressed the highest levels of the three markers. Furthermore, MDSCs potently inhibited T cell proliferation and activity, which was restored by the blockade of CD39-CD73 and PD1-PDL-1 axis, suggesting the potent immunosuppressive activity of these cells in CRC in an ADO-pathway and PD1/PDL-1 dependent manner [111].

In accordance with other authors, the paper suggests the use of ectonucleotidases and PD-1/PDL-1 inhibitors as potential therapy in association with FOLFOX-bevacizumab. Another investigation aimed to characterize MDCSs and had similar results. Indeed, the number of $Lin^{-/low}HLA\text{-}DR^{-}CD11b^{+}CD33^{+}$ (MDSCs) in peripheral blood was markedly increased in CRC patients compared with healthy donors. The MDSCs levels were correlated with tumor metastasis and, among other findings, these cells had high CD39 expression. *In vitro* tests also showed the immunosuppressive activity and the potential to inhibit $CD3^{+}$T cells proliferation of MDSCs from two stage IV CRC patients [112].

In order to determine the expression and role of CD39 in CRC progression and metastasis, an investigation analyzed it in stromal and epithelial cells *in vivo* and in tumor tissue and the results were surprisingly different from the others. All mice CD39 null died precociously and, in terms of tumor size, the primary tumor model mice did not have any differences among wild-type, heterozygous CD39 and human transgenic CD39 mice. In the metastatic model, the metastasis of htCD39 livers had significantly more growth associated with higher liver injury markers, which suggests that the overexpression of the human enzyme in mice promotes tumor progression and metastasis. Furthermore, tumor cells of the primary CRC model expressed $P2Y_2$, P2X7 and NTPDase2 (CD39L1). On the other hand, metastasis had high CD39 expression in tumor infiltrates in the three groups of mice in epithelial cells, macrophages and in stromal cells. Both primary and metastasis had high CD39 expression in the tumor border. About macrophages polarization, CD39 inhibitor increased CD11b and Ly6C expression in M1 TAM and F4/80 in macrophages *in vitro*. And CD39 inhibitor promote smaller tumor growth, Ly6C and MHC II in F4/80+, increased CD8 T cells in the spleen, CD4 in the blood, and Caspase-3 expression, comparated with control group *in vivo*. In the tumor tissue from CRC patients the results showed lower CD39 levels when compared to non-neoplastic tissue and, mainly in early stages, CD39 expression was less than normal borden, while medium stage (T3N±M1) expressed levels comparable to normal borden. Opposed to the *in vivo* experiment, $P2Y_2$ and P2X7 expression were markedly decreased in tumor tissue than normal borders [113]. However, different of these found, higher CD39 expression in tumor tissue vs. normal tissue was observed in other study, where CD39 inhibition *in vitro* decreased tumor cells proliferation [114].

In or hand, the presence of variant allele CD39 in mCRC patients samples was favorable to chemotherapy response plus bevacizumab these patients, suggest single nucleotide polymorphisms (SNPs) as predictors to prognosis [115].

Another finding that is distinct from the others is the investigation of the NTPDase5 (ENTPD5/PCPH or CD39L4), which is the only member of the NDPDase family that was described as a proto-oncoprotein and its gene is known as mt-PCPH. The ATP levels in HCT-116 and HCT-15 cells after transfection with myc-tagged mt-PCPH were significantly lower, but *in vitro* and *in situ* tests showed lack of PCPH activity, indicating that it might act through protein-protein

interactions [116]. The studies, therefore, evidence that the CD39 expression is extensively associated with a more immunosuppressive profile of the immune cells, mainly Treg but also MDSCs. Nevertheless, the mechanisms of the CD39 mediated suppression and its correlation with tumor progression and prognosis in CRC patients could be deeper investigated. In addition, the levels and impact of the enzyme might vary according to the cells expressing it, considering Kunzli et. all results and more studies are needed to determine CD39 in tumor, stromal and epithelial cells within the tumor and the tumor borders [113].

4.1.2. Ectonucleotidases - CD73 participation in CRC progression

CD73 is also an enzyme anchored to the plasma membrane in the C-terminal portion by a residue of glycosyl-phosphatidylinositol (GPI) and can also be found in the form soluble in the cytosol or in the extracellular medium [94, 117]. Physiologically, CD73 is widely distributed in different tissues, being expressed in the colon, kidney, brain, liver, heart, lung, vascular endothelium, spleen, lymph nodes and bone marrow [118]. In the immune system, CD73 is found on the surface of Treg lymphocytes, neutrophils, MDSCs, DC, NK cells and macrophages [26]. Pathologically, CD73 is overexpressed in several types of tumors, including CRC [119–124].

CD73 expression was reduced under normoxia and hypoxia conditions in a highly liver-metastatic human colorectal cancer cell subline (SW48LM2 cells) when compared with less malignant tumor cells. In addition, mCRC cells exhibited a decrease of extracellular nucleoside levels (ADO, guanosine and INO) and a parallel increase of nucleotides (AMP, GMP and IMP), which indicate that loss of CD73 expression and the changes in purine metabolism as beneficial for tumor progression [125]. By the other hand, the gene expression analyzed from tumor tissue that received cetuximab treatment or FOLFIRI/FOLFOX, or the combination of both, correlated low CD73 expression with better progression free survival (PFS) [126, 127]. When KRAS status was analyzed, in the wild type group, CD73 levels were predictive of PFS benefit from cetuximab and OS benefit from cetuximab, while no predictive effects for CD73 were observed in KRAS mutated patients. Also, CD73 exhibited no correlation between plasma protein levels and tumor mRNA expression levels [127].

In contrast, higher expression of CD73 in CRC cells was observed in several studies [128–132]. CD73 overexpression was associated with high malignant potential, increased cell proliferation, increased the cell cycle protein D1, its transcription regulation molecule β-catenin and epidermal growth factor receptor (EGFR) [130], familiar historicity of cancer, lymph nodes metastasis, nerve invasion, distant metastasis and advance tumor staging [131]. In liver metastasis, the CD73 expression also was associated with worse pathological features and poorer response to preoperative therapy [132]. About the prognosis, OS was lower in patients with $CD73^{high}$ tumor expression when compared to those $CD73^{low}$ [128, 129, 131–133]. Furthermore, the CD73 expression was significantly lower in of chemotherapy responders patients, the RAS-MAPK-inhibition induces CD73 upregulation and immunosuppressive response in TME [133]. For this reason, some authors suggest CD73 as a biomarker for poor prognosis [129, 130]. Besides that, the inhibition of CD73 in a mouse model of colitis-associated tumorigenesis reduced histological damage of the colon, and it was significantly increased with ADO receptor agonist (NECA), with increased TNF-α and IL-6 [134]. Furthermore, using the same model, the CD73 inhibitor (AB680) generated unlock of anticancer immune response, increased CD8+T cells activation and improved Treg and exhausted T cells [135]. In a study with a microRNA (miR30a) that inhibits cell proliferation and promotes apoptosis of tumor cells was downregulated in CRC, *in vitro*, *in vivo* and tumor tissue, the authors suggest that microRNA acts through the downregulation of CD73 expression [136]. The CD28 coexpression was also evaluated, and knockdown reduce CD73 expression in CD8+ T cells *in vitro* [137].

Cancer associated fibroblasts (CAFs) expressed high levels of CD73 in colorectal cancer and were strongly correlated with poor prognosis. The results describe the CD73 immune checkpoint as a feedforward circuit, which is enhanced by ADO release in tumor cell death. ADO binds to A2B receptors, increasing CD73 expression and A2A receptors, inducing the immune suppression in the tumor. Targeting this circuit significantly improved antitumor immunity in CAF-rich tissues [21]. The CD73 expression was evaluated also in tumor and stromal tissue and was overexpressed in both, the increased CD73 in the stromal compartment was correlated with less advanced tumors and better outcome. These findings suggest that different cells can overexpress CD73 and its function

and correlated outcome can variate according to the localization, the cell type and the interaction involved [129].

The extracellular vesicles also evaluated and presented enriched CD73 level, in vitro the CD73 inhibition caused reactive of proliferative and cytotoxic capacity of T cells [138].

A study showed that CD73 expression was decreased under fasting protocol induction, followed by a decrease of *in vitro* and *in vivo* CRC growth, decreased adenosine levels and M2-TAM polarization. In addition, the fasting protocol promoted *in vitro* autophagia which was CD73 suppression-dependent in CT26 cells, and the impairment of M2-TAM polarization was associated to inactivation of JAK1/STAT3 pathway, suggesting that antitumor immunity induced by fasting is mediated by ADO signaling blockage [139].

In summary, there is more evidence that CD73 expression is favorable to CRC growth and metastasis. However, the effect of CD73 on tumor promotion is dependent on tumor stage and on its expression in a specific tumor/non-tumor cell that compose TME. Finally, considering that the majority of the investigations were focused in lymphocytes, additional studies to determine the CD73 expression and function in innate immune cells present in the TME of CRC is also needed.

4.2. Purinoceptors - General Aspects

Purinergic signaling is initiated by the release of nucleotides and nucleosides into the extracellular space. ATP under normal conditions is at the millimolar range in the cytoplasm (3–10 mM) and at the nanomolar range in the extracellular side [140]. However, ATP can be released through the plasma membrane by lytic (cell death) and nonlytic mechanisms (vesicles and membrane channels formed by the pannexin-1/connexins) [141, 142]. Following their release into the extracellular space, the nucleotides and nucleosides exert their effects through interactions with specific membrane receptors called purinergic receptors or purinoceptors [143]. These receptors are responsible for message transmission. Purinoceptors are divided into two groups, the P1R, which has the main endogenous agonist, ADO, and the P2R, which are sensitive to multiple di- and triphosphate nucleosides, such as ATP, ADP, UTP, and UDP [144]. P1R are receptors coupled to G protein divided into four subtypes, A1R, A2AR, A2BR,

and A3R distributed among various cell types [144]. When P1 receptors are stimulated they control events related to cell proliferation, angiogenesis, immunomodulation, cell migration and chemoresistance [145]. These receptors comprise a conserved and complex communication system present in various tissues and cells. P1R are expressed in heart, lung, liver, testis, muscle, spinal cord, spleen, intestine, immune system and brain in different levels and ratios [146–148]. However, in certain conditions, for example an establishment of a pathological situation like a tumor development or an persistent exacerbated inflammation, the cells may answer by adjusting the expression of these receptors [149, 150].

4.2.1. ATP and ADO as agonists of purinorreceptor-mediated protumor actions

The role of extracellular ATP is still not well consolidated in CRC, since conflict data are found in the literature and it might be due to different functions depending on the receptor and the type of cell involved. Studies have shown that the high concentration of ATP and P2R agonists reduced cell viability and the proliferation at the S phase of cell cycle through the inhibition of protein kinase C (PKC) in CRC cells line [151, 152]. In contrast, *in vitro* investigation indicated that the Caco-2 exposition to ATP increased multidrug resistance-associated protein 2 (MRP2) expression, which conferred resistance to etoposide, cisplatin and doxorubicin and enhanced survival of the cells [153]. The study also suggests that the mechanism is mediated by P2Y receptors, but further investigation is needed to elucidate which receptor might be involved [154].

Indeed, the ATP-based chemotherapy response assay is a method used to measure tumor response to therapy, since the tumor cells quickly deplete ATP levels. Peripheral blood samples from advanced CRC patients in adjuvant chemotherapy with FOLFOX or Mayo clinic regimen were tested for ATP levels. The responsive groups (>40% ATP reduction) for both treatments positively correlated to OS and PFS, showing the ATP as an important marker and a key to individualized chemotherapy [155]. However, *in vivo* tests with a therapy based on inducing immunogenic cell death (ICD) resulted in enhanced ATP secretion, as other molecules, like dangerous signals by the tumor, which correlated to tumor reduction and increased immune response of $CD8^+$ T cells [153].

The ATP levels can be used for many objectives it can be leaked out of the cell by membrane channels, by damage in the cell membrane or death cell, which explains the variance in results observed. The studies *in vitro* showed the opposite role of ATP, it can be desfavorable for tumor progression at the same time that it can be favorable for chemotherapy resistance, the last one was also shown in tumor samples. The increase of ATP secretion in *in vivo* studies was caused by cell damage induced by the treatment, with increased immunogenicity and improved immune system response. Keeping open the question of the benefit or harm of ATP for this type of tumor.

The only investigation of ADO as the main purinergic molecule found in this research was a adenosine-related single nucleotide polymorphism (SNP) analysis from a cohort with 451 mCRC patients. Indeed, three SNPs were positively associated with OS in the group treated with FOLFIRI plus bevacizumab (CD39 rs11188513, CD73 rs2229523, and A2BR rs2015353). This data suggests the adenosine pathway is not only prognostic but also predictive for bevacizumab-based chemotherapy [156].

4.2.2. The P1 receptors (A1, A2A, A2B, A3) and its relationship with CRC progression

P1R differ in their affinity for the agonist. A1R, A2AR and A3R are receptors of high affinity and A2BR low affinity for ADO [148]. Accordingly, under physiological concentrations adenosine is present at low levels in both the intracellular and extracellular space (nanomolar range) and mediate effects via A2AR, A1R, and A3R. When adenosine concentrations reach elevated levels in the micromolar range such as in the inflammatory situation or in the tumor microenvironment, A2B assumes the role of being the principal receptor mediating adenosine signaling [157].

A1R and A3R are coupled to the Gi or Go proteins, and their activation leads to a decrease in intracellular cyclic AMP (cAMP) levels, whereas A2AR and A2BR are coupled to Gs protein, resulting in increased levels of intracellular cAMP. The stimulation of A1R and A3R can also trigger the release of Ca^{2+} ions from intracellular stores, as well as A2B receptor stimulation can also activate phospholipase C [144, 157]. Of note, all the P1R are coupled to MAPK/ERK signaling pathways [158].

Studies have shown that P1R is differentially expressed in CRC cell lines and in CRC tissues as well [159, 160]. Interestingly, the A2B receptor seems to exhibit a dual role in CRC progression. Indeed, A2B receptor expression was considerably higher than A1, A2A and A3 in CRC tissues and in five CRC cell lines (DLD1, SW480, HCT-15, LOVO, and COLO205), while no significant difference was detected for the other P1 receptors. Furthermore, A2B was overexpressed under hypoxia conditions, and no difference was detected in A1, A2A or A3 expression in normoxic or hypoxic conditions. It is possible that the overexpression of A2B in hypoxia contributes to cancer cell growth and angiogenesis probably by stimulating HIF-1alpha [150]. In contrast, the expression of A2B was associated with Saos-2, Phoenix Eco, U2OS, and HCT116 cell death. The activation of p53 in response to cell stresses in TetOn-p53-WT Saos-2 directly stimulates the A2B gene expression which in turn contributes to p53-induced apoptosis. Moreover, A2B receptor is involved in hypoxia and chemotherapy-induced cell death in Saos-2 by regulating Bcl-2 family - a group of factors that mediates intrinsic cell death [161].

Finally, the PBS-603, an A2B receptor antagonist, induced acute increase of ROS, and mitochondrial oxygen consumption, which changed the cell metabolism and increased the *in vitro* sensitivity to chemotherapeutic agents. However, the cytotoxic activity was independent of A2B activation, as its knockdown in cancer cells did not confirm the effects triggered by PBS-603 [162]. These data evidentiate the controversial effect of the A2B in CRC, which was related to both proliferation and apoptosis. The results show different perspectives of the association of A2B expression and hypoxia conditions and further analysis are needed to properly elucidate its role and also its selective agonists/antagonists.

On the other hand, other analysis found high A2A expression and associated worst tumor features and prognosis and the presence of TILs [160, 163]. In 204 tumor specimens from CRC patients, both PDL-1 and A2A expressions were higher in tumor than in adjacent non-tumor tissue. PD-L1 expression was correlated with lymph node metastasis and tumor TNM stage, while A2A levels were correlated with tumor size, tumor invasion, and TNM stage. Also, the high expressions of PD-L1 and A2A were inversely correlated with OS, showing the independent prognostic predictor value of both immunosuppressive markers in

CRC [160]. In RNA-seq and whole exome seq data investigated from 453 colon adeno-carcinomas (COAD) and rectal adenocarcinomas (READ), the results showed association between tumor associated lymphocytes (TILs) and the co-inhibitory immune checkpoints involved in the inactivation of the TILs was investigated. Among the 9 signatures of immune checkpoints analyzed, the TIL load positively correlated with the expression of adenosine A2A receptor in COAD, in accordance with the potential role of this receptor to favor the tumor [163].

The A1 receptor was investigated in one study involving metformin - an anti-diabetic drug- treatment. Upregulation of A1 expression at mRNA and protein levels was observed in HCT116 and SW480 cells treated with metformin and related to induction of apoptosis. The effect was dependent on mTOR-inactivation, a key player of cell survival and proliferation pathways of cancer cells, which results in AMPK activation, leading to cell cycle arrest and apoptosis [159].

Using [18F]FE@SUPPY as a PET-tracer for A3, higher expression of the receptor was found in HT-29 cells and in human CRC tissue compared to normal adjacent mucosa, but lower expression was detected *in vivo* with HT-29 xenograft, due to the inefficacy of the tracer in this model [164]. On the other hand, a A3 receptor agonist (N6-(2,2-diphenylethyl)-2-hexynyladenosine) caused proliferation inhibition in dose and time dependent manner in Caco-2 cells, and the study suggests that it can be a novel anti-cancer agent. But the observed - cell death in an apoptotic or autophagy-dependent manner, cell growth inhibition, migration inhibition and induction of higher ROS level - was A3 receptor independent effect, as the A3 knockdown did not reverse/prevent the effect induced by A3 agonist [165]. The role of A3 receptors, therefore, is still not comprehended in CRC, since the effect of the agonist tested wasn't due to the receptor mechanism. Although the first study evidentiated the high expression of A3 in cells and tumors, the authors had a different objective other than investigating the receptor effects.

4.2.3. The P2 receptors - P2X participation on CRC development

P2Rs comprise two major categories of receptors, P2X1-7, ionotropic receptors that are sensitized by extracellular ATP, and $P2Y_{1,2,4,6,11–14,}$ metabotropic receptors stimulated by ATP, ADP, UTP, UDP, and UDP-glucose) [144].

P2XR receptors have intracellular N- and C-subunits that bind to protein kinases and also have two transmembrane regions, involved in the channel activation and ionic pore coats. P2XR subtypes differ in their rates of desensitization; ion conductivity; and sensitivity to agonists, antagonists, and allosteric modulators [143, 166].

P2X1 receptor is widely expressed in smooth muscle and mediates the neurotransmitter actions of ATP and Ca^{2+} influx in platelet aggregation [167, 168]. P2X2 receptors are widely expressed in central and peripheral neurons and have been implicated in neurotransmission [169–171]. P2X3 receptors are expressed in sensory neurons [172–174]. The receptors P2X4 and P2X6 are expressed in the central nervous system, endothelial cells and thymus [141, 175–177]. P2X7 receptor is expressed in immune cells, pancreas, skin, and microglia and mediates a response against high concentrations of ATP leading to cell death [166, 178–184]. P2XR cell signaling is mediated by gating primarily $Na^{+,}$ K^{+}, and Ca^{2+} and, occasionally Cl^{-} [185, 186].

P2X7 expression analysis in human tumor tissues and adjacent tissue it was seen that this receptor expression was higher in tumors, mainly in highly malignant tumors, which was identified as an independent variable of worse prognostic, correlationated with tumor size, lymph node metastasis, and TNM stage. In addition, it was verified that the activation of Akt and NF-κB by a P2X7 agonist accelerated CRC proliferation [187]. Zhang et al, found that both low and high expressions were found in CRC tissue compared with normal tissue, but, similar to previous study, the higher expression of P2X7 correlated to shorter survival [188]. Furthermore, P2X7 is more expressed in mCRC tissues than in primary tumors, suggesting the association with metastasis. The higher expression of the receptor was also found in CRC cell lines compared with normal colon cell lines and in metastatic-derived CRC cell lines compared with primary tumor-derived [188]. Both authors suggest P2X7 as a target to CRC treatment [187, 188]. Using the P2X7 inhibitor (A438079) or P2X7 suppression the results were: inhibits proliferation, invasion, migration, promotes apoptosis, increase the number of tumor spheres *in vitro*. The inhibitor also inhibits tumor growth *in vivo* [189] and P2X7 overexpression increase the growth and weight of tumor by increase of proliferation, angiogenesis and CSCs properties, increased macrophages infiltration, TAM recruitment hypoxic site, HIF-1α and HIF-1β, and reduced

apoptosis rates *in vivo* [190]. It was shown that NK depletion or Treg transfer promoted high P2X7 expression in mice with T cell down regulated and spontaneous bowel tumor, besides that, the mice had increased tumor growth, multiplicities of colon tumor and intestinal polyp formation, correlated with less survival [191]. In contrast, the P2X7 blockade in a colitis-associated cancer model *in vivo* promoted Treg infiltration, increased neutrophils, epithelial cell growth and protection against apoptosis, suggesting a protective factor of P2X7 [192].

The construction of a prognostic risk prediction model for CRC, by analysis of colon cancer tissue and normal colon tissue from three independent datasets (GEO and TCGA), found P2X5 between the four more important high risk genes. The differentially expressed genes found are mainly involved in protein transport, apoptotic and neurotrophin signaling pathways [193].

Briefly, just two receptors (P2X7 and P2X5) had papers included, showing the lack of information about P2X receptors in CRC. So far, P2X7 was seen mainly as a bad factor to CRC progression, with large studies showing it robustly.

4.2.4. The P2 receptors - P2Y participation on CRC development

$P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ are activated by ATP and ADP, $P2Y_2$ and $P2Y_4$ are activated also by UTP , $P2Y_6$ is activated by UDP and $P2Y_{14}$ receptor is activated by UDP-glucose [170]. Activation of the protein Gq mediated by P2YR leads to the stimulation of phospholipase C, production of inositol-(1,4,5)-trisphosphate and diacylglycerol, increases intracellular Ca^{2+} levels and diacylglycerol stimulates protein kinase C and may inhibit adenylate cyclase [194]. P2YR are expressed in a variety of tissues and organs: lung, kidney, pancreas, adrenal gland, heart, vascular endothelium, skin, muscles and brain.

The purinergic receptors are much more complicated to investigate considering the methodologic limitations and the complexity of the subtypes and their activation and mechanisms of action according to the cell expressing it. Few studies of the P2YR were found and only included $P2Y_2$, $P2Y_6$ and $P2Y_{12}$. The $P2Y_2$ was investigated in one *in vitro* study, which analyzed the resistance to apoptosis mediated by the treatment with ursolic acid in HT-29 cells. The results showed the treatment induced ATP production, which is probably released and binds to $P2Y_2$ receptors. The receptor then activates tyrosine kinase Src and

leads to p38 phosphorylation. The p38 pathway induces the expression of COX-2 and results in chemoresistance [195].

The $P2Y_6$ receptor was analyzed in two articles, one *in vitro* and one both *in vitro* and *in vivo*. The role of $P2Y_6$ receptors in cell migration were investigated in lung and colon cancer cell lines and stimulation of Caco-2 cells monolayer with a $P2Y_6$ selective agonist (MRS2693) induced the filling in the plaque of the cells and focal adhesions and filopodia. The addiction of $P2Y_6$ antagonist (MRS2578) had the opposite effect, showing a potential role of this receptor in the tumor growth [196]. Meanwhile, the treatment of HT-29 cells with the $P2Y_6$ agonist prevented the apoptotic effect of TNF-α, and an increase of X-linked inhibitor of apoptosis protein (XIAP) expression was shown, correlated with AKT phosphorylation [197]. *In vivo* experiments with $P2Y_6^{+/+}$ and $P2Y_6^{-/-}$ mice showed, in knockout animals, the number and size of colorectal tumors was significantly reduced. Besides that, $P2Y_6^{-/-}$ group had a significantly less dysplastic score and reduced vascularization when compared to $P2Y_6^{+/+}$, which indicates the role of these receptors in tumorigenesis. The expression of the transcriptional factor related to various types of cancers β-catenin was investigated to further understand the effect of $P2Y_6$ downregulation. β-catenin was observed in plasma membranes of $P2Y_6^{-/-}$ derived cells, while in plasma membrane, cytosolic, perinuclear and nuclear staining of $P2Y_6^{+/+}$. Furthermore, the presence of β-catenin in the nucleo correlated with the abnormal expression of proto-oncogene c-MYC. These data indicate the expression of $P2Y_6$ is associated with the localization of β-catenin related to proliferative and invasive phenotypes and poor outcomes in CRC patients [197]. However, the authors indicate the cautious interpretation of their results, since the number of samples was small and further investigations are needed.

The $P2Y_{12}$ receptor in platelets is well known for its role in arterial thrombosis and its antagonists are used for the prevention and treatment in cardiovascular diseases. The efficacy of one of $P2Y_{12}$ antagonist (ticagrelor) therapy for breast and CRC was tested, since the platelets participate in cancer-associated thrombosis and in metastasis. Ticagrelor significantly inhibited formation of tumor cell-induced platelet aggregation *in vitro* and, in the patients, the treatment also reduced its aggregation and activation. These findings suggest the role of $P2Y_{12}$

in the platelets aggregation in cancer and its inhibition as a potential therapy for patients with high risk of cancer-associated thrombosis [198].

5. Concluding remarks

The increased expression of CD73 on immune cells or resident cells, together or not with the increased expression of CD39, leads to blocking the response of the effector immune cells and tumor progression in CCR. When CD73 was expressed by the tumor cell, it was related to tumor progression and decreased survival, while achieving a better response to chemotherapy; while the decreased expression of this enzyme was associated with lower ADO levels, decreased macrophage M2 polarization, increased autophagy, increased levels of AMP, IL-6 and TNFα. egarding the P1 family receptors, A1 expression was related to less tumor regression; A2A with tumor progression, lower survival and increased PDL-1 expression; A2B with increased proliferation but also increased apoptosis; and A3 had no association. The P2X and P2Y families: P2X5 had a lower prognosis; P2X7 decreased survival, increased tumor progression and possible protective factor. P2Y2 increased apoptosis but increased resistance to chemotherapy; P2Y6 increased migration and increased resistance to chemotherapy; P2Y12 increased platelet aggregation. ATP at high levels had greater tumor progression and chemoresistance, and decreased survival, while showing less proliferation. The Figure 3 summarizes the main findings of the current study.

Bias of this work is only in English language papers and published after 2009 were included. Is possible that important papers in different idioms and older than 2009 were lost. Furthermore, one article chosen cannot be found, which forced us to exclude it.

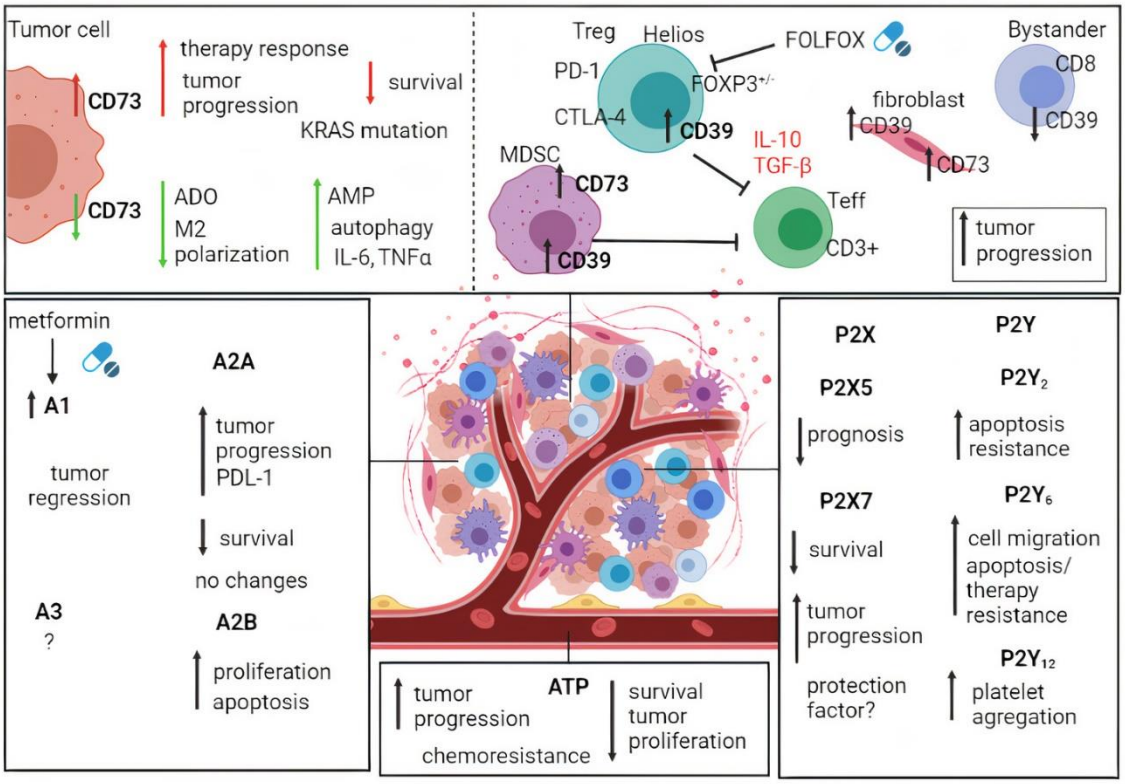


Figure 3. Hallmarks of purinergic signaling in colorectal cancer progression. Abbreviations: ATP (adenosine triphosphate); AMP (adenosine monophosphate); ADO (adenosine); IL-6 (interleukin-6), IL-10 (interleukin-10), TNFα (tumor necrosis factor alpha), TGFβ (Transforming growth factor beta), Treg (T regulatory lymphocyte), Teff (T effector lymphocyte), MDSC (myeloid-derived suppressor cell), FOLFOX (5-FU e leucovorina com oxaliplatina, PD-1, CTLA-4); A1, A2A, A2B, A3 (P1 purinergic receptor sensitized by adenosine) P2X5, P2X7 (P2 purinergic receptor sensitized by ATP); P2Y2, P2Y6, P2Y12 (P2 purinergic receptor selective for ATP/UTP, UDP, and ADP as agonist, respectively); ADP (adenosine diphosphate); INO (inosine); NAD+ (nicotinamide adenine dinucleotide); PNX1, P2X (ionotropic purinergic receptors), P2Y (metabotropic purinergic receptors), P1 (adenosine metabotropic receptors), E-NTPDase1/CD39 (ecto-nucleoside triphosphate diphosphohydrolase 1), ENTs (nucleoside equilibrative transporters); CNTs (concentrative nucleoside transporters); ADA (adenosine deaminase).

<u>Open Questions</u>

- There are a large number of studies investigating the participation of T cells on CRC progression. However, little is known about the role of other cells, mainly the innate immune cells such as macrophages, neutrophils and MDSCs. The macrophages have been studied for its importance in the TME and it has been robustly correlated with purine signaling, yet is still neglected in CRC.
- In addition, another immune parameter that could be more studied is the correlation between cell exhaustion and the purinergic signaling, since their presence and role is well established in cancer and these cells are targets for immunotherapies.
- Considering that the TME is fundamental to tumor progression and the site of CRC is not sterile, the microbiome presence certainly impacts the tumor development as well as the response to chemo-radio-immunotherapy. Although there is interest in the role of microorganisms, we did not find studies about microbiome correlated with the purinergic system in CRC. It is needed to better know how the microenvironment affects enzymes and receptors purine to discover possible targets.
- Considering the TNM system presents flaws in the staging of CRC patients, the purine molecules and enzymes can be used as potential additional biomarkers for a better understanding of the tumor progression and the response to chemo-radio-immunotherapy.
- Regarding the studies that analyzed ectonucleotidases expression, the majority did not show the localization of the enzymes: tumor cell, stroma cell, immune cell. This focus is necessary because the specific cell expression profile may correlate with better or worse prognosis for patients.
- Due to the difficulty in analyzing the function of the receptors, many studies have failed to prove that the effects shown were mediated by purinoceptors.

Funding

The authors would like to thank the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS; process number 21/2551-0000078-3), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; code

001), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – process numbers 312187/2018-1), Santa Casa de Misericórdia de Porto Alegre, and Universidade Federal de Ciências da Saúde de Porto Alegre. G.G. Roliano, V.T. Brunetto, and E. Braganhol were recipients of CAPES, FAPERGS, and CNPq fellowships.

Conflict of Interest

Authors declare no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

All authors are in agreement with the content of the manuscript and with the submission.

Data availability

It is not applicable.

References:

1. Siegel RL, Miller KD, Goding Sauer A, et al (2020) Colorectal cancer statistics, 2020. CA Cancer J Clin 70:145–164. https://doi.org/10.3322/CAAC.21601
2. Müller M, Ibrahim A, Arends M (2016) Molecular pathological classification of colorectal cancer. Virchows Arch 469:125–134. https://doi.org/10.1007/S00428-016-1956-3
3. Gian L de'Angelis, Lorena B, Cinzia A, et al (2018) Microsatellite instability in colorectal cancer. Acta Bio Medica Atenei Parm 89:97. https://doi.org/10.23750/ABM.V89I9-S.7960
4. Buccafusca G, Proserpio I, Tralongo AC, et al (2019) Early colorectal cancer: diagnosis, treatment and survivorship care. Crit Rev Oncol Hematol 136:20–30. https://doi.org/10.1016/J.CRITREVONC.2019.01.023
5. Dekker E, Tanis PJ, Vleugels JLA, et al (2019) Colorectal cancer. Lancet

394:1467–1480. https://doi.org/10.1016/S0140-6736(19)32319-0

6. Mármol I, Sánchez-de-Diego C, Dieste AP, et al (2017) Colorectal carcinoma: A general overview and future perspectives in colorectal cancer. Int J Mol Sci 18:. https://doi.org/10.3390/IJMS18010197
7. Dzunic M, Petkovic I, Cvetanovic A, et al (2019) Current and future targets and therapies in metastatic colorectal cancer. J BUON 24:1785–1792
8. Cremolini C, Loupakis F, Antoniotti C, et al (2015) FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment of patients with metastatic colorectal cancer: updated overall survival and molecular subgroup analyses of the open-label, phase 3 TRIBE study. Lancet Oncol 16:1306–1315. https://doi.org/10.1016/S1470-2045(15)00122-9
9. Piawah S, Venook AP (2019) Targeted therapy for colorectal cancer metastases: A review of current methods of molecularly targeted therapy and the use of tumor biomarkers in the treatment of metastatic colorectal cancer. Cancer 125:4139–4147. https://doi.org/10.1002/CNCR.32163
10. Arnold D, Lueza B, Douillard JY, et al (2017) Prognostic and predictive value of primary tumour side in patients with RAS wild-type metastatic colorectal cancer treated with chemotherapy and EGFR directed antibodies in six randomized trials. Ann Oncol 28:1713–1729. https://doi.org/10.1093/ANNONC/MDX175
11. American Cancer Society (2021) American Cancer Society. Cancer Facts & Figures 2021. Atlanta: American Cancer Society; 2021
12. Robinson J, Newcomb P, Hardikar S, et al (2017) Stage IV colorectal cancer primary site and patterns of distant metastasis. Cancer Epidemiol 48:92–95. https://doi.org/10.1016/J.CANEP.2017.04.003
13. Jin K, Gao W, Lu Y, et al (2012) Mechanisms regulating colorectal cancer cell metastasis into liver (Review). Oncol Lett 3:11–15. https://doi.org/10.3892/OL.2011.432
14. Liu Q, Ma Y, Luo D, et al (2018) Real-world study of a novel prognostic scoring system: for a more precise prognostication and better clinical treatment guidance in stages II and III colon cancer. Int J Colorectal Dis 33:1107–1114. https://doi.org/10.1007/s00384-018-3071-1

15. Puppa G, Sonzogni A, Colombari R, Pelosi G (2010) TNM staging system of colorectal carcinoma: a critical appraisal of challenging issues. Arch Pathol Lab Med 134:837–852. https://doi.org/10.5858/134.6.837
16. Marks K, West N, Morris E, Quirke P (2018) Clinicopathological, genomic and immunological factors in colorectal cancer prognosis. Br J Surg 105:e99–e109. https://doi.org/10.1002/BJS.10756
17. Eaden J, Abrams K, Mayberry J (2001) The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut 48:526–535. https://doi.org/10.1136/GUT.48.4.526
18. Herrinton L, Liu L, Levin T, et al (2012) Incidence and mortality of colorectal adenocarcinoma in persons with inflammatory bowel disease from 1998 to 2010. Gastroenterology 143:382–389. https://doi.org/10.1053/J.GASTRO.2012.04.054
19. Sillo TO, Beggs AD, Morton DG, Middleton G (2019) Mechanisms of immunogenicity in colorectal cancer. Br J Surg 106:1283–1297. https://doi.org/10.1002/BJS.11204
20. Koi M, Carethers JM (2017) The colorectal cancer immune microenvironment and approach to immunotherapies. Futur Oncol 13:1633–1647. https://doi.org/10.2217/FON-2017-0145
21. Yu M, Guo G, Huang L, et al (2020) CD73 on cancer-associated fibroblasts enhanced by the A2B-mediated feedforward circuit enforces an immune checkpoint. Nat Commun 11:. https://doi.org/10.1038/S41467-019-14060-X
22. Gonzalez H, Hagerling C, Werb Z (2018) Roles of the immune system in cancer: from tumor initiation to metastatic progression. Genes Dev 32:1267–1284. https://doi.org/10.1101/GAD.314617.118
23. Lam, Khiem C; Araya, Romina E; Huang, April; Chen, Quanyi; Di Modica, Martina; Rodrigues, Richard R; Lopès, Amélie; Johnson, Sarah B.; Schwarz, Benjamin; Bohrnsen, Eric; Cogdill, Alexandria P.; Bosio, Catharine M.; Wargo, Jennifer A.; Lee, Maxwell P.; Gol RS (2021) Microbiota triggers STING-type I IFN-dependent monocyte reprogramming of the tumor microenvironment. Cell 184:5338–5356
24. Allard B, Longhi MS, Robson SC, Stagg J (2017) The ectonucleotidases CD39 and CD73: Novel checkpoint inhibitor targets. Immunol Rev

276:121–144. https://doi.org/10.1111/imr.12528

25. Allard D, Chrobak P, Allard B, et al (2019) Targeting the CD73-adenosine axis in immuno-oncology. Immunol. Lett. 205:31–39
26. Azambuja JH, Ludwig N, Braganhol E, Whiteside TL (2019) Inhibition of the Adenosinergic Pathway in Cancer Rejuvenates Innate and Adaptive Immunity. Int J Mol Sci 20:5698. https://doi.org/10.3390/ijms20225698
27. Burnstock G, Boeynaems J-M (2014) Purinergic signalling and immune cells. Purinergic Signal 10:529–564. https://doi.org/10.1007/s11302-014-9427-2
28. Ferrari D, McNamee EN, Idzko M, et al (2016) Purinergic Signaling During Immune Cell Trafficking. Trends Immunol 37:399–411. https://doi.org/10.1016/j.it.2016.04.004
29. Burnstock G (2009) Purinergic signalling: Past, present and future. Brazilian J Med Biol Res 42:3–8. https://doi.org/10.1590/S0100-879X2008005000037
30. Robson SC, Sévigny J, Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signal 2:409–430. https://doi.org/10.1007/s11302-006-9003-5
31. Yegutkin GG (2008) Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Biochim Biophys Acta - Mol Cell Res 1783:673–694. https://doi.org/10.1016/j.bbamcr.2008.01.024
32. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: The next generation. Cell 144:646–674
33. Karin M (2005) Inflammation-activated protein kinases as targets for drug development. Proc Am Thorac Soc 2:386–390. https://doi.org/10.1513/PATS.200504-034SR
34. Arneth B (2019) Tumor Microenvironment. Medicina (Kaunas) 56:. https://doi.org/10.3390/MEDICINA56010015
35. Gajewski T, Schreiber H, Fu Y (2013) Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol 14:1014–1022. https://doi.org/10.1038/NI.2703
36. Snoderly H, Boone B, Bennewitz M (2019) Neutrophil extracellular traps

in breast cancer and beyond: current perspectives on NET stimuli, thrombosis and metastasis, and clinical utility for diagnosis and treatment. Breast Cancer Res 21:. https://doi.org/10.1186/S13058-019-1237-6

37. Cassetta L, Pollard J (2018) Targeting macrophages: therapeutic approaches in cancer. Nat Rev Drug Discov 17:887–904. https://doi.org/10.1038/NRD.2018.169
38. Mantovani A, Marchesi F, Malesci A, et al (2017) Tumour-associated macrophages as treatment targets in oncology. Nat Rev Clin Oncol 14:399–416. https://doi.org/10.1038/nrclinonc.2016.217
39. Mizuno R, Kawada K, Itatani Y, et al (2019) The role of tumor-associated neutrophils in colorectal cancer. Int J Mol Sci 20:. https://doi.org/10.3390/IJMS20030529
40. Popěna I, Abols A, Saulite L, et al (2018) Effect of colorectal cancer-derived extracellular vesicles on the immunophenotype and cytokine secretion profile of monocytes and macrophages. Cell Commun Signal 16:. https://doi.org/10.1186/S12964-018-0229-Y
41. Sica A, Schioppa T, Mantovani A, Allavena P (2006) Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: Potential targets of anti-cancer therapy. Eur J Cancer 42:717–727. https://doi.org/10.1016/J.EJCA.2006.01.003
42. Gouveia-Fernandes S (2020) Monocytes and Macrophages in Cancer: Unsuspected Roles. In: Advances in Experimental Medicine and Biology. Springer, pp 161–185
43. Wang J, Li D, Cang H, Guo B (2019) Crosstalk between cancer and immune cells: Role of tumor-associated macrophages in the tumor microenvironment. Cancer Med 4709–4721. https://doi.org/10.1002/cam4.2327
44. Najmeh S, Cools-Lartigue J, Rayes RF, et al (2017) Neutrophil extracellular traps sequester circulating tumor cells via β1-integrin mediated interactions. Int J Cancer 140:2321–2330. https://doi.org/10.1002/IJC.30635
45. Granot Z, Jablonska J (2015) Distinct Functions of Neutrophil in Cancer and Its Regulation. Mediators Inflamm 2015:. https://doi.org/10.1155/2015/701067

46. Germann M, Zangger N, Sauvain M, et al (2020) Neutrophils suppress tumor-infiltrating T cells in colon cancer via matrix metalloproteinase-mediated activation of TGF β . EMBO Mol Med 12:. https://doi.org/10.15252/EMMM.201910681
47. Xiong Y, Wang K, Zhou H, et al (2018) Profiles of immune infiltration in colorectal cancer and their clinical significant: A gene expression-based study. Cancer Med 7:4496–4508. https://doi.org/10.1002/CAM4.1745
48. Malesci A, Bianchi P, Celesti G, et al (2017) Tumor-associated macrophages and response to 5-fluorouracil adjuvant therapy in stage III colorectal cancer. Oncoimmunology 6:. https://doi.org/10.1080/2162402X.2017.1342918
49. Ye L, Zhang T, Kang Z, et al (2019) Tumor-infiltrating immune cells act as a marker for prognosis in colorectal cancer. Front Immunol 10:. https://doi.org/10.3389/FIMMU.2019.02368
50. Bronte V, Brandau S, Chen SH, et al (2016) Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nat Commun 7:. https://doi.org/10.1038/NCOMMS12150
51. Gabrilovich DI (2017) Myeloid-derived suppressor cells. Cancer Immunol Res 5:3–8. https://doi.org/10.1158/2326-6066.CIR-16-0297
52. Raulet DH, Vance RE (2006) Self-tolerance of natural killer cells. Nat Rev Immunol 6:520–531. https://doi.org/10.1038/NRI1863
53. Elliott JM, Yokoyama WM (2011) Unifying concepts of MHC-dependent natural killer cell education. Trends Immunol 32:364–372. https://doi.org/10.1016/J.IT.2011.06.001
54. Morvan MG, Lanier LL (2016) NK cells and cancer: You can teach innate cells new tricks. Nat Rev Cancer 16:7–19. https://doi.org/10.1038/NRC.2015.5
55. Holt D, Ma X, Kundu N, Fulton A (2011) Prostaglandin E 2 (PGE 2) suppresses natural killer cell function primarily through the PGE 2 receptor EP4. Cancer Immunol Immunother 60:1577–1586. https://doi.org/10.1007/S00262-011-1064-9
56. Pietra G, Manzini C, Rivara S, et al (2012) Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. Cancer Res 72:1407–1415.

https://doi.org/10.1158/0008-5472.CAN-11-2544

57. Hoskin DW, Mader JS, Furlong SJ, et al (2008) Inhibition of T cell and natural killer cell function by adenosine and its contribution to immune evasion by tumor cells (Review). Int J Oncol 32:527–535. https://doi.org/10.3892/ijo.32.3.527
58. Richards JO, Chang X, Blaser BW, et al (2006) Tumor growth impedes natural-killer-cell maturation in the bone marrow. Blood 108:246–252. https://doi.org/10.1182/blood-2005-11-4535
59. Guillerey C, Huntington ND, Smyth MJ (2016) Targeting natural killer cells in cancer immunotherapy. Nat Immunol 17:1025–1036. https://doi.org/10.1038/NI.3518
60. Halama N, Braun M, Kahlert C, et al (2011) Natural killer cells are scarce in colorectal carcinoma tissue despite high levels of chemokines and cytokines. Clin Cancer Res 17:678–689. https://doi.org/10.1158/1078-0432.CCR-10-2173
61. Lee J-C, Lee K-M, Kim D-W, Heo DS (2004) Elevated TGF-β1 Secretion and Down-Modulation of NKG2D Underlies Impaired NK Cytotoxicity in Cancer Patients. J Immunol 172:7335–7340. https://doi.org/10.4049/JIMMUNOL.172.12.7335
62. Speiser DE, Ho PC, Verdeil G (2016) Regulatory circuits of T cell function in cancer. Nat Rev Immunol 16:599–611. https://doi.org/10.1038/NRI.2016.80
63. Fridman WH, Pagès F, Sautès-Fridman C, Galon J (2012) The immune contexture in human tumours: Impact on clinical outcome. Nat Rev Cancer 12:298–306. https://doi.org/10.1038/NRC3245
64. Malissen B, Bongrand P (2015) Early T cell activation: integrating biochemical, structural, and biophysical cues. Annu Rev Immunol 33:539–561. https://doi.org/10.1146/ANNUREV-IMMUNOL-032414-112158
65. Sallusto F, Geginat J, Lanzavecchia A (2004) Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 22:745–763. https://doi.org/10.1146/ANNUREV.IMMUNOL.22.012703.104702
66. Tosolini M, Kirilovsky A, Mlecnik B, et al (2011) Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in

patients with colorectal cancer. Cancer Res 71:1263–1271. https://doi.org/10.1158/0008-5472.CAN-10-2907

67. Sakaguchi S, Yamaguchi T, Nomura T, Ono M (2008) Regulatory T Cells and Immune Tolerance. Cell 133:775–787. https://doi.org/10.1016/J.CELL.2008.05.009
68. Pandiyan P, Zheng L, Ishihara S, et al (2007) CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. Nat Immunol 8:1353–1362. https://doi.org/10.1038/NI1536
69. Peggs KS, Quezada SA, Chambers CA, et al (2009) Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. J Exp Med 206:1717–1725. https://doi.org/10.1084/JEM.20082492
70. Nguyen LT, Ohashi PS (2015) Clinical blockade of PD1 and LAG3-potential mechanisms of action. Nat Rev Immunol 15:45–56. https://doi.org/10.1038/NRI3790
71. Tanaka A, Sakaguchi S (2017) Regulatory T cells in cancer immunotherapy. Cell Res 27:109–118. https://doi.org/10.1038/CR.2016.151
72. Shang B, Liu Y, Jiang SJ, Liu Y (2015) Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: A systematic review and meta-analysis. Sci Rep 5:. https://doi.org/10.1038/SREP15179
73. Saito T, Nishikawa H, Wada H, et al (2016) Two FOXP3 + CD4 + T cell subpopulations distinctly control the prognosis of colorectal cancers. Nat Med 22:679–684. https://doi.org/10.1038/NM.4086
74. Betts G, Jones E, Junaid S, et al (2012) Suppression of tumour-specific CD4 + T cells by regulatory T cells is associated with progression of human colorectal cancer. Gut 61:1163–1171. https://doi.org/10.1136/GUTJNL-2011-300970
75. Frey DM, Droeser RA, Viehl CT, et al (2010) High frequency of tumor-infiltrating FOXP3+ regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients. Int J Cancer 126:2635–2643. https://doi.org/10.1002/IJC.24989
76. Salama P, Phillips M, Grieu F, et al (2009) Tumor-infiltrating FOXP3+ T

regulatory cells show strong prognostic significance in colorectal cancer. J Clin Oncol 27:186–192. https://doi.org/10.1200/JCO.2008.18.7229

77. Quinn DI, Shore ND, Egawa S, et al (2015) Immunotherapy for castration-resistant prostate cancer: Progress and new paradigms. Urol Oncol Semin Orig Investig 33:245–260. https://doi.org/10.1016/J.UROLONC.2014.10.009
78. Emens LA (2018) Breast cancer immunotherapy: Facts and hopes. Clin Cancer Res 24:511–520. https://doi.org/10.1158/1078-0432.CCR-16-3001
79. Eroglu Z, Zaretsky JM, Hu-Lieskovan S, et al (2018) High response rate to PD-1 blockade in desmoplastic melanomas. Nature 553:347–350. https://doi.org/10.1038/NATURE25187
80. Menon S, Shin S, Dy G (2016) Advances in cancer immunotherapy in solid tumors. Cancers (Basel) 8:. https://doi.org/10.3390/CANCERS8120106
81. Brahmer JR, Tykodi SS, Chow LQM, et al (2012) Safety and Activity of Anti–PD-L1 Antibody in Patients with Advanced Cancer. N Engl J Med 366:2455–2465. https://doi.org/10.1056/NEJMOA1200694)
82. Tintelnot J, Stein A (2019) Immunotherapy in colorectal cancer: Available clinical evidence, challenges and novel approaches. World J Gastroenterol 25:3920–3928. https://doi.org/10.3748/WJG.V25.I29.3920
83. Ganesh K, Stadler ZK, Cercek A, et al (2019) Immunotherapy in colorectal cancer: rationale, challenges and potential. Nat Rev Gastroenterol Hepatol 16:361–375. https://doi.org/10.1038/s41575-019-0126-x
84. Wherry EJ (2011) T cell exhaustion. Nat Immunol 12:492–499. https://doi.org/10.1038/NI.2035
85. Zajac AJ, Blattman JN, Murali-Krishna K, et al (1998) Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med 188:2205–2213. https://doi.org/10.1084/jem.188.12.2205
86. McLane LM, Abdel-Hakeem MS, Wherry EJ (2019) CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer. Annu Rev Immunol 37:457–495. https://doi.org/10.1146/annurev-immunol-041015-055318
87. Milner JJ, Toma C, Yu B, et al (2017) Runx3 programs CD8+ T cell

residency in non-lymphoid tissues and tumours. Nature 552:253–257. https://doi.org/10.1038/NATURE24993

88. Barber DL, Wherry EJ, Masopust D, et al (2006) Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 439:682–687. https://doi.org/10.1038/NATURE04444
89. Saleh R, Taha RZ, Toor SM, et al (2020) Expression of immune checkpoints and T cell exhaustion markers in early and advanced stages of colorectal cancer. Cancer Immunol Immunother 69:1989–1999. https://doi.org/10.1007/S00262-020-02593-W
90. Kim CG, Jang M, Kim Y, et al (2019) VEGF-A drives TOX-dependent T cell exhaustion in anti-PD-1-resistant microsatellite stable colorectal cancers. Sci Immunol 4:. https://doi.org/10.1126/SCIIMMUNOL.AAY0555
91. Di J, Liu M, Fan Y, et al (2020) Phenotype molding of T cells in colorectal cancer by single-cell analysis. Int J Cancer 146:2281–2295. https://doi.org/10.1002/IJC.32856
92. Zimmermann H (2009) Prostatic acid phosphatase, a neglected ectonucleotidase. Purinergic Signal 5:273–275. https://doi.org/10.1007/s11302-009-9157-z
93. Zimmermann H, Zebisch M, Sträter N (2012) Cellular function and molecular structure of ecto-nucleotidases. Purinergic Signal 8:437–502. https://doi.org/10.1007/s11302-012-9309-4
94. Allard D, Allard B, Gaudreau PO, et al (2016) CD73-adenosine: A next-generation target in immuno-oncology. Immunotherapy 8:145–163
95. Ferretti E, Horenstein A, Canzonetta C, et al (2019) Canonical and non-canonical adenosinergic pathways. Immunol Lett 205:25–30. https://doi.org/10.1016/J.IMLET.2018.03.007
96. Bagheri S, Saboury A, Haertlé T (2019) Adenosine deaminase inhibition. Int J Biol Macromol 141:1246–1257. https://doi.org/10.1016/J.IJBIOMAC.2019.09.078
97. Gao Z, Wang X, Zhang H, et al (2021) The roles of adenosine deaminase in autoimmune diseases. Autoimmun Rev 20:. https://doi.org/10.1016/J.AUTREV.2020.102709
98. Hu G, Wu P, Cheng P, et al (2017) Tumor-infiltrating CD39+ γδTregs are novel immunosuppressive T cells in human colorectal cancer.

Oncoimmunology 6:. https://doi.org/10.1080/2162402X.2016.1277305

99. Sundström P, Stenstad H, Langenes V, et al (2016) Regulatory T cells from colon cancer patients inhibit effector T-cell migration through an adenosine-dependent mechanism. Cancer Immunol Res 4:183–193. https://doi.org/10.1158/2326-6066.CIR-15-0050
100. Khaja ASS, Toor SM, Salhat HE, et al (2017) Intratumoral FoxP3+Helios+ regulatory T Cells upregulating immunosuppressive molecules are expanded in human colorectal cancer. Front Immunol 8:. https://doi.org/10.3389/FIMMU.2017.00619
101. Parodi A, Battaglia F, Kalli F, et al (2013) CD39 is highly involved in mediating the suppression activity of tumor-infiltrating CD8+ T regulatory lymphocytes. Cancer Immunol Immunother 62:851–862. https://doi.org/10.1007/S00262-013-1392-Z
102. Strasser K, Birnleitner H, Beer A, et al (2019) Immunological differences between colorectal cancer and normal mucosa uncover a prognostically relevant immune cell profile. Oncoimmunology 8:. https://doi.org/10.1080/2162402X.2018.1537693
103. Scurr M, Ladell K, Besneux M, et al (2014) Highly prevalent colorectal cancer-infiltrating LAP + Foxp3 - T cells exhibit more potent immunosuppressive activity than Foxp3 + regulatory T cells. Mucosal Immunol 7:428–439. https://doi.org/10.1038/MI.2013.62
104. Timperi E, Pacella I, Schinzari V, et al (2016) Regulatory T cells with multiple suppressive and potentially pro-tumor activities accumulate in human colorectal cancer. Oncoimmunology 5:. https://doi.org/10.1080/2162402X.2016.1175800
105. Zhulai G, Churov A V., Oleinik EK, et al (2018) Activation of cd4+cd39+ T cells in colorectal cancer. Bull Russ State Med Univ 7:47–53. https://doi.org/10.24075/BRSMU.2018.027
106. Takatori H, Kawashima H, Matsuki A, et al (2015) Helios Enhances Treg Cell Function in Cooperation With FoxP3. Arthritis Rheumatol 67:1491–1502. https://doi.org/10.1002/ART.39091
107. Zhan Y, Zheng L, Liu J, et al (2021) PLA2G4A promotes right-sided colorectal cancer progression by inducing CD39+γδ Treg polarization. JCI Insight 6:. https://doi.org/10.1172/jci.insight.148028

108. Simoni Y, Becht E, Fehlings M, et al (2018) Bystander CD8+ T cells are abundant and phenotypically distinct in human tumour infiltrates. Nature 557:575–579. https://doi.org/10.1038/S41586-018-0130-2
109. Gallerano D, Ciminati S, Grimaldi A, et al (2020) Genetically driven CD39 expression shapes human tumor-infiltrating CD8+ T-cell functions. Int J Cancer 147:2597–2610. https://doi.org/10.1002/ijc.33131
110. Rodin W, Sundström P, Ahlmanner F, et al (2021) Exhaustion in tumor-infiltrating Mucosal-Associated Invariant T (MAIT) cells from colon cancer patients. Cancer Immunol Immunother 70:3461–3475. https://doi.org/10.1007/s00262-021-02939-y
111. Limagne E, Euvrard R, Thibaudin M, et al (2016) Accumulation of MDSC and Th17 cells in patients with metastatic colorectal cancer predicts the efficacy of a FOLFOX-bevacizumab drug treatment regimen. Cancer Res 76:5241–5252. https://doi.org/10.1158/0008-5472.CAN-15-3164
112. Zhang B, Wang Z, Wu L, et al (2013) Circulating and Tumor-Infiltrating Myeloid-Derived Suppressor Cells in Patients with Colorectal Carcinoma. PLoS One 8:. https://doi.org/10.1371/JOURNAL.PONE.0057114
113. Künzli BM, Bernlochner MI, Rath S, et al (2011) Impact of CD39 and purinergic signalling on the growth and metastasis of colorectal cancer. Purinergic Signal 7:231–241. https://doi.org/10.1007/S11302-011-9228-9
114. Zhao Y, Chen X, Ding Z, et al (2021) Identification of Novel CD39 Inhibitors Based on Virtual Screening and Enzymatic Assays. J Chem Inf Model. https://doi.org/10.1021/acs.jcim.1c00590
115. Gaibar M, Galán M, Romero-Lorca A, et al (2021) Genetic variants of ANGPT1, CD39, FGF2 and MMP9 linked to clinical outcome of bevacizumab plus chemotherapy for metastatic colorectal cancer. Int J Mol Sci 22:1–16. https://doi.org/10.3390/ijms22031381
116. MacCARTHY CM, Notario V (2013) The ENTPD5/mt-PCPH oncoprotein is a catalytically inactive member of the ectonucleoside triphosphate diphosphohydrolase family. Int J Oncol 43:1244–1252. https://doi.org/10.3892/IJO.2013.2052
117. Beavis PA, Stagg J, Darcy PK, Smyth MJ (2012) CD73: A potent suppressor of antitumor immune responses. Trends Immunol 33:231–237. https://doi.org/10.1016/j.it.2012.02.009

118. Yang J, Liao X, Yu J, Zhou P (2018) Role of CD73 in Disease: Promising Prognostic Indicator and Therapeutic Target. Curr Med Chem 25:2260–2271. https://doi.org/10.2174/0929867325666180117101114
119. Allard B, Turcotte M, Stagg J (2012) CD73-Generated Adenosine: Orchestrating the Tumor-Stroma Interplay to Promote Cancer Growth. J Biomed Biotechnol 2012:1–8. https://doi.org/10.1155/2012/485156
120. Antonioli L, Yegutkin GG, Pacher P, et al (2016) Anti-CD73 in Cancer Immunotherapy: Awakening New Opportunities. Trends in Cancer 2:95–109
121. Azambuja JH, Gelsleichter NE, Beckenkamp LR, et al (2019) CD73 Downregulation Decreases In Vitro and In Vivo Glioblastoma Growth. Mol Neurobiol 56:. https://doi.org/10.1007/s12035-018-1240-4
122. Gao Z wei, Wang H ping, Lin F, et al (2017) CD73 promotes proliferation and migration of human cervical cancer cells independent of its enzyme activity. BMC Cancer 17:1–8. https://doi.org/10.1186/s12885-017-3128-5
123. Li H, Lv M, Qiao B, Li X (2019) Blockade pf CD73/adenosine axis improves the therapeutic efficacy of docetaxel in epithelial ovarian cancer. Arch Gynecol Obstet. https://doi.org/10.1007/s00404-019-05139-3
124. Yang Q, Du J, Zu L (2013) Overexpression of CD73 in prostate cancer is associated with lymph node metastasis. Pathol Oncol Res 19:811–814. https://doi.org/10.1007/s12253-013-9648-7
125. Matsuyama M, Wakui M, Monnai M, et al (2010) Reduced CD73 expression and its association with altered purine nucleotide metabolism in colorectal cancer cells robustly causing liver metastases. Oncol Lett 1:431–436. https://doi.org/10.3892/OL_00000076
126. Cushman SM, Jiang C, Hatch AJ, et al (2014) Gene expression markers of efficacy and resistance to cetuximab treatment in metastatic colorectal cancer: Results from CALGB 80203 (Alliance). Clin Cancer Res 21:1078–1086. https://doi.org/10.1158/1078-0432.CCR-14-2313
127. Hatch AJ, Sibley AB, Starr MD, et al (2016) Blood-based markers of efficacy and resistance to cetuximab treatment in metastatic colorectal cancer: results from CALGB 80203 (Alliance). Cancer Med 5:2249–2260. https://doi.org/10.1002/CAM4.806
128. Wu XR, He XS, Chen YF, et al (2012) High expression of CD73 as a poor

prognostic biomarker in human colorectal cancer. J Surg Oncol 106:130–137. https://doi.org/10.1002/JSO.23056

129. Zhang B, Song B, Wang X, et al (2015) The expression and clinical significance of CD73 molecule in human rectal adenocarcinoma. Tumor Biol 36:5459–5466. https://doi.org/10.1007/s13277-015-3212-x
130. Wu R, Chen Y, Li F, et al (2016) Effects of CD73 on human colorectal cancer cell growth in vivo and in vitro. Oncol Rep 35:1750–1756. https://doi.org/10.3892/OR.2015.4512
131. Wang G, Fu S, Li D, Chen Y (2019) Expression and clinical significance of serum NT5E protein in patients with colorectal cancer. Cancer Biomarkers 24:461–468. https://doi.org/10.3233/CBM-182207
132. Messaoudi N, Cousineau I, Arslanian E, et al (2020) Prognostic value of CD73 expression in resected colorectal cancer liver metastasis. Oncoimmunology 9:. https://doi.org/10.1080/2162402X.2020.1746138
133. Terp MG, Gammelgaard OL, Vever H, et al (2021) Sustained compensatory p38 MAPK signaling following treatment with MAPK inhibitors induces the immunosuppressive protein CD73 in cancer: combined targeting could improve outcomes. Mol Oncol 15:3299–3316. https://doi.org/10.1002/1878-0261.13046
134. Liu H, Lu W, He H, et al (2019) Inflammation-dependent overexpression of c-Myc enhances CRL4 DCAF4 E3 ligase activity and promotes ubiquitination of ST7 in colitis-associated cancer. J Pathol 248:464–475. https://doi.org/10.1002/PATH.5273
135. Kim M, Min YK, Jang J, et al (2021) Single-cell RNA sequencing reveals distinct cellular factors for response to immunotherapy targeting CD73 and PD-1 in colorectal cancer. J Immunother Cancer 9:. https://doi.org/10.1136/jitc-2021-002503
136. Xie M, Qin H, Luo Q, et al (2017) MicroRNA-30a regulates cell proliferation and tumor growth of colorectal cancer by targeting CD73. BMC Cancer 17:. https://doi.org/10.1186/S12885-017-3291-8
137. Lai YP, Kuo LC, Lin BR, et al (2021) CD28 engagement inhibits CD73-mediated regulatory activity of CD8+ T cells. Commun Biol 4:. https://doi.org/10.1038/s42003-021-02119-9
138. Ploeg EM, Ke X, Britsch I, et al (2021) Bispecific antibody CD73xEpCAM

selectively inhibits the adenosine-mediated immunosuppressive activity of carcinoma-derived extracellular vesicles. Cancer Lett 521:109–118. https://doi.org/10.1016/j.canlet.2021.08.037

139. Sun P, Wang H, He Z, et al (2017) Fasting inhibits colorectal cancer growth by reducing M2 polarization of tumor-associated macrophages. Oncotarget 8:74649–74660. https://doi.org/10.18632/oncotarget.20301
140. Burnstock G (2006) Historical review: ATP as a neurotransmitter. Trends Pharmacol Sci 27:166–76. https://doi.org/10.1016/j.tips.2006.01.005
141. García-Alcocer G, Padilla K, Rodríguez A, et al (2012) Distribution of the purinegic receptors P2X(4) and P2X(6) during rat gut development. Neurosci Lett 509:92–95. https://doi.org/10.1016/J.NEULET.2011.12.044
142. D'Amico D, Valdebenito S, Eugenin E (2021) The role of Pannexin-1 channels and extracellular ATP in the pathogenesis of the human immunodeficiency virus. Purinergic Signal. https://doi.org/10.1007/S11302-021-09817-3
143. Di Virgilio F, Adinolfi E (2017) Extracellular purines, purinergic receptors and tumor growth. Oncogene 36:293–303. https://doi.org/10.1038/onc.2016.206
144. Burnstock G (2018) Purine and purinergic receptors: https://doi.org/101177/2398212818817494 2:239821281881749. https://doi.org/10.1177/2398212818817494
145. Kazemi MH, Raoofi Mohseni S, Hojjat-Farsangi M, et al (2018) Adenosine and adenosine receptors in the immunopathogenesis and treatment of cancer. J Cell Physiol 233:2032–2057. https://doi.org/10.1002/jcp.25873
146. Borea PA, Dalpiaz A, Varani K, et al (1996) Binding thermodynamics at A1 and A2A adenosine receptors. Life Sci 59:1373–88
147. Borea PA, Varani K, Vincenzi F, et al (2014) The A3 Adenosine Receptor: History and Perspectives. Pharmacol Rev 67:74–102. https://doi.org/10.1124/pr.113.008540
148. Borea PA, Gessi S, Merighi S, et al (2018) Pharmacology of Adenosine Receptors: The State of the Art. Physiol Rev 98:1591–1625. https://doi.org/10.1152/physrev.00049.2017
149. Dahan R, Ravetch J V. (2016) Co-targeting of Adenosine Signaling Pathways for Immunotherapy: Potentiation by Fc Receptor Engagement.

Cancer Cell 30:369–371. https://doi.org/10.1016/j.ccell.2016.08.007

150. Ma DF, Kondo T, Nakazawa T, et al (2010) Hypoxia-inducible adenosine A2B receptor modulates proliferation of colon carcinoma cells. Hum Pathol 41:1550–1557. https://doi.org/10.1016/J.HUMPATH.2010.04.008
151. Yaguchi T, Saito M, Yasuda Y, et al (2010) Higher concentrations of extracellular ATP suppress proliferation of Caco-2 human colonic cancer cells via an unknown receptor involving PKC inhibition. Cell Physiol Biochem 26:125–134. https://doi.org/10.1159/000320518
152. Dillard, Clémentine; Borde, Chloé; Mohammad, Ammara; Puchois, Virginie; Jourdren, Laurent; Larsen, Anette K.; Sabbah, Michèle; Maréchal, Vincent; Escargueil, Alexandre E.; Pramil E (2021) Expression Pattern of Purinergic Signaling Components in Colorectal Cancer Cells and Differential Cellular Outcomes Induced by Extracellular ATP and Adenosine. 11472
153. Wen Y, Chen X, Zhu X, et al (2019) Photothermal-Chemotherapy Integrated Nanoparticles with Tumor Microenvironment Response Enhanced the Induction of Immunogenic Cell Death for Colorectal Cancer Efficient Treatment. ACS Appl Mater Interfaces 11:43393–43408. https://doi.org/10.1021/ACSAMI.9B17137
154. Vinette V, Placet M, Arguin G, Gendron F-P (2015) Multidrug Resistance-Associated Protein 2 Expression Is Upregulated by Adenosine 5'-Triphosphate in Colorectal Cancer Cells and Enhances Their Survival to Chemotherapeutic Drugs. PLoS One 10:e0136080. https://doi.org/10.1371/journal.pone.0136080
155. Kim CD, Kim SH, Jung SH, Kim JH (2019) Clinical value of an adenosine triphosphate-based chemotherapy response assay in resectable stage III colorectal cancer. Ann Surg Treat Res 97:93–102. https://doi.org/10.4174/ASTR.2019.97.2.93
156. Tokunaga R, Cao S, Naseem M, et al (2019) Prognostic Effect of Adenosine-related Genetic Variants in Metastatic Colorectal Cancer Treated With Bevacizumab-based Chemotherapy. Clin Colorectal Cancer 18:e8–e19. https://doi.org/10.1016/J.CLCC.2018.09.003
157. Sheth S, Brito R, Mukherjea D, et al (2014) Adenosine Receptors: Expression, Function and Regulation. Int J Mol Sci 15:2024.

https://doi.org/10.3390/IJMS15022024

158. Zou Y, Yang R, Li L, et al (2021) Purinergic signaling: a potential therapeutic target for depression and chronic pain. Purinergic Signal 2021 1:1–10. https://doi.org/10.1007/S11302-021-09801-X
159. Lan B, Zhang J, Zhang P, et al (2017) Metformin suppresses CRC growth by inducing apoptosis via ADORA1. Front Biosci - Landmark 22:248–257. https://doi.org/10.2741/4484
160. Wu Z, Yang L, Shi L, et al (2019) Prognostic impact of adenosine receptor 2 (A2aR) and programmed cell death ligand 1 (PD-L1) expression in colorectal cancer. Biomed Res Int 2019:. https://doi.org/10.1155/2019/8014627
161. Long JS, Crighton D, O'Prey J, et al (2013) Extracellular adenosine sensing-a metabolic cell death priming mechanism downstream of p53. Mol Cell 50:394–406. https://doi.org/10.1016/J.MOLCEL.2013.03.016
162. Mølck C, Ryall J, Failla LM, et al (2016) The A2b adenosine receptor antagonist PSB-603 promotes oxidative phosphorylation and ROS production in colorectal cancer cells via adenosine receptor-independent mechanism. Cancer Lett 383:135–143. https://doi.org/10.1016/J.CANLET.2016.09.018
163. Kitsou M, Ayiomamitis GD, Zaravinos A (2020) High expression of immune checkpoints is associated with the TIL load, mutation rate and patient survival in colorectal cancer. Int J Oncol 57:237–248. https://doi.org/10.3892/IJO.2020.5062
164. Balber T, Singer J, Berroterán-Infante N, et al (2018) Preclinical in Vitro and in Vivo Evaluation of [18F]FE@SUPPY for Cancer PET Imaging: Limitations of a Xenograft Model for Colorectal Cancer. Contrast Media Mol Imaging 2018:. https://doi.org/10.1155/2018/1269830
165. Marucci G, Santinelli C, Buccioni M, et al (2018) Anticancer activity study of A3 adenosine receptor agonists. Life Sci 205:155–163. https://doi.org/10.1016/J.LFS.2018.05.028
166. Gusic M, Benndorf K, Sattler C (2021) Dissecting activation steps in P2X7 receptors. Biochem Biophys Res Commun 569:112–117. https://doi.org/10.1016/J.BBRC.2021.06.101
167. Oury C, Wéra O (2021) P2X1: a unique platelet receptor with a key role in

thromboinflammation. Platelets 32:902–908. https://doi.org/10.1080/09537104.2021.1902972

168. Fong Z, Griffin C, Large R, et al (2021) Regulation of P2X1 receptors by modulators of the cAMP effectors PKA and EPAC. Proc Natl Acad Sci U S A 118:. https://doi.org/10.1073/PNAS.2108094118
169. Grohmann M, Schumacher M, Günther J, et al (2021) BAC transgenic mice to study the expression of P2X2 and P2Y 1 receptors. Purinergic Signal 17:449–465. https://doi.org/10.1007/S11302-021-09792-9
170. Schneider R, Leven P, Glowka T, et al (2021) A novel P2X2-dependent purinergic mechanism of enteric gliosis in intestinal inflammation. EMBO Mol Med 13:. https://doi.org/10.15252/EMMM.202012724
171. Kong Y, Wang Y, Wu D, et al (2020) Involvement of P2X2 receptor in the medial prefrontal cortex in ATP modulation of the passive coping response to behavioral challenge. Genes Brain Behav 19:. https://doi.org/10.1111/GBB.12691
172. King B (2021) P2X3 receptors participate in purinergic inhibition of gastrointestinal smooth muscle. Auton Neurosci 234:. https://doi.org/10.1016/J.AUTNEU.2021.102830
173. Xia L, Luo H, Ma Q, et al (2021) GPR151 in nociceptors modulates neuropathic pain via regulating P2X3 function and microglial activation. Brain. https://doi.org/10.1093/BRAIN/AWAB245
174. Lu R, Metzner K, Zhou F, et al (2021) Functional Coupling of Slack Channels and P2X3 Receptors Contributes to Neuropathic Pain Processing. Int J Mol Sci 22:1–18. https://doi.org/10.3390/IJMS22010405
175. de Baaij J, Blanchard M, Lavrijsen M, et al (2014) P2X4 receptor regulation of transient receptor potential melastatin type 6 (TRPM6) Mg2+ channels. Pflugers Arch 466:1941–1952. https://doi.org/10.1007/S00424-014-1440-3
176. Padilla K, Gonzalez-Mendoza D, Berumen L, et al (2016) Differential gene expression patterns and colocalization of ATP-gated P2X6/P2X4 ion channels during rat small intestine ontogeny. Gene Expr Patterns 21:81–88. https://doi.org/10.1016/J.GEP.2016.08.002
177. Yu Q, Zhao Z, Sun J, et al (2010) Expression of P2X6 receptors in the enteric nervous system of the rat gastrointestinal tract. Histochem Cell

Biol 133:177–188. https://doi.org/10.1007/S00418-009-0659-0

178. Mishra A, Behura A, Kumar A, et al (2021) P2X7 receptor in multifaceted cellular signalling and its relevance as a potential therapeutic target in different diseases. Eur J Pharmacol 906:. https://doi.org/10.1016/J.EJPHAR.2021.174235
179. Grassi F, De Ponte Conti B (2021) The P2X7 Receptor in Tumor Immunity. Front cell Dev Biol 9:. https://doi.org/10.3389/FCELL.2021.694831
180. Rabelo I, Arnaud-Sampaio V, Adinolfi E, et al (2021) Cancer Metabostemness and Metabolic Reprogramming via P2X7 Receptor. Cells 10:. https://doi.org/10.3390/CELLS10071782
181. Campagno K, Lu W, Jassim A, et al (2021) Rapid morphologic changes to microglial cells and upregulation of mixed microglial activation state markers induced by P2X7 receptor stimulation and increased intraocular pressure. J Neuroinflammation 18:. https://doi.org/10.1186/S12974-021-02251-7
182. Sidoryk-Węgrzynowicz M, Strużyńska L (2021) Astroglial and Microglial Purinergic P2X7 Receptor as a Major Contributor to Neuroinflammation during the Course of Multiple Sclerosis. Int J Mol Sci 22:. https://doi.org/10.3390/IJMS22168404
183. Purohit R, Bera A (2021) Pannexin 1 plays a pro-survival role by attenuating P2X7 receptor-mediated Ca 2+ influx. Cell Calcium 99:. https://doi.org/10.1016/J.CECA.2021.102458
184. Hirayama Y, Anzai N, Koizumi S (2021) Mechanisms underlying sensitization of P2X7 receptors in astrocytes for induction of ischemic tolerance. Glia 69:2100–2110. https://doi.org/10.1002/GLIA.23998
185. Di Virgilio F, Sarti AC, Grassi F (2018) Modulation of innate and adaptive immunity by P2X ion channels. Curr Opin Immunol 52:51–59. https://doi.org/10.1016/j.coi.2018.03.026
186. Di Virgilio F (2015) P2X receptors and inflammation. Curr Med Chem 22:5–24. https://doi.org/10.2174/0929867322666141210155311
187. Qian F, Xiao J, Hu B, et al (2017) High expression of P2X7R is an independent postoperative indicator of poor prognosis in colorectal cancer. Hum Pathol 64:61–68.

https://doi.org/10.1016/j.humpath.2017.03.019

188. Zhang Y, Ding J, Wang L (2019) The role of P2X7 receptor in prognosis and metastasis of colorectal cancer. Adv Med Sci 64:388–394. https://doi.org/10.1016/J.ADVMS.2019.05.002
189. Zhang Y, Li F, Wang L, Lou Y (2021) A438079 affects colorectal cancer cell proliferation, migration, apoptosis, and pyroptosis by inhibiting the P2X7 receptor. Biochem Biophys Res Commun 558:147–153. https://doi.org/10.1016/j.bbrc.2021.04.076
190. Yang Z, Zhang M, Peng R, et al (2020) The prognostic and clinicopathological value of tumor-associated macrophages in patients with colorectal cancer: a systematic review and meta-analysis. Int J Colorectal Dis 35:1651–1661. https://doi.org/10.1007/S00384-020-03686-9
191. Janakiram NB, Mohammed A, Bryant T, et al (2015) Adoptive transfer of regulatory T cells promotes intestinal tumorigenesis and is associated with decreased NK cells and IL-22 binding protein. Mol Carcinog 54:986–998. https://doi.org/10.1002/MC.22168
192. Hofman P, Cherfils-Vicini J, Bazin M, et al (2015) Genetic and pharmacological inactivation of the purinergic P2RX7 receptor dampens inflammation but increases tumor incidence in a mouse model of colitis-associated cancer. Cancer Res 75:835–845. https://doi.org/10.1158/0008-5472.CAN-14-1778
193. Gao P, He M, Zhang C, Geng C (2018) Integrated analysis of gene expression signatures associated with colon cancer from three datasets. Gene 654:95–102. https://doi.org/10.1016/J.GENE.2018.02.007
194. von Kügelgen I (2021) Molecular pharmacology of P2Y receptor subtypes. Biochem Pharmacol 187:. https://doi.org/10.1016/J.BCP.2020.114361
195. Limami Y, Pinon A, Leger DY, et al (2012) The P2Y 2/Src/p38/COX-2 pathway is involved in the resistance to ursolic acid-induced apoptosis in colorectal and prostate cancer cells. Biochimie 94:1754–1763. https://doi.org/10.1016/J.BIOCHI.2012.04.006
196. Girard M, Dagenais Bellefeuille S, Eiselt É, et al (2020) The P2Y6 receptor signals through Gαq/Ca2+/PKCα and Gα13/ROCK pathways to

drive the formation of membrane protrusions and dictate cell migration. J Cell Physiol 235:9676–9690. https://doi.org/10.1002/JCP.29779

197. Placet M, Arguin G, Molle CM, et al (2018) The G protein-coupled P2Y6 receptor promotes colorectal cancer tumorigenesis by inhibiting apoptosis. Biochim Biophys Acta - Mol Basis Dis 1864:1539–1551. https://doi.org/10.1016/J.BBADIS.2018.02.008
198. Wright JR, Chauhan M, Shah C, et al (2020) The TICONC (Ticagrelor-Oncology) Study: Implications of P2Y12 Inhibition for Metastasis and Cancer-Associated Thrombosis. JACC CardioOncology 2:236–250. https://doi.org/10.1016/J.JACCAO.2020.04.009

CAPÍTULO II: Investigation of the impact of obese serum exposition on colorectal cancer cell malignity.

ROLIANO, G.G.[1], AZAMBUJA, J.H.[2], DE SOUZA, P.O.[3], DORNELES G.[1], BOEIRA. M.[1], MARTINS, I.[1], BRUNETTO, V. T.[1,5], KALIL, A.N.[1,4], PERES, A.[3], BRAGANHOL, E.[1,3,5]*

[1]Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil.

[2]Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA.

[3]Programa de Pós-Graduação em Biociências, UFCSPA, Porto Alegre, RS, Brazil.

[4]Serviço de Cirurgia Oncológica do Hospital Santa Rita da Santa Casa de Misericórdia, Porto Alegre, RS, Brazil.

[5]Instituto de Cardiologia do Rio Grande do Sul/Fundação Universitária do Instituto de Cardiologia (IC-FUC), Porto Alegre, RS, Brazil.

*Corresponding Author

Elizandra Braganhol (E-mail: ebraganhol@ufcspa.edu.br)

Universidade Federal de Ciências da Saúde (UFCSPA)

Sarmento Leite St, 245 – Main Building – Room 304

Zipcode: 90.050-170 - Porto Alegre, RS, Brazil

Phone: +55 51 3303 8762

As normas da revista *BMC Edocrine Disorders*, para a publicação do mauscrito estão disponíveis em: https://bmcendocrdisord.biomedcentral.com/submission-guidelines/preparing-your-manuscript/research-article

INTRODUCTION

Weight gain is a worldwide problem, with obesity taxes increasing year after year [1]. Obesity is defined by accumulation of fat on the body, commonly measured by height and weight, using Body Mass Index (BMI) [2]. Unfortunately this condition is associated with a variety of pathologies, including resistance to insulin, arterial hypertension and heart diseases, as a consequence of fat accumulation and further impairment of the biological systems [3]. A hallmark of obesity is the establishment of a chronic inflammatory process, which has been associated with a microenvironment favorable to colorectal cancer (CRC) progression [4–6].

CRC is the third most common type of cancer in the world, characterized by genomic instability, with mutations on genes how KRAS and BRAF and by the presence of microsatellite instability, that are important to therapy directionament [7–9]. The CRC usually begins as polyps in the colon and rectun which progressively undergo malignant transformation and the cancer establishment in a process that takes approximately ten years [10]. CRC incidence is higher in the developed countries, being associated with unhealthy diet, sedentary lifestyle, overweight and obesity [11]. In addition, chronic inflammatory diseases, such as Crohn's Disease and ulcerative colitis contribute to the increased incidence of CRC [6, 12, 13].

Tumor microenvironment is composed of infiltrated immune cells. Macrophages have a main role in innate immunity. When in presence of tumor cells mediators lose the antitumor potential. leading to an anti-inflammatory profile (type-M2 phenotype), decreasing Th1 response and increasing Th2 [14–17]. This fact is established in many tumors, but in CRC it remains inconclusive [18–22].

Obesity promotes a systemic and chronic inflammatory process, which is related to increased incidence of cancer. However, little is known about the contribution of blood circulating factors on CRC progression. We hypothesize that the chronic inflammatory process promoted by obesity induces a systemic increase of serum cytokine levels, which in turn favor tumor malignity. Therefore, in the current study we investigated the impact of the exposition of serum from healthy and obese individuals on human CRC cell lines growth.

MATERIAL AND METHODS

SUBJECTS. The Ethic Committee of the UFCSPA (2.034.065) approved this study. All participants, 22 Caucasian men, were informed about the study and signed the informed consent. Participants were healthy, no smokers, age ranging from 18 to 30 years and free of illness and injury. The body mass index (BMI) was used to determinate the assigned to two experimental groups: obese (OB; BMI ≥ 30 Kg/m^2) and non-obese (NOB; BMI < 29,9 Kg/m^2) [2]. Qualified professional performed all anthropometric evaluations, and all tests were performed three times and the average was used for analysis. Body mass (Kg) and height (meters) were determined by a semi-analytical scale (Welmy, Santa BarbaraD'Oeste, Brazil), with capacity for 200 Kg and a stadiometer attached (Welmy, Santa Barbara D'Oeste, Brazil) with accuracy of 0.1 Kg and 0.005 cm, respectively. BMI (Kg/m^2) was defined as body mass (Kg) divided by the square of height (m^2). Blood samples (approximately 10 mL) were collected from the antecubital vein into serum separator tubes (Becton–Dickinson, BD, Juiz de Fora, MG, Brazil), centrifuged for 10 min at 1048 *g*, and the obtained serum was frozen at -80°C and reserved for cytokine determination, biochemical parameters analysis, and for cell culture experiments.

BIOCHEMICAL DETERMINATIONS. To assess the biochemical profile, blood samples were collected from OB and NOB groups of individuals, glucose, cholesterol, triglycerides, HDL, LDL (Labtest, Brazil, ref 133, 76, 87, 13) , and leptin levels (Quantikine ELISA; R&D Systems, Inc., MN, USA) were determined as described by the manufacturers.; Data were expressed as mg/dL (glucose, cholesterol, triglycerides, HDL, LDL) or ng/mL (leptin) and were determined in a microplate reader (SpectraMax® M3 Molecular Devices).

CELL CULTURE PROCEDURES. Human colorectal cancer HCT116 and Caco-2 cell lines were obtained from ATCC (American Type Cell Collection; Rockville, Maryland, USA). The cell lines were grown in high glucose Dulbecco's modified Eagle's medium (DMEM; HCT116) and RPMI-1640 Medium (Caco-2) (Gibco BRL, Gaithersburg, Maryland, USA, 31600-034 and 12657-029) respectively, containing 8.4 mM HEPES (pH 7.4) (Sigma Chemical Co., St. Louis, MO, USA-7365-45-9), 23.8 mM $NaHCO_3$ - (Sigma Chemical Co., St. Louis, MO), 0.1% fungizone (Gibco BRL, Gaithersburg, Maryland, USA - 15290- 018), 100 U/L penicillin/streptomycin (Gibco BRL, Gaithersburg, Maryland, USA - 15140-122). Supplemented with 10% or 20% (v/v) fetal

bovine serum (FBS) for HCT116 and Caco-2 cell lines, respectively (Gibco BRL, Gaithersburg, Maryland, USA -12657-029). The cell lines were cultured at 37ºC, a minimum relative humidity of 95%, and a 5% CO_2 atmosphere. For the experiments the cell lines were seeded in culture plates and exposed to OB or NOB serum (10% v/v) for 2, 24, 48 or 72 h, as described below. Cells cultured at standard condition (DMEM/10% FBS or RPMI-1640/20% FBS) were considered controls.

MTT ASSAY. HCT116 and Caco-2 were seeded in 96 well plates ($10x10^3$ cells/well to HCT116 and $8x10^3$ cells/well to Caco-2) in DMEM/10% FBS and RPMI-1640/20% FBS, respectively. The cells were exposed to OB or NOB serum, as described above. Following 48 h of treatment, the cell viability was assessed by 3(4,5-dimethyl)-2,5diphenyltetrazolium bromide (MTT) assay (Sigma Chemical Co., St. Louis, MO, USA - M2128). The absorbance was determined at 492 nm on a microplate reader (SpectraMax® M3 Molecular Devices). Data were expressed as percentage of control.

SRB ASSAY. HCT116 and Caco-2 cell lines were seeded in 96 well plates ($10x10^3$ cells/well HCT116, $8x10^3$ cells/well Caco-2) in DMEM/10% FBS and RPMI-1640/20% FBS, respectively. The cells were exposed to OB or NOB serum, as described above. The cell proliferation was determined after 48 h of treatment by Sulforhodamine B (SRB) assay (Sigma Chemical Co., St. Louis, MO, USA - 230162). The absorbance was determined at 530 nm on a microplate reader (SpectraMax® M3 Molecular Devices). Data were expressed as as percentage of control.

ADHESION ASSAY. Caco-2 were seeded in 96 well plates ($2x10^4$ cells/well) in RPMI-1640/10% FBS. The cells were exposed to OB or NOB serum, as described above. After 2 h, the cells were fixed with 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA - P6148), and the number of adherent cells was measured using 0.5% (w/v) violet crystal staining (Sigma Chemical Co., St. Louis, MO, USA - V5265). The absorbance was determined at 590 nm on a microplate reader (SpectraMax® M3 Molecular Devices). Data were expressed as percentage of control.

PROPIDIUM IODIDE ASSAY. Cell damage was assessed by fluorescent image analysis of PI uptake. Caco-2 cells were seeded in 24 well plates ($8x10^3$ cells/well) and they were exposed to OB or NOB serum, as described above. At the end of treatment, CRC cells were incubated with PI (7.5 μM) for 30 min. PI fluorescence was excited at 515–560 nm using an inverted microscope (Olympus ,Tokyo, Japan) fitted with a

standard rhodamine filter. Images were captured using a digital camera connected to the microscope (20x magnification).

CELL MIGRATION ASSAY. HCT116 cell line was seeded in 12-well plates ($5x10^3$ cells/well). Following 72 h, cell monolayer wound was performed with a P200 pipette tip in SFB reduced conditions (DMEM/0.5% FBS).Than, DMEM/0.5% SFB medium was replaced by a fresh one in presence of treatments (OB and NOB serum -10%). Control cells were exposed to DMEM/0.5% FBS. Following 0, 6, 18, 24 and 48 h of scratch, images were captured using an inverted microscope (40x magnification) connected to a digital camera (Olympus BX-50 with optical lens, Japan).Cell migration was determined by measuring the width of the wound divided by two and by subtracting this value from the initial half-width distance of the wound. Data were expressed as the percentage of migration compared to DMEM/0.5% FBS.

ELISA ASSAY. The cytokines IL-6 (ref. 900-K16), IL-10 (ref. 900-K21), TNFα (300-01A) were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using commercial reagents (Peprotech Inc., NJ, USA).

STATISTICAL ANALYSIS. Statistics were performed using software Graphpad Prism 8 and data were expressed as mean ± standard error (SEM) and were subjected to one-way analysis of variance (ANOVA) followed by Tukey-Kramer *post-hoc* test (for multiple comparisons) or Test *t* student when appropriate. Differences were considered significant for a p-value of $p<0.05$ (Prism GraphPad Software, San Diego, USA).

RESULTS AND DISCUSSION

To determine the impact of obesity in parameters of CRC cell malignancy, OB and NOB subjects were recruited to participate in the study. The experimental design applied is detailed in material and methods and in Figure 1. Figure 2 shows the main characteristics of the participants. The OB and NOB groups were composed by male individuals, the age ranged from 20 to 30 years old, the height and weight ranged from

1.62 to 1.82 m and 62.5 Kg to 139.5 Kg, respectively (Fig. 2A). The BMI ranged from 21.48 to 29.65 and 30.17 to 46.61 for NOB and OB groups, respectively. The NOB group was composed by eutrophic and overweight individuals, while the OB group exhibited individuals with increasing obesity grades (I, II, and III), as indicated in the heatmap (Fig. 2B). As expected, the biochemical parameters including serum triglycerides, cholesterol, HDL, LDL, glucose, and leptin levels were altered in OB, and those subjects exhibited higher triglycerides (TRI 96-253 *versus* 84-180 mg/dL), cholesterol (CHO 130-281 *versus* 102-255 mg/dL), leptin (LEP 38-84 *versus* 11-49 ng/mL), and glucose (GLU 63-165 *versus* 79-135 mg/dL) levels when compared to the NOB group, respectively. Taken together, the biochemical parameters exhibited by OB groups are according to the high prevalence of diabetes type 2 and cardiovascular diseases as already described in the literature [23].

Next, the circulating cytokines levels in OB and NOB groups were determined. As shown in Figure 3 (panels A-C), TNFα and IL-6 levels were 1.9 and 1.3 times higher while the IL-10 levels were 1.5 decreased when compared to NOB ($p<0.0001$, $p=0.0328$, and $p=0.0061$, respectively). Of note, TNFα and IL-6 are proinflammatory cytokines, and their increased levels have been described in OB individuals as a result of the chronic inflammatory process associated to high-fat tissue accumulation [24]. In addition, the 1.5 times decrease of the antiinflammatory IL-10 levels and the 2.8 times increase of TNFα/IL-10 ratio in OB group perpetuate the chronic inflammatory condition associated with obesity (Fig. 3, panel C and G, respectively) [5]. Indeed, the chronic inflammatory condition established in OB individuals is related to the increased risk to develop a variety of diseases, including cancer [3, 25, 26]. Therefore, the impact of OB and NOB serum exposition on cytokine release by CRC cell culture was further investigated. Interestingly, TNFα levels were 42% higher in Caco-2 when compared to HCT-116 cell cultures (Fig. 3D). The exposition of cell cultures to the serum of OB and NOB serum did not promote a differential effect on TNFα, IL-6 or IL-10 secretion by cancer cells (Fig. 3 D-F). However, the proinflammatory TNFα and anti-inflammatory IL-10 ratio was 34% higher in HCT-116 exposed to OB when compared to NOB serum (Fig. 3 I, $p=0.0052$), suggesting the prevalence of an inflammatory condition, which is can be beneficial for tumor progression. Finally, the genetic characteristics of Caco-2 (p53 mutated and KRAS wild type) and HCT-116 (p53 wild type and KRAS mutated), certainly contribute to the differential cytokine release exhibited by these cells.

In line with the relationship among obesity, chronic inflammatory conditions and cancer, we determined whether the exposition of CRC cell lines to OB and NOB serum may impact biological parameters related to tumor development, including cell adhesion, migration and viability. As shown in Figure 4 (panel A), the OB serum induced a ~29% increase of Caco-2 adhesion when compared to control (p=0.01). However, no significant difference on cell migration was observed on HCT-116-treated cultures for 48 h of analysis (Fig. 4B and C). Similarly, nor cell viability and proliferation, neither cell death parameters were altered in Caco-2 and HCT-116 cells exposed to OB or NOB serum, as assessed by MTT, SRB, and PI staining assays (Fig. 5A-E).

Taken together, these data indicate that the OB serum exposition alters the TNFα and IL-10 release and modulates the cell adhesion properties of CRC cells. Nonetheless, no alterations were observed in cell viability, proliferation, migration and cell death parameters at the experimental conditions tested. Additional experiments are required to conclude whether the soluble factors present in the serum from OB individuals are able to directly impact tumor progregression.

ACKNOWLEDGEMENTS

This study was supported by the Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - Processo 312187/2018-1), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; Processo 001), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS - Processo 21/2551-0000078-3); G.G. Roliano and V.B. Toniazzo were recipients of CAPES or CNPq fellowships.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES:

1. Global Burden of Disease Study 2015 (GBD 2015) Obesity and Overweight Prevalence 1980-2015 | GHDx. http://ghdx.healthdata.org/record/ihme-data/gbd-2015-obesity-and-overweight-prevalence-1980-2015. Accessed 28 Sep 2021.

2. Obesity. https://www.who.int/health-topics/obesity#tab=tab_1. Accessed 25 Sep

2021.

3. Ma Y, Yang Y, Wang F, Zhang P, Shi C, Zou Y, et al. Obesity and risk of colorectal cancer: a systematic review of prospective studies. PLoS One. 2013;8. doi:10.1371/JOURNAL.PONE.0053916.

4. Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, et al. Role of tumor microenvironment in tumorigenesis. J Cancer. 2017;8:761–73. doi:10.7150/JCA.17648.

5. Andersen CJ, Murphy KE, Fernandez ML. Impact of Obesity and Metabolic Syndrome on Immunity. Adv Nutr. 2016;7:66. doi:10.3945/AN.115.010207.

6. Balkwill FR, Mantovani A. Cancer-related inflammation: Common themes and therapeutic opportunities. Semin Cancer Biol. 2012;22:33–40. doi:10.1016/j.semcancer.2011.12.005.

7. Cancer. https://www.who.int/news-room/fact-sheets/detail/cancer. Accessed 25 Sep 2021.

8. Atreya CE, Yaeger R, Chu E. Systemic Therapy for Metastatic Colorectal Cancer: From Current Standards to Future Molecular Targeted Approaches. Am Soc Clin Oncol Educ B. 2017;37:246–56.

9. Mármol I, Sánchez-de-Diego C, Dieste AP, Cerrada E, Yoldi MJR. Colorectal carcinoma: A general overview and future perspectives in colorectal cancer. Int J Mol Sci. 2017;18.

10. Bye WA, Nguyen TM, Parker CE, Jairath V, East JE. Strategies for detecting colon cancer in patients with inflammatory bowel disease. Cochrane Database Syst Rev. 2017;2017. doi:10.1002/14651858.CD000279.PUB4.

11. Obesity and overweight. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight. Accessed 25 Sep 2021.

12. Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. Cell. 2010;140:883–99. doi:10.1016/j.cell.2010.01.025.

13. Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell. 2005;7:211–7. doi:10.1016/j.ccr.2005.02.013.

14. Qian BZ, Pollard JW. Macrophage Diversity Enhances Tumor Progression and Metastasis. Cell. 2010;141:39–51.

15. Oh SA, Li MO. TGF- : Guardian of T Cell Function. J Immunol. 2013;191:3973–9. doi:10.4049/jimmunol.1301843.

16. Ng THS, Britton GJ, Hill E V., Verhagen J, Burton BR, Wraith DC. Regulation of adaptive immunity; the role of interleukin-10. Front Immunol. 2013;4 MAY:1–13.

17. Noy R, Pollard JW. Tumor-Associated Macrophages: From Mechanisms to

Therapy. Immunity. 2014;41:49–61. doi:10.1016/j.immuni.2014.06.010.

18. Yang Z, Zhang M, Peng R, Liu J, Wang F, Li Y, et al. The prognostic and clinicopathological value of tumor-associated macrophages in patients with colorectal cancer: a systematic review and meta-analysis. Int J Colorectal Dis. 2020;35:1651–61. doi:10.1007/S00384-020-03686-9.

19. Zhang X, Chen Y, Hao L, Hou A, Chen X, Li Y, et al. Macrophages induce resistance to 5-fluorouracil chemotherapy in colorectal cancer through the release of putrescine. Cancer Lett. 2016;381:305–13. doi:10.1016/J.CANLET.2016.08.004.

20. Malesci A, Bianchi P, Celesti G, Basso G, Marchesi F, Grizzi F, et al. Tumor-associated macrophages and response to 5-fluorouracil adjuvant therapy in stage III colorectal cancer. Oncoimmunology. 2017;6. doi:10.1080/2162402X.2017.1342918.

21. Larionova I, Tuguzbaeva G, Ponomaryova A, Stakheyeva M, Cherdyntseva N, Pavlov V, et al. Tumor-Associated Macrophages in Human Breast, Colorectal, Lung, Ovarian and Prostate Cancers. Front Oncol. 2020;10. doi:10.3389/FONC.2020.566511.

22. Wang H, Tian T, Zhang J. Tumor-Associated Macrophages (TAMs) in Colorectal Cancer (CRC): From Mechanism to Therapy and Prognosis. Int J Mol Sci. 2021;22. doi:10.3390/IJMS22168470.

23. Kushi LH, Byers T, Doyle C, Bandera E V., McCullough ML, McTiernan A, et al. American Cancer Society Guidelines on Nutrition and Physical Activity for cancer prevention: reducing the risk of cancer with healthy food choices and physical activity. CA Cancer J Clin. 2006;56:254–81. doi:10.3322/CANJCLIN.56.5.254.

24. Rock CL, Thomson C, Gansler T, Gapstur SM, McCullough ML, Patel A V., et al. American Cancer Society guideline for diet and physical activity for cancer prevention. CA Cancer J Clin. 2020;70:245–71. doi:10.3322/CAAC.21591.

25. Rajala MW, Scherer PE. Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. Endocrinology. 2003;144:3765–73. doi:10.1210/EN.2003-0580.

26. Grant RW, Dixit VD. Adipose tissue as an immunological organ. Obesity (Silver Spring). 2015;23:512. doi:10.1002/OBY.21003.

LEGEND TO FIGURES:

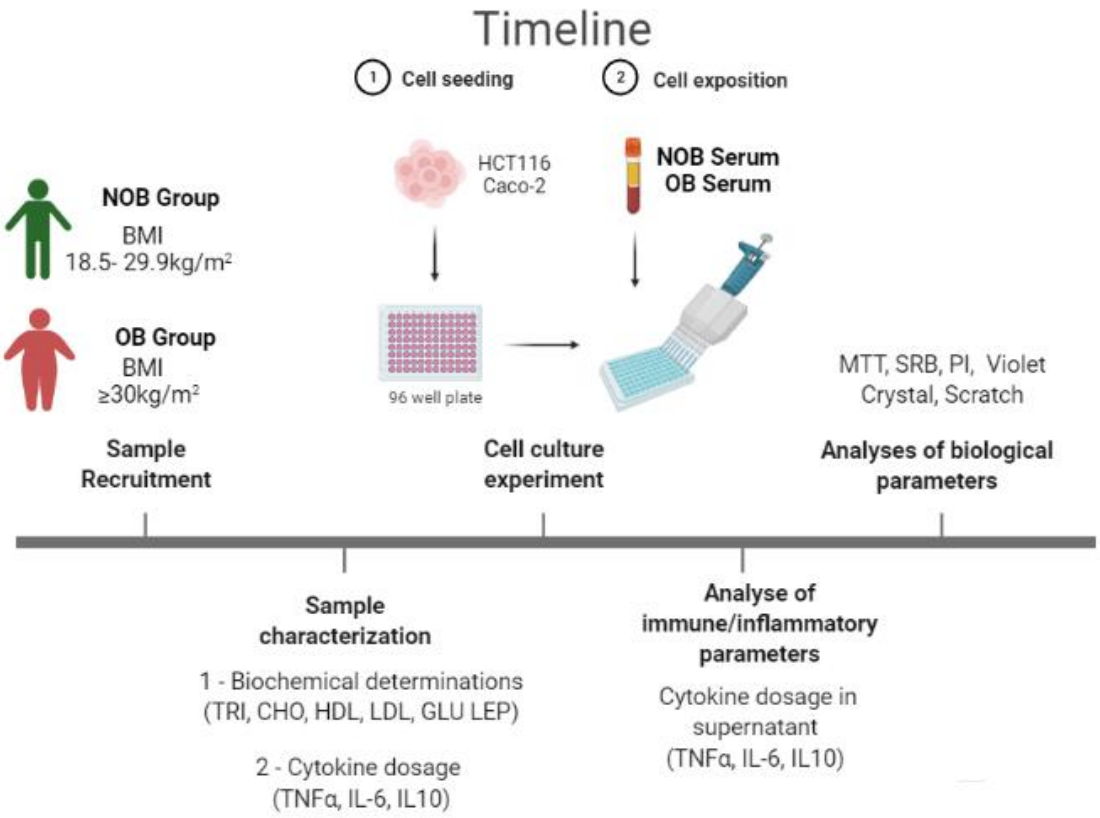


Figure 1. Design of experimental protocol. First, the blood samples from the male participants were collected and further characterized as OB and NOB according to data of weight, height; biochemical profile was performed; and circulating cytokine levels were determined by ELISA. Afterwards, human Caco-2 and HCT116 colorectal cancer cell lines were cultured and exposed to serum from OB and NOB participants and the following parameters were analysed: TNFα, IL-10 and IL-6 levels (ELISA); cell viability (MTT), proliferation (SRB), migration (scratch wound), adhesion (crystal violet staining), and cell death (PI staining). Abbreviations: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); SRB (sulforhodamine B); PI (propidium iodide); OB (obese); NOB (non-obese); BMI (Body mass index); TRI (triglycerides); CHO (cholesterol); HDL (high density lipoprotein); LDL (low density lipoprotein); GLU

(glucose); LEP (leptin); ELISA (Enzyme Linked Immuno Sorbent Assay); TNFα (tumor necrosis factor alpha); IL-6 (interleukin 6); IL-10 (interleukin 10).

A

	Identification	Age	Height	Weight
NON-OBESE	A	24	1.71	62.8
	B	21	1.67	62.5
	C	27	1.76	70.5
	D	20	1.82	76
	E	21	1.75	73
	F	21	1.78	78.2
	G	22	1.62	64.9
	H	24	1.77	79.5
	I	24	1.74	78.2
	J	18	1.79	85.1
	K	24	1.82	98.2
OBESE	A	23	1.73	90.3
	B	23	1.82	100
	C	22	1.81	101.5
	D	26	1.67	87
	E	28	1.75	99.6
	F	25	1.75	100
	G	25	1.69	94
	H	18	1.75	105.6
	I	28	1.72	103
	J	24	1.76	110
	K	30	1.73	139.5

B

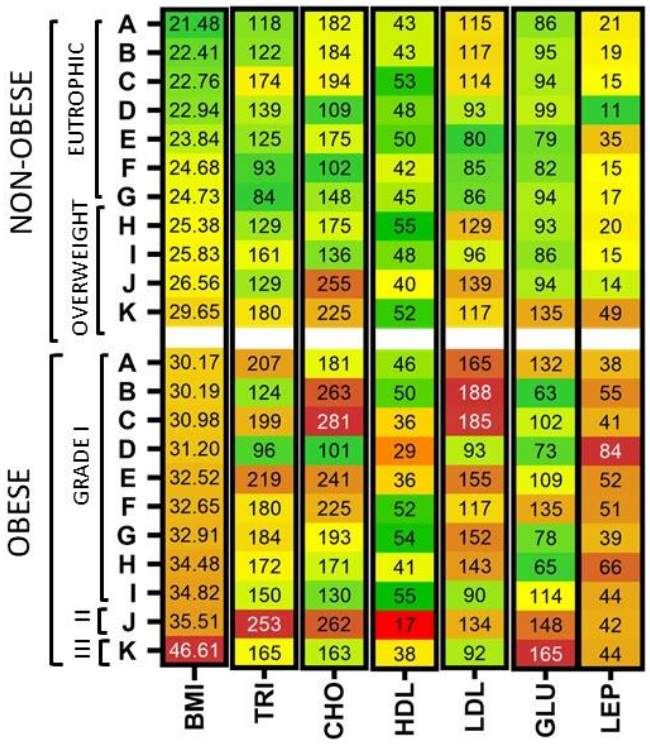


Figure 2. Description of sample characteristics. (A) Table of subjects data. Subjects were grouped according to BMI stage as obese (OB; BMI ≥ 30; N=11) and non-obese (NOB; BMI ≤ 29.9; N=11) and they were indetifed by letters (as indicated); **(B)** heatmap representation of biochemical profile (GLU, TRI, CHO, HDL, LDL, LEP levels) as indicated. Abbreviations: BMI (body mass index); OB (obese); NOB (non-obese); GLU (glucose); TRI (triglycerides); CHO (cholesterol); HDL (high density lipoprotein); LDL (low density lipoprotein); LEP (leptin).

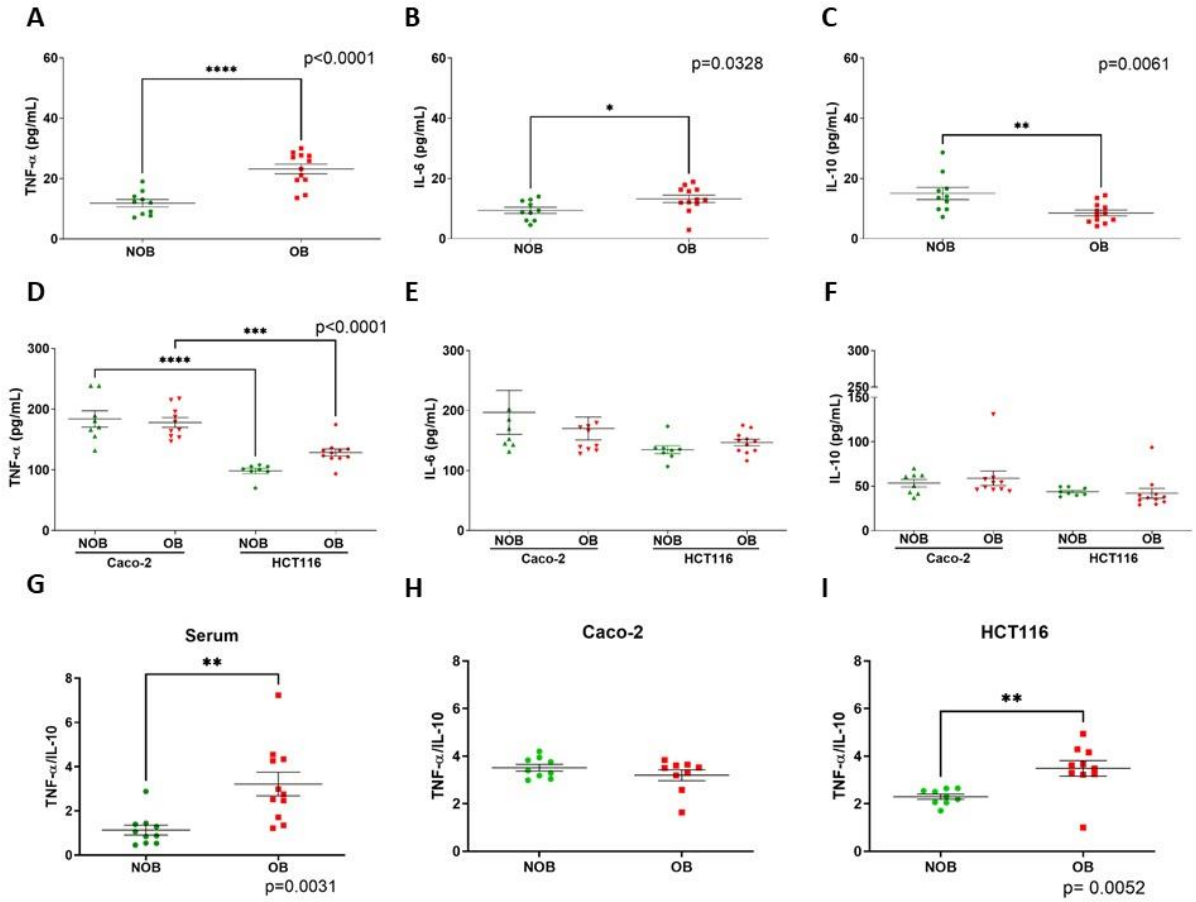


Figure 3. Analysis of cytokine levels in the circulation of the subjects and in the supernatant of colorectal cell cultures exposed to the OB and NOB serum. **(A)** TNFα, **(B)** IL-6, **(C)** IL-10 circulating levels in the subjects (N=11 per group); **(D)** TNFα, **(E)** IL-6, **(F)** IL-10 levels in the supernatant of control or cell cultures exposed to OB or NOB serum, as indicated. **(G, H, I)** TNFα/IL-10 ratio in serum of subjects. Data represent the average ± standard error of at least two experiments performed in duplicate, analyzed by Student's *t*-test (graphs A, B, C, G, H and I) or ANOVA followed by Tukey's *post-hoc* (graphs D, E and F). *, **, *** and ***Significantly different among experimental groups, as indicated (p=0.0328, p=0.0061, p=0.0010 and p<0.0001, respectively). Abbreviations: OB (obese); NOB (non-obese).

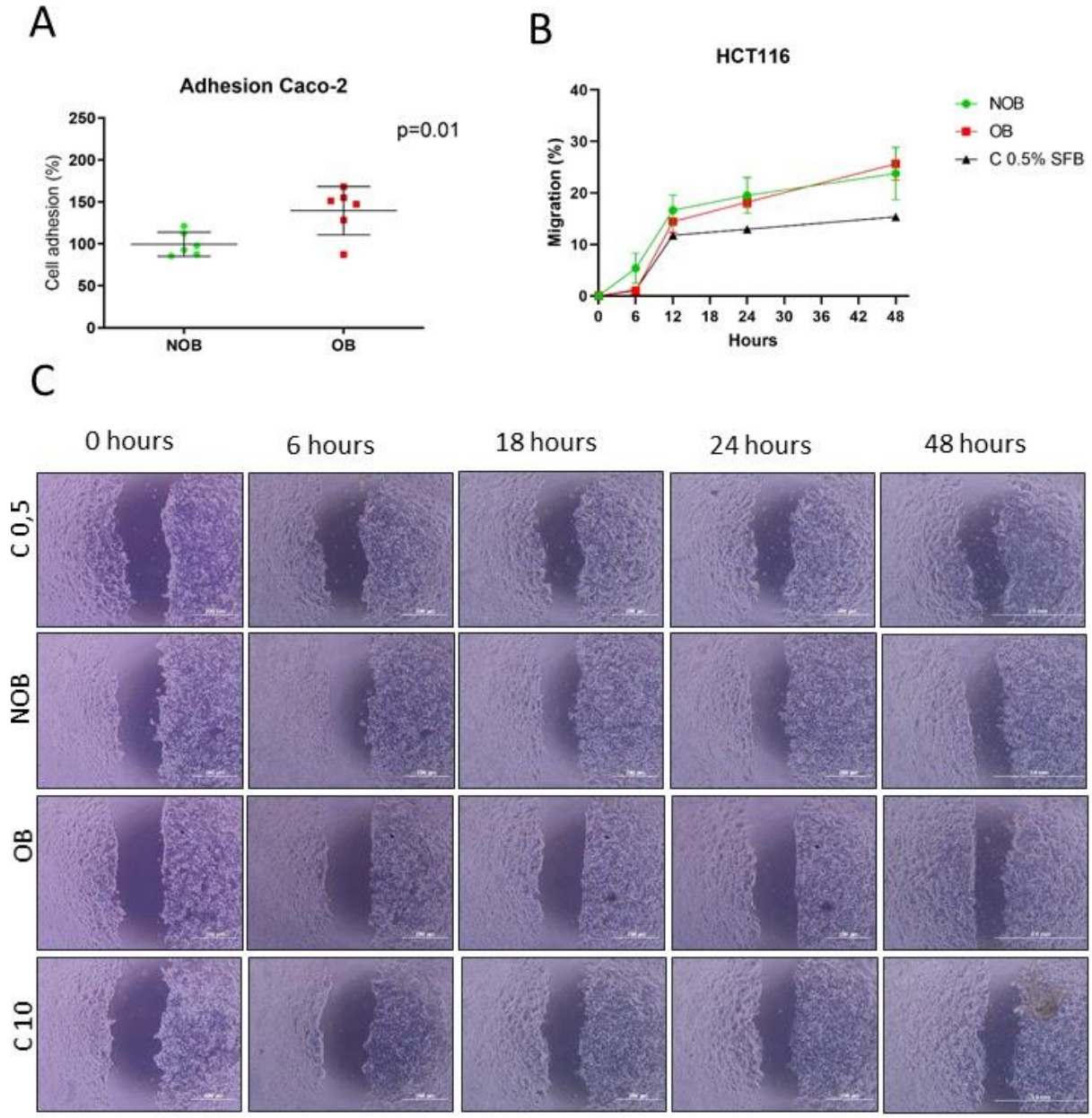


Figure 4. Determination of cell adhesion and migration in colorectal cancer cell lines exposed to OB and NOB serum. **(A)** Caco-2 cell adhesion was evaluated following 2 h of cell seed in presence of OB or NOB serum. **(B)** HCT16 cell migration was determined by scratch-wound assay in cultures exposed to OB or NOB serum (as indicated) for 48 h; **(C)** Representative images from migration assay; images were captured in an optical microscope coupled to a digital camera (magnification 40x) . Data represent the average ± standard error of one representative experiment

performed in triplicate and analyzed by Student's t-test (graph A; p=0.01) or one-way ANOVA followed by Tukey's post-hoc (graph B; non-statistical difference was observed). Abbreviations: OB (obese); NOB (non-obese).

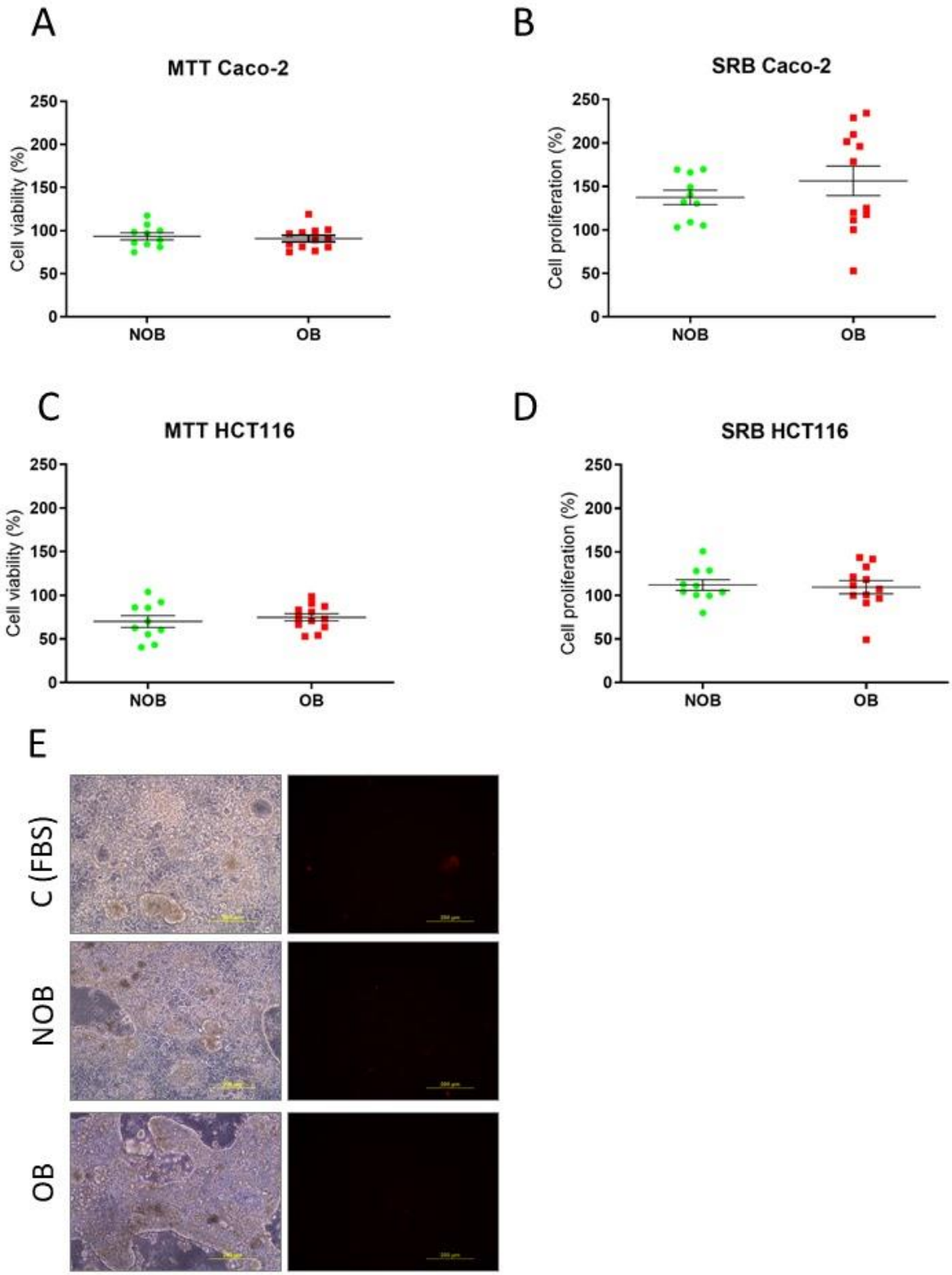


Figure 5. Evaluation of cell viability, proliferation and necrosis cell death in colorectal cancer cell lines exposed to OB and NOB serum. **(A, C)** Caco-2 and HCT116 cell viability, respectively, was evaluated following 48 h of exposition to OB or NOB serum by MTT assay. **(B, D)** Caco-2 and HCT116 cell proliferation, respectively, was evaluated following 48 h of exposition to OB or NOB serum by SRB assay. **(E)** Caco-2 cells were exposed to OB or NOB serum for 72 h and further incubated with PI, as described in material and methods. Representative images were captured in an optical microscope (standard rhodamine filter, 515-560nm fluorescence) coupled to a digital camera (magnification 20x). Data represent the average ± standard error of at least 3 experiments performed in triplicate and analyzed by Student's t-test (non-statistical difference was observed). Abbreviations: OB (obese); NOB (non-obese); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); SRB (sulforhodamine B); PI (propidium iodide).

CAPÍTULO III: Colorectal cancer immunomodulatory effects in macrophages

ROLIANO, G.G.[1], AZAMBUJA, J.H.[2], DAL PRÁ, M.[3], DA CRUZ, L.L.P.[1], DE SOUZA, P.O.[3], FACHEL, F.N.S.[4], BRUNETTO, V. T.[1,3], KALIL, A.N.[1,5], BRAGANHOL, E.[1,3,5]*

[1]Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil.

[2]Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA.

[3]Programa de Pós-Graduação em Biociências, UFCSPA, Porto Alegre, RS, Brazil.

[4] Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, UFRGS, Brazil.

[5]Serviço de Cirurgia Oncológica do Hospital Santa Rita da Santa Casa de Misericórdia, Porto Alegre, RS, Brazil.

[6]Instituto de Cardiologia do Rio Grande do Sul/Fundação Universitária do Instituto de Cardiologia (IC-FUC), Porto Alegre, RS, Brazil.

*Corresponding Author

Elizandra Braganhol (E-mail: ebraganhol@ufcspa.edu.br)

Universidade Federal de Ciências da Saúde (UFCSPA)

Sarmento Leite St, 245 – Main Building – Room 304

Zipcode: 90.050-170 Porto Alegre, RS, Brazil

Phone: +55 51 3303 8762

As normas da revista *Frontiers in Immunology*, para a publicação do manuscrito estão disponíveis em: https://bmcendocrdisord.biomedcentral.com/submission-guidelines/preparing-your-manuscript/research-article

INTRODUCTION

Colorectal cancer (CRC) is the fifth (colon) and eighth (rectum) cancer in news cases and new death by cancer in the world [1]. It is a cancer that can be prevented by diagnosing colonoscopy and removing polyps in the intestine (early stage of cell change) [2], the treatment is cirurgical remove and chemotherapy with fluoropyrimidine (5-fluorouracil) plus oxaliplatin or irinotecan [3]. Several risk factors can lead to the development of this cancer, and inflammatory bowel diseases are one of them [4], it is known, that the immune/inflammatory system plays a role in tumor initiation process [5–9]. CRC can progress to metastasis, which is the main cause of death for patients [10, 11], the tumor microenvironment is a key molecular step in this process [12].

Macrophages have the physiological function of destroying pathogens or altered cells [13, 14]. They have an important ability to switch through a spectrum of activation states depending on stimulus, to proinflammatory (M1/classical) or anti-inflammatory (M2/alternative) phenotypes [14–16]. The M1 polarization is induced by interferon-γ (IFN-γ), tumor necrosis factor (TNF), or lipopolysaccharide (LPS) [13]. M1 macrophage participate in the recruitment of Th1, Tc1 and NK cells, creating an immune response against tumor cells mediated by higher production of pro-inflammatory cytokines such as interleukin-12 (IL-12), IL-6, IL-8, TNF, IL-1β and nitric oxide (NO) and reactive oxygen species (ROS) and higher antigen-presenting capacity [13, 14]. In turn, the M2 macrophage profile is related to anti-inflammatory cytokine production, as IL-10, arginase activity and mannose receptors expression (CD204, CD206) [14, 17]. This phenotype is induced by IL-4, which is produced by Th2 cells, eosinophils, basophils or macrophages themselves [14].

The term “tumor-associated macrophages” (TAM) is relative to macrophages that end up being corrupted by tumor cells and start to play in favor of the tumor progression [5, 18, 19]. TAMs are predominantly immunosuppressive in nature and share several similarities with M2 macrophages, particularly those related to IL-10 release, cell proliferation induction, and tissue repair signaling activation [19].

Despite the evidence connecting cancer and inflammation, the participation of the innate immune cells, more specifically macrophages, in CRC progression is still poorly understood. Considering that tumor cells shape the behavior of macrophages to contribute to tumor growth, there are possibly unknown mechanisms that generate this

environment enriched in immunosuppressive factors. The elucidation of these pathways may be important to better understand the mechanisms of CRC progression, thus, create therapeutic strategies for the clinical management of patients.

MATERIAL AND METHODS

MATERIALS

The chemical components used in this study can be found in the supplementary Table 1. Other chemicals and kits were purchased from standard commercial suppliers with analytical grade and used as per manufacturer's instructions.

CELL CULTURE

CELL LINE

Human colorectal cancer (HCT116) cell line was obtained from ATCC (American Type Cell Collection; Rockville, Maryland, USA). The cell line was grown in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 8.4 mM HEPES, 23.8 mM $NaHCO_3$ (pH 7.4), 0.1% fungizone, 100 U/L penicillin/streptomycin 0.5 U/mL, and supplemented with 10% (v/v) fetal bovine serum (FBS). The cell line was incubated at 37ºC in a humidified incubator with 5% CO_2.

PRODUCTION OF HCT116 CONDITIONED MEDIUM (CM)

To prepare HCT116 conditioned medium (CM), cells were seeded at high density in 75 cm^2 culture flasks. Once the sub-confluence stage was achieved, cell medium was replaced by fresh high glucose DMEM/10% FBS, and cells were cultured for an additional 24 h. The CM was collected, centrifuged (1,000 *g* – 5 min) and stored at -80°C until use.

PRIMARY HUMAN MACROPHAGE CULTURES

Primary human macrophages were obtained from differentiation of circulating monocytes as previously described [20] with minor modifications. Briefly, monocytes were isolated from total blood of umbilical cord using Histopaque®-1077 density gradient; cells were seeded in 6 multi-well plates (7×10^6 cells/well) and differentiated

into macrophages by stimulation with GM-CSF (50 ng/mL) for 7 days. Subsequently, mature human macrophages (Mφ) were exposed to HCT116-CM or directly co-cultured with HCT116 (7×10^3 cells/well). The analyses were performed 24 or 48 h following exposure. HCT116 cancer cells and human macrophages cultured in DMEM/10% FBS and RPMI/10% FBS, respectively, under the same conditions were considered controls. All procedures used in the present study were approved by the Ethic Committee of the HCPA (2.476.898). Cord blood bags were acquired by the Umbilical Cord Blood Bank of the HCPA, when considered waste materials were used for monocyte isolation.

BRDU ASSAY. Mφ and HCT116 were co-cultured as described above. For DNA synthesis analysis, the culture medium (RPMI/10% FBS) was replaced by fresh RPMI/0.5% FBS. After 22 h of starvation, cells were exposed to 10 μM bromodeoxyuridine (BrdU) for 2 h incubation in RPMI/10% FBS. Cells were then incubated with anti-BrdU-PE antibody (1:50 dilution; BD Biosciences) and the percentage of BrdU-incorporating cells was determined by flow cytometry (FACSCanto II Flow Cytometer; BD Biosciences).

CYTOKINE PRODUCTION. Mφ and HCT116 were co-cultured as described above. Cytokines (TNF, IL-10, IL-6, IL-1β and IL-8) were determined in the supernatant of cell cultures using Cytometric Bead Array (CBA). All analyses were performed by flow cytometry (FACSCanto II Flow Cytometer; BD Biosciences) following the manufacturer's instructions.

DCFH-DA assay. Intracellular formation of reactive oxygen species (ROS) was determined by the dichlorofluorescein diacetate (DCFH-DA) assay according to previous study (Ali et al.,1992). Mφ and HCT116 were co-cultured as described above. Then the culture medium was removed, and cells were incubated with 1 μM DCFH-DA in serum-free DMEM. After 30 min incubation at 37°C, cells were washed with PBS and images were captured using a digital camera connected to a microscope (Olympus IX71, Japan, 485/520 nm). Fluorescence was quantified in ImageJ (version 1.52a, National Institutes of Health, USA). The results were expressed as arbitrary units.

PROPIDIUM IODIDE ASSAY. Cell death by necrosis was evaluated using fluorescent image analysis obtained from a propidium iodide (PI) incorporation assay. Mφ and

HCT116 were co-cultured as described above. Following the exposure, the medium was removed, and PI (7.5 µM, final concentration) was added. Cells were then incubated for 60 min, and phase contrast and fluorescence microphotographs were captured using a digital camera connected to an inverted microscope (Olympus IX71, JP) fitted with a standard Rhodamine filter excited at 515 and 560 nm.

NO Determination. Nitric oxide (NO) production was evaluated by measuring nitrite levels in the supernatant of cell culture, using a colorimetric reaction with Griess reagent according to Stuehr and Nathan [21]. Following the treatments, 5% sulfanilamide (w/v) in phosphoric acid was added to cell-culture supernatants (1:1 ratio) and then mixed. After 10 min of incubation at RT (25±1°C), samples were mixed with Griess reagent 0.1% (w/v) (N-[1-naphthyl] ethyl-enediamine dihydrochloride) (1:1:1 ratio). Following an additional 10 min in the dark at RT, the absorbance was measured in a spectrophotometer SpectraMax® M2 (Molecular Devices, US) at 540 nm. Results were expressed in µM NO/mg protein and were obtained by comparing the results with a standard curve of known concentrations of sodium nitrate.

MACROPHAGE POLARIZATION ANALYSIS. Mϕ and HCT116 were co-cultured as described above. Cells were washed with PBS and carefully detached with cold EDTA solution (2.5 µM) using a cell scraper. The cell viability of detached cells was determined by cell counting using trypan-blue staining. Samples were then incubated with HLA-DR (PE Mouse Anti-Human HLA-DR; 555812, dilution 1:5,BD Pharmingen™), CD206 (FITC Mouse Anti-Human CD206,551135, dilution 1:5, BD Pharmingen™) and CD163 (BV461 Mouse Anti-human CD163, 562643, dilution 1:20 BD Horizon™) (Grugan et al, 2012). The expression of membrane markers was determined by flow cytometry (FACS Canto II, BD Biosciences); 10,000 events were collected per sample and the data obtained were evaluated using the FlowJo_vX.0.7 software.

RHODAMINE-123 ASSAY. Cells were washed with PBS and carefully detached with cold EDTA solution (2.5 µM) using a cell scraper. Cells (2×10^5 cells/mL) were incubated with rhodamine 123 (5 µg/mL) prepared in DMEM 10%FBS according to the manufacturer's instructions for 30 min at RT. Subsequently cells were washed once with DMEM/10%FBS and analyzed by flow cytometry (FACSCanto II, BD Biosciences). Mitochondrial fluorescence was generated by excitation from the primary laser that

emitted at 488 nm and collection at the FL1 position after passing through a DF530/30 filter.

STATISTICAL ANALYSIS

Data were expressed as mean ± S.D. and were subjected to one-way analysis of variance (ANOVA) followed by either Tukey–Kramer post-hoc test (for multiple comparisons), Bonferroni test, or Student's t-test as appropriate (GraphPad Prism 5 Software). Difference between mean values were considered significant when $P<0.05$.

RESULTS AND DISCUSSION

To determine the impact of tumor cells in macrophage polarization we develop a primary culture of human macrophages and we add a colorectal cancer cell line (HCT116) in this culture. Control macrophages showed expected morphology cell morphology (Fig. 1A), but in co-culture with tumor cell had more stretched characteristics, remembering a “fried egg”, and had tropism to HCT116, circling the cell (Fig. 1C), indicating that somehow the macrophage answered to tumor cell presence. Morphology of HCT116 when shown in low density (Fig. 1B).

To evaluate the presence of cell death by necrosis in the macrophages exposed to HCT116-CM or co-cultured with tumor cells, PI assay was performed. The incorporation of propidium iodide was not different in the groups (Fig. 2A), indicating that neither the HCT116-CM nor HCT116 induce necrosis in macrophages after 24h of exposure. Taken together, this dataset shows macrophages and tumor cells can cohabit the same environment without causing death and that tumor cells release signals that attract macrophages and modify their morphology.

Then, we perform a proliferation assay with BrdU (Fig. 2B), and when co-cultured with macrophages in DMEM/0.5% FBS, the HCT116 cells tend to increase by 5 times the cell proliferation. This means that macrophages induce cell proliferation in tumor cells, however, this data represents a single experiment that needs to be repeated.

Next, as the initial results indicated that macrophages were promoting *in vitro* tumor cell progression, we decided to evaluate parameters of oxidative stress. Reactive

oxygen species (ROS) levels in macrophage culture were analyzed by the DCFH-DA assay. In the representative images it can be observed that, when co-cultured with tumor cells, macrophage reduces the intensity of fluorescence (Fig. 3A), basically being seen as the fluorescence of the tumor cell. The intensity of fluorescence tended to increase in the HCT116 group, and decreased when the macrophage was present (Fig. 3C). The decrease of ROS observed in macrophages co-cultured with HCT116 could be associated with immunosuppressive macrophage polarization, a characteristic of TAM [22, 23]. The macrophage morphology, the increase of tumor proliferation induced by macrophages and the decreased ROS production may converge to tumor survival. Therefore, additional experiments were performed in order to better understand the impact of macrophages on CRC progression.

The potential of the mitochondrial membrane of macrophages exposed to HCT-CM was assessed by Rho123 assay. The HCT116-CM tend to decrease the median fluorescence intensity of macrophages when compared to control (Mφ DMEM/0.5% FBS) (Fig. 3C and 3D). The level of NO was further determined (Fig. 4A). The nitrites production was the same in 24h to all groups (macrophage, HCT116, conditioned medium and macrophage-HCT166 co-culture), but in 48h, nitrites levels increased significate in the co-culture group, when compared to macrophage alone ($p<0.0001$). NO is a strong inductor of angiogenesis and may be related to cancer progression [24]. To evaluate the cytokine release profile exhibited by macrophage in the presence or absence of HCT116 tumor cells, we performed flow cytometry using CBA kit. Interestingly, macrophages co-cultured with HCT116 tumor cells exhibited an increase of 2,7, 1,9, 1,8 and 2,6 times of TNF, IL10, IL6, IL1β, respectively, while the level of IL-8 was decreased by 2,7 times, when compared to control (macrophages cultured alone) (Fig. 5). Considering the protumor role of the cytokines TNF, IL10, and IL6, these results suggest that CRC cells induce a M2-*like* macrophage polarization, which is known to TAM.

Finally, the expression of macrophage polarization markers was determined by flow cytometry. In line with the TAM phenotype, macrophages co-cultured with HCT116 exhibited increased expression of CD206 and CD163 ($p=0.026$ and 0.034, respectively) when compared to control (Mφ cultured alone), while the expression of HLA-DR was not different among the groups (Fig. 6A-C).

In summary, these data suggest that CRC cells induce a M2-like macrophage polarization, which promotes tumor progression. However, additional experiments and an increase of experimental “N” are needed to better understand the CRC cells-macrophage crosstalk and the further impact in the cancer cell proliferation as well as stimulus to metastasis.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

This study was supported by the Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - Processo 312187/2018-1), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; Processo 001), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS - Processo 21/2551-0000078-3); G.G. Roliano and V.B. Toniazzo were recipients of CAPES or CNPq fellowships.

REFERENCES

1. Sung H, Ferlay J, Siegel R, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71:209–49. doi:10.3322/CAAC.21660.

2. Schmoll HJ, Cutsem E Van, Stein A, Valentini V, Glimelius B, Haustermans K, et al. ESMO Consensus Guidelines for management of patients with colon and rectal cancer. A personalized approach to clinical decision making. Ann Oncol. 2012;23:2479–516. doi:10.1093/ANNONC/MDS236.

3. Weinberg BA, Marshall JL, Hartley M, Salem ME. A paradigm shift from one-size-fits-all to tailor-made therapy for metastatic colorectal cancer. Clin Adv Hematol Oncol. 2016;14:116–28.

4. Jess T, Rungoe C, Peyrin-Biroulet L. Risk of Colorectal Cancer in Patients With Ulcerative Colitis: A Meta-analysis of Population-Based Cohort Studies BACKGROUND & AIMS: Patients with ulcerative colitis. 2012. doi:10.1016/j.cgh.2012.01.010.

5. Balkwill FR, Mantovani A. Cancer-related inflammation: Common themes and therapeutic opportunities. Semin Cancer Biol. 2012;22:33–40. doi:10.1016/j.semcancer.2011.12.005.

6. Gupta RB, Harpaz N, Itzkowitz S, Hossain S, Matula S, Kornbluth A, et al. Histologic Inflammation Is a Risk Factor for Progression to Colorectal Neoplasia in Ulcerative Colitis: A Cohort Study. Gastroenterology. 2007;133:1099–105.

7. Itzkowitz SH, Harpaz N. Diagnosis and management of dysplasia in patients with inflammatory bowel diseases. Gastroenterology. 2004;126:1634–48.

8. Itzkowitz SH, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. Am J Physiol Gastrointest Liver Physiol. 2004;287. doi:10.1152/AJPGI.00079.2004.

9. Rutter M, Saunders B, Wilkinson K, Rumbles S, Schofield G, Kamm M, et al. Severity of Inflammation Is a Risk Factor for Colorectal Neoplasia in Ulcerative Colitis. Gastroenterology. 2004;126:451–9.

10. Miller KD, Nogueira L, Mariotto AB, Rowland JH, Yabroff KR, Alfano CM, et al. Cancer treatment and survivorship statistics, 2019. CA Cancer J Clin. 2019;69:363–85. doi:10.3322/CAAC.21565.

11. Atreya CE, Yaeger R, Chu E. Systemic Therapy for Metastatic Colorectal Cancer: From Current Standards to Future Molecular Targeted Approaches. Am Soc Clin Oncol Educ B. 2017;37:246–56.

12. Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, et al. Role of tumor microenvironment in tumorigenesis. J Cancer. 2017;8:761–73. doi:10.7150/JCA.17648.

13. MANTOVANI A, SICA A, SOZZANI S, ALLAVENA P, VECCHI A, LOCATI M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 2004;25:677–86. doi:10.1016/j.it.2004.09.015.

14. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep. 2014;6:13. doi:10.12703/P6-13.

15. Locati M, Mantovani A, Sica A. Macrophage Activation and Polarization as an Adaptive Component of Innate Immunity. Adv Immunol. 2013;120:163–84.

16. Wadwa RP, Kinney GL, Ogden L, Snell-Bergeon JK, Maahs DM, Cornell E, et al. Soluble interleukin-2 receptor as a marker for progression of coronary artery calcification in type 1 diabetes. Int J Biochem Cell Biol. 2006;38:996–1003. doi:10.1016/J.BIOCEL.2005.09.015.

17. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med. 1989;170:2081. doi:10.1084/JEM.170.6.2081.

18. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 2004 41. 2004;4:71–8. doi:10.1038/nrc1256.

19. Qian B, Pollard JW. Macrophage Diversity Enhances Tumor Progression and Metastasis. Cell. 2010;141:39. doi:10.1016/J.CELL.2010.03.014.

20. Parisi MM. PADRONIZAÇÃO DE TÉCNICA DE PURIFICAÇÃO DE MONÓCITOS COMO MODELO DE CULTURA CELULAR PARA ESTUDO DA DIFERENCIAÇÃO IN VITRO DE MACRÓFAGOS. Dissertação (Mestrado em Bioquímica) Programa de Pós-Graduação em Ciências Biológicas ICBS. 2014.

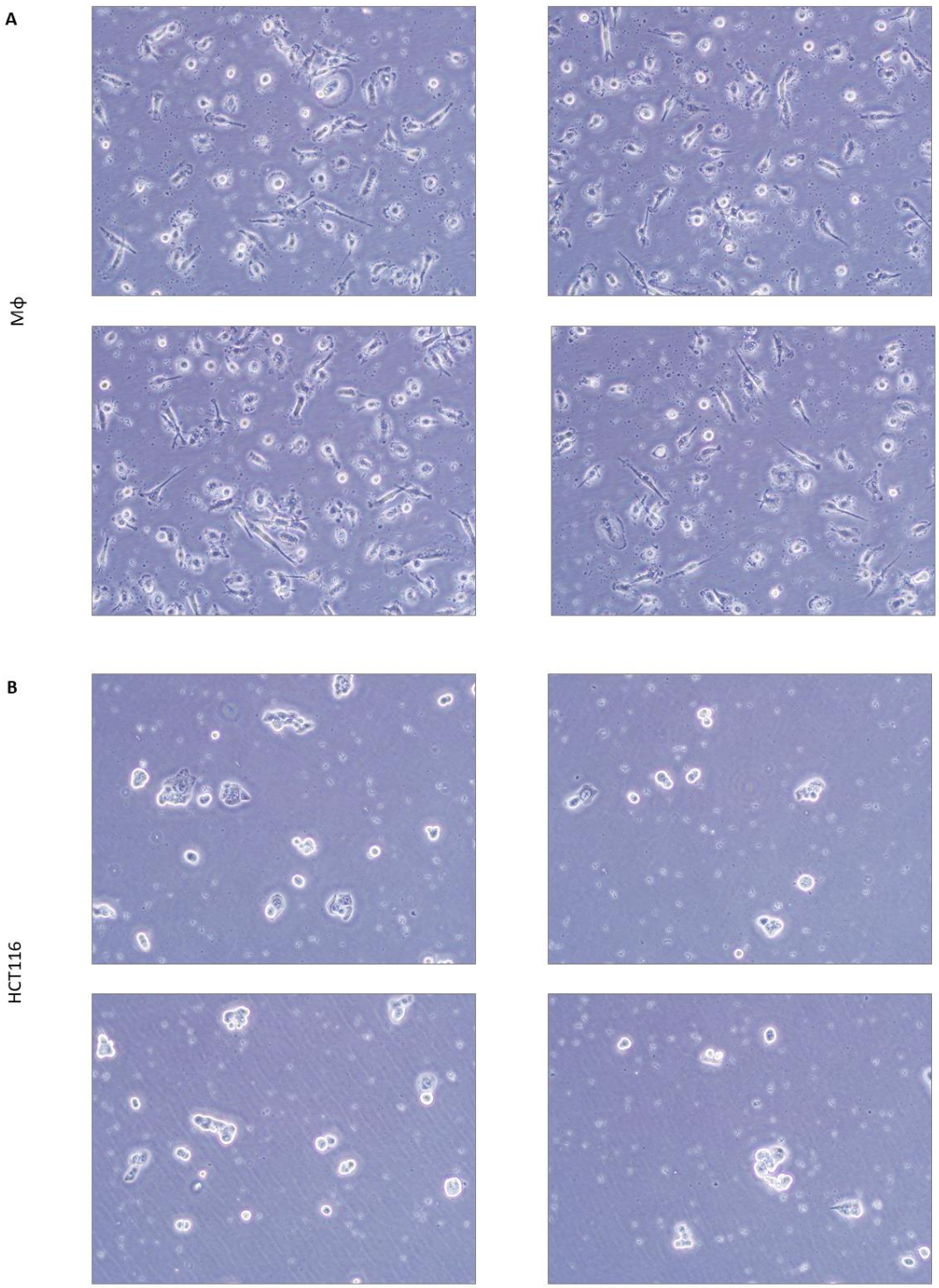
21. Stuehr D, Nathan C. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J Exp Med. 1989;169:1543–55. doi:10.1084/JEM.169.5.1543.

22. Lee H, Surh Y. Resolvin D1-mediated NOX2 inactivation rescues macrophages undertaking efferocytosis from oxidative stress-induced apoptosis. Biochem Pharmacol. 2013;86:759–69. doi:10.1016/J.BCP.2013.07.002.

23. Simões RL, De-Brito NM, Cunha-Costa H, Morandi V, Fierro IM, Roitt IM, et al. Lipoxin A4 selectively programs the profile of M2 tumor-associated macrophages which favour control of tumor progression. Int J Cancer. 2017;140:346–57. doi:10.1002/IJC.30424.

24. Ghimire K, Altmann H, Straub A, Isenberg J. Nitric oxide: what’s new to NO? Am J Physiol Cell Physiol. 2017;312:C254–62. doi:10.1152/AJPCELL.00315.2016.

FIGURES AND LEGENDS:



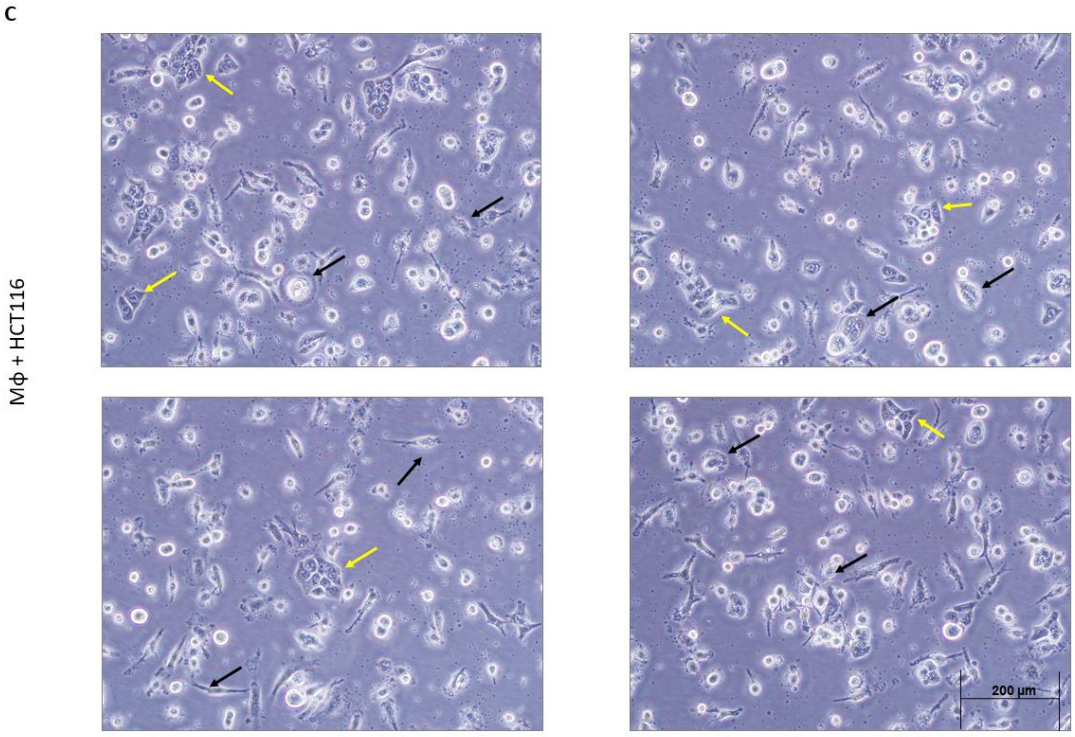


Figure 1: Representative images of human macrophages (A), cell line HCT116 (B) and both in co-culture (C). The black arrows indicate macrophages and yellow arrows indicate tumoral cell HCT116. In co-culture the macrophages appear to be activated by a morphology more stretched (similar to fried egg), while alone this aspect was less observed.

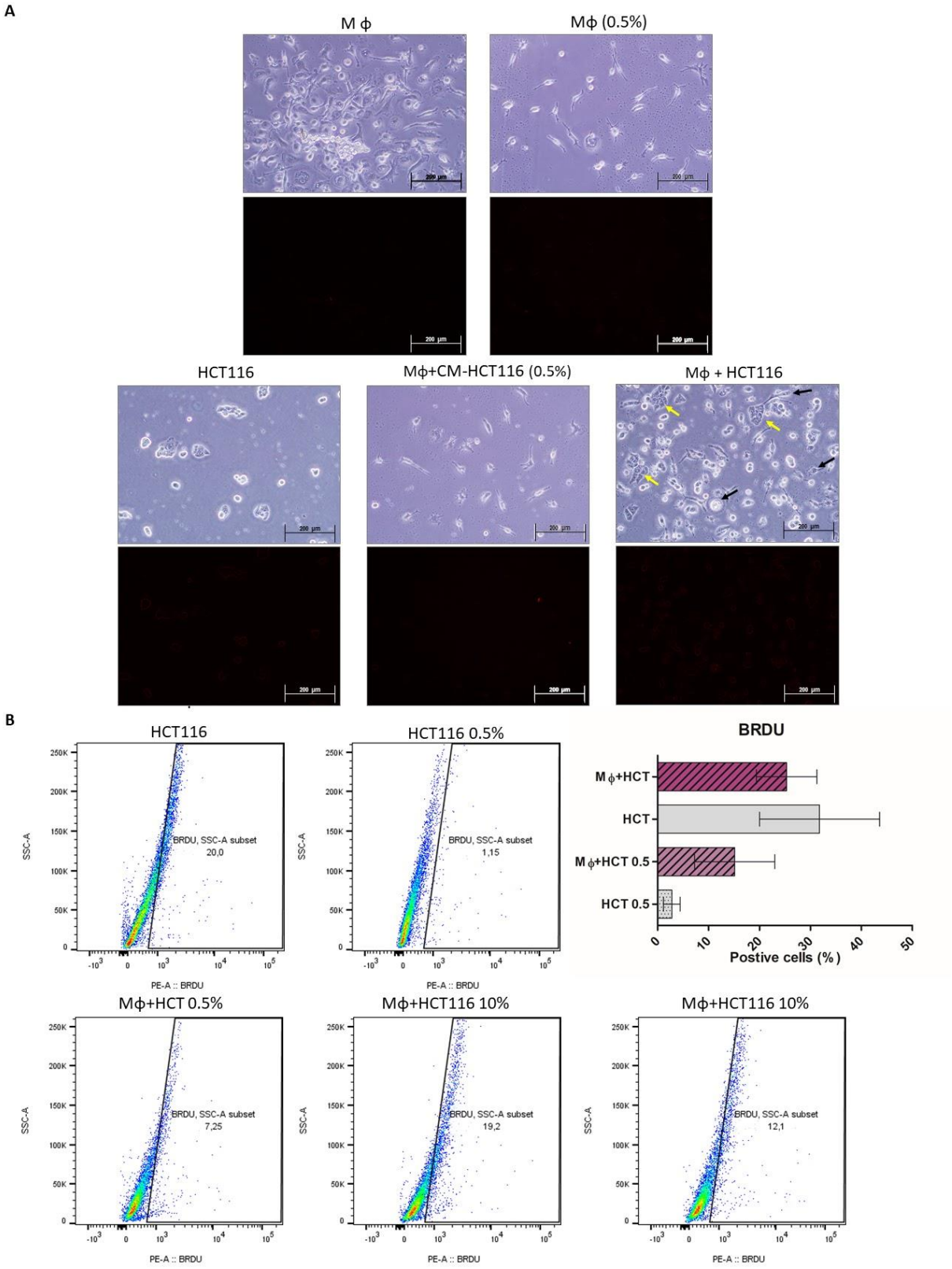


Figure 2: Representative images of Propidium Iodide assay (A), the controls macrophages, with 10%FBS or 0.5%FBS, respectively (higher) had similar results that experimental groups, HCT116 10%FBS, Conditioned medium-HCT116 0.5%FBS and macrophages-HCT116 co-culture, respectively (bottom). Without any significant

difference between them (data not shown). Proliferation BRDU assay (B), the HCT116 10%FBS (first graphic) had 20% of positive cells, HCT116 0.5%FBS (second graphic) had 1.15%, the macrophages-HCT116 0.5%FBS co-culture had 7.25% (fourth graphic) and macrophages-HCT116 10%FBS co-culture had 19.2 and 12.1% (duplicate – fifth and last graphic). The thirty graphic is the percentage of positives cells bar representation.

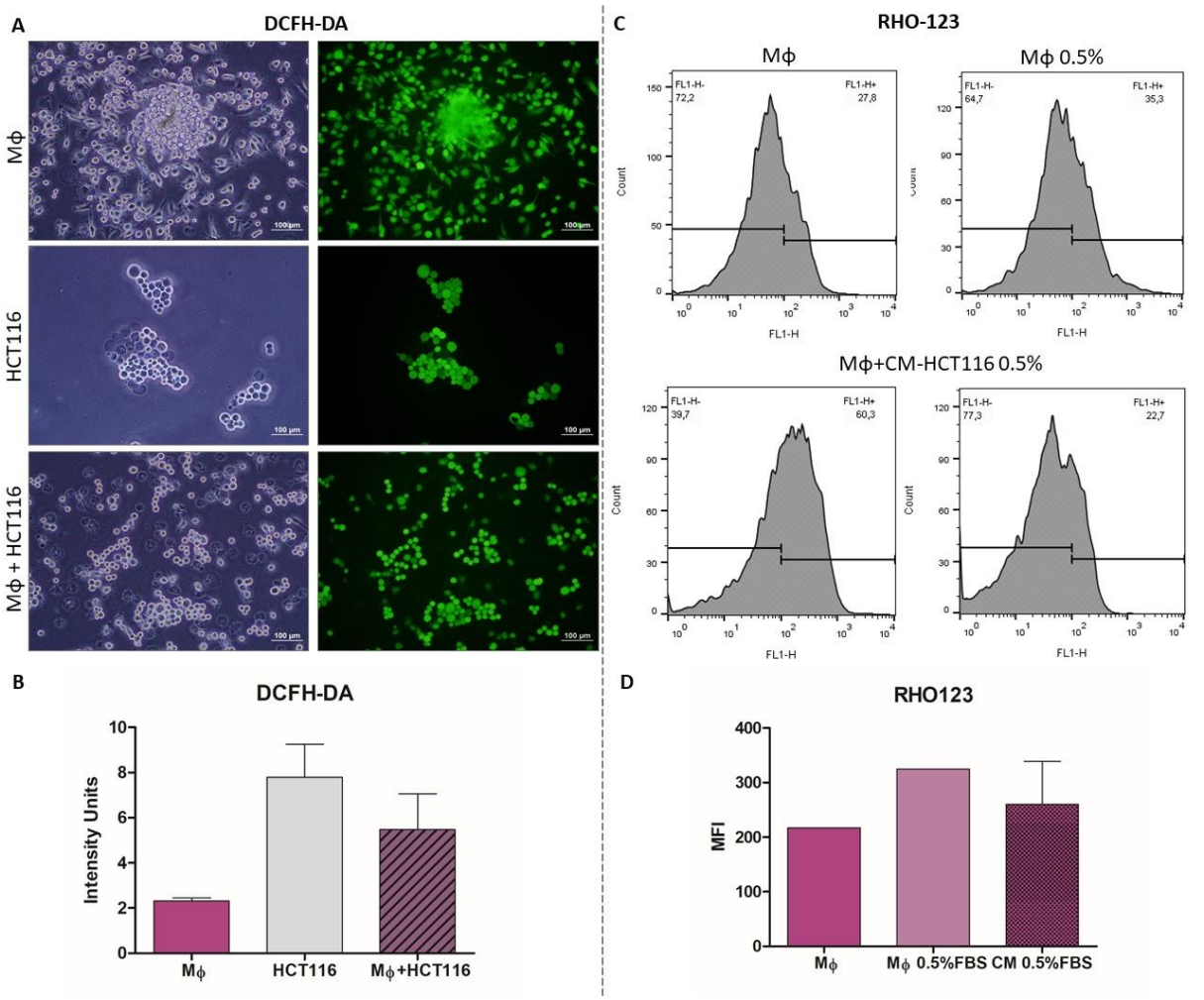


Figure 3: Representative images (A) and Intensity Units graph (B) of reactive oxygen species (ROS). Apparently macrophages alone produce less ROS, and when co-culture with HCT116 the ROS levels increase, nonetheless are not significant statistics. The membrane potential was analyzed by rhodamine123 assay (C and D), with no difference between the controls (macrophages 10%FBS and 0.5%FBS) and experimental group (CM 0.5%FBS).

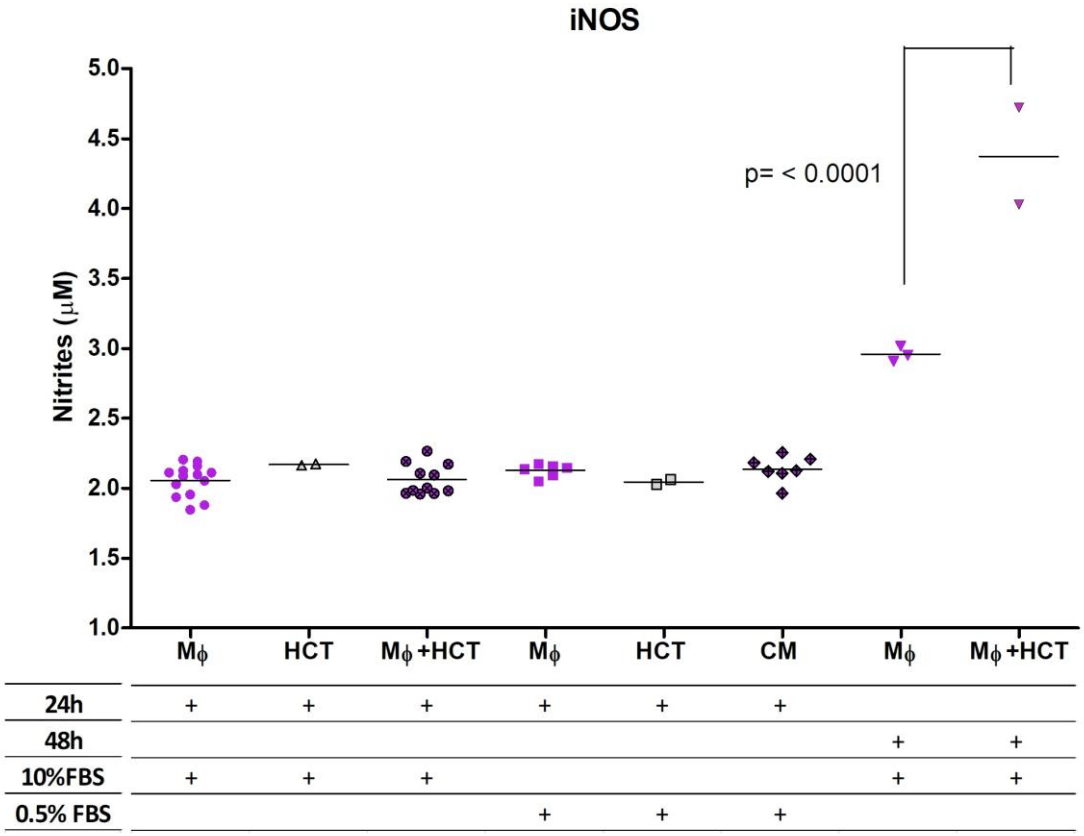


Figure 4: Nitric Oxide determination was performed in two times (24h and 48h) and in the following conditions: macrophage with 10% and 0.5%FBS, macrophage with conditioned medium 0.5%FBS; HCT116 with 10% and 0.5%FBS; macrophage co-cultured with HCT116 (A). The only significant difference was in time 48h, increased NO in co-culture compared with macrophage alone ($p<0.0001$). The proliferation assay was performed with BrdU assay, HCT116 10%FBS and co-culture macrophages and HCT116 10%FBS presented increased marcation to BrdU. While, as expected, macrophages 0.5%FBS, HCT116 0.5%FBS and macrophage-HCT116 0.5%FBS didn't have a proliferation market.

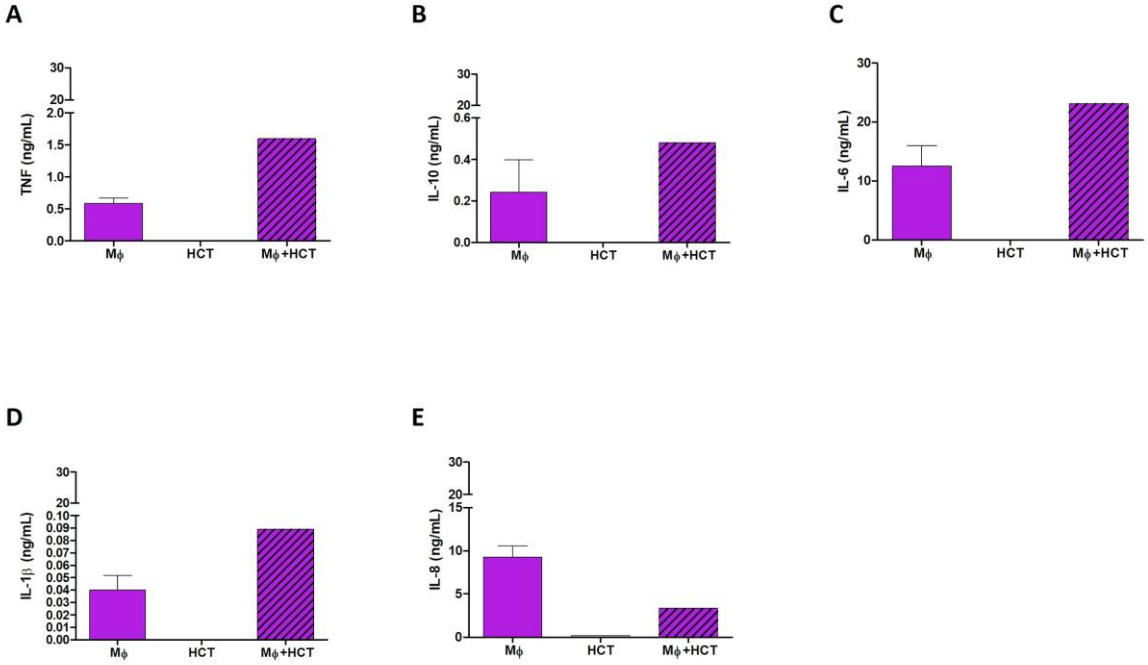


Figure 5: To analysis of cytokines production of cultures, the cytometry with CBA kit was performed, tumoral cell HCT116 expressed little or none cytokine investigated, while macrophages biased to express less pro-inflammatory cytokines (A,C and D) and more anti-inflammatory cytokine (E). On the contrary of macrophage alone, co-culture with HCT116 is biased to express more pro-inflammatory and less anti-inflammatory cytokine.

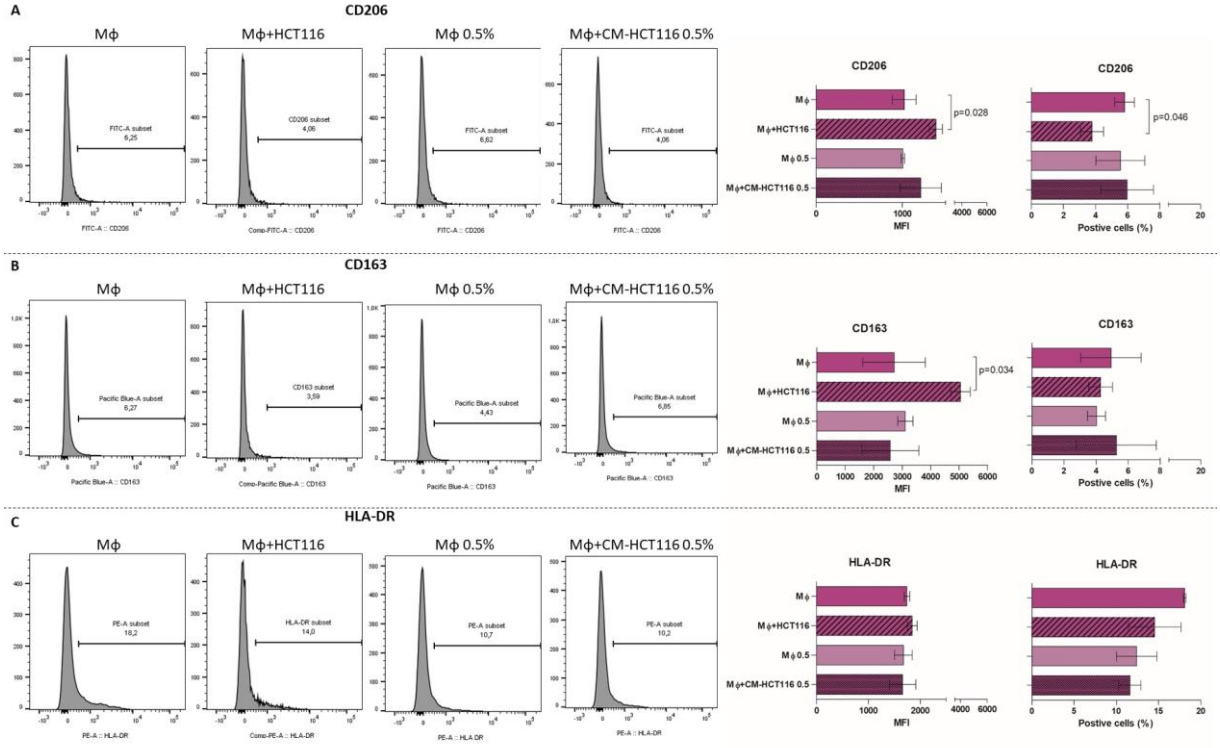


Figure 6: To analyze macrophage polarization in contact with tumor cell HCT116, M1 (HLA-DR(C)) and M2 (CD206 (A) and CD163 (B)) markers were used. In median fluorescence intensity analyze, CD206 and CD163 had increased in co-culture group (p=0.026 and 0.034), and CD206 had decreased in positive cell marker in co-culture group, HLA-DR not had changes (D).

9 CONCLUSÃO

Ao final desse trabalho, foi possível identificar que ao reunir os estudos sobre a sinalização purinérgica, seus receptores, enzimas e produtos (ATP, ADO), alguns conceitos já estão bastante fortalecidos, como o papel da enzima CD73 que gera o bloqueio da resposta imune e leva a progressão do CCR, assim como os receptores da família P1. Enquanto que os receptores P2Y e P2X apresentaram dualidade. Notamos a necessidade de mais estudos para ampliar o conhecimento sobre a função do sistema purinérgico no CCR. Quanto aos resultados experimentais, os indivíduos obesos apresentaram maiores alterações, nas análises bioquímicas, assim como aumento nos níveis séricos de TNF-α, IL-6 (pró-inflamatórios) e redução de IL-10 (anti-inflamatório), exibindo características de inflamação associada a obesidade. Quando as células tumorais foram expostas aos soros de ambos os grupos, e os sobrenadantes foram analisados, as duas linhagens responderam de forma diferente na liberação de mediadores solúveis e alguns parâmetros celulares. Mais experimentos são necessários para testar a hipótese de que o soro de indivíduos obeso é capaz de impactar na progressão tumoral, mas possivelmente as células respondem diferentemente por possuírem características moleculares distintas. Os experimentos de co-cultivo com macrófagos humanos apresentaram vantagens para progressão das células tumorais, exibindo fenótipo misto característico de TAM, no qual mais experimentos são necessários para melhor compreender a função dos macrófagos na alteração do microambiente tumoral do câncer colorretal.