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INVESTIGAÇÃO DA AÇÃO DOS RECEPTORES PURINÉRGICOS $P2Y_2$ E $P2Y_{12}$ NA PROLIFERAÇÃO DE CÉLULAS DE CARCINOMA ESCAMOSO E ADENOCARCINOMA DE ESÔFAGO

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Orientadora: Profª Dr Fernanda Bueno Morrone

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RESUMO

O câncer de esôfago, em geral, é diagnosticado em estágio avançado, o que leva a um prejuízo nas terapias empregadas, resultando em uma alta taxa de mortalidade. É dividido em dois subtipos: adenocarcinoma e carcinoma de células escamosas; os dois subtipos diferem quanto a histologia, etiologia e epidemiologia. A sinalização purinérgica utiliza nucleotídeos e nucleosídeos no meio extracelular como moléculas sinalizadoras e já foi relacionada com diferentes tipos de carcinomas. Essas moléculas ligam-se a receptores para adenosina acoplados a proteína G, caracterizados como P1 (A_1, A_{2A}, A_{2B} e A_3), a receptores ionotrópicos P2X (P2X1-P2X7), e ainda a receptores P2Y ($P2Y_{1, 2, 4, 6, 11, 12,13\ 14}$), acoplados a proteína G. Diante das evidências descritas na literatura e a falta de dados correlacionando a sinalização purinérgica com câncer de esôfago, analisamos o papel dos receptores $P2Y_2$ ($P2Y_2R$) e $P2Y_{12}$ ($P2Y_{12}R$) nos processos de proliferação, capacidade de formação de colônias, migração, adesão e atividade enzimática das ectonucleotidases e vias de sinalização envolvidas, após o estímulo com nucleotídeos. Para isso, utilizamos as células Kyse-30 e Kyse-450, representativas de carcinoma de células escamosas, e a linhagem OE-33, representativa de adenocarcinoma. Também, verificamos a expressão do $P2Y_2R$ em biópsias de pacientes com carcinoma de células escamosas e adenocarcinoma, comparadas com tecidos não neoplásicos. Observamos que as amostras de biópsia expressam o receptor $P2Y_2$, porém em diferentes intensidades de marcação. As linhagens celulares expressam $P2Y_2$ e $P2Y_{12}R$, possuem diferentes respostas frente ao estímulo com os nucleotídeos ADP, ATP e UTP, porém o bloqueio desses receptores leva à diminuição da proliferação, formação de população policlonal, adesão e migração. Quanto às vias relacionadas à ação do $P2Y_2R$, verificamos a ativação de ERK1/2 e Akt em diferentes tempos após o estímulo com ATP e UTP. Os dados apresentados neste estudo demonstram que a modulação de receptores purinérgicos $P2Y_2$ e $P2Y_{12}$, pode tornar-se uma ferramenta promissora para alcançar eficácia no tratamento do câncer de esôfago.

Palavras-chave: adenocarcinoma, carcinoma de células escamosas, esôfago, receptores purinérgicos P2Y, nucleotídeos.

ABSTRACT

Esophageal cancer, in general, is diagnosed at an advanced stage, which leads to impairment in the therapies employed, resulting in a high mortality rate. It is classified into two subtypes: adenocarcinoma and squamous cell carcinoma; both differ in histology, etiology and epidemiology. Purinergic signaling uses nucleotides and nucleosides in the extracellular medium as signaling molecules, and has been linked to different types of carcinomas. These molecules bind to G-protein coupled adenosine receptors, characterized as P1 (A1, A2A, A2B and A3), to P2X ionotropic receptors (P2X1-P2X7), and to P2Y receptors ($P2Y_{1, 2, 4, 6, 11, 12, 13\ 14}$), coupled to G protein. Given the evidence described in the literature and the lack of data correlating purinergic signaling with esophageal cancer, we analyzed the role of $P2Y_2$ ($P2Y_2R$) and $P2Y_{12}$ ($P2Y_{12}R$) receptors in the processes of proliferation, colony formation capacity, migration, adhesion, enzymatic activity of the ectonucleotidases and signaling pathways after the nucleotide stimulation. For this, we used the Kyse-30 and Kyse-450 cells, representative of squamous cell carcinoma, and the OE-33 line, representative of adenocarcinoma. Also, we verified the expression of $P2Y_2R$ in biopsies of patients with squamous cell carcinoma and adenocarcinoma, compared to non-neoplastic tissues. We observed that the biopsy specimens express the $P2Y_2$ receptor, but at different labeling intensities. The cell lines expressing $P2Y_2$ and $P2Y_{12}R$, have different responses to the stimulus with the nucleotides ADP, ATP and UTP, but the blockade of these receptors leads to a decrease in proliferation, polyclonal population formation, adhesion and migration. Regarding the pathways related to the action of $P2Y_2R$, we verified the activation of ERK1 / 2 and Akt at different times after the stimulation with ATP and UTP. The data presented in this study demonstrate that the modulation of purinergic receptors $P2Y_2$ and $P2Y_{12}$ may become a promising tool for achieving efficacy in the treatment of esophageal cancer.

Keywords: adenocarcinoma, esophagus, squamous cell carcinoma, purinergic receptors P2Y, nucleotides.

LISTA DE ABREVIATURAS

ADP – Adenosina disfosfato

ADO – Adenosina

AMP – Adenosina monofosfato

ATP – Adenosina trifosfato

cAMP – Monofosfato cíclico de adenosina

CD39 – Ecto-nucleosídeo trifosfato difosfohidrolase

CD73 – Ecto-5'-nucleotidase

CDKN2A – Quinase dependente de ciclina 2A

EAC – Adenocarcinoma de esôfago

EMT – Transição epitélio-mesenquimal

ERK – Quinase reguladora de sinal extracelular

ESCC – Carcinoma de células escamosas de esôfago

GPCR – Receptor acoplado à proteína G

HER2 – Receptor do fator de crescimento epidérmico humano 2

HIF-1α – fator induzido por hipóxia 1α

IL-8 – Interleucina-8

IP3 – Inositol-3-fosfato

MAPKs – Quinases ativadas por mitógeno

MLCK – Miosina quinase de cadeia leve

MMP-9 – Metaloproteinases de matriz-9

mTORC1 – *mammalian target of rapamycin complex-1*

PI3K – Quinase fosfotidilinositol-3

PKC – Proteína quise C

PLC – Fosfolipase C

RB – Proteína associada ao retinoblastoma

RE – retículo endoplasmático

SMAD4 – mediador da transtução de sinal via TGFβ

TGFβ – Fator de transformação do crescimento beta

TNFAIP3 – Gene que codifica proteína 3 induzida por TNF- α

TNF- α – Fator de necrose tumoral alpha

TP53 – gene que codifica para a proteína p53

UDP – Uridina difosfato

UTP – Uridina trifosfato

VE-caderina – Caderina vascular endotelial

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1.1 Câncer de esôfago

O câncer de esôfago divide-se em dois tipos histológicos principais: carcinoma de células escamosas (ESCC) e adenocarcinoma (EAC), classificados de acordo com a capacidade de invasão tumoral (submucosa ou intramucosa), a presença de metástases em linfonodos regionais e a presença de metástases distantes (Ajani *et al.*, 2011). O ESCC desenvolve-se a partir das células escamosas epiteliais que compõem o revestimento interno do esôfago. Danos químicos e físicos recorrentes à mucosa esofágica, favorecem o aparecimento de lesões e aumentam o risco de desenvolver ESCC (Napier *et al.*, 2014). O EAC tem um desenvolvimento mais lento, onde em um primeiro momento o epitélio escamoso esofágico é substituído por mucosa colunar do tipo intestinal, essa transformação já é caracterizada como uma condição pré-neoplásica, e é conhecida como esôfago de Barret (Spechler e Souza, 2014). Esse tipo tumoral surge de um epitélio colunar e o fator de risco que mais tem influência em seu desenvolvimento é o refluxo gastroesofágico (El-Serag *et al.*, 2002; Hvid-Jensen *et al.*, 2011). A Figura 1 representa a progressão das alterações que ocorrem no epitélio esofágico até o aparecimento tumoral (Smyth *et al.*, 2017).

O tipo mais prevalente é o carcinoma de células escamosas, responsável por 90% dos casos deste tipo tumoral no mundo, sua maior incidência ocorre no Oriente, África Oriental e América do Sul (Zhang, 2013). Porém, nos últimos quarenta anos, a incidência de EAC aumentou de forma alarmante em países industrializados ocidentais. O câncer de esôfago possui maior incidência em homens (ESCC: 3,3 homens:1 mulher; EAC: 7 homens:1 mulher) (Arnold *et al.*, 2015) e o número de casos aumenta com a idade. Dados obtidos no Reino Unido, por exemplo, em um levantamento feito pelo *National*

Comprehensive Cancer Network (2016), mostraram que 56% dos casos de câncer de esôfago ocorrem em pacientes com mais de 70 anos.

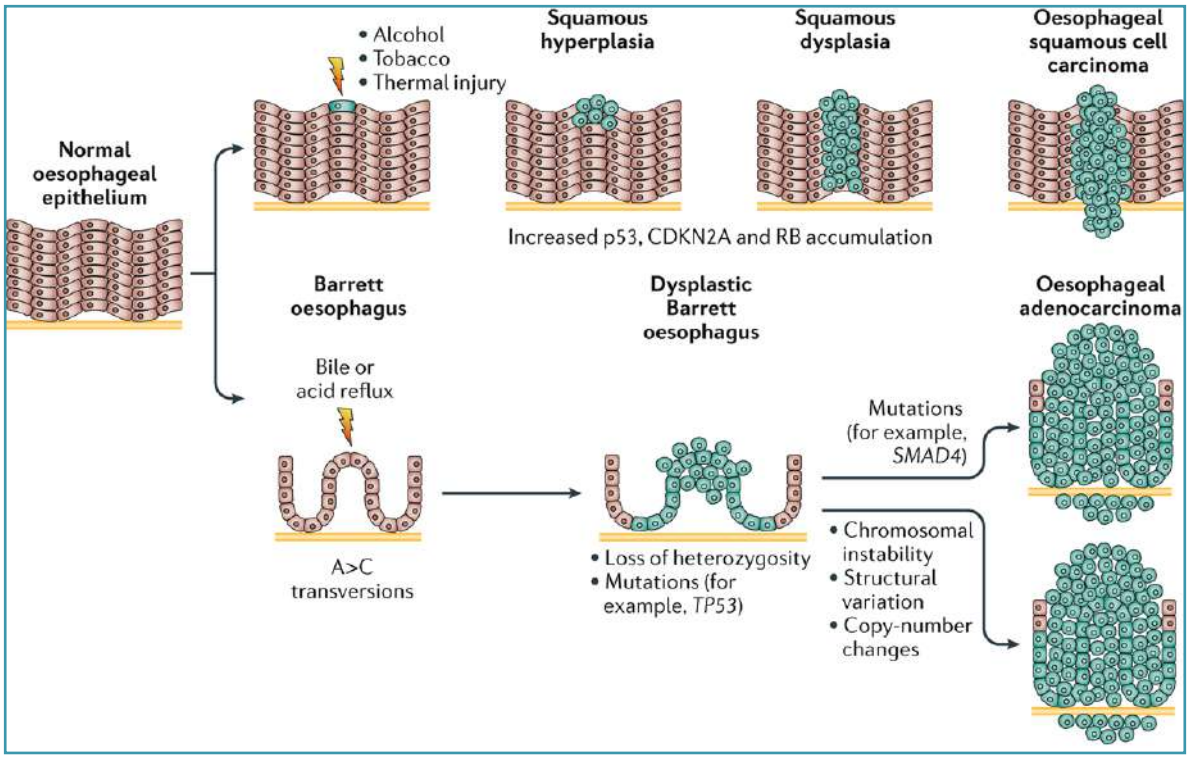

Figura 1: Transformação do epitélio esofágico em ESCC e EAC. A exposição crônica da mucosa esofágica a danos provoca lesões no epitélio. Por fim ocorre o acúmulo de alterações moleculares que favorecem o aparecimento do fenótipo maligno. CDKN2A, inibidor de quinase dependente de ciclina 2A; RB, proteína associada a retinoblastoma. Figura adaptada (Smyth *et al.*, 2017).

A estimativa do Instituto Nacional do Câncer (INCA) para 2018 é de 10.790 novos casos, sendo que a incidência entre os homens se encontra em sexto lugar no Brasil (INCA, 2018). A Tabela 1 apresenta as principais características do ESCC e do EAC. O prognóstico, de maneira geral, não é favorável devido ao fato de a doença ser diagnosticada, na grande maioria, já em estágio avançado e a expectativa de vida para 10 a 20% dos pacientes é em torno de 5 anos na população ocidental (Siegel *et al.*, 2016). Em 2012, estimou-se que 400 mil pessoas foram a óbito anualmente devido a essa patologia em todo o mundo (Torre *et al.*, 2015). Porém, lesões displásicas precursoras podem ser identificadas por simples

endoscopia, e quando isto ocorre, o tratamento local é eficiente, não havendo necessidade de ressecção esofágica extensa nem tratamento oncológico intensivo (Lordick *et al.*, 2016).

Tabela 1: Características do carcinoma de células escamosas e adenocarcinoma de esôfago.

	ESCC	EAC
Fatores de risco	Tabagismo, etilismo, injúria térmica, baixa ingesta de nutrientes, consumo excessivo de carne vermelha e de carne processada.	Refluxo crônico, esôfago de Barret, obesidade visceral.
Localização anatômica	Mais comum no terço superior e médio.	Mais comum no esôfago distal.
Sintomas	Tosse, rouquidão, dor ao deglutir, dor no peito, inchaço de nódulos linfáticos.	Dor epigástrica, dor no peito, vômito e soluço.
Diagnóstico	Endoscopia e análise histopatológica.	Endoscopia e análise histopatológica.
Tratamento	Quimio e radioterapia combinadas, esofagectomia.	Quimioterapia neoadjuvante, cirurgia, quimio e radioterapia combinadas.
Comorbidades associadas	Cirrose hepática, metástases (vias aéreas, sistema digestivo), aterosclerose.	Obesidade, aterosclerose.

Tabela adaptada (Morita *et al.*, 2010; Verschuur e Siersema, 2010; Qu *et al.*, 2013; Smyth *et al.*, 2017).

O desenvolvimento do câncer de esôfago é multifatorial, e envolve o acúmulo de alterações genéticas e epigenéticas, levando à ativação de oncogenes e inativação ou perda de supressores tumorais. Algumas características genéticas diferem entre os dois tipos tumorais, e ainda existem diferenças na frequência de genes alterados dentro do mesmo tipo tumoral. Esse fator está intimamente ligado com a etnia, indivíduos negros possuem diferentes alterações histológicas do que as presentes em indivíduos brancos e apresentam as menores taxas de sobrevivência após a esofagectomia (Greenstein *et al.*, 2008).

Hábitos culturais de regiões específicas como consumo de chimarrão e chás em altas temperaturas e maior ingestão de carne vermelha, na região Sul do Brasil, Argentina e Uruguai, mascar *quid betel* (folha de pimenteira) e alto consumo de carnes processadas no Sul e Sudeste da Ásia, também já foram relacionados com o aumento do risco para desenvolver a doença (Chung *et al.*, 2010; Qu *et al.*, 2013).

A hiperqueratinização do epitélio (*Tylosis*) é uma alteração genética autossômica dominante causada por uma mutação, caracterizada pela hiperqueratose palmar e plantar. Essa mutação gera um risco acumulado de 90% no desenvolvimento de ESCC depois dos 70 anos de idade (Blaydon *et al.*, 2012). A expressão anormal da proteína tumoral p53 já foi encontrada em tecidos adjacentes à displasia ou ao ESCC, níveis aumentados do inibidor de quinase dependente de ciclina 2A (CDKN2A) e da proteína associada ao retinoblastoma (RB) são características acentuadas no ESCC. Essas alterações podem ser detectadas até mesmo nas lesões precursoras e quando esses três componentes (p53, CDKN2A, RB) apresentam-se em níveis elevados, pode-se inferir que está ocorrendo uma progressão da inflamação para o ESCC (Fagundes *et al.*, 2005; Muller *et al.*, 2014; Liu *et al.*, 2017).

A busca por marcadores que possam auxiliar a distinguir alterações displásicas de um tumor já estabelecido é um desafio para os pesquisadores. No entanto, existem dois genes candidatos para ocupar essa função: TNFAIP3 (que codifica a proteína 3 induzida pelo fator de necrose tumoral) e o CHN (que codifica a quimerina 1) aumentam sua expressão durante a transição do tecido normal para displasia e carcinoma (Couch *et al.*, 2016).

Quanto à progressão do esôfago de Barret para o EAC, sugere-se que danos oxidativos ao DNA tenham forte influência nessa transição (Vaninetti *et al.*, 2008). Esse tipo tumoral desenvolve-se em microambiente inflamatório e com baixa diversidade de microbiota (Fels Elliott *et al.*, 2017). Dois mecanismos genéticos foram descritos por contribuir para

essa transição: 1) Perda gradual de genes supressores de tumor CDKN2A e TP53, mutações no SMAD4 e a quebra de enzimas modificadoras da cromatina, tais eventos ocorrem no início da progressão tumoral. 2) Instabilidade cromossômica provocada pela aneuploidia na região cromossômica codificante para TP53, que ocasiona a perda na regulação da p53 (Stachler *et al.*, 2015).

A carga de mutações pontuais encontradas no EAC invasivo é muito elevada, ficando atrás apenas do câncer de pulmão e melanoma (Weaver *et al.*, 2014). No entanto, alterações estruturais também possuem grande relevância (amplificações e exclusões) na prática clínica. A amplificação de maior interesse ocorre em genes que codificam receptores tirosina quinase, como o HER2 (receptor do fator de crescimento epidérmico humano 2), reguladores do ciclo celular e fatores de transcrição (Dulak *et al.*, 2012; Dulak *et al.*, 2013). Essas alterações quando acumuladas, estão associadas a resistência às terapias empregadas (Secrier *et al.*, 2016).

A principal maneira de prevenir o ESCC é evitando o tabagismo, o consumo excessivo de álcool e de bebidas em temperaturas acima de 70 °C , já a prevenção do EAC encontra-se relacionada a adquirir hábitos saudáveis de alimentação (maior consumo de frutas e vegetais) e a prática de exercícios para evitar a obesidade (Murphy *et al.*, 2017).

O principal tratamento empregado nos casos de pacientes com tumores em estadiamento avançado e metastáticos é a ressecção cirúrgica. E quando esta não é possível, são aplicados tratamentos paliativos na tentativa de melhorar a qualidade de sobrevida dos pacientes, contudo, nessas situações a taxa de sobrevida é menor que um ano (Verschuur e Siersema, 2010; Backemar *et al.*, 2016). A quimioterapia combinada com radioterapia tem sido usada tanto como tratamento neoadjuvante quanto adjuvante e tem se mostrado eficaz no aumento do tempo de sobrevida dos pacientes (Maluf *et al.*, 2002; Van Hagen *et al.*, 2012). A combinação dos tratamentos usuais com imunoterapia

tem levado a uma melhora na sobrevida, no entanto, não se mostra suficiente para alcançar a cura desses pacientes a longo prazo (Goode e Smyth, 2016). Desse modo, investir em programas de prevenção, diagnóstico precoce e a descoberta de novos alvos terapêuticos e novas terapias são fundamentais para alcançar o aumento das taxas de sobrevida dos pacientes acometidos pelo câncer de esôfago.

1.2 Sistema Purinérgico

A sinalização purinérgica utiliza nucleotídeos de adenina como principais moléculas sinalizadoras no meio extracelular (Burnstock, 1972). As ectonucleotidases são as enzimas responsáveis por regular os níveis de nucleotídeos presentes no meio extracelular através de sua hidrólise em seus respectivos nucleosídeos (Robson *et al.*, 2006). Tanto o difosfato de adenosina (ADP) quanto o monofosfato de adenosina (AMP) podem resultar da atividade das enzimas CD39 (ecto-nucleosídeo trifosfato difosfohidrolase) que convertem ATP ou ADP em AMP, ou ainda da enzima CD73 (5'-ectonucleotidase) que converte AMP a adenosina (Zimmermann *et al.*, 2012).

Essas moléculas são liberadas e se ligam a receptores purinérgicos. A adenosina irá ligar-se a receptores metabotrópicos denominados como P1. Outra classe de receptores são os receptores P2, divididos em receptores associados a canais iônicos, conhecidos como P2X, ou receptores P2Y que são acoplados a proteínas G os quais variam seus ligantes de acordo com o receptor específico(Burnstock, 2007).

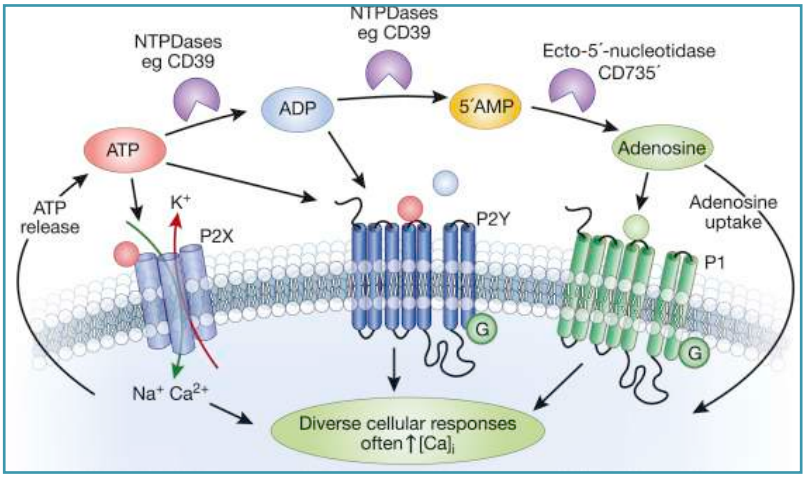

Figura 2: Componentes do sistema purinérgico. Representação dos receptores purinérgicos P1, P2X e P2. Ação das ectonucleotidases (NTPDases) hidrolisando nucleotídeos/nucleosídeos. Agonistas preferenciais de cada receptor. Figura adaptada (Menzies *et al.*, 2017).

Nucleosídeos como a adenosina (ADO) e nucleotídeos (ATP, ADP, UTP, UDP) extracelulares estão envolvidos em inúmeros processos celulares. Possuem a capacidade de estimular ou inibir as células frente a importantes processos como: proliferação, morte, diferenciação, adesão, migração, secreção de mediadores inflamatórios e de fatores de crescimento (Bours *et al.*, 2011; Antonioli, Blandizzi*, et al.*, 2013; Idzko *et al.*, 2014).

O ATP é a molécula sinalizadora do sistema purinérgico com a maior capacidade de se ligar a diferentes receptores P2 (Burnstock, 2004). Uma vez liberado no espaço extracelular, o ATP vai atuar como uma molécula sinalizadora primária controlando diferentes funções, tanto em um senário fisiológico quanto patológico (Verkhratsky e Burnstock, 2014). O ATP pode ser liberado no espaço extracelular de diferentes maneiras, as células saudáveis fazem essa liberação principalmente através de canais permeáveis ou por exocitose em forma de vesículas. Sabe-se que células inflamatórias e tumorais possuem capacidade de liberar grandes quantidades de ATP (Burnstock, 2006a), e isso ocorre via canais de panexina ou conexina. Essa liberação exagerada ocorre principalmente em resposta a estresse mecânico, injúria, isquemia e hipóxia (Burnstock, 2017).

Em condições basais, uma célula possui ATP na concentração de 3-5mM em seu espaço intracelular, e quando liberado, uma concentração mil vezes inferior já é capaz ativar seus receptores no meio extracelular. Assim, uma pequena quantidade liberada é suficiente para desencadear uma resposta celular, sem alteração significativa no estoque

intracelular necessário para geração de energia. Uma vez liberada, essa molécula possui meia-vida de poucos segundos antes de ser hidrolisada pelas ectonucleotidases (Fitz, 2007).

1.2.1 Receptores P1

São receptores acoplados à proteína G (GPCR) e divididos em quatro subtipos: A_1, A_{2A}, A_{2B} e A_3. Apresentam sete domínios transmembrana formados por estruturas em α-hélice compostas por 21 a 28 aminoácidos hidrofóbicos, com a cauda N-terminal voltada para o meio extracelular e a C-terminal para o meio intracelular. Esse grupo de receptores utiliza como agonista a adenosina (metabólito do ATP) (Ralevic e Burnstock, 1998).

Os receptores A_1 e A_3 são acoplados a proteínas $G_{i/o}$, que possuem funções inibitórias sobre a enzima adenilato ciclase, enquanto que os receptores A_{2A} e A_{2B} são acoplados a proteína G_s e ativam a produção de AMPc (adenosina monofosfato cíclico). Os receptores A_1 e A_3 exercem função sobre a enzima fosfolipase C (PLC), estimulando a síntese de inositol-3-fosfato (IP3) (Ralevic e Burnstock, 1998; Abbracchio *et al.*, 2009). Os receptores P1 são amplamente expressos, no entanto, sua afinidade para a molécula de adenosina varia conforme o subtipo. Os receptores A_1 e A_{2A} têm maior afinidade pelo nucleosídeo (Km < 30nm), ao passo que os receptores A_3 e A_{2B} apresentam baixa afinidade (1-20 μM) (Ham e Evans, 2012).

1.2.2 Receptores P2X

Existem sete subtipos de receptores do tipo P2X já descritos ($P2X_{1-7}$) e todos eles são considerados canais catiônicos não-seletivos, quando são ativados pelo ATP formam canais na membrana plasmática das células que permitem a passagem de K^+, Na^+ e Ca^{2+} (Burnstock, 2004). Esses receptores apresentam duas porções transmembrana com uma

alça extracelular e as caudas N-terminal e C-terminal localizam-se na região intracelular. Na alça extracelular localizam-se os sítios de ligação para ATP, antagonistas e moduladores destes receptores e a cauda C-terminal apresenta uma importante função relacionada com a dessensibilização (Khakh, B. S. *et al.*, 1999).

Os receptores P2X são expressos principalmente no sistema nervoso, células de músculo liso, ao passo que os receptores P2Y estão presentes em uma ampla variedade de tecidos, entre eles o sistema gastrointestinal e epitelial (Dubyak e El-Moatassim, 1993; Roman e Fitz, 1999). A Tabela 2 mostra os subtipos de receptores P2, seus respectivos ligantes e o principal mecanismo de transdução de sinal.

1.2.3. Receptores P2Y

Já foram descritos oito subtipos de receptores da família P2Y ($P2Y_{1,2,4,6,11,12,13,14}$). Por serem acoplados à proteína G da mesma maneira que os receptores P1, os receptores P2Y apresentam sete domínios transmembrana, com a cauda N-terminal voltada para o meio extracelular e a C-terminal voltada para o meio intracelular (Burnstock, 2006b). Os P2YR podem ativar a fosfolipase C (PLC), o que leva à liberação de cálcio no meio intracelular ou ainda afetar a adenilato ciclase ocasionando em alteração nos níveis de cAMP (Burnstock, 2004).

Quanto à ligação destes receptores à proteína-G, eles são subdivididos em dois grupos: $P2Y_{1,2,4,6,11}$ e $P2Y_{12,13,14}$. O primeiro grupo apresenta-se acoplado à proteína-G_q/G_{11}, e regula a atividade da PLC, controlando assim a liberação de Ca^{2+} a partir do retículo endoplasmático, mediada por IP3. O segundo grupo modula a ação de canais iônicos e inibe a ação da enzima adenilato ciclase via proteína-$G_{i/o}$ (Burnstock, 2006b; Verkhratsky *et al.*, 2009).

É importante citar que a exposição continuada dos GPCRs aos seus agonistas pode

iniciar um processo de dessensibilização, o que diminui a sua atividade e leva à internalização ou a endocitose (Erb e Weisman, 2012). Esse receptor pode tornar-se ativo novamente, e sabe-se que a cinética entre dessensibilização e recuperação da atividade do receptor é necessária para ativar a expressão de genes relacionados com o crescimento e desenvolvimento celular (Ng *et al.*, 2012).

A dessensibilização dos GPCRs é geralmente mediada pela família de quinases GRK, que vão fosforilar resíduos de serina/treonina presentes nos domínios intracelulares do receptor, e em alguns casos podem promover a ligação de β-arrestinas. As β-arrestinas possuem capacidade de regulação de funções como a dessensibilização e endocitose do receptor e ainda podem interagir com MAPKs (quinases ativadas por mitógenos) e interferir em vias se sinalização intracelular (Shenoy e Lefkowitz, 2011).

Nos últimos anos, muitos grupos de pesquisa têm voltado sua atenção para a sinalização purinérgica e seu papel na promoção e progressão tumoral. Pelo fato de serem expressos em diversos órgãos e atuarem de diferentes maneiras em cada localização, desenvolver terapias utilizando-se desses receptores torna-se um desafio.

Tabela 2: Agonistas e mecanismo de ação dos receptores P2.

Receptor P2	Agonista natural	Mecanismo de transdução de sinal
P2X1-P2X6	ATP	Canais seletivos: Na^{+}, K^{+}, Ca^{+2}.
P2X7	ATP	Canais seletivos: Na^{+}, K^{+}, Ca^{+2} e formação de poro permeável de solutos (até 900 Da).
$P2Y_1$	ADP	$G_{q/11}$ (aumento de IP3/DAG).
$P2Y_2$	ATP e UTP	$G_{q/11}$ (aumento de IP3/DAG).
$P2Y_4$	UTP	$G_{q/11}$ (aumento de IP3/DAG) e G_i (inibição da síntese de cAMP).
$P2Y_6$	UDP	$G_{q/11}$ (aumento de IP3/DAG).
$P2Y_{11}$	ATP	$G_{q/11}$ (aumento de IP3/DAG) e G_s (aumento de cAMP).
$P2Y_{12}$	ADP	G_i (inibição da síntese de cAMP).
$P2Y_{13}$	ADP	G_i (inibição da síntese de cAMP).
$P2Y_{14}$	UDP-glicose	G_i (inibição da síntese de cAMP).

IP3: inositol trifosfato; DAG: diacilglicerol; cAMP: adenosina 3',5'-monofosfato cíclico. Tabela adaptada (Di Virgilio e Adinolfi, 2017).

1.3 Vias de Sinalização Relacionadas à Ativação de $P2Y_2R$ e $P2Y_{12}R$

As principais vias de sinalização envolvidas com a ativação do receptor $P2Y_2$, que é foco deste estudo, são através da modulação de PLC β e IP3 que irão ocasionar mudanças na concentração de Ca^{+2} intracelular e ativação de vias de fosforilação de proteínas quinases ativadas por mitógeno (MAPKs) (Burnstock, 2004; Von Kugelgen e Harden, 2011; Burnstock, 2014).

O Ca^{+2} presente no retículo endoplasmático (RE) atua como segundo mensageiro, e possui capacidade de regular diferentes funções celulares, incluindo a proliferação, adesão, migração e sobrevivência (Erb e Weisman, 2012; Tsai e Meyer, 2012). Isso irá acontecer como resposta a estímulos físicos, químicos e biológicos provenientes do meio (Wei *et al.*, 2012; Melchionda *et al.*, 2016).

Medidas da concentração de Ca^{+2} intracelular realizadas em diferentes tipos celulares (normais e tumorais), demonstram que os nucleotídeos têm capacidade de aumentar de forma transiente a concentração de Ca^{+2} intracelular. Os eventos de sinalização via liberação de Ca^{+2} vão impactar em funções mitocondriais através do estímulo para a produção de ATP ou através de liberação de citocromo c e outros fatores proapoptóticos que irão culminar em morte celular (Rong e Distelhorst, 2008). O $P2Y_2R$ quando estimulado por ATP e UTP possui a capacidade de elevação dos níveis de Ca^{+2} intracelular, e como resultado há fosforilação da via ERK1/2 resultando em aumento na migração celular (Chen *et al.*, 2004; Chadet *et al.*, 2014).

Em hepatócitos, apenas ATP e o UTP tiveram capacidade de regular o metabolismo do glicogênio e a proliferação celular, através da modulação de receptores P2Y com consequente aumento dos níveis intracelulares de Ca^{+2} e ativação de ERK1/2 (Dixon *et al.*, 2005). Em células HeLa a ativação do $P2Y_2R$ leva a proliferação celular, resultante da atuação conjunta das vias ERK1/2 e PI3K em induzir a expressão da proteína c-Fos a qual atua como um proto-oncogene (Muscella *et al.*, 2003). A sinalização mediada pelo Ca^{+2} citosólico também pode estimular a atividade de AKT, resultando em acúmulo nuclear de β-catenina, a qual pode induzir a tumorigênese (Chai *et al.*, 2015).

A ativação do $P2Y_2R$ pelo ATP dá suporte às células tumorais para a transição epitélio-mesenquimal (EMT), onde as células inicialmente epiteliais mudam de fenótipo para facilitar a migração e invasão para outros tecidos, e as vias ERK1/2 e proteína quinase C (PKC) ativadas pelo ATP irão contribuir para esse processo de invasão tumoral (Chadet *et al.*, 2014; Eun *et al.*, 2015). Em câncer de próstata os nucleotídeos ATP e UTP foram capazes de ativar o $P2Y_2R$ juntamente com o receptor de crescimento epidérmico (EGFR), esses dois receptores atuaram em conjunto promovendo a proliferação, migração e invasão tumoral *in vitro* e *in vivo* via ativação de ERK1/2 (Li *et al.*, 2013; Li *et al.*, 2015).

De maneira interessante, o $P2Y_2R$ pode interagir tanto com a β-arrestina1 quanto com a β-arrestina2, e isso irá depender de qual nucleotídeo está ativando o receptor. O ATP possui forte interação com a β-arrestina1 e fraca interação com β-arrestina2, ao passo que quando o agonista é o UTP, este tem forte interação com ambas (Hoffmann *et al.*, 2008). Essa diferença de interação com as β-arrestinas, suporta a ideia de que a quantidade necessária de ATP para que haja dessensibilização do $P2Y_2R$ seja dez vezes maior do que a de UTP (Velazquez *et al.*, 2000).

Com relação ao outro receptor que também é estudado nesta tese, a ativação do $P2Y_{12}R$ vai inibir a ação da adenilato ciclase e a geração de AMP cíclico (Burnstock, 2004; Von Kugelgen e Harden, 2011; Burnstock, 2014). Lee e colaboradores (2012), identificaram em células microgliais que o recrutamento de β-arrestina é necessário para que ocorra a ativação de ERK1/2 após o estímulo com ADP no $P2Y_{12}R$, e os eventos posteriores a essa ativação irão favorecer a adesão celular.

De fato, o mecanismo de sinalização via $P2Y_{12}$ é amplamente estudado em plaquetas, e por isso melhor elucidado nestas células (Guidetti *et al.*, 2008; Wu *et al.*, 2010). A ativação do $P2Y_{12}R$ contribui para a estabilização de eventos trombóticos de forma dependente da fosforilação das vias PI3K/Akt (Garcia *et al.*, 2010). Do mesmo modo, por estar acoplado à proteína G inibitória, a ativação do $P2Y_{12}R$ leva a uma regulação positiva de outras vias de sinalização intracelular como a ERK1/2, miosina quinase de cadeia leve (MLCK) e quinases da família Src (Leon *et al.*, 2003). A MLCK encontra-se envolvida com a produção da força necessária para que ocorra o movimento de migração, regulando de forma negativa o movimento migratório celular através da manutenção da tensão da membrana (Totsukawa *et al.*, 2004; Chen *et al.*, 2014).

Outros estudos também sugerem que a ativação do $P2Y_{12}R$ nas plaquetas através do ADP, ocorre de maneira independente de PI3K/Akt. Pelo fato da rapamicina inibir a via

mTORC1 (*mammalian target of rapamycin complex-1*) e essa inibição resultar em retração do coágulo, estabilidade do trombo e diminuir o espalhamento das plaquetas (Aslan *et al.*, 2011). Acredita-se que a trombina (presente no interior das plaquetas e nos tecidos) ativa a via mTORC1 nas plaquetas através da liberação de ADP que irá ativar $P2Y_{12}R$ mediado pela ativação de PKC (Moore *et al.*, 2014).

Ainda em plaquetas, foi verificado que após o estímulo do $P2Y_{12}R$ por ADP há ativação de ERK1/2 induzida por trombina, sendo essencial para a produção de tromboxano A2. Porém, isso não ocorre quando o $P2Y_1R$ é ativado pelo ADP (Shankar *et al.*, 2006). A figura 3 resume as principais vias de sinalização elucidadas na literatura após a ativação dos receptores $P2Y_1$, $P2Y_2$, $P2Y_{12}$.

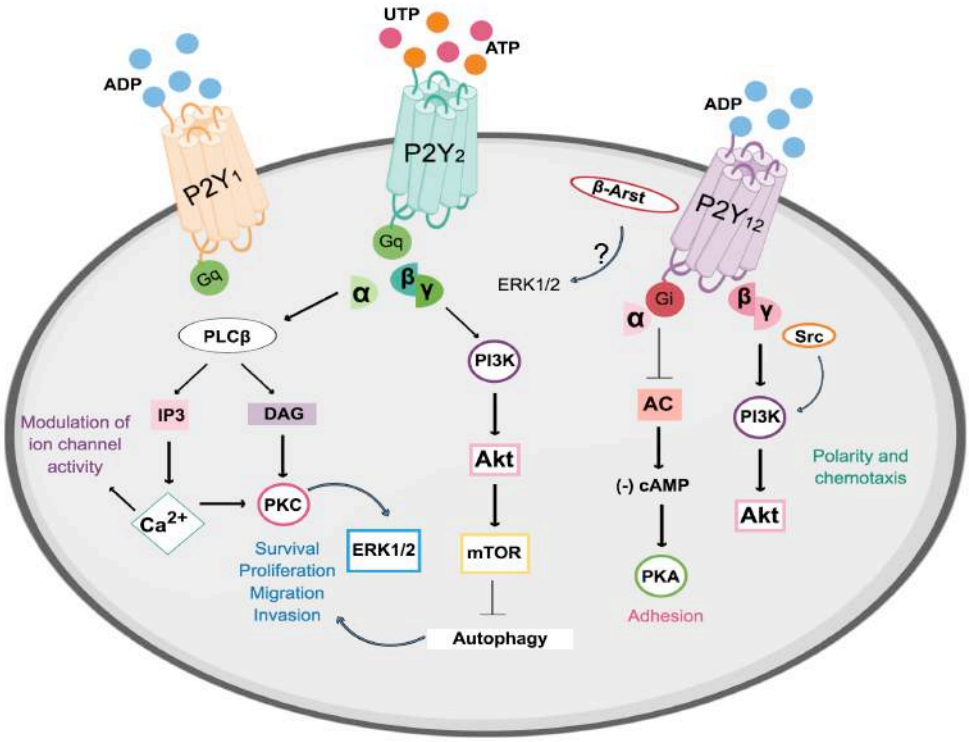

Figura 3: Sinalização mediada pela ativação de $P2Y_1R$, $P2Y_2R$, $P2Y_{12}R$. Sabe-se que a proteína G é composta de 3 subunidades (αβγ), que são dissociadas com a sua ativação e irão desencadear diferentes respostas. Os receptores $P2Y_1$ e $P2Y_2$ estão acoplados à proteína Gq, e por isso compartilham as vias de sinalização desencadeada pela ligação de seus agonistas. O $P2Y_{12}R$ por estar acoplado a proteína Gi, atua principalmente através da inibição da adenilato ciclase, inibindo cAMP. O mecanismo pelo qual a ERK1/2 exerce suas funções após a ativação do $P2Y_{12}R$ ainda não está bem elucidado, mas sabe-se que β-arrestinas (β-Arst) quando recrutadas, podem levar a ativação de ERK1/2. As vias de sinalização ativadas por esses receptores vão desempenhar funções que atribuirão às células capacidade de proliferação, migração, adesão e sobrevivência. **Fonte:** ilustração original baseada em revisão bibliográfica.

1.4 Sistema Purinérgico e Câncer

O microambiente tumoral é um sítio de produção e liberação de ATP extracelular e de conversão desta molécula em adenosina, essas reações geram um meio rico para fatores promotores de crescimento e imunomodulatórios (Wilhelm *et al.*, 2010). A sinalização purinérgica está envolvida no desenvolvimento de vários tipos de cânceres, entre eles: melanoma, carcinoma epitelial intestinal, mama e gliomas (Morrone *et al.*, 2003; Coutinho-Silva *et al.*, 2005; White, Butler*, et al.*, 2005; Gehring *et al.*, 2012; Sarangi *et al.*, 2013). O ATP é uma molécula abundante presente no microambiente tumoral e pode agir promovendo o crescimento tumoral ou pode ter função antineoplásica (Rapaport e Fontaine, 1989; Spychala, 2000). Além disso, há mais de uma década, sabe-se que diferentes receptores purinérgicos podem regular funções celulares como proliferação, diferenciação e apoptose (White, Ryten*, et al.*, 2005).

Burnstock (2013), em um apanhado geral de estudos já publicados, propôs que os receptores $P2Y_1$ e $P2Y_2$ encontram-se principalmente envolvidos com eventos de proliferação celular, através da alteração dos níveis intracelulares de cAMP, através da modulação da adenilato cyclase, ou pelo aumento dos níveis intracelulares de cálcio via PLC. O sistema purinérgico exerce uma função no crescimento de alguns tipos de tumores, evidenciado através da indução de proliferação de gliomas por ATP *in vitro* (Morrone *et al.*, 2003), e extremamente baixa atividade das ectoenzimas em gliomas quando comparadas aos astrócitos (Wink *et al.*, 2003). Estudos *in vivo* mostraram que a expressão de ectoenzimas que degradam o ATP altera o crescimento tumoral dependendo do produto de degradação desta molécula (Morrone *et al.*, 2006).

Os P2YR estão amplamente distribuídos pelos tecidos e estão relacionados com a regulação de sistemas como: esquelético, imune, cardiovascular, pulmonar, renal, digestivo, endócrino e nervoso (Abbracchio *et al.*, 2009). Muitos estudos têm

demonstrado que os P2YR estão superexpressos em tecidos tumorais de diferentes órgãos (Tabela 3).

Tabela 3: Expressão dos P2YR em diferentes tipos tumorais.

Tipo tumoral	P2YR relacionado
Melanoma	1, 2, 4 e 6
Colorretal	1, 2, 4, 6, 12
Esôfago	2
Pulmão	2 e 6
Próstata	1, 2, 6 e 11
Cérebro	1 e 12
Mama	2, 12
Ovário	2
Endometrial	2
Tireoide	1, 2
Malignidades hematológicas	11

Tabela adaptada (Burnstock e Di Virgilio, 2013).

É possível observar que o $P2Y_2R$ é mais frequentemente expresso em diferentes tipos tumorais. O ATP e UTP são os ligantes fisiológicos que ativam os $P2Y_2R$, sendo este o único receptor que aceita tanto adenina quanto uracila como nucleobase no agonista 5'-trifosfato. No entanto, sob altas concentrações podem haver ligações cruzadas utilizando-se de outro nucleotídeo, o UDP-glicose como via de ativação, sendo este um agonista total para $P2Y_2R$ (Ko *et al.*, 2008).

2. JUSTIFICATIVA

O carcinoma de esôfago apresenta-se como uma doença de alta morbidade e letalidade. Por não apresentar sinais iniciais há um prejuízo no diagnóstico, e a confirmação da doença acontece já em estado avançado. Dessa forma, há um comprometimento da qualidade de vida e do tratamento desses pacientes e novas terapias com o intuito de melhorar o prognóstico precisam ser desenvolvidas. Além disso, segundo levantamento feito pelo INCA em 2018, a região Sul do Brasil é onde há maior incidência dessa patologia.

Nucleotídeos são moléculas importantes que regulam muitas funções patofisiológicas no espaço extracelular, através da ativação de receptores purinérgicos (Burnstock 2011). Estudos realizados pelo nosso grupo de pesquisa têm revelado evidências de uma relação entre a sinalização purinérgica em diferentes neoplasias (Morrone et al. 2003; Morrone et al. 2005; Morrone et al. 2006; Stella et al. 2010; Gehring et al. 2012). Um trabalho recentemente publicado pelo nosso grupo demonstrou o envolvimento do receptor P2X7 na redução da viabilidade mitocondrial e taxa de migração de linhagens celulares de esôfago tratadas com altas concentrações de ATP. Essas células também apresentaram elevada atividade da enzima responsável pela hidrólise de ATP (Santos, A. A., Jr. *et al.*, 2017).

Desse modo, devido às diferentes formas que os receptores purinérgicos podem influenciar em uma grande variedade de tumores, e a escassa literatura sobre a atuação do P2YR em um tipo de câncer com alta taxa de mortalidade como o de esôfago, vimos a oportunidade de compreender o papel principalmente que o $P2Y_2R$ exerce nesta neoplasia. No entanto, devido ao fato das plaquetas estarem fortemente relacionadas com a formação do microambiente tumoral e na formação de metástases, o fato do $P2Y_{12}R$ possuir importante papel na agregação plaquetária, e já existir um fármaco disponível no

mercado como o bissulfato de clopidogrel (antagonista de $P2Y_{12}R$), também consideramos válida a investigação da modulação deste receptor nas células de câncer de esôfago.

É de suma importância que entendamos a relação entre as vias que levam ao desenvolvimento deste tipo específico de câncer para que novas abordagens terapêuticas possam ser definidas com o intuito de melhorar a expectativa e a qualidade de vida da população acometida por esta doença. A partir de uma melhor compreensão dos mecanismos e fatores associados também se torna possível alertar a população em geral sobre as medidas preventivas que podem ser tomadas, afim de diminuir a incidência desta patologia.

3. OBJETIVOS

Investigar o papel dos receptores purinérgicos $P2Y_2$ e $P2Y_{12}$ na proliferação e migração do carcinoma de células escamosas e adenocarcinoma de esôfago.

3.1 Objetivos específicos

a) Verificar a expressão do $P2Y_2R$ em biópsias de pacientes câncer de esôfago e em amostras de pacientes sem a neoplasia.
b) Identificar a expressão dos receptores purinérgicos P2Y2 em linhagens celulares humanas de câncer de esôfago (câncer de células escamosas: Kyse-30 e Kyse-450; e adenocarcinoma: OE-33).
c) Determinar o envolvimento dos receptores $P2Y_2$ com os eventos de proliferação, formação de colônias, migração, e adesão celular, utilizando a modulação farmacológica por meio de antagonistas e agonista específicos.
d) Investigar as vias de fosforilação (Erk/Akt) pelas quais os receptores $P2Y_2$ exercem sua função.
e) Identificar a expressão dos receptores purinérgicos $P2Y_{12}$ em linhagens celulares humanas de câncer de esôfago (Kyse-30 e Kyse-450).
f) Determinar o envolvimento dos receptores $P2Y_{12}$ com os eventos de proliferação e autofagia em linhagens celulares humanas de câncer de esôfago, utilizando modulação farmacológica de antagonista e agonista específicos.
g) Avaliar a atividade ATPásica e ADPásica em linhagens celulares humanas de câncer de esôfago.

4. RESULTADOS

4.1 CAPÍTULO I

Artigo de revisão submetido à *Current Medicinal Chemistry*.

P2 Purinergic Receptors Involvement in Esophageal Diseases

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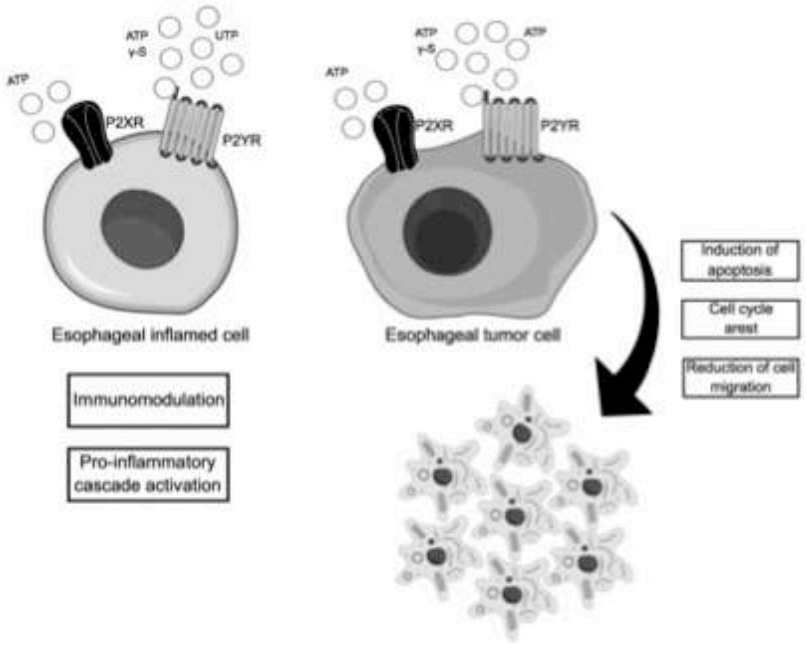
ABSTRACT

The signaling of nucleotides and nucleosides was widely investigated during the last decades in several conditions of distinct organs. It is already known that purinergic receptors expression exists in healthy tissues, and in most cases, there is an increase of the expression in pathological conditions. However, in relation to the esophagus and its pathologies, the purinergic context is poorly understood. In this review, we compiled information reported so far regarding the involvement of P2 purinergic receptors in esophageal diseases such as gastroesophageal reflux disease and esophageal carcinoma. Mainly, we discuss the effects of pharmacological modulation and characterization of these receptors in different esophageal cell lines and its patterns of expression in esophageal tissue samples, both in healthy and altered tissues. We propose that the study of the role of P2 purinergic receptors and their mechanisms of action may help to explore their use as pharmacological targets in the future.

Keywords: P2 Purinergic Receptors; P2XR; P2YR; Extracellular Nucleotides; Gastroesophageal Reflux Disease; Esophageal Carcinoma; Adenocarcinoma.

Graphical Abstract



Esophageal inflamed and esophageal tumor cells express the P2X ion channel coupled receptors and P2Y G-protein coupled receptors. Extracellular nucleotides may act activating these receptors that are able to trigger several cellular responses. Cell responses depend on the cell type and the specific receptor activated.

1. INTRODUCTION

Purinergic signaling was elucidated for de first time in 1972, with identification of membrane receptors that responds to purine nucleosides and nucleotides (Burnstock, 1972). Later, these receptors were identified as P1 receptors (A_1, A_{2A}, A_{2B} and A_3) (Fredholm, Bertil B *et al.*, 2001) and P2 receptors(Abbracchio e Burnstock, 1994). Additionally, P2 receptors were divided in two families: P2X ionotropic receptors (P2X1-7R) and P2Y G protein-coupled receptors ($P2Y_{1,2,4,6,11,12,13,14}R_)$ (Abbracchio e Burnstock, 1994; Burnstock, 2004). Adenosine binds to P1 receptors; ATP activates P2XR, while P2YR differ on ligands preference according to each receptor subtype, with ATP and its metabolites as natural ligands: ATP ($P2Y_2$ and $P2Y_{11}$), ADP ($P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$), UDP ($P2Y_6$ and $P2Y_{14}$), UTP ($P2Y_2$ and $P2Y_4$), while UDP-glucose and other nucleotides sugars activate $P2Y_{14}$ (Franke *et al.*, 2012; Jacobson *et al.*, 2015).

The P2XR are considered as non-selective cation channels. When they are activated by ATP they form channels on the plasma membrane of the cells that allow the passage of K^+, Na^+ and Ca^{2+} (Burnstock, 2004). Besides presenting two transmembrane portions with an extracellular loop, these receptors have N-terminal and C-terminal tails located in the intracellular region. In the extracellular loop, the binding sites for ATP, antagonists and modulators are located, whereas the C-terminal tail has an important function related to receptor desensitization (Khakh, Baljit S. *et al.*, 1999; Burnstock, 2004).

Unlike the structure of P2XR, P2YR have seven transmembrane domains, with the N-terminal tail facing the extracellular medium, while the C-terminal towards the intracellular medium. P2YR can activate phospholipase C (PLC), which leads to the release of calcium in the intracellular environment or even affect adenylate cyclase causing changes in cAMP levels (Burnstock, 2004; 2006a). As for the coupling of these receptors to the G-protein, they are subdivided into two groups: $P2Y_{1,2,4,6}$ and $P2Y_{12,13,14}$. The first group is coupled to the $G_{q/11}$ protein, and regulates the activity of the PLC, thus controlling the release of Ca^{2+} from the endoplasmic reticulum, mediated by inositol. The second group modulates the action of ion channels and inhibits the action of the adenylate cyclase enzyme via protein $G_{i/0}$ (Burnstock, 2004; 2006b; Verkhratsky *et al.*, 2009) (Figure 1).

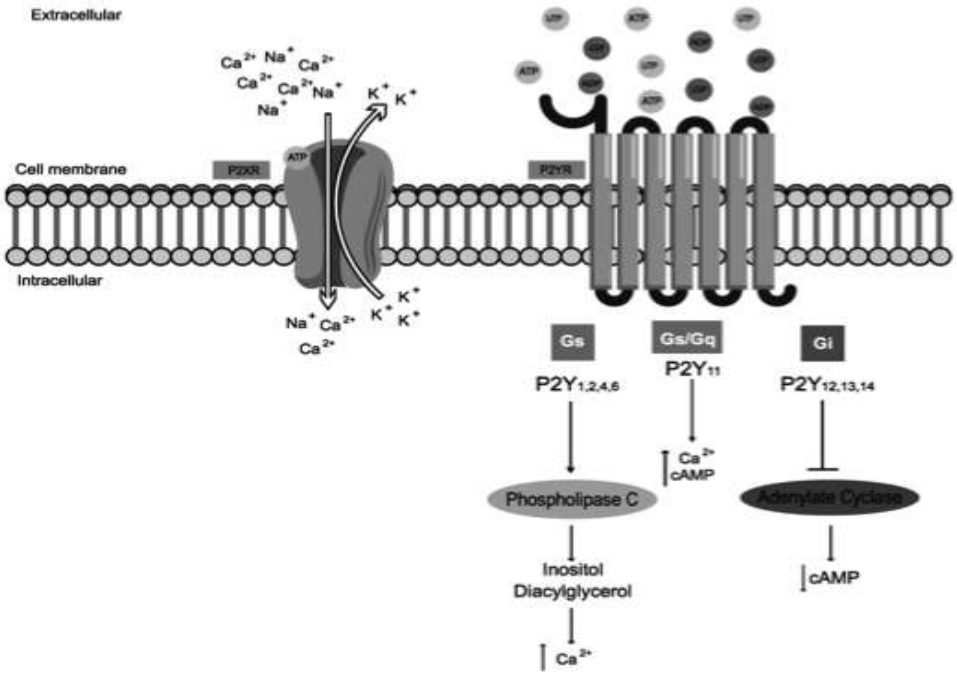


Figure. 1: Representation of P2R mechanisms of action. The P2XR are ionotropic receptors that when activated by ATP allow the transit of cations such as K+, Na+ and Ca2+ between the extra and intracellular media. The effects promoted by this activation are mediated by the concentration of these ions. The P2YR are activated by ADP, ATP, UDP and/or UTP, depending on each receptor subtype. They are metabotropic receptors and can be coupled to different G proteins. P2YR coupled to the Gs protein ($P2Y1_{,2,4,6,11}$) activate phospholipase C, which through inositol increases intracellular Ca2+ levels. The P2Y11 receptor can also couple to the Gq protein, and by stimulating the activity of the adenylate cyclase enzyme, increases the levels of the second messenger cAMP. In contrast, receptors coupled to Gi protein ($P2Y_{12,13,14)}$ inhibit the adenylate cyclase enzyme and there is a consequent decrease in cAMP levels.

The ATP molecule reaches the extracellular space through its release by healthy cells (epithelial cells, endothelial cells, platelets, mast cells and terminal nerves) and by apoptotic, necrotic and tumor cells (Burnstock e Di Virgilio, 2013; Cekic e Linden, 2016). This release occurs via pannexin 1 channels, conexins, secretory granules, ABC transporters, exocytosis (Chekeni *et al.*, 2010; Lazarowski, 2012), and even through a large pore formed on the plasma membrane by P2X7R (Volonte *et al.*, 2012). Outside of the cell, ATP becomes a messenger molecule (Di Virgilio e Adinolfi, 2017), promoting different responses according to their binding to specific receptors present on the cell surface membrane (Zimmermann, 2016).

ATP and the other nucleotides signaling are controlled by ectoenzymes capable of metabolizing ATP to adenosine. Initially, CD39 (ectonucleoside triphosphate diphosphohydrolase 1, E-NTPDase1) converts ATP and ADP into AMP, which is dephosphorylated into adenosine by CD73 (ecto-5'-nucleotidase, Ecto5´NTase) (Antonioli, Pacher*, et al.*, 2013; Kanthi, Y. M. *et al.*, 2014). Later, adenosine can be metabolized to inosine by

adenosine deaminase (ADA); recaptured into the intracellular space by the cell membrane nucleoside transporters named Equilibrative Nucleoside Transporters (ENTs) and Concentrative Nucleoside Transporters (CNTs) (Antonioli, Blandizzi*, et al.*, 2013); or can be phosphorylated into AMP by adenosine kinase (Baldwin *et al.*, 2004; Gray *et al.*, 2004). Each receptor differs in the amount of agonist required for its activation, for example P2X7R requires higher levels of ATP for its activation than other receptors that are activated with physiologic concentrations of natural agonists (Stagg e Smyth, 2010). Healthy and injured tissues differ as to the release of ATP; in inflamed or tumor microenvironment, the level of ATP is higher than the same tissue in physiological condition (Pellegatti *et al.*, 2008). Furthermore, tumor cells may be more sensitive to the effects caused by ATP than normal cells, depending on the expressed P2 receptors on cell surface (Burnstock e Di Virgilio, 2013). Regarding the inflammatory processes, ATP signaling is important, since its release in response to cell damage can culminate in the generation of chemotactic signals, immune cells activation, release of inflammatory mediators and cell death (Jacob *et al.*, 2013). Many authors have demonstrated the interaction of purinergic receptors in different systems; both in health and in pathological processes the receptors seems to have influence (Burnstock, 2014; Burnstock *et al.*, 2014; Cekic e Linden, 2016; Pedata *et al.*, 2016; Magni *et al.*, 2017). The majority of these studies show that purinergic receptors have potential to be pharmacological targets. Of note, purinergic signaling and their receptors have been recently related in several types of cancer and there is mounting interest in the role of nucleosides and nucleotides in therapeutic potential for cancer treatment (Burnstock e Di Virgilio, 2013). Burnstock (2013)(Burnstock, 2013) in a wide-ranging review of previous studies, proposed that the activation of $P2Y_1$ and $P2Y_2$ receptors are mainly involved with events of cell proliferation, by altering intracellular levels of cAMP, through modulating adenylate cyclase or by increasing intracellular calcium levels via PLC; while P2X7R is more related to cytotoxic effects (Wang *et al.*, 2004; Fang *et al.*, 2013).

The esophagus is a muscular tube; in adult humans, it measures around 18-25 cm that connects the pharynx to the stomach. Its epithelium varies according to the location, the upper part is composed by striated muscle, the lower part by smooth muscle, and in the middle there is a mixture of striated and smooth muscles (Staller e Kuo, 2013). Different illnesses can affect the esophagus, such as esophagitis, gastroesophageal reflux disease (GERD), Barrett's esophagus (BE), adenocarcinoma and squamous cell carcinoma (di Pietro and Fitzgerald, 2013). Recently, Burnstock *et al*. (2017) (Burnstock *et al.*, 2017) reviewed the role of the purinergic system in several pathologies affecting the gastrointestinal tract, including some occurring in the esophagus, nevertheless, there is still few studies linking the interaction of the purinergic receptors with the development of these diseases.

In this review, we discuss information reported regarding the involvement of P2 purinergic receptors in some esophageal diseases such as GERD and esophageal carcinoma.

2. P2 RECEPTORS AND THE ESOPHAGUS

It has been described that purinergic receptors are broadly distributed in the gastrointestinal tract (Burnstock *et al.*, 2017), and nucleotides and nucleosides can exert effects on the digestive system; therefore, the components of the purinergic system could play a role in the regulation of important functions such as in the salivary and intestinal epithelial secretion, enteric neurotransmission and motility (Lavoie *et al.*, 2011). In addition, some authors have demonstrated that there is expression of different purinergic receptors in the esophagus in both physiological and pathological conditions *in vitro*, and in human and animal model (Maaser *et al.*, 2002; Lecea *et al.*, 2011; Ma *et al.*, 2011; Shieh *et al.*, 2014; Santos, A. A. *et al.*, 2017; Wu *et al.*, 2017).

Wu *et al.* (2015) (Wu *et al.*, 2015) showed that normal epithelial cells of esophagus could release ATP in extracellular space after stimulation with weak acid. In physiological context, the ATP was recognized as a ciliary beat stimulating molecule (Levin *et al.*, 1997) and P2Y receptors have an important contribution in the regulation of esophageal motility (Wan *et al.*, 2016). Wu *et al*. (2017), demonstrated the role of different purinergic receptors and their agonists on IL-8 production in normal esophageal epithelial cells. The authors described that UTP and ATP-γ-S are capable of increasing IL-8 concentrations, and the crucial participation of $P2Y_2R$ was confirmed when the $P2Y_2R$ was blocked and UTP was unable to modulate IL-8 production. Beyond that, it was also suggested that the signaling through ERK activation pathway could be important in this process (Wu *et al.*, 2017).

3. GASTROESOPHAGEAL REFLUX DISEASE (GERD)

Acid regurgitation, heartburn and epigastric pain could be considered indicative symptoms of GERD (Pettit, 2005). It is one of the most common disorders that chronically affects upper gastrointestinal tract, with prevalence being estimated to be 15-20% globally (Keshteli *et al.*, 2017). This condition is described as a complication associated with reflux, when the lower esophageal sphincter is ineffective, allowing the scape of stomach content and chronic exposure of esophageal mucosa, resulting in tissue injury (El-Serag *et al.*, 2002; Vakil *et al.*, 2006).

It is believed that the development of GERD occurs through the following events: (i) death of superficial esophageal cells, (ii) generation of an inflammatory response that, starting in the epithelium, progresses to ulcerations in the submucosa; and (iii) the loss of superficial esophageal cells induce the progenitor cell hyperplasia in the basal layer of the squamous epithelium, which confers the histological characteristics of the disease (Souza *et al.*, 2017).

Multiple risk factors are involved with GERD such as alcohol use, family history, smoking, eating habits (Keshteli *et al.*, 2017), *Helicobacter pylori* infection, obesity, and the use of medications that disturbs esophageal function (Pandolfino *et al.*, 2008). Moreover, in most severe cases, it may progress to BE (Riley *et al.*, 2017), being a condition developed by about 10% of patients diagnosed with GERD (Huang e Yu, 2016). Due to chronic damage caused by the reflux, BE is characterized by a metaplasic change of the native squamous epithelial lining to a columnar epithelium (Tan *et al.*, 2017).

GERD is a risk factor for several diseases which are not directly attached to the esophageal tract whereby laryngitis, reflux asthma syndrome, interstitial lung disease, and otitis media (Wijarnpreecha *et al.*, 2017). However, more importantly, GERD and BE are one of the major risk factors for esophageal adenocarcinoma (EAC) (Rajendra e Sharma, 2017).

Although is well known the intimate role of purinergic signaling in inflammation and cancer (Burnstock *et al.*, 2017) to date, there are no studies investigating any direct form of relation between the purinergic system and BE and EAC. It has been previously described that inflammation caused by esophageal reflux is closely linked to the release of inflammatory cytokines, demonstrating the major involvement of HIF-2α and the participation of Interleukine-8 (Souza *et al.*, 2017). Likewise, different studies suggested that chronic mucosal inflammation associated with GERD is characterized by increase of IL-8 expression, and authors recognized an important function of this cytokine in pathogenesis of GERD (Isomoto *et al.*, 2003).

Using HET-1A cell line, representative of normal human esophageal tissue, it was previously demonstrated that the exposure to pH 5 triggers the release of ATP by these cells (Ma *et al.*, 2011). Ma *et al.* (2012) (Ma *et al.*, 2012) proposed that the ATP released by the cells induce the upregulation of inflammatory mediators, among them IL-8. In addition, by treatment with suramin (P2R antagonist) and ATP-γ-S (P2R agonist), it is possible to infer the involvement of P2R in this inflammatory process and consequent tissue injury.

Purinergic receptors expression were characterized in biopsies samples of patients with GERD, and P2X3R and P2X7R genes expression were significantly elevated in this condition when compared with asymptomatic patients and healthy controls (Shieh *et al.*, 2014). On the other hand, it was reported in the same study, that purinergic receptors $P2Y_{1,2,6,12}$ had similar gene expression among patients with GERD, asymptomatic and healthy ones; so the authors suggest a potential involvement of P2X3R and P2X7R in inflammatory sensitization of human esophagus (Shieh *et al.*, 2014). In this context, it is possible to infer that ATP signaling via P2X receptors may contribute to the inflammatory condition induced by the release of cytokines. It would be of great importance further studies that elucidate the effects of specific agonists and antagonists for those P2X

receptors subtypes on the esophageal cells inflammatory response.

4. ESOPHAGEAL CARCINOMA

Recently, it was estimated that are diagnosed 456,000 new cases of esophageal cancer and that 400,000 deaths occur annually worldwide (Ferlay *et al.*, 2015; Dong e Thrift, 2017). Differing according to their risk factors and demographic distributions, the two main types of esophageal cancer are EAC and esophageal squamous cell carcinoma (ESCC) (Ma *et al.*, 2012; Torre *et al.*, 2015). ESCC occurs in the upper third of esophagus and develops from squamous epithelial cells (Zhang *et al.*, 2012). Although its incidence is decreasing in the same time EAC is rising, the globally incidence of ESCC still higher than EAC (Smyth *et al.*, 2017). It corresponds to more than 90% of esophageal cancer diagnoses and the overall incidence increase with age (Zhang, 2013; Napier *et al.*, 2014). ESCC is a very aggressive illness and the survival rates are low, only 10-20% of patients reach 5-year of survival (Lagergren *et al.*, 2017). The main risk factors comprise alcohol use, tobacco smoking, low consumption of fruit and vegetables and high consumption of hot beverages and red meat (Morita *et al.*, 2010; Napier *et al.*, 2014)(Morita *et al.*, 2010; Napier *et al.*, 2014).

To date, ESCC is the only type of esophageal cancer that has, although few, studies characterizing purinergic receptors role in this tumor progression processes, such as proliferation and cell death. Maaser et al. (2002), using a human oesophageal squamous carcinoma cell line (Kyse-140), demonstrated that ATP and the hydrolysis resistant ATP derivative (ATP-γ-S) treatments can decline cell proliferation in dose-dependent manner and trigger cell cycle arrest in S-phase; equally after 48 h with ATP treatment induced a intensification of caspase-3 activity as well as apoptosis through labeling specific DNA strand breaks, thereby the authors suggest that cell death occurs via $P2Y_2R$ activation.

Likewise, using the TE-13 cell line of human esophageal squamous carcinoma cells, Wang *et al*. (2005) found a inhibitory effect of ATP on cell proliferation after 48 h of treatment, cell cycle delay in S-phase and an increase in apoptosis rate in a dose-dependent manner, then authors propose that final metabolite of ATP degradation, adenosine can also contribute to these results. Furthermore, a recent study revealed that three different human ESCC cell lines (Kyse-30, Kyse-450 and OE-21) displayed P2X3R, P2X4R, P2X5R, P2X6R, P2X7R expression, while P2X1R is expressed by Kyse-30 and Kyse-450 and P2X2 was expressed only by Kyse-450. Biopsies of esophagus reveal positive nuclear staining for P2X7R and staining is higher in ESCC than esophagitis sample. Authors also found that cell lines of ESCC displayed an increase of enzymatic degradation of ATP than ADP similarly the AMP hydrolysis is elevated in Kyse-450 cell line. The treatment with high ATP

concentrations had toxic effects and reduced cell number, viability, migration and clonogenic capacity, suggesting that occurred through activation of the P2X7 receptor (Santos *et al.*, 2017).

Table 1 summarizes the effect of *in vitro* experiments with pharmacologic modulation of P2R in esophageal cells.

Table 1: Effect of P2R pharmacologic modulation in esophageal cells *in vitro*.

Cell lineage	Cell type	P2R involved	Agonists	Effect	Mechanism	Reference
HEECs	Primary human esophageal epithelial cells	$P2Y_2$	ATP-γ-S UTP	Pro-inflammatory cascade activation	ERK	(Wu *et al.*, 2017)
Kyse-140	ESCC	$P2Y_2$	ATP ATP-γ-S	Growth inhibition; cell cycle arrest and induction of apoptosis	DNA strand breaks; Increase Caspase 3	(Maaser *et al.*, 2002)
TE-13	ESCC	Not shown	ATP	Inhibition of cell proliferation, cell cycle arrest; Increase of apoptotic rate	Cell cycle delay in S-phase or G0/G1 phase; Apoptotic events (such as chromatin condensation, fragment nuclei and apoptotic body)	(Wang *et al.*, 2005)
Kyse-30, Kyse-450, OE-21	ESCC	P2X7	ATP	Reduction of cell number, viability, migration and clonogenic capacity	Not shown	(Santos, A. A. *et al.*, 2017)

CONCLUSION

There is a growing interest in researching new pharmacological targets through the modulation of components of the purinergic system for different illnesses (Burnstock, 2017). Purinergic signaling has been extensively studied in several inflammatory comorbidities and in different types of cancer, including the ones that affect the esophageal tract, showing an important role in those conditions. It has been shown by distinct research groups that there is expression of different P2 receptors in esophagus and their activation by nucleotides, mainly by ATP, exert effects in both physiological and pathological settings. In this context, esophageal squamous cell carcinoma is the more elucidated disorder studied, and in all cases different doses of ATP have an important inhibitory effect upon cell cycle, proliferation and migration. Furthermore, regarding inflammatory esophageal diseases, such as GERD, the involvement of P2R in the release of inflammatory cytokines has also been demonstrated. However, important pathologies, which are raising in Western industrialized countries during the last decades, like Barrett's esophagus and adenocarcinoma (Smyth *et al.*, 2017) need to be explored in the purinergic context. Of note, it is essential to elucidate the mechanisms of action by which the activation or blockade of purinergic receptors can exert their effects. It is important to note that researchers cited here used different lineages with different origins for the same pathology, which may explain why the cells respond in a similar way using different concentrations of nucleotides. In this review, we present for the first time, a compilation of a wealth of information relating esophageal diseases and the role of P2 purinergic receptors. Although it is a field with lack of information, it is favored the possibility of new studies of these receptors as a possible pharmacological target in the treatment of these diseases in the future.

List of Abbreviations

ATP = Adenosine Triphosphate

ADP = Adenosine Diphosphate

UDP = Uridine Diphosphate

UDP-glucose = Uridine Diphosphate glucose

UTP = Uridine Triphosphate

PLC = Phospholipase C

cAMP = Cyclic adenosine 3',5'-monophosphate

ADA = Adenosine deaminase

ENTs = Equilibrative nucleoside transporters

CNTs = Concentrative nucleoside transporters

AMP = Adenosine monophosphate

GERD = Gastroesophageal reflux disease

IL-8 = Interleukine 8

ATP-γ-S = Adenosine 5′-O-3-thiotriphosphate

ERK = Extracellular signal-regulated kinase

BE = Barrett's esophagus

EAC = Esophageal adenocarcinoma

HIF-2α = Hypoxia-inducible factor 2 alpha

ESCC = Esophageal squamous cell carcinoma

HEECs = Human esophageal epithelial cells

Conflict of Interest

The authors are responsible for the content, writing of the paper and are all in accordance with the publication. The authors declare no conflict of interest regarding this manuscript.

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REFERENCES

[1] Burnstock, G., Purinergic nerves. *Pharmacological reviews,* **1972**, *24*, (3), 509-581.

[2] Fredholm, B.B.; IJzerman, A.P.; Jacobson, K.A.; Klotz, K.-N.; Linden, J., International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacological reviews,* **2001**, *53*, (4), 527-552.

[3] Abbracchio, M.P.; Burnstock, G., Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacology & therapeutics,* **1994**, *64*, (3), 445-475.

[4] Burnstock, G., Introduction: P2 receptors. *Current topics in medicinal chemistry,* **2004**, *4*, (8), 793-803.

[5] Franke, H.; Verkhratsky, A.; Burnstock, G.; Illes, P., Pathophysiology of astroglial purinergic signalling. *Purinergic signalling,* **2012**, *8*, (3), 629-657.

[6] Jacobson, K.A.; Paoletta, S.; Katritch, V.; Wu, B.; Gao, Z.-G.; Zhao, Q.; Stevens, R.C.; Kiselev, E., Nucleotides Acting at P2Y Receptors: Connecting Structure and Function. *Molecular Pharmacology,* **2015**, *88*, (2), 220-230.

[7] Khakh, B.S.; Bao, X.R.; Labarca, C.; Lester, H.A., Neuronal P2X transmitter-gated cation channels change their ion selectivity in seconds. *Nature neuroscience,* **1999**, *2*, 322.

[8] Burnstock, G., Pathophysiology and therapeutic potential of purinergic signaling. *Pharmacological reviews,* **2006**, *58*, (1), 58-86.

[9] Burnstock, G., Purinergic P2 receptors as targets for novel analgesics. *Pharmacology & therapeutics,* **2006**, *110*, (3), 433-454.

[10] Verkhratsky, A.; Krishtal, O.A.; Burnstock, G., Purinoceptors on neuroglia. *Molecular neurobiology,* **2009**, *39*, (3), 190-208.

[11] Burnstock, G.; Di Virgilio, F., Purinergic signalling and cancer. *Purinergic signalling,* **2013**, *9*, (4), 491-540.

[12] Cekic, C.; Linden, J., Purinergic regulation of the immune system. *Nature reviews. Immunology,* **2016**, *16*, (3), 177-192.

[13] Chekeni, F.B.; Elliott, M.R.; Sandilos, J.K.; Walk, S.F.; Kinchen, J.M.; Lazarowski, E.R.; Armstrong, A.J.; Penuela, S.; Laird, D.W.; Salvesen, G.S.; Isakson, B.E.; Bayliss, D.A.; Ravichandran, K.S., Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. *Nature,* **2010**, *467*, (7317), 863-867.

[14] Lazarowski, E.R., Vesicular and conductive mechanisms of nucleotide release. *Purinergic signalling,* **2012**, *8*, (3), 359-373.

[15] Volonte, C.; Apolloni, S.; Skaper, S.D.; Burnstock, G., P2X7 receptors: channels, pores and more. *CNS & neurological disorders drug targets,* **2012**, *11*, (6), 705-721.

[16] Di Virgilio, F.; Adinolfi, E., Extracellular purines, purinergic receptors and tumor growth. *Oncogene,* **2017**, *36*, (3), 293-303.

[17] Zimmermann, H., Extracellular ATP and other nucleotides-ubiquitous triggers of intercellular messenger release. *Purinergic signalling,* **2016**, *12*, (1), 25-57.

[18] Antonioli, L.; Pacher, P.; Vizi, E.S.; Hasko, G., CD39 and CD73 in immunity and inflammation. *Trends in molecular medicine,* **2013**, *19*, (6), 355-367.

[19] Kanthi, Y.M.; Sutton, N.R.; Pinsky, D.J., CD39: Interface between vascular thrombosis and inflammation. *Current atherosclerosis reports,* **2014**, *16*, (7), 425.

[20] Antonioli, L.; Blandizzi, C.; Pacher, P.; Hasko, G., Immunity, inflammation and cancer: a leading role for adenosine. *Nature reviews. Cancer,* **2013**, *13*, (12), 842-857.

[21] Baldwin, S.A.; Beal, P.R.; Yao, S.Y.; King, A.E.; Cass, C.E.; Young, J.D., The equilibrative nucleoside transporter family, SLC29. *Pflugers Archiv : European journal of physiology,* **2004**, *447*, (5), 735-743.

[22] Gray, J.H.; Owen, R.P.; Giacomini, K.M., The concentrative nucleoside transporter family, SLC28. *Pflugers Archiv : European journal of physiology,* **2004**, *447*, (5), 728-734.

[23] Stagg, J.; Smyth, M.J., Extracellular adenosine triphosphate and adenosine in cancer. *Oncogene,* **2010**, *29*, (39), 5346-5358.

[24] Pellegatti, P.; Raffaghello, L.; Bianchi, G.; Piccardi, F.; Pistoia, V.; Di Virgilio, F., Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. *PloS one,* **2008**, *3*, (7), e2599.

[25] Jacob, F.; Perez Novo, C.; Bachert, C.; Van Crombruggen, K., Purinergic signaling in inflammatory cells: P2 receptor expression, functional effects, and modulation of inflammatory responses. *Purinergic signalling,* **2013**, *9*, (3), 285-306.

[26] Burnstock, G.; Evans, L.C.; Bailey, M.A., Purinergic signalling in the kidney in health and disease. *Purinergic signalling,* **2014**, *10*, (1), 71-101.

[27] Burnstock, G., Purinergic signalling in the gastrointestinal tract and related organs in health and disease. *Purinergic signalling,* **2014**, *10*, (1), 3-50.

[28] Pedata, F.; Dettori, I.; Coppi, E.; Melani, A.; Fusco, I.; Corradetti, R.; Pugliese, A.M., Purinergic signalling in brain ischemia. *Neuropharmacology,* **2016**, *104*, 105-130.

[29] Magni, G.; Riccio, D.; Ceruti, S., Tackling chronic pain and inflammation through the purinergic system. *Current medicinal chemistry,* **2017**.

[30] Burnstock, G., Purinergic signalling: pathophysiology and therapeutic potential. *The Keio journal of medicine,* **2013**, *62*, (3), 63-73.

[31] Wang, Q.; Wang, L.; Feng, Y.H.; Li, X.; Zeng, R.; Gorodeski, G.I., P2X7 receptor-mediated apoptosis of human cervical epithelial cells. *American journal of physiology. Cell physiology,* **2004**, *287*, (5), C1349-1358.

[32] Fang, J.; Chen, X.; Zhang, L.; Chen, J.; Liang, Y.; Li, X.; Xiang, J.; Wang, L.; Guo, G.; Zhang, B.; Zhang, W., P2X7R suppression promotes glioma growth through epidermal growth factor receptor signal pathway. *The international journal of biochemistry & cell biology,* **2013**, *45*, (6), 1109-1120.

[33] Staller, K.; Kuo, B. In *Principles of Deglutition: A Multidisciplinary Text for Swallowing and its Disorders*. Shaker, R.; Belafsky, P.C.; Postma, G.N.; Easterling, C., Eds.; Springer New York: New York, NY, **2013**, pp 269-286.

[34] Burnstock, G.; Jacobson, K.A.; Christofi, F.L., Purinergic drug targets for gastrointestinal disorders. *Current opinion in pharmacology,* **2017**, *37*, 131-141.

[35] Lavoie, E.G.; Gulbransen, B.D.; Martin-Satue, M.; Aliagas, E.; Sharkey, K.A.; Sevigny, J., Ectonucleotidases in the digestive system: focus on NTPDase3 localization. *American journal of physiology. Gastrointestinal and liver physiology,* **2011**, *300*, (4), G608-620.

[36] Maaser, K.; Hopfner, M.; Kap, H.; Sutter, A.P.; Barthel, B.; von Lampe, B.; Zeitz, M.; Scherubl, H., Extracellular nucleotides inhibit growth of human oesophageal cancer cells via P2Y(2)-receptors. *British journal of cancer,* **2002**, *86*, (4), 636-644.

[37] Shieh, K.R.; Yang, S.C.; Tseng, H.L.; Yi, C.H.; Liu, T.T.; Chen, C.L., Evidence for up-regulation of purinergic receptor genes associating with TRPV1 receptors and neurotrophic factors in the inflamed human esophagus. *Current molecular medicine,* **2014**, *14*, (9), 1205-1214.

[38] Ma, J.; Altomare, A.; Rieder, F.; Behar, J.; Biancani, P.; Harnett, K.M., ATP: a mediator for HCl-induced TRPV1 activation in esophageal mucosa. *American journal of physiology. Gastrointestinal and liver physiology,* **2011**, *301*, (6), G1075-1082.

[39] Lecea, B.; Gallego, D.; Farre, R.; Opazo, A.; Auli, M.; Jimenez, M.; Clave, P., Regional functional specialization and inhibitory nitrergic and nonnitrergic coneurotransmission in the human esophagus. *American journal of physiology. Gastrointestinal and liver physiology,* **2011**, *300*, (5), G782-794.

[40] Santos, A.A.; Cappellari, A.R.; de Marchi, F.O.; Gehring, M.P.; Zaparte, A.; Brandão, C.A.; Lopes, T.G.; da Silva, V.D.; Pinto, L.F.R.; Savio, L.E.B.; Moreira-Souza, A.C.A.; Coutinho-

Silva, R.; Paccez, J.D.; Zerbini, L.F.; Morrone, F.B., Potential role of P2X7R in esophageal squamous cell carcinoma proliferation. *Purinergic signalling,* **2017**, 1-14.

[41] Wu, L.; Oshima, T.; Fukui, H.; Watari, J.; Miwa, H., Adenosine triphosphate induces P2Y2 activation and interleukin-8 release in human esophageal epithelial cells. *Journal of gastroenterology and hepatology,* **2017**, *32*, (7), 1341-1347.

[42] Wu, L.; Oshima, T.; Shan, J.; Sei, H.; Tomita, T.; Ohda, Y.; Fukui, H.; Watari, J.; Miwa, H., PAR-2 activation enhances weak acid-induced ATP release through TRPV1 and ASIC sensitization in human esophageal epithelial cells. *American journal of physiology. Gastrointestinal and liver physiology,* **2015**, *309*, (8), G695-702.

[43] Levin, R.; Braiman, A.; Priel, Z., Protein kinase C induced calcium influx and sustained enhancement of ciliary beating by extracellular ATP. *Cell calcium,* **1997**, *21*, (2), 103-113.

[44] Wan, H.X.; Hu, J.H.; Xie, R.; Yang, S.M.; Dong, H., Important roles of P2Y receptors in the inflammation and cancer of digestive system. *Oncotarget,* **2016**, *7*, (19), 28736-28747.

[45] Pettit, M., Gastroesophageal reflux disease: clinical features. *Pharmacy world & science : PWS,* **2005**, *27*, (6), 417-420.

[46] Keshteli, A.H.; Shaabani, P.; Tabibian, S.R.; Saneei, P.; Esmaillzadeh, A.; Adibi, P., The relationship between fruit and vegetable intake with gastroesophageal reflux disease in Iranian adults. *Journal of research in medical sciences : the official journal of Isfahan University of Medical Sciences,* **2017**, *22*, 125.

[47] Vakil, N.; van Zanten, S.V.; Kahrilas, P.; Dent, J.; Jones, R., The Montreal Definition and Classification of Gastroesophageal Reflux Disease: A Global Evidence-Based Consensus. *The American journal of gastroenterology,* **2006**, *101*, 1900.

[48] El-Serag, H.B.; Mason, A.C.; Petersen, N.; Key, C.R., Epidemiological differences between adenocarcinoma of the oesophagus and adenocarcinoma of the gastric cardia in the USA. *Gut,* **2002**, *50*, (3), 368-372.

[49] Souza, R.F.; Bayeh, L.; Spechler, S.J.; Tambar, U.K.; Bruick, R.K., A new paradigm for GERD pathogenesis. Not acid injury, but cytokine-mediated inflammation driven by HIF-2alpha: a potential role for targeting HIF-2alpha to prevent and treat reflux esophagitis. *Current opinion in pharmacology,* **2017**, *37*, 93-99.

[50] Pandolfino, J.E.; Kwiatek, M.A.; Kahrilas, P.J., The Pathophysiologic Basis for Epidemiologic Trends in Gastroesophageal Reflux Disease. *Gastroenterology Clinics of North America,* **2008**, *37*, (4), 827-843.

[51] Riley, C.A.; Wu, E.L.; Hsieh, M.C.; Marino, M.J.; Wu, X.C.; McCoul, E.D., Association of Gastroesophageal Reflux With Malignancy of the Upper Aerodigestive Tract in Elderly Patients. *JAMA otolaryngology-- head & neck surgery,* **2017**.

[52] Huang, F.L.; Yu, S.J., Esophageal cancer: Risk factors, genetic association, and treatment. *Asian journal of surgery,* **2016**.

[53] Tan, W.K.; di Pietro, M.; Fitzgerald, R.C., Past, present and future of Barrett's oesophagus. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology,* **2017**, *43*, (7), 1148-1160.

[54] Wijarnpreecha, K.; Panjawatanan, P.; Thongprayoon, C.; Jaruvongvanich, V.; Ungprasert, P., Association between gastroesophageal reflux disease and nonalcoholic fatty liver disease: A meta-analysis. *Saudi journal of gastroenterology : official journal of the Saudi Gastroenterology Association,* **2017**, *23*, (6), 311-317.

[55] Rajendra, S.; Sharma, P., Barrett Esophagus and Intramucosal Esophageal Adenocarcinoma. *Hematology/oncology clinics of North America,* **2017**, *31*, (3), 409-426.

[56] Isomoto, H.; Wang, A.; Mizuta, Y.; Akazawa, Y.; Ohba, K.; Omagari, K.; Miyazaki, M.; Murase, K.; Hayashi, T.; Inoue, K.; Murata, I.; Kohno, S., Elevated levels of chemokines in esophageal mucosa of patients with reflux esophagitis. *The American journal of gastroenterology,* **2003**, *98*, (3), 551-556.

[57] Ma, J.; Altomare, A.; Guarino, M.; Cicala, M.; Rieder, F.; Fiocchi, C.; Li, D.; Cao, W.; Behar, J.; Biancani, P.; Harnett, K.M., HCl-induced and ATP-dependent upregulation of TRPV1 receptor expression and cytokine production by human esophageal epithelial cells. *American journal of physiology. Gastrointestinal and liver physiology,* **2012**, *303*, (5), G635-645.

[58] Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D.M.; Forman, D.; Bray, F., Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer. Journal international du cancer,* **2015**, *136*, (5), E359-386.

[59] Dong, J.; Thrift, A.P., Alcohol, smoking and risk of oesophago-gastric cancer. *Best practice & research. Clinical gastroenterology,* **2017**, *31*, (5), 509-517.

[60] Torre, L.A.; Bray, F.; Siegel, R.L.; Ferlay, J.; Lortet-Tieulent, J.; Jemal, A., Global cancer statistics, 2012. *CA: a cancer journal for clinicians,* **2015**, *65*, (2), 87-108.

[61] Zhang, H.Z.; Jin, G.F.; Shen, H.B., Epidemiologic differences in esophageal cancer between Asian and Western populations. *Chinese journal of cancer,* **2012**, *31*, (6), 281-286.

[62] Smyth, E.C.; Lagergren, J.; Fitzgerald, R.C.; Lordick, F.; Shah, M.A.; Lagergren, P.; Cunningham, D., Oesophageal cancer. *Nature reviews. Disease primers,* **2017**, *3*, 17048.

[63] Zhang, Y., Epidemiology of esophageal cancer. *World journal of gastroenterology,* **2013**, *19*, (34), 5598-5606.

[64] Napier, K.J.; Scheerer, M.; Misra, S., Esophageal cancer: A Review of epidemiology, pathogenesis, staging workup and treatment modalities. *World journal of gastrointestinal oncology,* **2014**, *6*, (5), 112-120.

[65] Lagergren, J.; Smyth, E.; Cunningham, D.; Lagergren, P., Oesophageal cancer. *Lancet,* **2017**.

[66] Morita, M.; Kumashiro, R.; Kubo, N.; Nakashima, Y.; Yoshida, R.; Yoshinaga, K.; Saeki, H.; Emi, Y.; Kakeji, Y.; Sakaguchi, Y.; Toh, Y.; Maehara, Y., Alcohol drinking, cigarette smoking, and the development of squamous cell carcinoma of the esophagus: epidemiology, clinical findings, and prevention. *International journal of clinical oncology,* **2010**, *15*, (2), 126-134.

[67] Wang, M.X.; Ren, L.M.; Shan, B.E., Inhibitory effects of extracellular adenosine triphosphate on growth of esophageal carcinoma cells. *World journal of gastroenterology : WJG,* **2005**, *11*, (38), 5915-5919.

[68] Burnstock, G., Purinergic Signalling: Therapeutic Developments. *Frontiers in pharmacology,* **2017**, *8*, 661.

4.2 CAPÍTULO II

Artigo Original a ser submetido Frontiers in *Pharmacology*.

$P2Y_2$ receptor activation leads to human esophageal cancer cells proliferation via ERK1/2 and Akt pathways

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Abstract

Background: Esophageal cancer is a worldwide prominent illness; that is divided in two main subtypes, squamous cell carcinoma and adenocarcinoma. Mortality rates are alarming, and the understanding of the mechanisms involved and possible therapeutic targets for this disease becomes essential. Purinergic signaling is involved in many diseases, and cancer research has demonstrated the participation of this system in several types of tumors. Here we focused in the purinergic receptor $P2Y_2$ expression in patient's biopsies samples and compared this influence in human esophageal squamous cell carcinoma with adenocarcinoma cell lines. *Methods:* 16 paraffin embedded blocks of ESCC and 7 of EAC were used to verify the $P2Y_2$ receptor expression. Three human esophageal cancer cell lines Kyse-30 and Kyse-450 (ESCC) and OE-33 (EAC) were used to perform *in vitro* analysis of cell proliferation, signaling pathways involved in $P2Y_2R$ activation, cell migration and adhesion. *Results:* we observe the $P2Y_2$ receptor dependence to survival that occurs via ERK1/2 and Akt activation. Nucleotides stimuli lead to an increase in cell proliferation and the $P2Y_2$ blockage can lead cells to death. *Conclusion:* Pharmacological modulation of $P2Y_2$ receptor showed a singular profile in different cell lines. Briefly, $P2Y_2R$ has important roles in the maintenance of living cells and is related to proliferation in esophageal cancer cells, moreover ATP and UTP are able to activate signaling pathways related with cell survival, proliferation and invasion. Our data also showed that pharmacological blocked of $P2Y_2R$ impair the capacity of proliferation in esophageal cancer cells and decreased in intracellular calcium levels.

Keywords: Esophagus; Purinergic Signaling; Nucleotides; Proliferation; Carcinoma.

Introduction

Esophageal cancer (EC) is a very aggressive illness that affects 450 000 patients worldwide, and ranks the 8th most common incident cancer in the world, and 6th in mortality in men (Pennathur *et al.*, 2013; Zhang, 2013). The high mortality rates are mainly due to poor diagnosis and 15%-25% of patients reaches five years of survival when they are diagnosed in early stages of disease (Pennathur *et al.*, 2013). There are two main subtypes of EC; esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) (Kamangar *et al.*, 2009). ESCC is the most incident type of esophagus cancer worldwide, however, cases of EAC have increased significantly over the years (Napier *et al.*, 2014). Beyond the cell type involved, epidemiological features, anatomic localization, risk factors and molecular characteristics differ between the subtypes (Lin *et al.*, 2016; The Cancer Genome Atlas Research, 2017).

Purinergic signaling (Burnstock, 2004) and their receptors have been related in several types of cancer and there is mounting interest in the role of nucleosides and nucleotides in therapeutic potential for cancer treatment (Burnstock e Di Virgilio, 2013). The purinergic receptors are classified in two major classes, P1 receptors that are G-protein coupled and divided in four subtypes (A_1, A_2A, A_2B and A_3) (Fredholm, Bertil B *et al.*, 2001), and P2-receptors distinguished in P2X or ionotropic receptors (P2XR) which are ligand-gated. P2XR contain an intrinsic ion channel with seven members described (P2X1-7) and P2Y or metabotropic receptors (P2YR) belonging to G-protein coupled receptors superfamily with eight members described in humans ($P2Y_{1,2,4,6,11,12,13,14}$) (Burnstock, 1978; Abbracchio e Burnstock, 1994). Purine and pyrimidine stimulates these receptors in extracellular space, here we focused in the P2YR that can be activated by different nucleotides according to its affinity for each receptor subtypes (Franke *et al.*, 2012). $P2Y_2R$ acts by altering intracellular levels of cAMP, modulating adenylate cyclase or increasing intracellular levels of calcium via PLC, and their main agonists are ATP and UTP (Burnstock, 2004).

Different P2YR subtypes are widely distributed in the body and they play an important role in physiological and pathological processes (Abbracchio *et al.*, 2006). Previous data showed that ATP is released in larger amounts by a higher metastatic cancer cell line when compared to a less aggressive or normal cell (Jin *et al.*, 2014). Maaser *et al*., (2002) demonstrated that the activation of $P2Y_2R$ by ATP at high concentrations leads to apoptosis and cell cycle arrest in esophageal squamous carcinoma cell line (Kyse-140). However, $P2Y_2R$ has also been related with tumor growth, invasion and metastasis in other types of cancer, for example, breast (Jin *et al.*, 2014), prostate (Li *et al.*, 2013) and hepatocellular (Xie *et al.*, 2014). Recently, the $P2Y_2R$ has become visible as a potential pharmacological target, promising results in cancer research field both through its blockage and when activated (Nylund *et al.*, 2007; Buzzi *et al.*, 2010; Schumacher *et al.*, 2013).

However, there is a scarce literature focusing on the purinergic system in esophageal pathologies such as esophagitis, Barrett's esophagus and carcinoma. Then, to provide a comprehensive assessment of the actions of $P2Y_2R$ in esophageal cancer, we analyzed by immunohistochemistry the $P2Y_2R$ expression in human biopsies of ESCC and EAC. Likewise, we explored if pharmacological modulation of $P2Y_2R$ with agonists and antagonist can affect tumor progression in human esophageal cancer cell lines of squamous cell carcinoma and adenocarcinoma. In addition, this study shows for the first time the relationship among the activation of $P2Y_2R$ and the proliferation, migration, and signaling pathways in esophageal cancer cells.

Materials and Methods

Agonists, Antagonist and Antibodies

The nucleotides (ATP and UTP) were purchased from Santa Cruz Biotechnology (CA, USA). The P2Y2R antagonist (AR-C 118925XX) was obtained from Tocris Bioscience (MO, USA), ERK inhibitor (PD98059) was obtained from Cayman Chemical (MI, USA) and PI3K inhibitor (LY294002) was obtained from Sigma Aldrich (MO, EUA). Anti-$P2Y_2$ Receptor was purchased from Alomone Labs (JRS, Israel). For pERK1/2 and pAkt cytometry was used Phosflow Kit from BD Biosciences (CA, USA).

Human Sample Characterization

This study was retrospective, from 2012 until 2017; we revised clinical data of thirty-four patients of both sexes, attended by the Oncology Service of São Lucas Hospital, Porto Alegre – Brazil. The paraffin blocks of esophageal biopsies were used to $P2Y_2R$ quantification by immunohistochemistry. The local Ethical Committee approved the study (number: 1.645.764). Patients were divided in three groups according to clinical data collection: healthy control (HC) group (17 cases), squamous cell carcinoma (60 cases) and adenocarcinoma (10 cases). The inclusion criteria for control group was (1) men and women aged 30 to 80 (2) report of pathological examination negative for malignancy. The exclusion criteria was defined as follows: (1) diagnosis of esophagitis, (2) diagnosis of Barret's esophagus (3) inadequate/absent biopsy sample. The inclusion criteria for cancer patients was: (1) men and women aged 30 to 80, (2) diagnosis of esophageal adenocarcinoma or squamous cell carcinoma, (3) esophagus as a primary site, (4) paraffin blocks available. The exclusion criteria was defined as follows: (1) clinical data incomplete, (2) inadequate/absent biopsy sample (3) tumor classified as

undifferentiated. When the inclusion and exclusion criteria were applied, the final number of participants was 10 cases of HC, 7 cases of EAC and 16 cases of ESCC.

Immunohistochemistry

As previously described, human histological samples of normal esophagus, ESCC and EAC were collected to verify the $P2Y_2R$ expression. The final number of biopsy samples collected to immunohistochemistry was, HC = 10 cases ESCC = 16 cases and EAC = 7 cases. Formalin-fixed, paraffin-embedded tumor blocks were used to obtain 2 μm thick sections, after the sectioned slides were deparaffinized in xylene and dehydrated in serial ethanol dilutions. The next steps were performed according to Braganhol et al., (2009), sections were incubated overnight at 4℃ with the rabbit polyclonal $P2Y_2R$ antibody (1:100), then sections were washed in PBS and incubated with secondary anti-rabbit IgG antibody at room temperature for 2 hours. Lastly, samples were incubated with 3,3'-diaminobenzidine (DAB) (Novolink chromogen) for 5 minutes, followed by haematoxylin counterstaining.

Cell Culture

Esophageal cancer cell lines, Kyse-30, Kyse-450 and OE-33, were kindly donated from Dr. Luis Felipe Ribeiro Pinto. Cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) (both purchased from Gibco, NY, USA), 50 units of penicillin/mL, 50 mg streptomycin/mL, and fungizone 250 μg/mL purchased from Sigma Aldrich (MO, EUA) at controlled cell incubator (37 °C, 5% CO2, and 95% humidity).

RNA Isolation and Real-time qPCR

The gene expression of purinergic receptor P2Y family ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{14}$) was determined by RT-qPCR. The total RNA was isolated from esophageal cancer cell lines (Kyse-30, Kyse-450, and OE-33) with TRIzol® Reagent (Life Technologies) in

accordance with the manufacturer's instructions. RNA purity (Abs 260/280nm ~2.0) and concentration were determined by Nanodrop® Lite and after treated with Deoxyribonuclease I (Sigma-Aldrich) to eliminate genomic DNA contamination in accordance with the manufacturer's instructions. The cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega, WI, USA) from 1 μg of the total RNA, following the manufacturer´s instruction. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis on the 7500 Real-time PCR System (Applied Biosystems, CA, EUA). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72°C for elongation. At the end of cycling protocol, a melting-curve analysis was included, and fluorescence measured from 60 to 99 °C to confirm the specificity of primers and absence of primer-dimers and showed in all cases one single peak. All real time assays were carried out in quadruplicate and, in all cases, substituting the templates for DNase/RNase-free distilled water in each PCR reaction included a reverse transcriptase negative control. *Gapdh*, *B2M* and *18S* were used as reference genes for normalization. The sequences of reverse and forward primers are in Table 1. The efficiency per sample was calculated using LinRegPCR 2016.1 Software (http://LinRegPCR.nl) and the stability of the references genes, and the optimal number of reference genes according to the pairwise variation (*V*) was analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Cq}$ method (Bustin *et al.*, 2013).

Cell Counting

Cells were seeded at density of 2 x 10^4 per well in 24-well plates. To perform these experiments, the tree cell lines are induced to G1-phase though FBS starvation by reducing its concentration of 10% to 5% and finally 0.5% for 24 hours. After that, cells were divided in six

treatment groups as follows: (1) Control group: received RPMI medium (0.5% SFB), (2) ATP (100 μM), (3) UTP: (100 μM), (4) AR-C 118925XX: at concentration of 20 μM, (5) AR-C 118925XX (20 μM) plus ATP (100 μM), (6) AR-C 118925XX (20 μM) plus UTP (100 μM). After 24 h of treatment, the medium was removed, the cells were washed with calcium and magnesium-free medium (CMF) and 100 μL of 0.23% trypsin/EDTA solution was used to detach cells. Absolut cell number was acquired by Countess FL cell counter (Life Technologies, CA, USA). This experiment was performed in triplicate for 3 times at least.

Clonogenic Assay

This experiment was performed according to Rafehi et al. (2011). Briefly, Kyse-30 and Kyse-450 (100 cells) and OE-33 (300 cells) were seeded in 24-well plates. Since it is a long-term experiment, we chose to use RPMI medium with 5% of FBS supplementation. After 24 hours, cells are treated with ATP (100 μM), UTP (50 and 100 μM) alone or in combination with AR-C 118925XX (20 μM), cells of control group received RPMI 5% FBS only. Every two days the medium was replaced, and the wells that received nucleotides initially, get a nucleotide pulse together with new medium. On the 7^{th} day, cells were washed twice with PBS and fixed with formalin 4% for 10 minutes; colonies were stained with 200 μL of crystal violet for 10 minutes, and then washed with PBS. Colonies with 50 cells or more were considered and counting was performed using the ImageJ software. This assay was performed in quadruplicate in two independent experiments.

Wound Healing Assay

To determine the cell capacity of migration, wound healing assay was performed according to previously studies (Valster *et al.*, 2005; Garay *et al.*, 2013). Kyse-30 (110×10^4), Kyse-450 (150×10^4) and OE-33 (150×10^4) cells were seeded on 24-well plates in RPMI 10% FBS for

24 hours at 37°C under 5% de CO2. After, cells were starved (5% FBS) for 24 hours and subsequently Kyse-30 and Kyse-450 were starved in 0.5% FBS for 24 hours; OE-33 cells do not resist FBS starvation to 0.5% for long periods, so with cell line the experiment was carried out with 5% FBS. Using a p200 pipette tip a line was scrapped into the well to simulate an injury. Cells were washed three times with PBS to remove the debris/serum and treated with ATP (100 μM), UTP (100 μM), AR-C 118925XX (20 μM), AR-C 118925XX (20 μM) plus ATP (100 μM), AR-C 118925XX (20 μM) plus UTP (100 μM), and controls received only media FBS 0.5% or 5% (OE-33). Kyse-450 and OE-33 were photographed at time point zero, 24 hours and 48 hours. Since the proliferation rate of the Kyse-30 lineage is higher than the other cell lines used in this study, we established different time points as follows: zero, 12 hours and 24 hours. All images were obtained via an inverted optical microscope (10 x objective) with a capturing digital image system (Olympus IX71), always in the same field. The images obtained were analyzed by using Adobe® Photoshop® CS5. This assay was conducted in duplicate in three independent experiments.

Adhesion Assay

To evaluate cell adhesion, Kyse-30, Kyse-450 and OE-33 were seeded (5 x 10^4 cells) in 96-well plates. For this experiment, the cells were treated together with plating and incubated for 2 hours at 37ºC. Treatment: (1) Control group: received RPMI medium (10% FBS), (2) ATP (50 and 100 μM), (3) UTP (50 and 100 μM), (4) AR-C 118925XX (20 μM), (5) AR-C 118925XX (20 μM) plus ATP (50 and 100 μM), (6) AR-C 118925XX (20 μM) plus UTP (50 and 100 μM). Passed the incubation time, cells were washed three times with PBS to remove non-adherent cells. Adherent cells were fixed with formalin for 10 minutes and stained with crystal violet (0.5%) diluted in methanol (20%). Then, cells were cleaned three times with Milli-Q™ water, and 100 μl of 10% acetic acid (v/v) was added in each well for elution. Lastly, the cell

adhesion was evaluated by measuring optical density (OD) at 570 nm in a plate reader (Spectra Max M2e, Molecular Devices) (Wang *et al.*, 2008). This experiment was conducted in triplicate in two independent experiments.

Determination of Signaling Pathways Phospho-AKT and Phospho-ERK1/2

In order to analyze whether nucleotide treatment activates intracellular signaling pathways, we will use the expression markers for phospho-ERK and phospho-AKT and measurements was executed using the BD Phosflow (BD Bioscience) protocol for adherent cells. Cells were plated (30×10^4) in 12-well plates and SFB was reduced to 0.5% for 24 hours. The cells were treated for 5, 15, 30 or 60 minutes with ATP and UTP at 100 μM concentration, the positive control received RPMI 10% SFB, and RPMI control 0.5% SFB, and maintained in incubation at 37°C. After treatment the cells will be trypsinized, and fixed with Phosflow Buffer for 10 minutes at 37°C. After, the cells were washed with PBS and permeabilized with Phosflow Perm Buffer for 30 minutes, conditioned on ice. After permeabilization the cells were washed twice and labeled with FITC anti-pERK 1/2 and anti-pAKT PE antibodies for 30 minutes conditioned on ice. Data were obtained using cytometer FACSCantoII (Beckton Dickinson) and BD FACSDiva software, subsequently analyzed using Flowjo v10. Flow cytometry was conducted in triplicate.

Enzymatic Activity

In order to analyze the ectonucleotidases activities we used the protocol described by Wink et al., (2003). 4×10^4 cells of Kyse-30, Kyse-450 and OE-33 were trypsinized and included to solution containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for Ectonucleotidase activities) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (CD73 activity) in a final volume of 200 μL. Samples were preincubated for 10 min at 37 °C be- fore starting the reaction with the addition of substrate (ATP, ADP or UTP) to a final concentration of 100 μM.

The reaction was stopped after 30 min with the addition of 200 μL of trichloroacetic acid at a final concentration of 5%. Samples were incubated on ice for 10 min and was added 1mL of a colorimetric solution containing 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added in order to determine the inorganic phosphate released (Pi). The quantification of Pi released was reached using a spectrophotometer at 630 nm (Spectra Max M2e, Molecular Devices). This assay was conducted in triplicate in three independent experiments and results were expressed as nmol Pi released/min/mg of protein.

Free intracellular calcium concentration analysis

This experiment was conducted according to Pillat et al. (2016). Kyse-30, Kyse-450 and OE-33 cells were plated (1 x 10^4 cells per well) in 96-well plates; the plates were black and had a flat bottom. Twenty-four hours after seeding cells were washed with PBS 1X and treated with AR-C 118925XX (20 μM) or medium alone (serum free), each well also received 5μM Fluo-3AM (Molecular Probes) in 0.5% Me2SO and 0.06% non-ionic pluronic acid surfactant F-127 (Sigma Aldrich) for 30 minutes at 37 ° C. Subsequently, the medium was removed and extracellular buffer (140 mM NaCl, 3 mM KCl, 1 mM MgCl 2, 2 mM CaCl 2, 10 mM Hepes and 10 mM glucose, pH 7.4) was added immediately prior to the Ca^{2+}. At the time of analysis, the cells were stimulated with nucleotides ATP or UTP (100 μM) in wells with $P2Y_2$ antagonist and in wells with medium only. The images were obtained through ECLIPSE-TiS Inverted Microscope (Nikon, Melville, NY) equipped with a 14-bit, high resolution, CoolSNAP HQ2 CCD camera (Photometrics, Tucson, AZ) and analyzed with NIS-Element software (Nikon). A xenon lamp at 488 nm excited fluo-3AM probe and fluorescence emission was detected using a band pass filter (515-530 nm) and we get images every second. Thirty cells were analyzed for each well, and each condition was performed in duplicate in three independent experiments. Time kinetics of free intracellular calcium ($[Ca^{2+}]i$) variations were composed from 300 images

acquired in 1-s intervals. The fluorescence intensities (F) were calibrated in a solution containing 5 mM ionophore (Fmax) and 10 μM BAPTA-AM (Fmin) to stipulate an assessment of the absolute variation in the intracellular calcium fluorescence using the subsequent equation ΔF/Fmin.

Statistical analysis

Data were analyzed by Student's t-test, one-way analysis of variance (one-way ANOVA), followed by Tukey or Dunnett's post hoc test or two-way analysis of variance (two-way ANOVA) followed by Bonferroni post-test for multiple comparisons, using GraphPad Software 6.0 (San Diego, CA, U.S.A.). Data are presented as mean ± SEM; p values <0.05 indicate statistical significance.

Results

$P2Y_2R$ immunostainig and clinical data of patients

Human samples of esophageal biopsies of normal esophagus, squamous cell carcinoma and adenocarcinoma were analyzed regarding $P2Y_2R$ expression by immunohistochemistry. Representative images of $P2Y_2R$ immunostainig in human biopsies demonstrated an area of normal appearing esophageal mucosa showing positivity for $P2Y_2R$ (**Fig**.**1A**), whereas immunoreactive receptors were markedly increased in ESCC (**Fig. 1B**). Figure **1C** depicts EAC cells showing membrane-augmented positivity for $P2Y_2R$. Socio-demographic and clinical data of patients are expressed in supplementary Table 1.

Esophageal cancer cell lines express $P2Y_2$ receptors

Since patients with esophageal cancer display relevant expression of $P2Y_2$ receptor in ESCC and EAC biopsies, we decided to investigate the expression of this receptor in different subtypes of esophageal cancer human cell lines. In order to evaluate the P2YR expression in Kyse-30, Kyse-450 and OE-33, we performed the RT-qPCR technique for eight receptor subtypes already described in humans (supplementary **Table 2**).

The results obtained demonstrate that the EAC OE-33 cell line has a higher expression of $P2Y_2R$ compared with Kyse-450 ($p<0.001$), although the *p*-value is not significant, we could observe a difference in mean of expression between the OE-33 (mean = 1.123) and Kyse-30 (mean = 0.9710) ($p= 0.178$). Kyse-30 showed higher expression than Kyse-450 ($p<0.05$) (**Fig.1D**).

Pharmacological blockage of $P2Y_2R$ decreases esophageal cancer cell number

In order to verify cell proliferation we performed cell-counting experiment, cells were treated with extracellular nucleotides and with the specific $P2Y_2R$ antagonist. Kyse-30 showed no significant difference in the cell number when treated with ATP or UTP (100 μM) (**Fig.2D**). In contrast, in Kyse-450 (**Fig.2E**) and OE-33 cells (**Fig.2F**) a significant increase was observed in cell number when compared to control ($p<0.05$). Moreover, the three cell lines responded in a similar way when received the $P2Y_2R$ antagonist AR-C 118925XX (20 μM), presenting a significant decrease in number of cells ($p<0.05$). And this effect was partially reversed by the subsequent addition of the $P2Y_2$ agonists ATP and UTP (100 μM).

UTP induces colony formation in Kyse-450 and OE-33 cell lines

Regarding to the formation of colonies, Kyse-30 (**Fig.3A**) cell line did not present significant changes in any of the treatments for 7 days. Conversely, the lineages Kyse-450 (**Fig.3B**) and OE-33 (**Fig.3C**) presented a similar behavior, showing a significant increase in the number of

colonies formation when treated with UTP (100 μM) alone or with AR-C 118925XX plus UTP (100 μM) in comparison the control ($p<0.01$). Furthermore, UTP treatment exerted a significant higher effect than ATP ($p<0.05$) in Kyse-450 cells. Also, treatment with AR-C 118925XX plus UTP induced a significant increase in colony number when compared with the group treated with AR-C 118925XX plus ATP in Kyse-450 ($p<0.01$) and OE-33 ($p<0.05$).

Extracellular nucleotides did not induce cell migration in tumor esophageal cells

We further investigated if the activation of $P2Y_2R$ with ATP or UTP was able to favor cell migration through wound-healing method. The nucleotides alone were not able to induce a significant difference in the cell migration of ESCC or EAC cell lines. When Kyse-30 and Kyse-450 cells were treated with $P2Y_2R$ antagonist there was a decrease in cell migration after 24h of treatment in comparison with the control ($p<0.001$) (**Fig.5A**). In the same way, Kyse-450 cells when treated with AR-C 118925XX showed a decrease in cellular migration capacity when compared to the control group at 24 hours ($p<0.05$), and this effect was maintained even with the subsequent addition of ATP ($p<0.05$) (**Fig.5B**). Regarding to OE-33 cell line, no significant difference was observed in cell migration in any treatments used (**Fig.5C**). Figure 5D shows representative images of the three cell lines studied at 0 and 24 hours after treatment.

Cell adhesion is impaired with $P2Y_2$ antagonism

We also evaluated the role of $P2Y_2R$ in cell adhesion capacity. The results demonstrated that UTP (100 μM) treatment lead to a significant increase of Kyse-30 cell adhesion when compared to control ($p<0.05$). In addition, treatments with AR-C 118925XX lead to a significant decrease in adherent cells when compared to control ($p<0.001$) (**Fig.6A**). The tested treatments did not alter Kyse-450 cell adhesion (**Fig.6B**). Furthermore, OE-33 lineage showed a significant decrease in cell adhesion when treated with the antagonist AR-C 118925XX, AR-

C 118925XX plus ATP, or with AR-C 118925XX plus UTP in both concentrations used were when compared to control group ($p<0.001$) (**Fig.6C**).

ATP and UTP promote activation of pERK1/2 and pAkt signaling pathways

In order to evaluate the signaling pathways involved in regulation of $P2Y_2$ receptor activities, we evaluated if these pathways are activated after ATP and UTP (100 μM) treatment in ESCC and EAC cells at different time-points. Kyse-30 treated with ATP at 3, 15, 30 and 60 minutes showed an increase in the expression of pAkt in comparison with control ($p<0.01$, $p<0.05$, $p<0.01$, $p<0.05$, respectively). Regarding pERK1/2, we observed a significant increase in its expression after 30 and 60 minutes with ATP treatment when compared to control (both $p<0.01$). When Kyse-30 received UTP pAkt expression was increased in relation to control in all time-points analyzed ($p<0.01$); whereas pERK1/2 expression was increased in time-points 15 and 30 minutes after UTP treatment in comparison with control ($p<0.01$) (**Fig.4A**). Conversely, Kyse-450 exhibited higher expression in pAkt after ATP treatment in 30 minutes ($p<0.05$), and pERK1/2 expression was increased in 30 and 60 minutes in comparison with control ($p<0.05$ and $p<0.01$, respectively) after ATP treatment. When Kyse-450 was treated with UTP, no significant difference was observed in pAkt pathway, nevertheless pERK1/2 expression presented an elevated expression after 3, 15 and 30 when compared to control ($p<0.01$) (**Fig.4B**). Once we stimulated the EAC OE-33 cell line with ATP, there was no significant difference in pAkt expression in any time-point tested. Instead, pERK1/2 expression was significantly elevated at 3, 15, 30, 60 minutes after ATP stimulus when compared with control ($p<0.01$, $p<0.01$, $p<0.01$, $p<0.05$, respectively). When we stimulated the cells with UTP, pAkt expression was elevated in 30 minutes in relation to control ($p<0.05$), and pERK1/2 expression was higher than control at 3, 15 and 30 minutes ($p<0.01$) (**Fig.4C**).

Cell lines of esophageal cancer present distinct profile of nucleotide hydrolysis

In order to understand how the nucleotide degradation occurs in three cell lines used in this study, we thus performed an evaluation of enzymatic capacity. Regarding to ATP hydrolysis to ADP realized by ectonucleotidases (CD39), adenocarcinoma cell line shown higher ATP hydrolysis compared to squamous cell carcinoma cell lines Kyse-30 ($P<0.05$) and Kyse-450 ($p<0.001$)(**Fig. 7A**). Regarding to ADP hydrolysis, Kyse-450 has lower capacity in hydrolyzes ADP to AMP than Kyse-30 ($p<0.001$) and OE-33 ($p<0.001$) (**Fig. 7B**). OE-33 also presents higher UTPase activity in comparison with Kyse-30 and Kyse-450 cells (both $p<0.05$) (**Fig. 7C**).

$P2Y_2$ response is dependent of intracellular calcium signaling

Taking into account the important role of calcium in cell signaling, we decided to measure the calcium free concentration after ATP or UTP stimuli. Non-stimulated cells were imaged for acquiring basal fluorescence values, prior to ATP or UTP (100 μM final concentration) stimuli. In Kyse-30 cells, after ATP or UTP stimuli, it was observed an elevation of intracellular calcium. Cells treated with $P2Y_2R$ antagonist showed a lower calcium concentration when compared to ATP or to AR-C 118925XX plus ATP ($p<0.05$), and to UTP or to AR-C 118925XX plus UTP ($P<0.05$) (**Fig.8A**). Concerning to Kyse-450 cell line, we did not observe significant differences between the treated groups (**Fig.8B**). OE-33 cell line did not respond to intracellular calcium measurements.

Discussion

The relationship between purinergic signaling and several types of cancer is broadly studied. Considering the high signaling potential of ATP and UTP as intra and extracellular messengers, and the large amounts of ATP existing in the tumor microenvironment (Burnstock, 2006a), the

understanding of signaling triggered by these molecules in esophageal cancer can explain how different mechanisms are involved in both, cell proliferation and death.

In this study, we showed, for the first time, the comparison among cells from two different histological types, human ESCC (Kyse-30 and Kyse-450) and EAC (OE-33), considering the role of $P2Y_2$ R in numerous cell processes that occur in cancer. First, we evaluated the gene expression of eight members of the P2Y receptors family. Taking into account the expression of $P2Y_2R$, focus of this study, EAC cell line OE-33 displays higher gene expression than ESCC cell lines, Kyse-30 and Kyse-450. Previous studies have described the association of P2Y receptors (specifically $P2Y_1$) in non-pathological functions related to muscle control (contraction and relaxation) and neurotransmission (Wan *et al.*, 2016). Also, $P2Y_2R$, expressed in primary human esophageal epithelial cells, are involved in pro-inflammatory responses when activated (Wu *et al.*, 2017).

Moreover, another cell line, Kyse-140, representative of human ESCC, expressed several purine receptors, including the $P2Y_2R$, and the activation of this receptor was related to activation of cell death mechanisms (Maaser *et al.*, 2002). In human hepatocellular carcinoma cells, the expression of $P2Y_2R$ was higher than normal hepatocytes (Xie *et al.*, 2014), evidencing that this receptor develops important function in the tumor microenvironment.

Reports demonstrate that $P2Y_2R$ activation by ATP and UTP causes cell migration and proliferation in MDA-MB-231and MCF-7 breast cancer cells, and also it is linked to pro-inflammatory cascade activation (Wagstaff *et al.*, 2000; Jin *et al.*, 2014). And in A-549 human lung cancer cells, ATP and UTP also supports cancer cells growth (Schafer *et al.*, 2003).

In cell counting concerning nucleotide stimulus, the cell lines utilized in this study responded in different ways. Kyse-30 did not shown significant response, meanwhile the Kyse-450 and OE-33 showed an increase in cell number after ATP and UTP stimulus. When ESCC and EAC cells were treated with $P2Y_2R$ antagonist the same effect was observed, a significant decrease

in cell number, which was not reversed by posterior addition of nucleotides. This result allows us to postulate that $P2Y_2R$ has an important role in maintenance of living cells and is related to proliferation in cell lines here analyzed. It is already been seen that ATP and UTP treatment after 24 and 48h were able to induce proliferation in different glioma cell lines (Morrone *et al.*, 2003).

The $P2Y_2$ receptor is mostly related to tumor growth, extravasation and invasion maintenance and metastasis promotion, in several types of cancer (Di Virgilio e Adinolfi, 2017). Xie et al. (2014) reported the involvement of $P2Y_2R$ and its activation through ATP in hepatocellular carcinoma cells, they also concluded that when $P2Y_2R$ is antagonized, ATP is unable to promote cell proliferation. Recently, our group demonstrated the involvement of P2X7 receptor in colony formation of ESCC treated with different concentrations of ATP (1-5 mM). The results obtained indicated that high concentrations of ATP, for long periods, are able to cause cell death (Santos, A. A., Jr. *et al.*, 2017).

Similarly, we aimed to investigate the role of $P2Y_2R$ in cell ability to form colonies. With the objective of evaluating how long lasts the effect of pharmacological blockade of $P2Y_2R$, the cells received the $P2Y_2R$ antagonist only on the first day of treatment; the cells received nucleotides every two days, and the experiment was finished on the 7^{th} day. Kyse-450 showed an increase in cell colonies number when stimulated with UTP, and OE-33 had an increase in the number of cell colonies after treated with both, ATP and UTP. Nonetheless, cells that received the $P2Y_2R$ antagonist did not show an inhibition effect; supporting an idea that pharmacological blockade of $P2Y_2R$ was lost over 7 days. Interestingly, the colonies formed into wells that received UTP were more consistent, which means that they were formed by a higher number of cells, than those that received ATP.

Cell migration is an important mechanism utilized by cancer cells to spread and generates metastasis, and studies have focused in the participation of $P2Y_2R$ in cell invasion and

migration. Li et al., (2015) proposed the involvement of $P2Y_2R$ activated by ATP/UTP in invasion and migration in different human prostate cancer cell lines, and these nucleotides activate epidermal growth factor receptor (EGFR) and ERK1/2. In human breast cancer cells, ATP and UTP also are able to stimulate cell migration mainly via ERK1/2, and when the $P2Y_2R$ is silenced this effect is lost (Chadet *et al.*, 2014). Here, although nucleotides have activated pathways related to cell migration (ERK1/2 and Akt), we did not observe significant increase in cell migration when nucleotides were utilized as treatment, on the other hand the $P2Y_2R$ antagonist lead to a decrease in cell migration in Kyse-30 and Kyse-450.

Here we observed that UTP increased cell adhesion in Kyse-30, furthermore the $P2Y_2R$ antagonism lead to a lower cell adhesion, this impairment might occur due to cell death occasioned by $P2Y_2R$ blockage. In MB-MDA-231 breast cancer cells, the activation of $P2Y_2R$ by ATP or UTP was capable to upregulate the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and this expression was inhibited in cells transfected with $P2Y_2R$ shRNA (Jin *et al.*, 2014). Also, in MB-MDA-231 the overexpression of CD73 was related to support cell adhesion, migration and invasion by producing large amounts of adenosine (Wang *et al.*, 2008).

Next, we assessed through cytometry the expression of ERK1/2 and pAkt signaling pathways after ATP and UTP (100 μM) stimulus. Our results showed that nucleotides exert activation in both signaling pathways, and Kyse-30 was more responsive to activation of Akt than another cell lines and its activation remained expressed for a longer period. Both nucleotides are able to induce the activation of ERK1/2, but ATP promotes more prolonged activation than UTP, this finding is in accordance with study carried out by Hoffmann et al., (2008), which elucidated that ATP and UTP nucleotides have different profiles of ERK activation.

Activation of signaling pathways such as ERK1/2 provide cell proliferation, differentiation and survival (Samatar e Poulikakos, 2014), and PI3K (Akt is main effector located downstream of

PI3K**)** are involved with cell survival, migration capacity, inflammation and cancer progression (Burgering e Coffer, 1995; Vanhaesebroeck *et al.*, 2010). Several studies have been demonstrated the nucleotide capacity in activates signaling pathways in different tumors. In MCF-7 cells UTP activates the phosphorylation of ERK1/2, but this did not occur when ERK1/2 was inhibited by PD98059 (Chadet *et al.*, 2014). In HeLa cells ATP and UTP evoked the activation of $P2Y_2$ and lead to ERK1/2 and PI3K phosphorylation (Muscella *et al.*, 2003). Jaques-Silva *et al*., (2004) when using ATP to treat glioma cells, observed increased cell proliferation that occurred via ERK and Akt after the purinergic receptors activation.

The expression and activity of the ectonucleotidases have been related to different stages of the tumor establishment such as proliferation, adhesion and invasion (Wang *et al.*, 2008). The purine metabolism focused here occurs through the enzymatic activity developed by the CD39 ectonucleotidase, that converts ATP to ADP, ADP to AMP and UTP to UDP (Zimmermann *et al.*, 2012). The ESCC representative cell line Kyse-450 was the lineage that exhibited lower capacity to hydrolyze the three extracellular nucleotides tested. This data suggested that $P2Y_2R$ agonists ATP and UTP are available for longer periods in extracellular space in these cells, and are capable of modulate the proliferation induction seen in this study.

Lastly, we evaluate the transient calcium concentration, Kyse-30 cell line exhibited a significant increase of intracellular calcium concentration after stimulation with ATP and UTP and the pharmacological inhibition of $P2Y_2R$ resulted in a decrease in calcium concentration. Elevation of intracellular calcium levels mediated by activation of the $P2Y_2$ receptor may result in phosphorylation of ERK1/2 and PI3K, important for cell proliferation, migration and survival (Muscella *et al.*, 2003; Chen *et al.*, 2004; Chadet *et al.*, 2014). In lung cancer cells, calcium signaling activated by ATP have ability in control proapoptotic and antiapoptitic proteins ratio and support cell survival (Song *et al.*, 2016).

Most of the studies are focused on ESCC, due to the fact that it is the type of higher incidence, however, we must also pay attention to esophageal adenocarcinoma, which has seen a considerable increase in the number of cases in the last years (Lagergren *et al.*, 2017). Considering the high rates of mortality in esophageal cancer, understanding the mechanisms that induce cell proliferation and death become fundamental in the search for new therapeutic targets. Taking together, our data indicates that $P2Y_2R$ plays distinct function in each cell line studied here. In Kyse-30 the purinergic receptor $P2Y_2$ is related with cell survivor maintenance, Akt signaling pathway shows important role for this maintenance. Nucleotides alone were not able to induce cell migration, but UTP treatment increase cell adhesion. $P2Y_2R$ also showed influence in calcium signaling when stimulated with ATP and UTP. Kyse-450 demonstrates an increase in cell number when received ATP and UTP, the main signaling pathway related is ERK1/2. These results allow us to postulate that $P2Y_2R$ blockage in human esophageal cancer cells could interfere in cell proliferation and as a consequence control tumor growth.

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Conflicts of interest

The authors declare no conflict of interest.

REFERENCES

ABBRACCHIO, M. P.; BURNSTOCK, G. Purinoceptors: are there families of P2X and P2Y purinoceptors? **Pharmacology & therapeutics,** v. 64, n. 3, p. 445-475, 1994.

ABBRACCHIO, M. P. et al. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. **Pharmacol Rev,** v. 58, n. 3, p. 281-341, Sep 2006.

BRAGANHOL, E. et al. Selective NTPDase2 expression modulates in vivo rat glioma growth. **Cancer Sci,** v. 100, n. 8, p. 1434-42, Aug 2009.

BURGERING, B. M.; COFFER, P. J. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. **Nature,** v. 376, n. 6541, p. 599-602, Aug 17 1995.

BURNSTOCK, G. A basis for distinguishing two types of purinergic receptor. In: STRAUB, R. e BOLIS, L. (Ed.). **Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach.** New York: Raven Press, 1978. p.107–118.

_____. Introduction: P2 receptors. **Curr Top Med Chem,** v. 4, n. 8, p. 793-803, 2004.

_____. Pathophysiology and therapeutic potential of purinergic signaling. **Pharmacol Rev,** v. 58, n. 1, p. 58-86, Mar 2006.

BURNSTOCK, G.; DI VIRGILIO, F. Purinergic signalling and cancer. **Purinergic Signal,** v. 9, n. 4, p. 491-540, Dec 2013.

BUSTIN, S. A. et al. The need for transparency and good practices in the qPCR literature. **Nat Methods,** v. 10, n. 11, p. 1063-7, Nov 2013.

BUZZI, N.; BOLAND, R.; RUSSO DE BOLAND, A. Signal transduction pathways associated with ATP-induced proliferation of colon adenocarcinoma cells. **Biochim Biophys Acta,** v. 1800, n. 9, p. 946-55, Sep 2010.

CHADET, S. et al. The activation of P2Y2 receptors increases MCF-7 breast cancer cells migration through the MEK-ERK1/2 signalling pathway. **Carcinogenesis,** v. 35, n. 6, p. 1238-47, Jun 2014.

CHEN, L. et al. ERK1/2 and p38 pathways are required for P2Y receptor-mediated prostate cancer invasion. **Cancer Lett,** v. 215, n. 2, p. 239-47, Nov 25 2004.

DI VIRGILIO, F.; ADINOLFI, E. Extracellular purines, purinergic receptors and tumor growth. **Oncogene,** v. 36, n. 3, p. 293-303, Jan 19 2017.

FRANKE, H. et al. Pathophysiology of astroglial purinergic signalling. **Purinergic Signal,** v. 8, n. 3, p. 629-57, Sep 2012.

FREDHOLM, B. B. et al. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. **Pharmacological reviews,** v. 53, n. 4, p. 527-552, 2001.

GARAY, T. et al. Cell migration or cytokinesis and proliferation?--revisiting the "go or grow" hypothesis in cancer cells in vitro. **Exp Cell Res,** v. 319, n. 20, p. 3094-103, Dec 10 2013.

HOFFMANN, C. et al. Agonist-selective, receptor-specific interaction of human P2Y receptors with beta-arrestin-1 and -2. **J Biol Chem,** v. 283, n. 45, p. 30933-41, Nov 7 2008.

JACQUES-SILVA, M. C. et al. ERK, PKC and PI3K/Akt Pathways Mediate Extracellular ATP and Adenosine-Induced Proliferation of U138-MG Human Glioma Cell Line. **Oncology,** v. 67, n. 5-6, p. 450-459, 2004.

JIN, H. et al. P2Y2 receptor activation by nucleotides released from highly metastatic breast cancer cells increases tumor growth and invasion via crosstalk with endothelial cells. **Breast Cancer Res,** v. 16, n. 5, p. R77, Aug 26 2014.

KAMANGAR, F. et al. Environmental causes of esophageal cancer. **Gastroenterol Clin North Am,** v. 38, n. 1, p. 27-57, vii, Mar 2009.

LAGERGREN, J. et al. Oesophageal cancer. **Lancet**, Jun 22 2017.

LI, W. H. et al. P2Y2 receptor promotes cell invasion and metastasis in prostate cancer cells. **Br J Cancer,** v. 109, n. 6, p. 1666-75, Sep 17 2013.

LI, W. H. et al. P2Y2 Receptor and EGFR Cooperate to Promote Prostate Cancer Cell Invasion via ERK1/2 Pathway. **PLoS One,** v. 10, n. 7, p. e0133165, 2015.

LIN, E. W. et al. The tumor microenvironment in esophageal cancer. **Oncogene,** v. 35, n. 41, p. 5337-5349, Oct 13 2016.

MAASER, K. et al. Extracellular nucleotides inhibit growth of human oesophageal cancer cells via P2Y(2)-receptors. **Br J Cancer,** v. 86, n. 4, p. 636-44, Feb 12 2002.

MORRONE, F. B. et al. Extracellular nucleotides and nucleosides induce proliferation and increase nucleoside transport in human glioma cell lines. **J Neurooncol,** v. 64, n. 3, p. 211-8, Sep 2003.

MUSCELLA, A. et al. Activation of P2Y2 receptor induces c-FOS protein through a pathway involving mitogen-activated protein kinases and phosphoinositide 3-kinases in HeLa cells. **J Cell Physiol,** v. 195, n. 2, p. 234-40, May 2003.

NAPIER, K. J.; SCHEERER, M.; MISRA, S. Esophageal cancer: A Review of epidemiology, pathogenesis, staging workup and treatment modalities. **World J Gastrointest Oncol,** v. 6, n. 5, p. 112-20, May 15 2014.

NYLUND, G. et al. P2Y2- and P2Y4 purinergic receptors are over-expressed in human colon cancer. **Auton Autacoid Pharmacol,** v. 27, n. 2, p. 79-84, Apr 2007.

PENNATHUR, A. et al. Oesophageal carcinoma. **Lancet,** v. 381, n. 9864, p. 400-12, Feb 2 2013.

PILLAT, M. M. et al. Glioblastoma-mesenchymal stem cell communication modulates expression patterns of kinin receptors: Possible involvement of bradykinin in information flow. **Cytometry A,** v. 89, n. 4, p. 365-75, Apr 2016.

RAFEHI, H. et al. Clonogenic assay: adherent cells. **J Vis Exp**, n. 49, Mar 13 2011.

SAMATAR, A. A.; POULIKAKOS, P. I. Targeting RAS-ERK signalling in cancer: promises and challenges. **Nat Rev Drug Discov,** v. 13, n. 12, p. 928-42, Dec 2014.

SANTOS, A. A., JR. et al. Potential role of P2X7R in esophageal squamous cell carcinoma proliferation. **Purinergic Signal**, Apr 11 2017.

SCHAFER, R. et al. ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. **Am J Physiol Lung Cell Mol Physiol,** v. 285, n. 2, p. L376-85, Aug 2003.

SCHUMACHER, D. et al. Platelet-derived nucleotides promote tumor-cell transendothelial migration and metastasis via P2Y2 receptor. **Cancer Cell,** v. 24, n. 1, p. 130-7, Jul 8 2013.

SONG, S. et al. ATP promotes cell survival via regulation of cytosolic [Ca2+] and Bcl-2/Bax ratio in lung cancer cells. **Am J Physiol Cell Physiol,** v. 310, n. 2, p. C99-114, Jan 15 2016.

THE CANCER GENOME ATLAS RESEARCH, N. Integrated genomic characterization of oesophageal carcinoma. **Nature,** v. 541, p. 169, 01/04/online 2017.

VALSTER, A. et al. Cell migration and invasion assays. **Methods,** v. 37, n. 2, p. 208-15, Oct 2005.

VANHAESEBROECK, B. et al. The emerging mechanisms of isoform-specific PI3K signalling. **Nat Rev Mol Cell Biol,** v. 11, n. 5, p. 329-41, May 2010.

WAGSTAFF, S. C. et al. Extracellular ATP activates multiple signalling pathways and potentiates growth factor-induced c-fos gene expression in MCF-7 breast cancer cells. **Carcinogenesis,** v. 21, n. 12, p. 2175-81, Dec 2000.

WAN, H. X. et al. Important roles of P2Y receptors in the inflammation and cancer of digestive system. **Oncotarget,** v. 7, n. 19, p. 28736-47, May 10 2016.

WANG, L. et al. Ecto-5'-nucleotidase promotes invasion, migration and adhesion of human breast cancer cells. **J Cancer Res Clin Oncol,** v. 134, n. 3, p. 365-72, Mar 2008.

WINK, M. R. et al. Altered extracellular ATP, ADP and AMP catabolism in glioma cell lines. **Cancer Lett,** v. 198, n. 2, p. 211-8, Aug 20 2003.

WU, L. et al. Adenosine triphosphate induces P2Y2 activation and interleukin-8 release in human esophageal epithelial cells. **J Gastroenterol Hepatol,** v. 32, n. 7, p. 1341-1347, Jul 2017.

XIE, R. et al. The P2Y2 nucleotide receptor mediates the proliferation and migration of human hepatocellular carcinoma cells induced by ATP. **J Biol Chem,** v. 289, n. 27, p. 19137-49, Jul 4 2014.

ZHANG, Y. Epidemiology of esophageal cancer. **World J Gastroenterol,** v. 19, n. 34, p. 5598-606, Sep 14 2013.

ZIMMERMANN, H.; ZEBISCH, M.; STRATER, N. Cellular function and molecular structure of ecto-nucleotidases. **Purinergic Signal,** v. 8, n. 3, p. 437-502, Sep 2012.

Table 1

Table 1. Primer sequences for RT-qPCR experiments included in the study

Gene	Forward primer	Reverse primer	Reference
18S	5'-GTAACCCGTTGAACCCCATT-3'	5'-CCATCCAATCGGTAGTAGCG-3'	Rho et al., 2010
B2M	5'-ACTGAATTCACCCCCACTGA-3'	5'-CCTCCATGATGCTGCTTACA-3'	Rho et al., 2010
Gapdh	5'-TGCACCACCAACTGCTTA-3'	5'-GGATGCAGGGATGATGTTC-3'	Rho et al., 2010
$P2Y_1$	5'-AGGGGTGTGGACGTCGTGGT-3'	5'-CCCAGCAGGCACCTCTTTGGA-3'	Designed by authors
$P2Y_2$	5'-GGGGAGCGAGACTTCCGGGGT-3'	5'-TCCACCACCTTGGGGGACCA-3'	Designed by authors
$P2Y_4$	5'-CATTGGCACCTGCCTTACTA-3'	5'-CCAGGGCATATTTCAGT-3'	Designed by authors
$P2Y_6$	5'-AGCTTCCTGCCTTTTCACAT-3'	5'-AAAGGCCTCCAATACAGTGC-3'	Designed by authors
$P2Y_{11}$	5'-CATGGCAGCCAACGTCTCGG-3'	5'-GGGCCACAGGAAGTCCCCCT-3'	Designed by authors
$P2Y_{12}$	5'-GCCTGGATCCGTTCATCTAT-3'	5'-GGGACAGAGATGTTGCAGAA-3'	Designed by authors
$P2Y_{13}$	5'– GACTGCCGCCATAAGAAGAC-3'	5'– CAGATCTGTTGAAGCCTTGC-3'	Designed by authors
$P2Y_{14}$	5'-TCACAGATGAAGGCCTAGACGCA-3'	5'-TGCCCAGTGAGCGTTTGTCGT-3'	Designed by authors

Paper Figure Legends

Fig.1 Representative images of human biopsies, healthy control (**A**), ESCC (**B**) and EAC (**C**) Brown staining indicates $P2Y_2R$ presence (All 200x). Expression of $P2Y_2R$ in human ESSC and EAC cell lines (**D**). One-Way ANOVA followed by Tukey post-test was used to calculate statistical difference between cell lines. Data showed as ± SEM ***p<0.001 and *p<0.05.

Fig.2 *Effects of agonists and antagonists of $P2Y_2R$ in cell number performed with Kyse-30, Kyse-450 and OE-33 cell lines.* Cell counting with Kyse-30 (**A**), Kyse-450 (**B**) and OE-33 (**C**) executed 24h after treatments with: ATP or UTP (100 μM), AR-C 118925XX (20 μM) alone and in combination with ATP or UTP (100 μM). One-Way ANOVA followed Tukey post-test calculates statistical differences between treatments; all data are present as ± SEM ***p<0.001 and *p<0.05.

Fig.3 *Colony formation capacity after treatment with antagonist and agonists of $P2Y_2R$.* Quantification of colony number after 7 days of treatment with ATP or UTP (100 μM), AR-C 118925XX (20 μM) (ARC in the graph) alone and in combination with ATP or UTP (100 μM), in Kyse-30 (**A**), Kyse-450 (**B**) OE-33 (**C**) and (**D**) Representative images of colony formation on tree cell lines. One-Way ANOVA followed Tukey post-test calculates statistical differences between treatments; all data are present as ± SEM ***p<0.001 and *p<0.05.

Fig.4 *Hound-healing assay*. Kyse-30 at 6, 12 and 24h after treatment (**A**), Kyse-450 (**B**) and OE-33 (**C**) at 24 after treatment as follows ATP or UTP (100 μM), AR-C 118925XX (ARC in the graph) (20 μM) alone or in combination with ATP or UTP (100 μM), and representative images of cell migration in Kyse-30 (**D**), Kyse-450 (**E**) and OE-33 (**F**). One-way ANOVA followed by Tukey post-test calculates statistical differences between time-points, which bar was compared to control, all data are present as ± SEM ***p<0.001 and *p<0.05.

Fig.5 *Evaluation of cell adhesion*. Kyse-30 (**A**), Kyse-450 (**B**) and OE-33 (**C**) after treated with ATP or UTP (50 and 100 μM), $P2Y_2$ antagonist AR-C 118925XX (ARC in the graph) (20 μM) alone or in combination with ATP or UTP (50 and 100 μM). One-Way ANOVA followed by Tukey post-test were carried out to calculate changes between the groups, *p<0.05. All data are present as ± SEM.

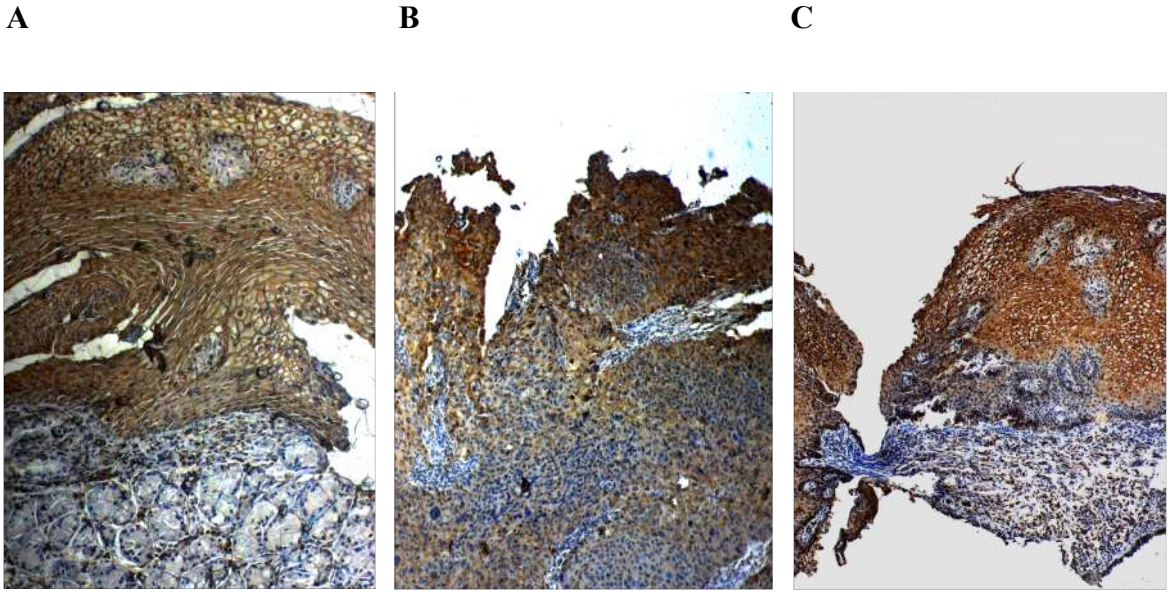
Fig.6 *Involvement of $P2Y_2$ in ERK1/2 and Akt cell signaling pathways.* Activation on signaling pathways expression after stimulus with agonists of $P2Y_2R$ ATP and UTP (100 μM), flow cytometry was performed at 3, 15, 30 and 60 minutes after nucleotide stimulation in Kyse-30 (**A**), Kyse-450 (**B**), and OE-33 (**C**). Two-Way ANOVA followed by Bonferroni post-test calculates statistical differences between time-points, which bar was compared to control, data are present as ± SEM ***p<0.001 and *p<0.05. data are present as ± SEM

Fig. 7 Basal enzymatic capacity in adenocarcinoma and squamous cell carcinoma cell lines. (**A**) ATPase capacity (**B**) ADPase capacity and (**C**) UTPase capacity. Samples were preincubated for 10 min at 37 °C be- fore starting the reaction with the addition of substrate (ATP, ADP or UTP) to a final concentration of 100 μM. The quantification of Pi released was reached using a spectrophotometer at 630 nm. One-Way ANOVA followed by Tukey post-test were carried out to calculate changes between the groups, data are present as ± SEM *p<0.05.

Fig. 8 Evaluation of intracellular calcium. Kyse-30 (**A**) and Kyse-450 (**B**) cells were pre-treated with only medium without FBS or AR-C 118925XX (ARC in the graph) (20 μM), during Calcium imaging cells were stimulated with ATP or UTP (100 μM). Transient $[Ca2+]_i$ was achieved by the change in fluorescence ($\Delta F/F_0$). EAC cell OE-33 did not shows basal dye fluorescence, making it impossible to carry out the experiment. One-Way ANOVA followed by Tukey post-test were used to assess differences between the groups, data are present as ± SEM ***p<0.001 and *p<0.05.

Paper Figures

Figure 1



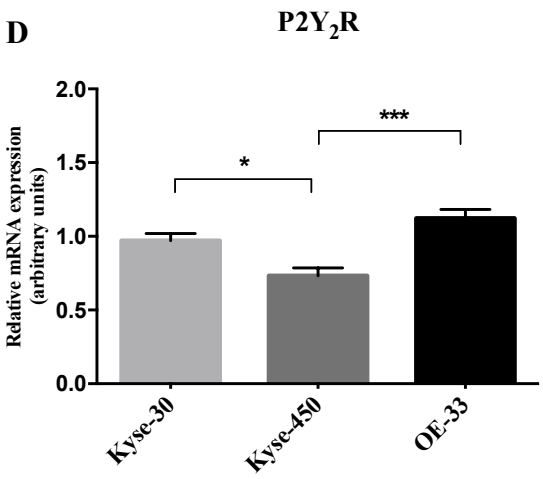
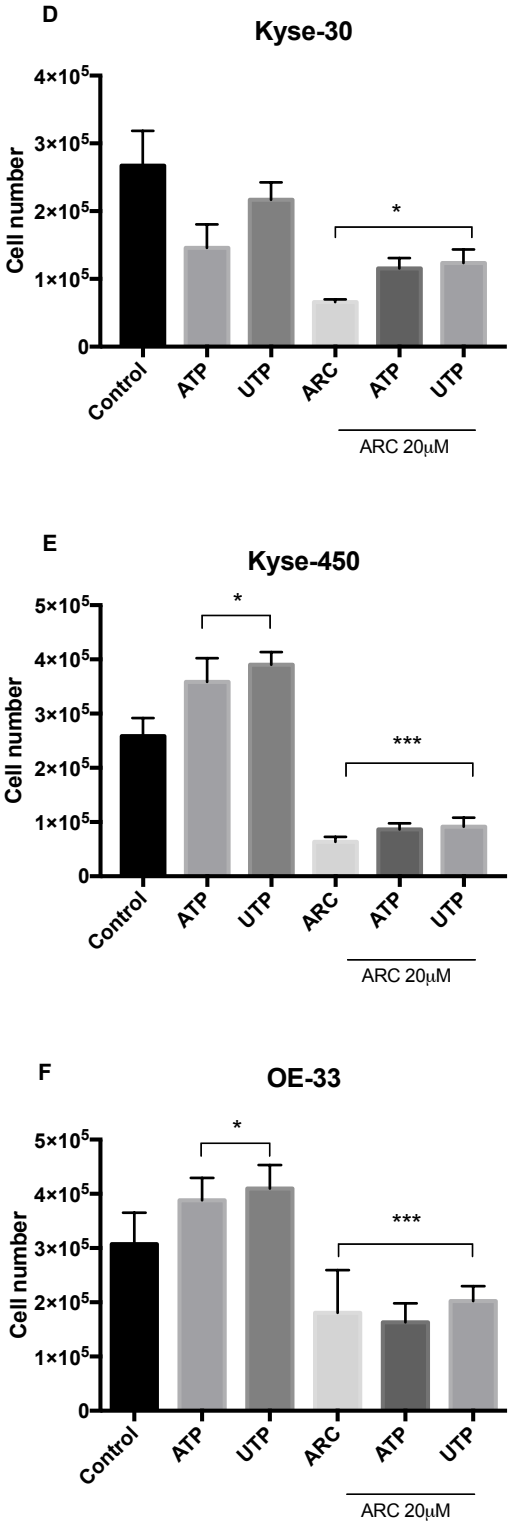


Figure 2


D
Kyse-30
Cell number
4×10^5
3×10^5
2×10^5
1×10^5
0
*
Control
ATP
UTP
ARC
ATP
UTP
ARC 20μM
E
Kyse-450
Cell number
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
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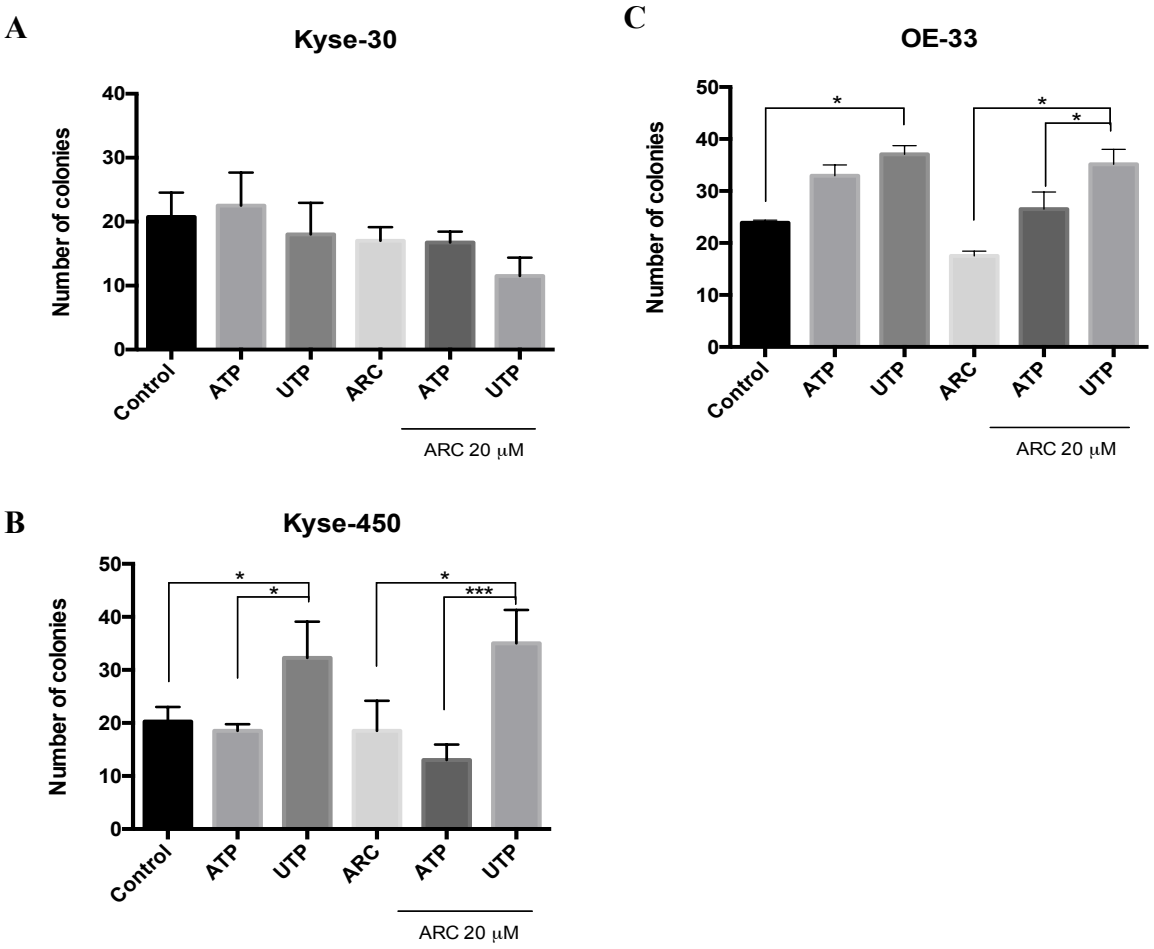
Control
ATP
UTP
ARC
ATP
UTP
ARC 20μM
F
OE-33
Cell number
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
*
***
Control
ATP
UTP
ARC
ATP
UTP
ARC 20μM

Figure 3


A
Kyse-30
Number of colonies
40
30
20
10
0
Control
ATP
UTP
ARC
ATP
UTP
ARC 20 μM
C
OE-33
Number of colonies
50
40
30
20
10
0
*
*
*
Control
ATP
UTP
ARC
ATP
UTP
ARC 20 μM
B
Kyse-450
Number of colonies
50
40
30
20
10
0
*
*
*
***
Control
ATP
UTP
ARC
ATP
UTP
ARC 20 μM

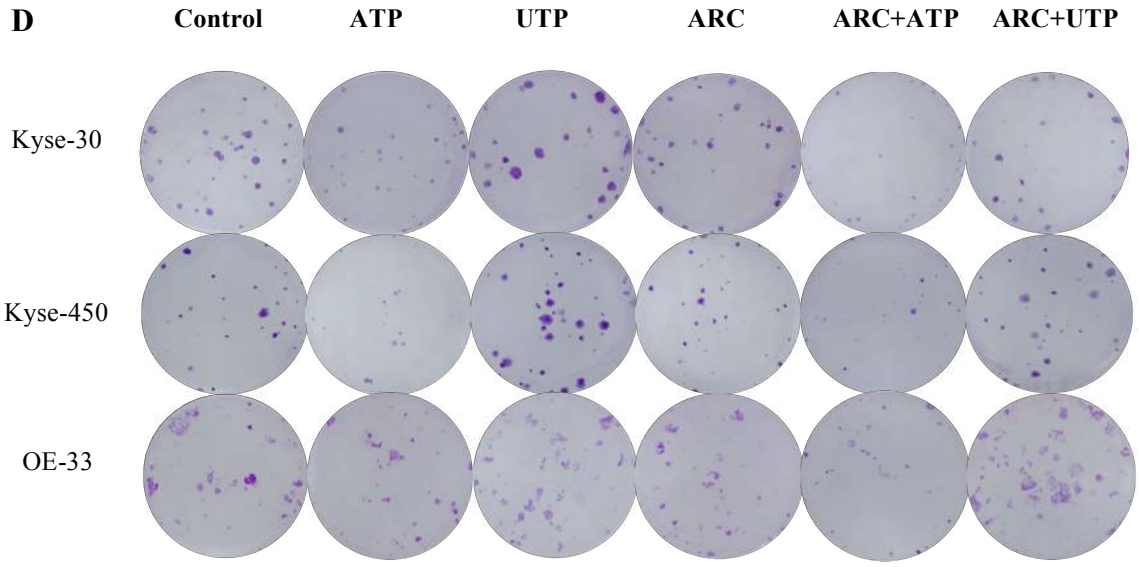

D
Control
ATP
UTP
ARC
ARC+ATP
ARC+UTP
Kyse-30
Kyse-450
OE-33

Figure 4

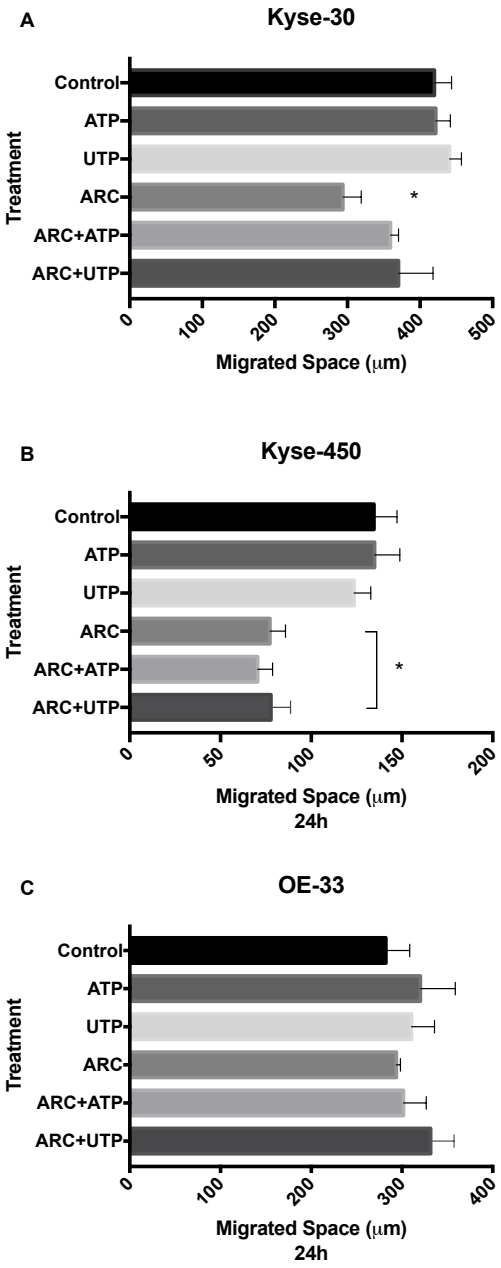

A
Kyse-30
Control
ATP
UTP
ARC
ARC+ATP
ARC+UTP
Treatment
*
0
100
200
300
400
500
Migrated Space (μm)
B
Kyse-450
Control
ATP
UTP
ARC
ARC+ATP
ARC+UTP
Treatment
*
0
50
100
150
200
Migrated Space (μm)
24h
C
OE-33
Control
ATP
UTP
ARC
ARC+ATP
ARC+UTP
Treatment
0
100
200
300
400
Migrated Space (μm)
24h

Figure 4

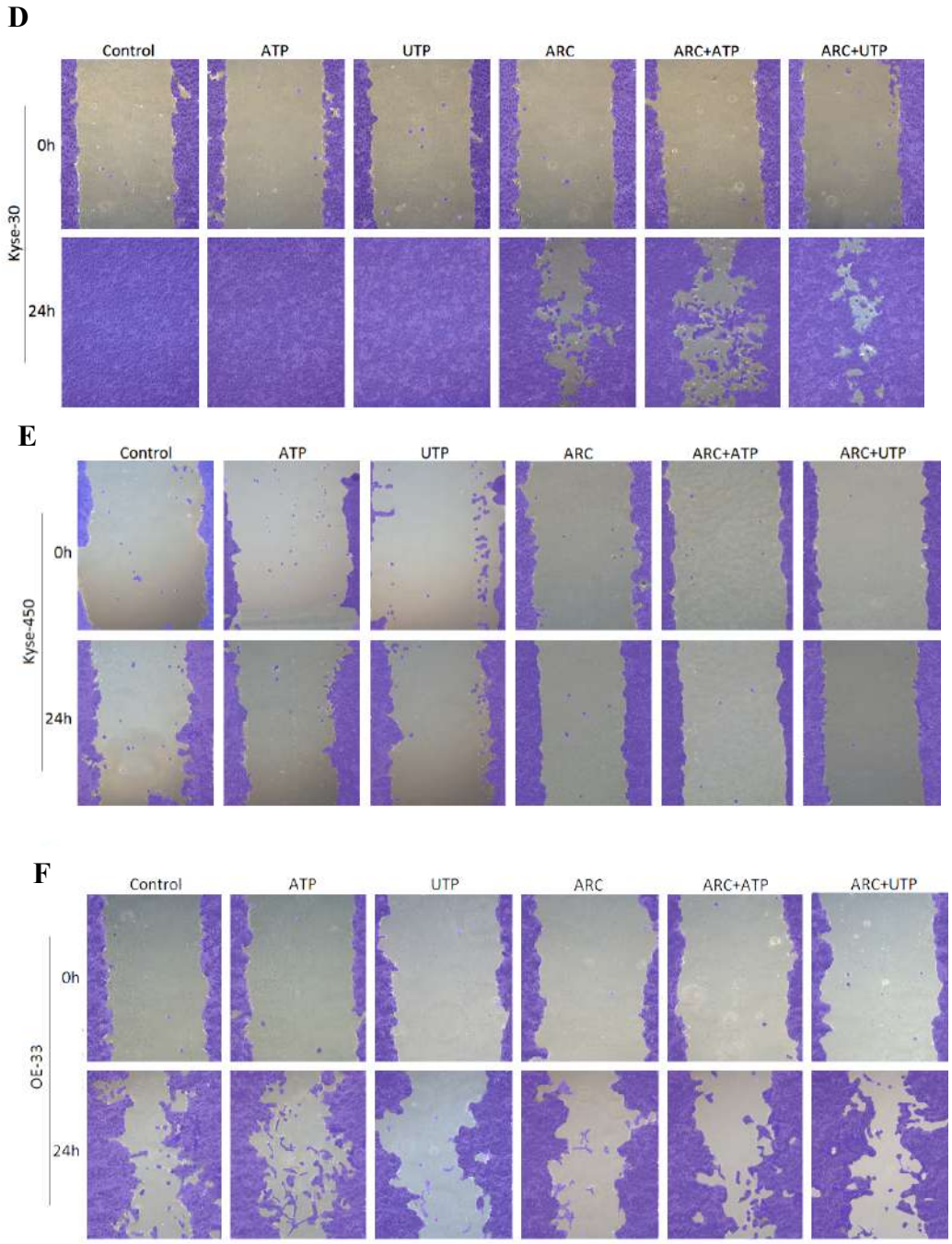

D
Control
ATP
UTP
ARC
ARC+ATP
ARC+UTP
0h
24h
Kyse-30
E
Control
ATP
UTP
ARC
ARC+ATP
ARC+UTP
0h
24h
Kyse-450
F
Control
ATP
UTP
ARC
ARC+ATP
ARC+UTP
0h
24h
OE-33

Figure 5

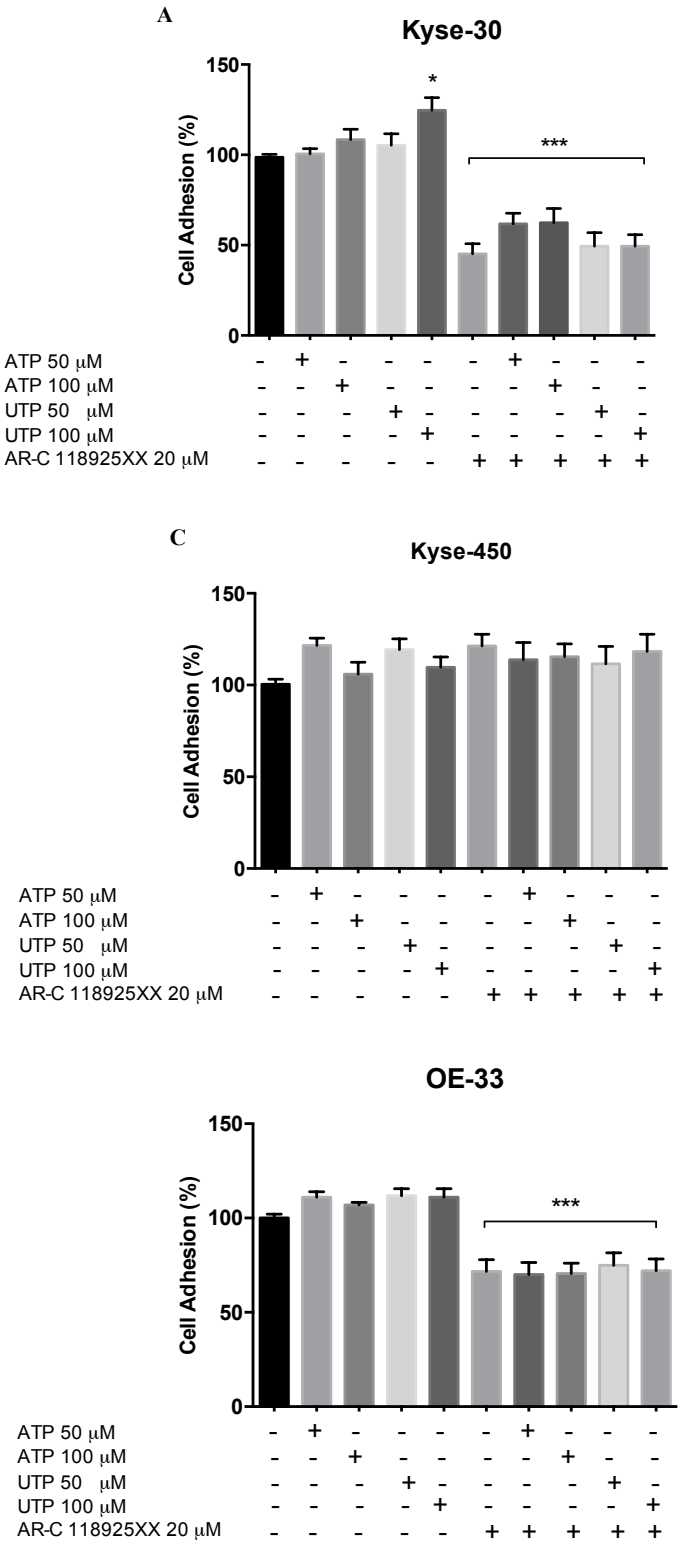

A
Kyse-30
Cell Adhesion (%)
150
100
50
0
*
***
ATP 50 μM - + - - - - + - - -
ATP 100 μM - - + - - - - + - -
UTP 50 μM - - - + - - - - + -
UTP 100 μM - - - - + - - - - +
AR-C 118925XX 20 μM - - - - - + + + + +
C
Kyse-450
Cell Adhesion (%)
150
100
50
0
ATP 50 μM - + - - - - + - - -
ATP 100 μM - - + - - - - + - -
UTP 50 μM - - - + - - - - + -
UTP 100 μM - - - - + - - - - +
AR-C 118925XX 20 μM - - - - - + + + + +
OE-33
Cell Adhesion (%)
150
100
50
0
***
ATP 50 μM - + - - - - + - - -
ATP 100 μM - - + - - - - + - -
UTP 50 μM - - - + - - - - + -
UTP 100 μM - - - - + - - - - +
AR-C 118925XX 20 μM - - - - - + + + + +

Figure 6

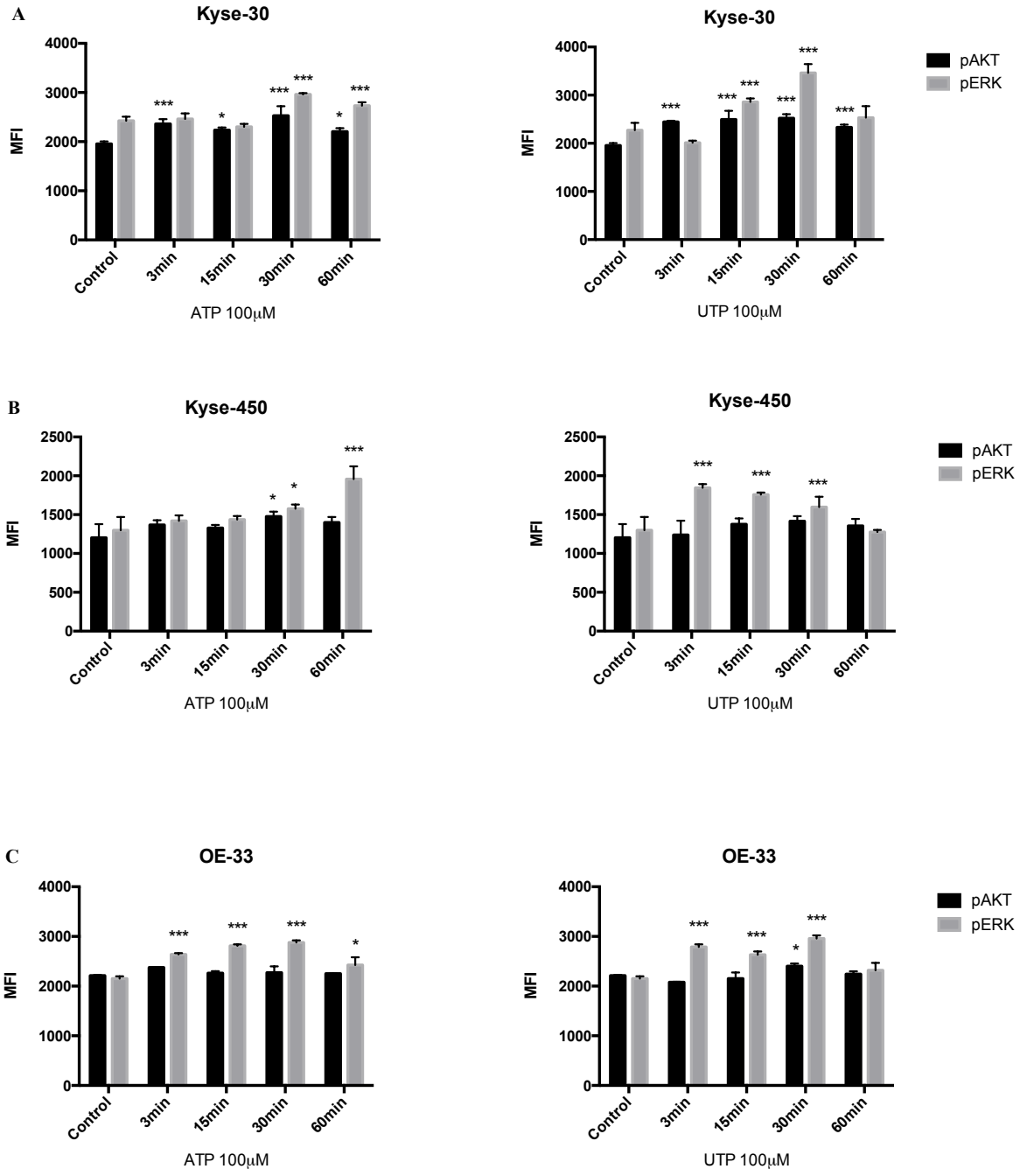

A
Kyse-30
MFI
4000
3000
2000
1000
0
Control
3min
15min
30min
60min
ATP 100μM
Kyse-30
UTP 100μM
pAKT
pERK
B
Kyse-450
2500
2000
1500
1000
500
ATP 100μM
UTP 100μM
C
OE-33
ATP 100μM
UTP 100μM

Figure 7

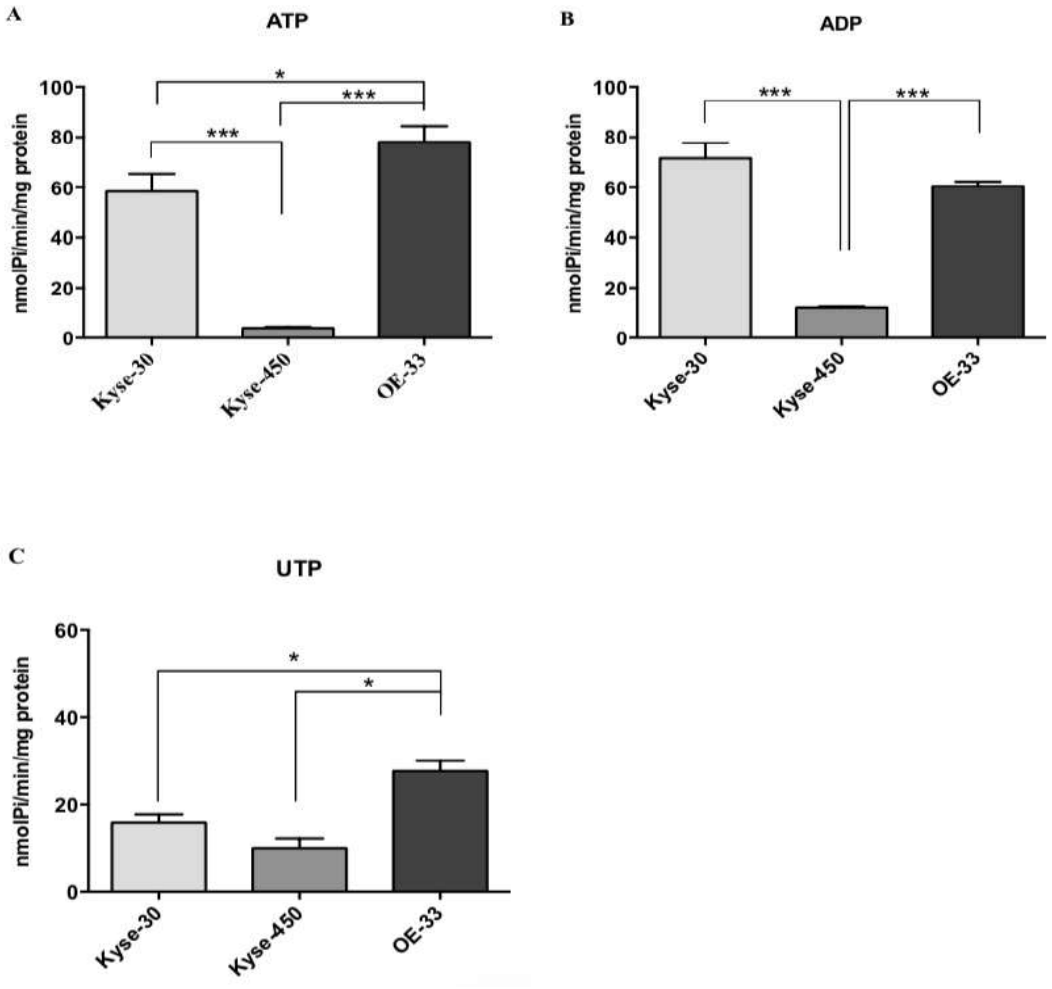
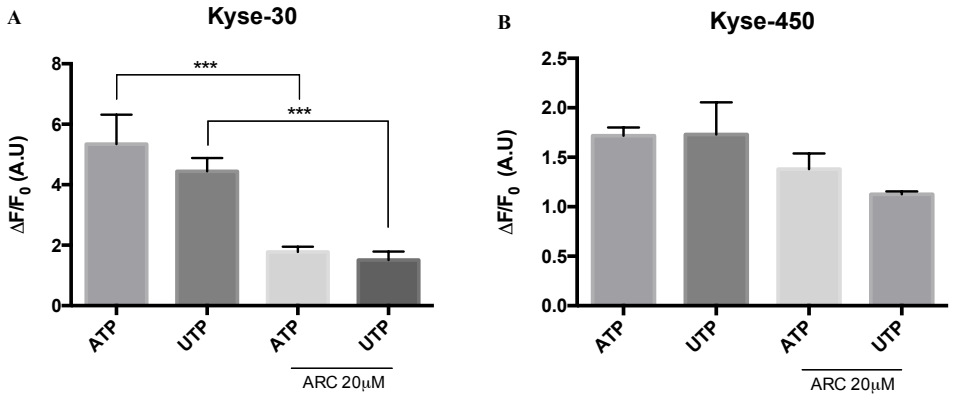

A
ATP
nmolPi/min/mg protein
100
80
60
40
20
0
*
***
***
Kyse-30
Kyse-450
OE-33
B
ADP
nmolPi/min/mg protein
100
80
60
40
20
0
***
***
Kyse-30
Kyse-450
OE-33
C
UTP
nmolPi/min/mg protein
60
40
20
0
*
*
Kyse-30
Kyse-450
OE-33

Figure 8


A
Kyse-30
***
***
ΔF/F0 (A.U)
8
6
4
2
0
ATP
UTP
ATP
UTP
ARC 20μM
B
Kyse-450
ΔF/F0 (A.U)
2.5
2.0
1.5
1.0
0.5
0.0
ATP
UTP
ATP
UTP
ARC 20μM

Supplementary Material

Table 1. Clinical data of esophageal cancer patients

Clinical data of patients (n= 60)	Adenocarcinoma (n=10)	Squamous cell carcinoma (n=50)
Histology	16,1%	83,9%
Age	75 (+/- 10,55)	63,65 (+/- 9,39)
Sex		
Men	60%	77,5%
Women	40%	22,5%
Dysphagia/odynophagia	50%	84%
Weight loss	30%	60%
Surgery		20%
Lymphadenectomy	Absent data	2%
Esophagectomy	1%	18%
Radiotherapy	40%	62%
Chemotherapy	40%	65%
Tobacco use	30%	60%
Alcohol use	Absent data	36%
Death	10%	83,7%

Data shown as n (%) or mean (SD).

Table 2. P2Y gene expression in esophageal cancer cell lines

Receptor	Kyse-30	Kyse-450	OE-33
$P2Y_1$	+++	+++	+
$P2Y_2$	+	+	++
$P2Y_4$	++	+	++
$P2Y_6$	++	++	absent
$P2Y_{11}$	++	+	+
$P2Y_{12}$	+	+	+
P2Y13	absent	absent	absent
$P2Y_{14}$	absent	+	absent

+ Low expression, ++ Moderate expression ++ High expression. The quantitative criteria as follows: ≥1= low expression, 1< and ≤3 = moderate expression, 3> = high expression.

4.3 CAPÍTULO III

Artigo em preparação.

Purinergic Receptor $P2Y_{12}$ Participates in Human Esophageal Squamous Cell Carcinoma Proliferation

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[4]Instituto Nacional do Câncer José Alencar Gomes da Silva, Praça Cruz Vermelha, 23. Centro, 20230-130, Rio de Janeiro, RJ, Brasil.

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ABSTRACT

Introduction: esophageal cancer is a prominent disease with high mortality rates due to late diagnosis and absence of specific symptoms. It is divided in esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC); these subtypes diverge in histological cell type affected, risk factors and outcome. Furthermore, the incidence changes according to geographic location, ESCC had higher prevalence in North China, Asia, Iran and South America, including the south of Brazil. Purinergic signaling is widely associated with several cancers, and different purinergic receptors are expressed in tumor tissues. The $P2Y_{12}$ purinergic receptor ($P2Y_{12}R$) is activated by ADP and broadly elucidated in platelets; these cells also had influence in tumor microenvironment mainly by promoting cancer cell metastasis and favors tumor growth. In this context, researches have suggested the $P2Y_{12}R$ blockade with different antagonists in cancer prevention and treatment. Here we used *in vitro* experiments to investigate the role of $P2Y_{12}R$ in ESCC cells proliferation after treatment with ADP and $P2Y_{12}R$ antagonist clopidogrel. Our results demonstrated that cells express the target receptors to ADP ($P2Y_1R$ and $P2Y_{12}R$). Moreover, ADP stimulus was capable of inducing direct cell proliferation in ESCC cells and the $P2Y_{12}R$ antagonist, clopidogrel, was able to decrease this capacity in different concentrations. In addition, we observe that clopidogrel treatment leads to an increase in cells with acridine orange dots, an indicative of cell autophagy.

Keywords: purinergic signaling, ADP, esophagus, proliferation, clopidogrel, squamous cell carcinoma.

Introduction

Esophageal cancer comprises two histological main types; esophageal squamous cell carcinoma (ESCC) that rises from epithelial cells and oesophageal adenocarcinoma (EAC) that emerge from glandular cells (Zhang, 2013). They have divergent risk factors and genetic predisposition. ESCC is the most common type in the world, but over the decades its incidence has been reduced and in contrast, the EAC rates have grown in well-developed countries in the past four decades (Napier *et al.*, 2014).

In Brazil, 96% of cases of esophagus are ESCC, and the southern region of the country has the highest incidence. In the southern of Brazil, the incidence of ESCC is higher than in the rest of the country, occupying the sixth most incident cancer in men and the fifteenth among women (Instituto Nacional Do Câncer, 2018). Dysplastic originator injuries can be discovered for both ESCC and EAC using endoscopy and noninvasive screening approaches, but routine screening is not currently praised in low risk areas or for low risk individuals (Van Laethem *et al.*, 2016).

Local ablative treatment of these dysplastic injuries results in outstanding long-term results, without the necessity for broad esophageal resection or severe oncological treatment, and some early cancers may also be treated successfully with endoscopic resection (Lordick *et al.*, 2016). Because there are no specific symptoms, and because there are no prevention and screening programs, esophageal cancer is commonly found at an advanced stage, which compromises treatment efficacy and decreases survival rates (Arnold *et al.*, 2015; Backemar *et al.*, 2016).

The purinergic system (Burnstock, 1972) and its receptors have been characterized over the years. The purinergic receptors are divided into G-coupled protein P1 receptors (A_1, A_2A, A_2B and A_3), activated by adenosine (Fredholm, B. B. *et al.*, 2001). Ionotropic P2X (1-7) receptors are activated by ATP (Burnstock, 2004; Abbracchio *et al.*, 2009). And, P2Y

(1,2,4,6,11,12,13,14) that can be activated by ADP, ATP, UDP or UTP, depending on the type of receptor stimulated (Burnstock, 2004). In the extracellular space, there are enzymes responsible for the degradation of the nucleotides in their respective nucleosides, named as ectonucleotidases. The CD39 (ectonucleoside triphosphate diphosphohydrolase 1, E-NTPDase1) hydrolyzes ATP and ADP into AMP, while CD73 (ecto-5'-nucleotidase, Ecto5´NTase) dephosphorylates AMP into adenosine (Antonioli, Pacher*, et al.*, 2013; Kanthi, Yogendra M. *et al.*, 2014).

A large number of studies have shown the relationship of the purinergic system in cancer scenery (Burnstock e Di Virgilio, 2013), participating in both pro-tumor and anti-tumor mechanisms in a wide-range of tumor types like colon cancer cells proliferation-induced by ATP (Buzzi *et al.*, 2010) or proliferation suppressed by ATP (Yaguchi *et al.*, 2010), in breast cancer $P2Y_2R$ supports cell migration (Chadet *et al.*, 2014) the $P2Y_{12}R$ inhibition lead a decrease metastasis (Gebremeskel *et al.*, 2015).

ADP stimulates the $P2Y_{12}R$, a Gi-coupled receptor that acts by modulation of adenylate cyclase leading in cAMP decrease and also activates P2Y1R, a Gq-coupled receptor that activates phospholipase β (PLC β) resulting in intracellular calcium increase (Burnstock, 2004). $P2Y_1R$ and $P2Y_{12}R$ were related with main functions performed by platelets, such as shape changes, aggregation, clot retraction and granule secretion (Kahner *et al.*, 2006; Hechler e Gachet, 2011; Jin *et al.*, 2014).

Taking into account the platelets participation in metastasis dissemination (Camerer *et al.*, 2004), researches have been considering $P2Y_{12}R$ role in several types of tumors like ovarian, breast, lung, tongue and pancreas (Sarangi *et al.*, 2013; Gebremeskel *et al.*, 2015; Tamagawa *et al.*, 2016; Cho *et al.*, 2017; Elaskalani *et al.*, 2017). Although the $P2Y_{12}$ have been well characterized in platelets and in different tumors, there are no studies addressing the function of this receptor in esophageal cancer.

Here we used two different cell lines representative of human ESCC (Kyse-30 and Kyse-450) to accesses ADP role in human esophageal cancer. The $P2Y_{12}R$ and $P2Y_1R$ expression was accessed by RT-qPCR, and we evaluated cell viability and proliferation, clonogenic capacity, and autophagy after pharmacological modulation with ADP and with the $P2Y_{12}R$ antagonist (clopidogrel).

Material and Methods

Reagents

The ADP was purchased from Santa Cruz Biotechnology (CA, USA) and the Clopidogrel Bisulfate was obtained from (Pharma Nostra, NED, EU) and was used as $P2Y_{12}R$ antagonist. Both were weighed and diluted in sterile water for injectable. Acridine orange was purchased from Sigma Aldrich (MO, EUA).

Cell Culture

Human ESCC (Kyse-30 and Kyse-450) cell lines were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) (both purchased from Gibco, NY, USA), 50 units of penicillin/mL, 50 mg streptomycin/mL, and fungizone 250 μg/mL purchased from Sigma Aldrich (MO, EUA) and incubated as standard conditions (37 °C, 5% CO2, and 95% humidity).

RNA Isolation and Real-time qPCR

The total RNA was isolated from esophageal cancer cell lines (Kyse-30 and Kyse-450) with TRIzol® Reagent (Life Technologies) in accordance with the manufacturer's instructions. Gene expressions of $P2Y_1R$ and $P2Y_{12}$ R were determined by RT-qPCR. RNA purity (Abs 260/280nm ~2.0) and concentration were determined by Nanodrop® Lite. The

Deoxyribonuclease I (Sigma-Aldrich) was used to eliminate genomic DNA contamination in accordance with the manufacturer's instructions. The cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega, WI, USA) from 1 μg of the total RNA, following the manufacturer´s instruction. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis on the 7500 Real-time PCR System (Applied Biosystems, CA, EUA). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72°C for elongation. Finally, a melting-curve analysis was included, and fluorescence measured from 60 to 99 °C to confirm the specificity of primers and absence of primer-dimers and exhibited in all cases one single peak. All experiments were carried out in quadruplicate and reverse transcriptase negative control was used by substituting the templates for DNase/RNase-free distilled water and included in each PCR reaction. *Gapdh*, *B2M* and *18S* were used as reference genes for normalization. The sequences of reverse and forward primers are in Table 1. The efficiency per sample was calculated using LinRegPCR 2016.1 Software (http://LinRegPCR.nl) and the stability of the references genes, and the optimal number of reference genes according to the pairwise variation (*V*) was analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Cq}$ method (Bustin *et al.*, 2013).

Cell viability

Cell mitochondrial viability was evaluated by MTT method (Mosmann, 1983). Esophageal cancer cells were seeded at $5x10^3$ cells per well in 96-well plates. The cells were treated with clopidogrel bisulfate (50, 100, 150, 200, 300, 500 μM) or 100 μM ADP only and clopidogrel bisulfate (50, 100, 150, 200, 300, 500 μM) for 20 minutes plus 100 μM ADP, in the control group the cells received only RPMI media. The experiment was carried out for 24, 48,

and 72 h. After the medium was removed, the cells were washed with calcium magnesium-free medium (CMF) and 100 µl of MTT solution (MTT 5 mg/ml in PBS in 90 % RPMI/10 % FBS) was added to the cells and incubated for 3 h. The media was removed, and formazan crystals were dissolved with 100 µL of dimethyl sulfoxide (DMSO). The absorbance was quantified at 540 nm (Spectra Max M2e, Molecular Devices). This assay was conducted in quadruplicate in four independent experiments, and results are expressed in percentage in relation to control.

Cell counting

Kyse-30 and Kyse-450 were seeded at density $2x10^4$ in 24-wells plate. Twenty-four hours after plating, cells were treated with 100 µM ADP, clopidogrel bisulfate (150, 300 and 500 µM) and clopidogrel bisulfate (150, 300 and 500 µM) plus 100 µM ADP, the control group received only RPMI medium. After 24 h of incubation the cells were washed with 200µl of PBS, trypsinized and counted in Cell Coutess II equipment (Life Technologies®). Cell counting was carried out in triplicate in three independent experiments and results were expressed in absolute cell number of living cells.

Clonogenic Assay

This assay allows the evaluation of polyclonal cells ability and it was conducted according to Rafehi *et al*., (2011) (Rafehi *et al.*, 2011). Kyse-30 and Kyse-450 (2×10^2) cells were seeded in a 24-wells plate. After 24 hours, the cells were treated with ADP (100 µM), clopidogrel bisulfate (150 and 300 µM) and clopidogrel bisulfate (150 and 300 µM) plus ADP (100 µM). The control group received only RPMI medium. The experiment was conducted for 7 days; during this time the cells received a pulse of ADP (100 µM) along with the medium exchange every 48 hours. After 7 days the cells were washed with PBS, fixed with 4% formalin for 20 minutes and stained with violet crystal for 10 minutes, washed twice with PBS and kept at room temperature for drying. The colony quantification was achieved by Image J software

and expressed as absolute number. This assay was carried out in triplicate.

Identification of Autophagic Cells

With the objective of evaluating whether $P2Y_{12}R$ blockage induces cell autophagy, we used cell staining with Acridine Orange (AO). This compound is permeable, and it is fixed in acidic vesicular organelles such as autolysosomes that are increased in autophagy process. AO is a green fluorophore and shifts to red fluorescence when trapped in autolysosomes, so this fluorescence can be quantified, and it allows us to estimate the cells that are in late step of autophagy (Thome *et al.*, 2016). Kyse-30 and Kyse-450 cells were plate at 4 x 10^4 density in 12-wells plate and treated with clopidogrel (150 and 300 μM), and the control group received only RPMI medium for 24 hours. Later, the treatment was drawn and a solution with 1 μg of AO per mL of RPMI medium (pH 7.4) was added in each well. Cells were incubated for 20 minutes in the dark at standard incubator. The pictures were captured via an inverted optical fluorescence microscope (20x objective) (Olympus IX71), the excitation/emission wavelength for green fluorescence (blue filter) was 480-500nm/509-543 and for red fluorescence (green filter) was (542.5-567.5)/ (579/631) excitation/emission, respectively. Pictures for each well were captured in the same field with blue and green filter. The images obtained were overlapped and orange and red dots were quantified, both with Image J software.

Statistical analysis

Data were analyzed by Student's T-test or one-way analysis of variance (one-way ANOVA) followed by Tukey post hoc test, using GraphPad Software 6.0 (San Diego, CA, U.S.A.). Data are presented as mean ± SEM; p values <0.05 indicate statistical significance.

Results

Esophageal cell lines express $P2Y_1$ and $P2Y_{12}$

To evaluate if the ESCC cells express the P2Y receptors activated by ADP, we verified the expression of $P2Y_1R$ and $P2Y_{12}R$. The results of RT-qPCR showed that both cell lines express P2Y1 and P2Y12 receptors. The $P2Y_1R$ expression was significantly higher in Kyse-30 when compared to Kyse-450 cells ($p<0.05$) (**Fig.1A**). Regarding to $P2Y_{12}R$, there was no significant difference in the expression levels of this receptors between the cells studied (**Fig.1B**).

$P2Y_{12}R$ blockage inhibited esophageal cancer cell proliferation

Since both ESCC cell lines studied express ADP binding receptors $P2Y_{12}$ and $P2Y_1$ receptors, and that these cells are able to hydrolyze ATP to ADP, we accessed the effects of ADP treatment and the antagonist in the cell mitochondrial viability. Kyse-30 and Kyse-450 cells were treated with $P2Y_{12}R$ agonist (ADP 100 μM) and with the antagonist (clopidogrel bisulfate) for 24, 48 and 72h and MTT assay was performed. In Kyse-30, at 24h, we observed that ADP alone increased cell viability. On the other hand, clopidogrel alone (100, 150, 200, 300 μM) decreased significantly cell viability when compared to control ($p<0.05$), and with clopidogrel 500 μM the cell viability was even lower ($p<0.001$). Interestingly, treatment with clopidogrel (100, 150, 200, 300, 500 μM) plus ADP also decreased cell viability in relation to control ($p<0.05$) (**Fig.2A**). After 48h all clopidogrel concentrations, except 50 μM alone or plus ADP, lead to a significant decrease in cell viability when compared to control ($p<0.001$) (**Fig.2B**). When cells were treated for 72h, clopidogrel at 100, 150, 200 μM ($p<0.05$) and 500 μM ($p<0.001$) or clopidogrel 50 μM plus ADP ($p<0.05$), 150, 200, 300 and 500 μM plus ADP ($p<0.001$) decreased in a significant manner cell viability in comparison with the control (**Fig.2C**). In Kyse-450 cells, after 24h of treatment, ADP induced an increase of viable cells, and only clopidogrel at 500 μM decreased significantly cell viability in comparison to control

($p<0.001$) (**Fig.2D**). After 48h, clopidogrel 100 μM ($p<0.05$) and 500 μM ($p<0.001$) induced a significant decrease in cell viability in relation to control, and treatment with clopidogrel (100, 150, 200, 300, 500 μM) plus ADP also induced a significant reduction in the viable cells (**Fig.2E**). Finally, after 72h of treatment, clopidogrel (200, 300 μM; $p<0.05$) and 500 μM ($p<0.001$) alone or clopidogreal 100-500 μM of clopidogrel in combination with ADP ($p<0.05$), diminished the viability of the cells (**Fig.2F**).

The cell counting was conducted with ESCC (Kyse-30 and Kyse-450) cell lines after 24h treatment with ADP 100 μM, clopidogrel (150, 300 and 500 μM) or clopidogrel in combination with ADP (100 μM). Kyse-30 cells treated with clopidogrel (150, 300 and 500 μM) displayed a significant decrease in cell number when compared to control ($p<0.001$) alone or with clopidogrel (300 and 500 μM) plus ADP ($p<0.001$) (**Fig.3A**). Kyse-450 showed a decrease in cell number when treated with clopidogrel 150 μM ($p<0.05$), 300 and 500 μM ($p<0.001$) compared to control. Interestingly ADP alone lead a significant increase in number of cell in comparison with the control ($p<0.05$), and this effect was abolished when the cells received clopidogrel (150, 300 and 500 μM) plus ADP ($p<0.05$; $p<0.001$; $p<0.001$, respectively) (**Fig.3B**).

ADP induces colony formation in ESCC cell lines

The capacity of ESCC cells to generate progeny was established after 7 days of treatment with ADP 100 μM, clopidogrel 150 and 300 μM alone or in combination with ADP. The cells received $P2Y_{12}R$ antagonist (clopidogrel) only in the first day of treatment. After, they received or not a pulse with ADP 100 μM every 48 hours together with the medium exchange, and control group received only medium. The experiment was conducted in this way in order to evaluate the duration of the receptor pharmacological blockade. Both cells responded in a similar manner; ADP lead an increase in colony formation ($p<0.05$), and

$P2Y_{12}R$ blockage caused a decrease in colony number ($p<0.05$) in comparison to control (**Fig. 4A** and **4B**). The treatments with clopidogrel 300 μM alone or clopidogrel plus ADP caused abolition in the formation of colonies. The **Fig.4C** was a representative image of the results in Kyse-30 and Kyse-450.

Treatment with $P2Y_{12}$ antagonist increases acridine orange staining

In order to confirm ADP-$P2Y_{12}$ effects in ESCC growth, cells were treated with the antagonist clopidogrel at 150 and 300 μM for 24 hours. Kyse-30 cell line treated with clopidogrel (150 and 300 μM) showed a significant increase in cells containing orange dots when compared to control ($p<0.05$) (**Fig.5A**). Although Kyse-450 had displayed high staining in the control group, the treatment with clopidogrel (150 and 300 μM) showed a significant increase in AO staining when compared to control ($p<0.001$) (**Fig.5B**). However, only this experiment does not allow us to support the idea that the cells shift to autophagy with the $P2Y_{12}R$ antagonism.

Discussion

Most of the available studies demonstrate the indirect action of $P2Y_{12}R$ on tumor cells through their modulation in platelets (Gay e Felding-Habermann, 2011). Previous studies using *in vivo* models of ovarian cancer have shown that platelets play pro-tumor functions and the number of platelets present is closely related to tumor growth and aggressiveness, resulting in poor prognosis and low responsiveness to treatment (Stone *et al.*, 2012; Bottsford-Miller *et al.*, 2015). Platelets are able to communicate with tumor cells to and from there, favor the spread of metastases and support the formation of a pre-metastatic niche (Labelle *et al.*, 2014). Moreover, activated platelets can secrete ATP granules that bind in others purinergic receptors and have influence in several cancers (Wagstaff *et al.*, 2000; Schafer *et*

al., 2003; Yaguchi *et al.*, 2010; Qian *et al.*, 2014).

Taking into account that esophageal cancer remain a worldwide health problem with high mortalities rates and high impact in patient's quality life and new therapies was needed (Lagergren *et al.*, 2017), we showed here, for the first time, the influence of $P2Y_{12}R$ activiation via ADP in esophageal squamous cancer cells proliferation. ESCC cells utilized in this study express both receptors ($P2Y_1R$ and $P2Y_{12}R$) that are activated by the nucleotide ADP. Furthermore, both cell lines presented ATPase and ADPase activities. Interstingly, Kyse-30 cells presented higher enzymatic capacity in hydrolyzing ATP than Kyse-450 cells, indicating that ADP remains longer in the extracellular medium, and that this nucleotide could be capable of activating P2Y receptors. Still, we observed that the pharmacological modulation of $P2Y_{12}R$ with its antagonist (clopidogrel) was able to decrease in cell viability and proliferation at different concentrations.

Previously data showed that $P2Y_{12}R$ expression is essential to maintaining ovarian cancer cell number, and its blockade increase apoptosis and decrease cell proliferation *in vitro,* and $P2Y_{12}R$ knockout mice had a decline in ovarian tumor growth (Cho *et al.*, 2017). In addition, $P2Y_{12}R$ pharmacological blockade with ticagrelor and clopidogrel (both platelet aggregation inhibitors) resulted in lower lung metastasis in animal model of melanoma and breast cancer and increase animal survival (Gebremeskel *et al.*, 2015).

Besides favoring the mesenchymal-epithelial transition, platelets can also inhibit the function of natural killer cells in struggling tumor cells (Nieswandt *et al.*, 1999; Palumbo *et al.*, 2005; Miyashita *et al.*, 2015). All these functions have been linked to the activation of $P2Y_{12}R$ present in platelets by ADP (Ballerini *et al.*, 2018). Our data showed that ADP acts in human esophageal cancer cells, occasioning in increased cell proliferation. On the other hand, the $P2Y_{12}R$ blockade was able in inhibit cell proliferation. Confirming these data, the $P2Y_{12}R$ activation by ADP also leads to an increase in cell capacity of generating polyclones and its

inhibition with clopidogrel impaired this capacity.

Autophagy is the cell ability in recycling its components in tightly regulated lysosomal digestion manner. This process usually occurs under cellular stress conditions such as oxidative stress, growth factors and nutrients deprivation and hypoxia (Boya *et al.*, 2013). Studies have demonstrated that autophagy can protect malignant cells and acts in favor of the tumor or can control tumor progression and may participate in chemoresistance (Qu *et al.*, 2003; Takamura *et al.*, 2011; Wei *et al.*, 2013). Our results demonstrated that $P2Y_{12}R$ blockade with the antagonist clopidogrel displayed more cells presenting acidic vesicular organelles stained by AO staining than the control group. These results could suggest that cells treated with clopidogrel can be entering in autophagy condition, but supplementary experiments are needed to confirm that.

Collectively, our data showed an important involvement of ADP-P2YR for esophageal cancer cells maintenance, by participating in fundamental proliferation processes. We concluded that $P2Y_{12}R$ stimulated by ADP acts as pro-tumor mechanism, but this effect on tumor growth needs to be better investigated, as well the mechanisms of cell death or autophagy.

References

[1] Zhang, Y., Epidemiology of esophageal cancer. *World journal of gastroenterology : WJG,* **2013**, *19*, (34), 5598-5606.

[2] Napier, K.J.; Scheerer, M.; Misra, S., Esophageal cancer: A Review of epidemiology, pathogenesis, staging workup and treatment modalities. *World journal of gastrointestinal oncology,* **2014**, *6*, (5), 112-120.

[3] Instituto Nacional do Câncer, I., **2018**.

[4] Van Laethem, J.L.; Carneiro, F.; Ducreux, M.; Messman, H.; Lordick, F.; Ilson, D.H.; Allum, W.H.; Haustermans, K.; Lepage, C.; Matysiak-Budnik, T.; Cats, A.; Schmiegel, W.; Cervantes, A.; Van Cutsem, E.; Rougier, P.; Seufferlein, T., The multidisciplinary management of gastro-oesophageal junction tumours: European Society of Digestive Oncology (ESDO): Expert discussion and report from the 16th ESMO World Congress on Gastrointestinal Cancer, Barcelona. *Dig Liver Dis,* **2016**, *48*, (11), 1283-1289.

[5] Lordick, F.; Mariette, C.; Haustermans, K.; Obermannova, R.; Arnold, D.; Committee, E.G., Oesophageal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol,* **2016**, *27*, (suppl 5), v50-v57.

[6] Arnold, M.; Soerjomataram, I.; Ferlay, J.; Forman, D., Global incidence of oesophageal cancer by histological subtype in 2012. *Gut,* **2015**, *64*, (3), 381-387.

[7] Backemar, L.; Wikman, A.; Djarv, T.; Johar, A.; Lagergren, P., Co-morbidity after oesophageal cancer surgery and recovery of health-related quality of life. *Br J Surg,* **2016**, *103*, (12), 1665-1675.

[8] Burnstock, G., Purinergic nerves. *Pharmacological reviews,* **1972**, *24*, (3), 509-581.

[9] Fredholm, B.B.; AP, I.J.; Jacobson, K.A.; Klotz, K.N.; Linden, J., International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacological reviews,* **2001**, *53*, (4), 527-552.

[10] Abbracchio, M.P.; Burnstock, G.; Verkhratsky, A.; Zimmermann, H., Purinergic signalling in the nervous system: an overview. *Trends in neurosciences,* **2009**, *32*, (1), 19-29.

[11] Burnstock, G., Introduction: P2 receptors. *Current topics in medicinal chemistry,* **2004**, *4*, (8), 793-803.

[12] Antonioli, L.; Pacher, P.; Vizi, E.S.; Hasko, G., CD39 and CD73 in immunity and inflammation. *Trends in molecular medicine,* **2013**, *19*, (6), 355-367.

[13] Kanthi, Y.M.; Sutton, N.R.; Pinsky, D.J., CD39: Interface between vascular thrombosis and inflammation. *Curr Atheroscler Rep,* **2014**, *16*, (7), 425.

[14] Burnstock, G.; Di Virgilio, F., Purinergic signalling and cancer. *Purinergic signalling,* **2013**, *9*, (4), 491-540.

[15] Buzzi, N.; Boland, R.; Russo de Boland, A., Signal transduction pathways associated with ATP-induced proliferation of colon adenocarcinoma cells. *Biochimica et biophysica acta,* **2010**, *1800*, (9), 946-955.

[16] Yaguchi, T.; Saito, M.; Yasuda, Y.; Kanno, T.; Nakano, T.; Nishizaki, T., Higher concentrations of extracellular ATP suppress proliferation of Caco-2 human colonic cancer cells via an unknown receptor involving PKC inhibition. *Cell Physiol Biochem,* **2010**, *26*, (2), 125-134.

[17] Chadet, S.; Jelassi, B.; Wannous, R.; Angoulvant, D.; Chevalier, S.; Besson, P.; Roger, S., The activation of P2Y2 receptors increases MCF-7 breast cancer cells migration through the MEK-ERK1/2 signalling pathway. *Carcinogenesis,* **2014**, *35*, (6), 1238-1247.

[18] Gebremeskel, S.; LeVatte, T.; Liwski, R.S.; Johnston, B.; Bezuhly, M., The reversible P2Y12 inhibitor ticagrelor inhibits metastasis and improves survival in mouse models of cancer. *International journal of cancer,* **2015**, *136*, (1), 234-240.

[19] Hechler, B.; Gachet, C., P2 receptors and platelet function. *Purinergic signalling,* **2011**, *7*, (3), 293-303.

[20] Jin, H.; Eun, S.Y.; Lee, J.S.; Park, S.W.; Lee, J.H.; Chang, K.C.; Kim, H.J., P2Y2 receptor activation by nucleotides released from highly metastatic breast cancer cells increases tumor growth and invasion via crosstalk with endothelial cells. *Breast Cancer Res,* **2014**, *16*, (5), R77.

[21] Kahner, B.N.; Shankar, H.; Murugappan, S.; Prasad, G.L.; Kunapuli, S.P., Nucleotide receptor signaling in platelets. *J Thromb Haemost,* **2006**, *4*, (11), 2317-2326.

[22] Camerer, E.; Qazi, A.A.; Duong, D.N.; Cornelissen, I.; Advincula, R.; Coughlin, S.R., Platelets, protease-activated receptors, and fibrinogen in hematogenous metastasis. *Blood,* **2004**, *104*, (2), 397-401.

[23] Elaskalani, O.; Falasca, M.; Moran, N.; Berndt, M.C.; Metharom, P., The Role of Platelet-Derived ADP and ATP in Promoting Pancreatic Cancer Cell Survival and Gemcitabine Resistance. *Cancers (Basel),* **2017**, *9*, (10).

[24] Sarangi, S.; Pandey, A.; Papa, A.L.; Sengupta, P.; Kopparam, J.; Dadwal, U.; Basu, S.; Sengupta, S., P2Y12 receptor inhibition augments cytotoxic effects of cisplatin in breast cancer. *Med Oncol,* **2013**, *30*, (2), 567.

[25] Cho, M.S.; Noh, K.; Haemmerle, M.; Li, D.; Park, H.; Hu, Q.; Hisamatsu, T.; Mitamura, T.; Mak, S.L.C.; Kunapuli, S.; Ma, Q.; Sood, A.K.; Afshar-Kharghan, V., Role of ADP receptors on platelets in the growth of ovarian cancer. *Blood,* **2017**, *130*, (10), 1235-1242.

[26] Tamagawa, T.; Shinoda, M.; Honda, K.; Furukawa, A.; Kaji, K.; Nagashima, H.; Akasaka, R.; Chen, J.; Sessle, B.J.; Yonehara, Y.; Iwata, K., Involvement of Microglial P2Y12 Signaling in Tongue Cancer Pain. *J Dent Res,* **2016**, *95*, (10), 1176-1182.

[27] Bustin, S.A.; Benes, V.; Garson, J.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.; Wittwer, C.T.; Schjerling, P.; Day, P.J.; Abreu, M.; Aguado, B.; Beaulieu, J.F.; Beckers, A.; Bogaert, S.; Browne, J.A.; Carrasco-Ramiro, F.; Ceelen, L.; Ciborowski, K.; Cornillie, P.; Coulon, S.; Cuypers, A.; De Brouwer, S.; De

Ceuninck, L.; De Craene, J.; De Naeyer, H.; De Spiegelaere, W.; Deckers, K.; Dheedene, A.; Durinck, K.; Ferreira-Teixeira, M.; Fieuw, A.; Gallup, J.M.; Gonzalo-Flores, S.; Goossens, K.; Heindryckx, F.; Herring, E.; Hoenicka, H.; Icardi, L.; Jaggi, R.; Javad, F.; Karampelias, M.; Kibenge, F.; Kibenge, M.; Kumps, C.; Lambertz, I.; Lammens, T.; Markey, A.; Messiaen, P.; Mets, E.; Morais, S.; Mudarra-Rubio, A.; Nakiwala, J.; Nelis, H.; Olsvik, P.A.; Perez-Novo, C.; Plusquin, M.; Remans, T.; Rihani, A.; Rodrigues-Santos, P.; Rondou, P.; Sanders, R.; Schmidt-Bleek, K.; Skovgaard, K.; Smeets, K.; Tabera, L.; Toegel, S.; Van Acker, T.; Van den Broeck, W.; Van der Meulen, J.; Van Gele, M.; Van Peer, G.; Van Poucke, M.; Van Roy, N.; Vergult, S.; Wauman, J.; Tshuikina-Wiklander, M.; Willems, E.; Zaccara, S.; Zeka, F.; Vandesompele, J., The need for transparency and good practices in the qPCR literature. *Nature methods,* **2013**, *10*, (11), 1063-1067.

[28] Wink, M.R.; Lenz, G.; Braganhol, E.; Tamajusuku, A.S.; Schwartsmann, G.; Sarkis, J.J.; Battastini, A.M., Altered extracellular ATP, ADP and AMP catabolism in glioma cell lines. *Cancer letters,* **2003**, *198*, (2), 211-218.

[29] Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods,* **1983**, *65*, (1-2), 55-63.

[30] Rafehi, H.; Orlowski, C.; Georgiadis, G.T.; Ververis, K.; El-Osta, A.; Karagiannis, T.C., Clonogenic assay: adherent cells. *Journal of visualized experiments : JoVE,* **2011**, (49).

[31] Thome, M.P.; Filippi-Chiela, E.C.; Villodre, E.S.; Migliavaca, C.B.; Onzi, G.R.; Felipe, K.B.; Lenz, G., Ratiometric analysis of Acridine Orange staining in the study of acidic organelles and autophagy. *J Cell Sci,* **2016**, *129*, (24), 4622-4632.

[32] Gay, L.J.; Felding-Habermann, B., Contribution of platelets to tumour metastasis. *Nature reviews. Cancer,* **2011**, *11*, (2), 123-134.

[33] Stone, R.L.; Nick, A.M.; McNeish, I.A.; Balkwill, F.; Han, H.D.; Bottsford-Miller, J.; Rupairmoole, R.; Armaiz-Pena, G.N.; Pecot, C.V.; Coward, J.; Deavers, M.T.; Vasquez, H.G.;

Urbauer, D.; Landen, C.N.; Hu, W.; Gershenson, H.; Matsuo, K.; Shahzad, M.M.; King, E.R.; Tekedereli, I.; Ozpolat, B.; Ahn, E.H.; Bond, V.K.; Wang, R.; Drew, A.F.; Gushiken, F.; Lamkin, D.; Collins, K.; DeGeest, K.; Lutgendorf, S.K.; Chiu, W.; Lopez-Berestein, G.; Afshar-Kharghan, V.; Sood, A.K., Paraneoplastic thrombocytosis in ovarian cancer. *The New England journal of medicine,* **2012**, *366*, (7), 610-618.

[34] Bottsford-Miller, J.; Choi, H.J.; Dalton, H.J.; Stone, R.L.; Cho, M.S.; Haemmerle, M.; Nick, A.M.; Pradeep, S.; Zand, B.; Previs, R.A.; Pecot, C.V.; Crane, E.K.; Hu, W.; Lutgendorf, S.K.; Afshar-Kharghan, V.; Sood, A.K., Differential platelet levels affect response to taxane-based therapy in ovarian cancer. *Clin Cancer Res,* **2015**, *21*, (3), 602-610.

[35] Labelle, M.; Begum, S.; Hynes, R.O., Platelets guide the formation of early metastatic niches. *Proc Natl Acad Sci U S A,* **2014**, *111*, (30), E3053-3061.

[36] Qian, Y.; Wang, X.; Liu, Y.; Li, Y.; Colvin, R.A.; Tong, L.; Wu, S.; Chen, X., Extracellular ATP is internalized by macropinocytosis and induces intracellular ATP increase and drug resistance in cancer cells. *Cancer letters,* **2014**, *351*, (2), 242-251.

[37] Schafer, R.; Sedehizade, F.; Welte, T.; Reiser, G., ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. *Am J Physiol Lung Cell Mol Physiol,* **2003**, *285*, (2), L376-385.

[38] Wagstaff, S.C.; Bowler, W.B.; Gallagher, J.A.; Hipskind, R.A., Extracellular ATP activates multiple signalling pathways and potentiates growth factor-induced c-fos gene expression in MCF-7 breast cancer cells. *Carcinogenesis,* **2000**, *21*, (12), 2175-2181.

[39] Lagergren, J.; Smyth, E.; Cunningham, D.; Lagergren, P., Oesophageal cancer. *Lancet,* **2017**.

[40] Miyashita, T.; Tajima, H.; Makino, I.; Nakagawara, H.; Kitagawa, H.; Fushida, S.; Harmon, J.W.; Ohta, T., Metastasis-promoting role of extravasated platelet activation in tumor. *J Surg Res,* **2015**, *193*, (1), 289-294.

[41] Nieswandt, B.; Hafner, M.; Echtenacher, B.; Mannel, D.N., Lysis of tumor cells by natural killer cells in mice is impeded by platelets. *Cancer research,* **1999**, *59*, (6), 1295-1300.

[42] Palumbo, J.S.; Talmage, K.E.; Massari, J.V.; La Jeunesse, C.M.; Flick, M.J.; Kombrinck, K.W.; Jirouskova, M.; Degen, J.L., Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. *Blood,* **2005**, *105*, (1), 178-185.

[43] Ballerini, P.; Dovizio, M.; Bruno, A.; Tacconelli, S.; Patrignani, P., P2Y12 Receptors in Tumorigenesis and Metastasis. *Frontiers in pharmacology,* **2018**, *9*, 66.

[44] Boya, P.; Reggiori, F.; Codogno, P., Emerging regulation and functions of autophagy. *Nat Cell Biol,* **2013**, *15*, (7), 713-720.

[45] Qu, X.; Yu, J.; Bhagat, G.; Furuya, N.; Hibshoosh, H.; Troxel, A.; Rosen, J.; Eskelinen, E.L.; Mizushima, N.; Ohsumi, Y.; Cattoretti, G.; Levine, B., Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *The Journal of clinical investigation,* **2003**, *112*, (12), 1809-1820.

[46] Takamura, A.; Komatsu, M.; Hara, T.; Sakamoto, A.; Kishi, C.; Waguri, S.; Eishi, Y.; Hino, O.; Tanaka, K.; Mizushima, N., Autophagy-deficient mice develop multiple liver tumors. *Genes Dev,* **2011**, *25*, (8), 795-800.

[47] Wei, Y.; Zou, Z.; Becker, N.; Anderson, M.; Sumpter, R.; Xiao, G.; Kinch, L.; Koduru, P.; Christudass, C.S.; Veltri, R.W.; Grishin, N.V.; Peyton, M.; Minna, J.; Bhagat, G.; Levine, B., EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance. *Cell,* **2013**, *154*, (6), 1269-1284.

[48] Rho, H.W.; Lee, B.C.; Choi, E.S.; Choi, I.J.; Lee, Y.S.; Goh, S.H., Identification of valid reference genes for gene expression studies of human stomach cancer by reverse transcription-qPCR. *BMC Cancer,* **2010**, *10*, 240.

Table 1. Primer sequences used in RT-qPCR.

Gene	Forward and Reverse primers	Reference
18S	**F-**5'GTAACCCGTTGAACCCCATT-3'	**(Rho *et al.*, 2010)**
	R-5'-CCATCCAATCGGTAGTAGCG-3'	
B2M	**F-**5'-ACTGAATTCACCCCCACTGA-3'	**(Rho *et al.*, 2010)**
	R-5'-CCTCCATGATGCTGCTTACA-3'	
Gapdh	**F-**5'-TGCACCACCAACTGCTTA-3'	**(Rho *et al.*, 2010)**
	R-5'-GGATGCAGGGATGATGTTC-3'	
$P2Y_1R$	**F-**5'-AGGGGTGTGGACGTCGTGGT-3'	Designed by authors
	R-5'-CCCAGCAGGCACCTCTTTGGA-3'	
$P2Y_{12}R$	**F-**5'-GCCTGGATCCGTTCATCTAT-3'	Designed by authors
	R-5'-GGGACAGAGATGTTGCAGAA-3'	

Paper Figure Legends

Fig.1 ESCC cells express $P2Y_1R$ and $P2Y_{12}R$. **A** $P2Y_1R$ expression was higher in Kyse-30 than Kyse-450 ($p<0.05$). **B** $P2Y_{12}R$ expression, there was no significant difference between ESCC cells. One-way ANOVA followed by Tukey post-hoc test was used in statistical analysis. Error bars represent ±SEM. * $p<0.05$, *** $p<0.001$.

Fig.2 ESCC cell viability accessed by MTT assay. The cells were treated with Clopidogrel at 50, 100, 150, 200, 300, 500 μM alone or plus 100 μM ADP and 100 μM ADP, control group received RPMI 10% FBS. Kyse-30 after 24 (**A**), 48 (**B**) and 72 h (**C**). Kyse-450 after 24 (**D**), 48 (**E**) and 72 h (**F**). Results are expressed as percentage relative to the untreated control. One-way ANOVA followed by Tukey post-hoc test was used in statistical analysis. Error bars represent ±SEM. * $p<0.05$, *** $p<0.001$.

Fig.3 Cell counting was carried out in ESCC cell lines after 24h of treatment with clopidogrel 150, 300 and 500 μM alone or plus 100 μM ADP and 100 μM ADP, control group received RPMI 10% FBS. **A** Kyse-30; **B** Kyse-450. One-way ANOVA followed by Tukey post-hoc test was used in statistical analysis. Error bars represent ±SEM. * $p<0.05$, *** $p<0.001$, # increase in relation to control ($p<0.001$).

Fig.4 Colony formation capacity in ESCC cells performed after 7-days treatment. Cells were treated with clopidogrel 150, 300 μM alone or plus 100 μM ADP and 100 μM ADP, control group received RPMI 10% FBS. Only colonies with 50 cells or more were considering counting. **A** Kyse-30 and **B** Kyse-450 cells. One-way ANOVA followed by Tukey post-hoc test was used in statistical analysis. Error bars represent ±SEM. * $p<0.05$, # increase in relation to control ($p<0.05$). **C** representative images of clonogenic assay.

Fig.5 Acridine Orange staining in ESCC cells after 24h of treatment with clopidogrel 150, 300 μM. Cells containing orange dots were counted and expressed in percentage of total cell number. **A** Kyse-30 and **B** Kyse-450 cells. **C** representative images of Acridine Orange staining in ESCC cells. T-test was performed to access significant differences. Error bars represent ±SEM. * $p < 0.05$, *** $p < 0.001$.

Paper Figures

Figure 1

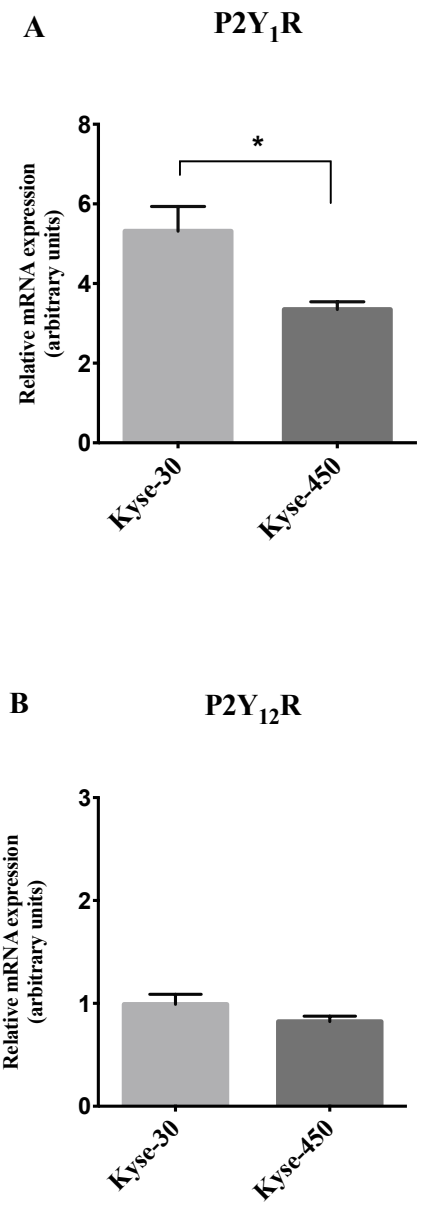


Figure 2

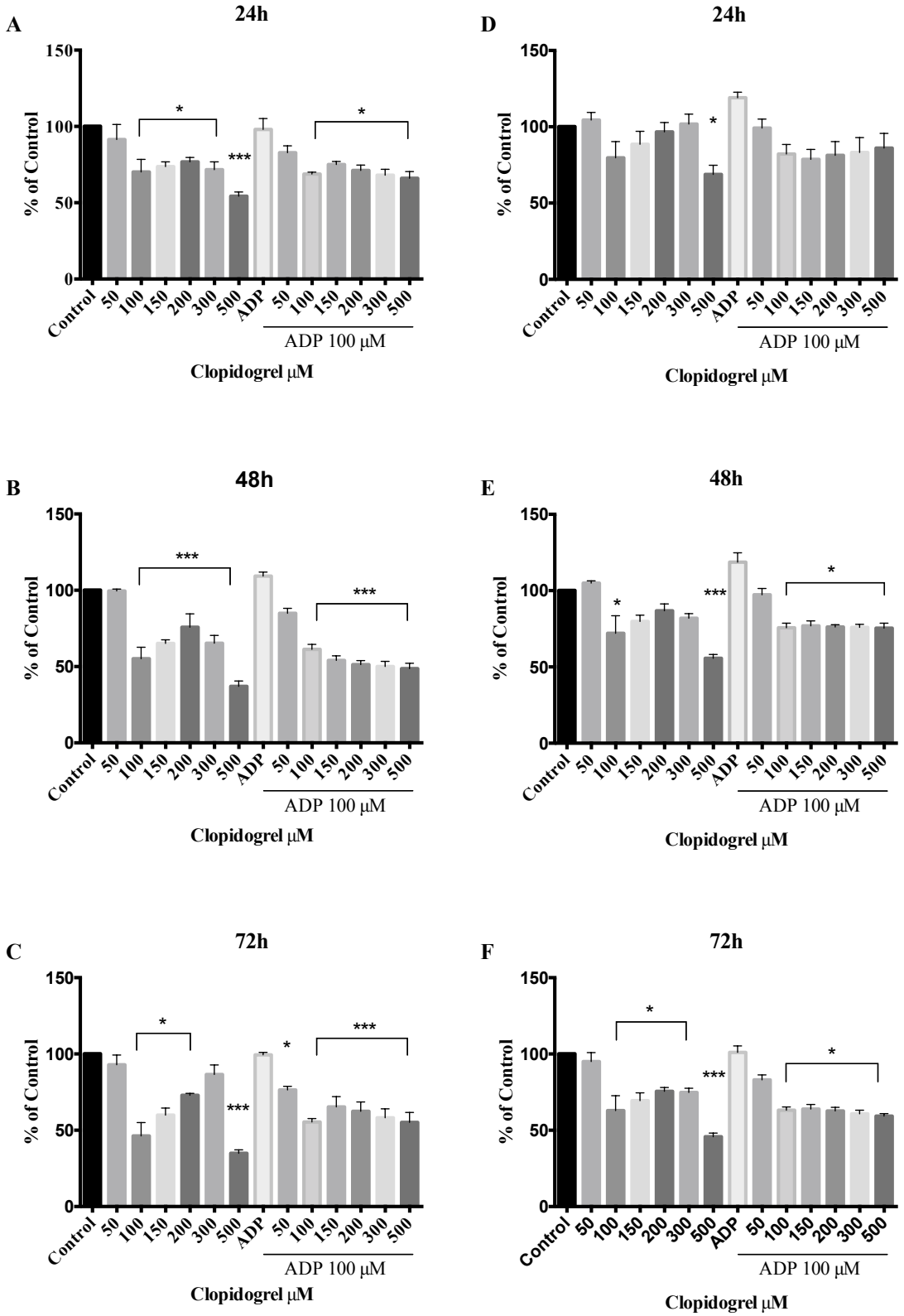

A
24h
% of Control
Control
50
100
150
200
300
500
ADP
ADP 100 µM
Clopidogrel µM
B
48h
C
72h
D
24h
E
48h
F
72h

Figure 3

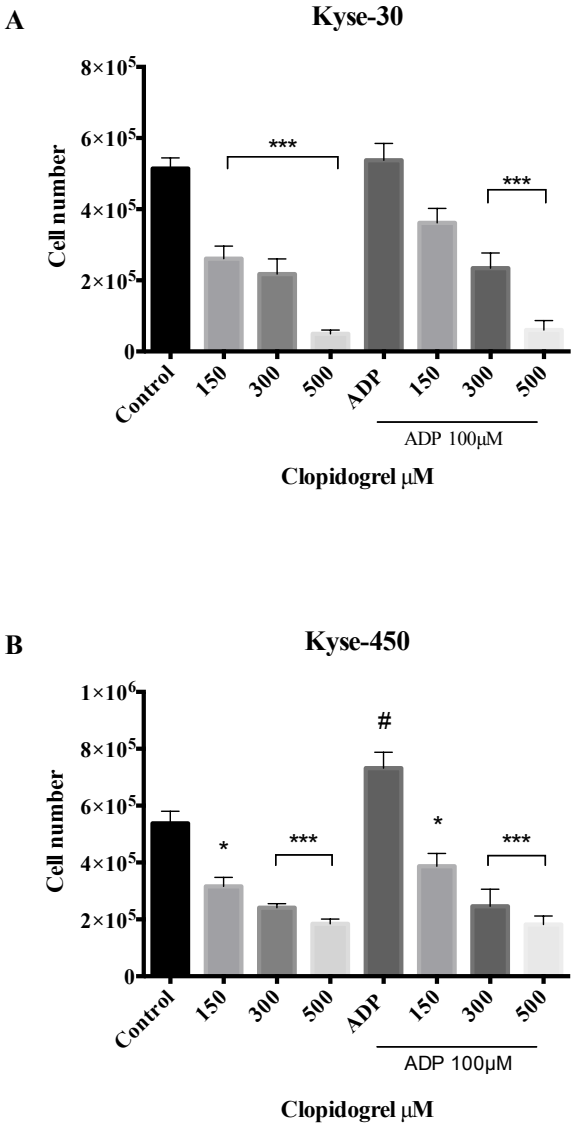
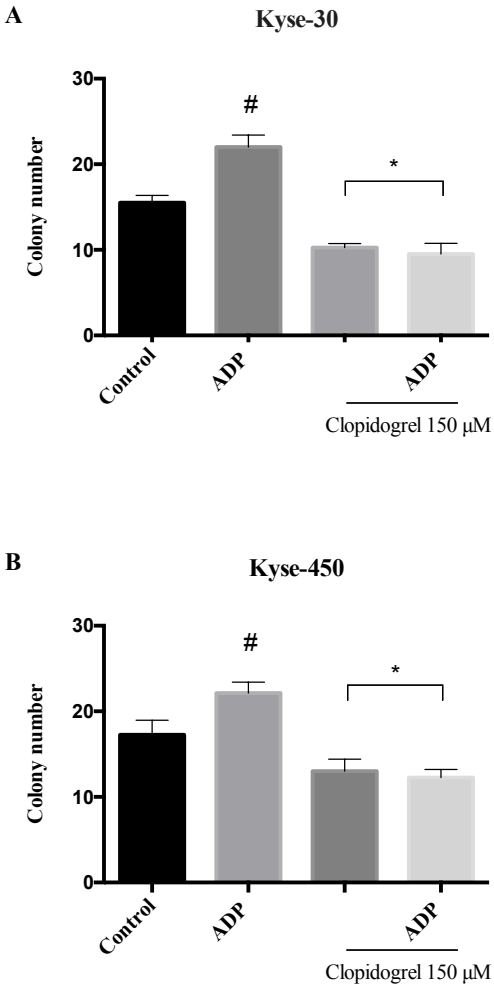

A
Kyse-30
Cell number
8×10^5
6×10^5
4×10^5
2×10^5
0
***
***
Control
150
300
500
ADP
150
300
500
ADP 100μM
Clopidogrel μM
B
Kyse-450
Cell number
1×10^6
8×10^5
6×10^5
4×10^5
2×10^5
0
#
*
***
*
***
Control
150
300
500
ADP
150
300
500
ADP 100μM
Clopidogrel μM

Figure 4


A
Kyse-30
Colony number
30
20
10
0
#
*
Control
ADP
ADP
Clopidogrel 150 μM
B
Kyse-450
Colony number
30
20
10
0
#
*
Control
ADP
ADP
Clopidogrel 150 μM

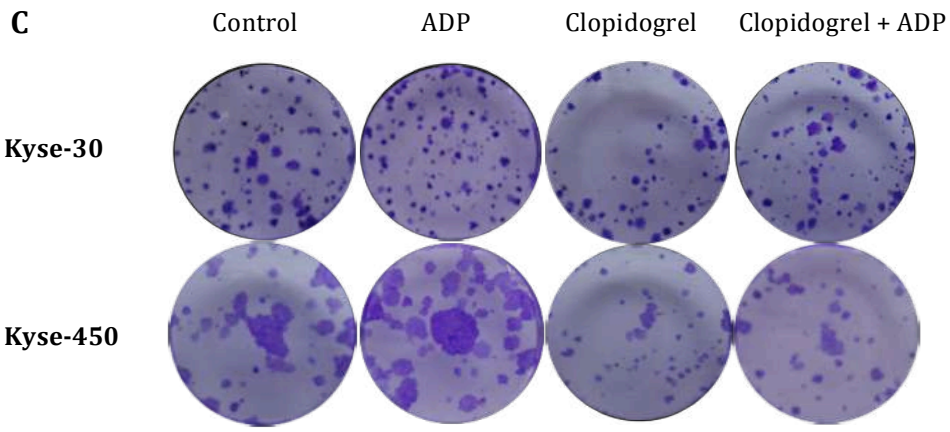
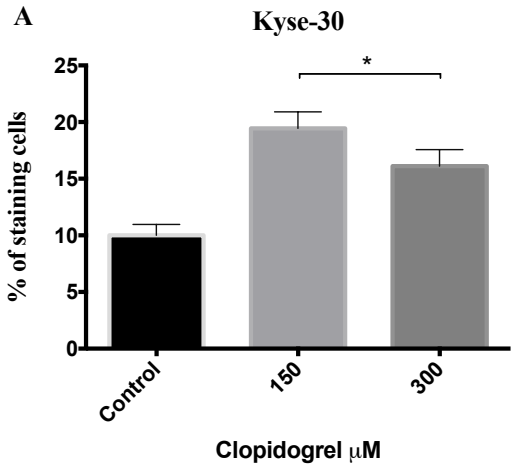
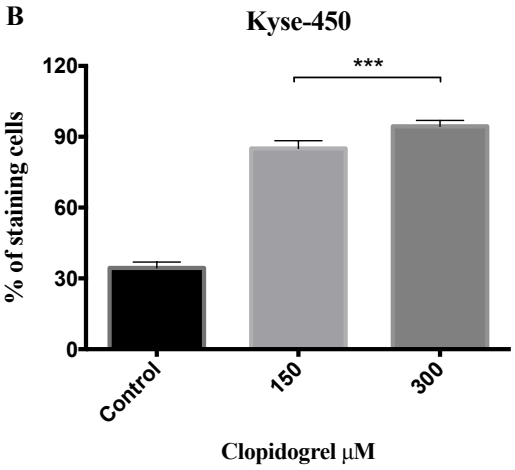
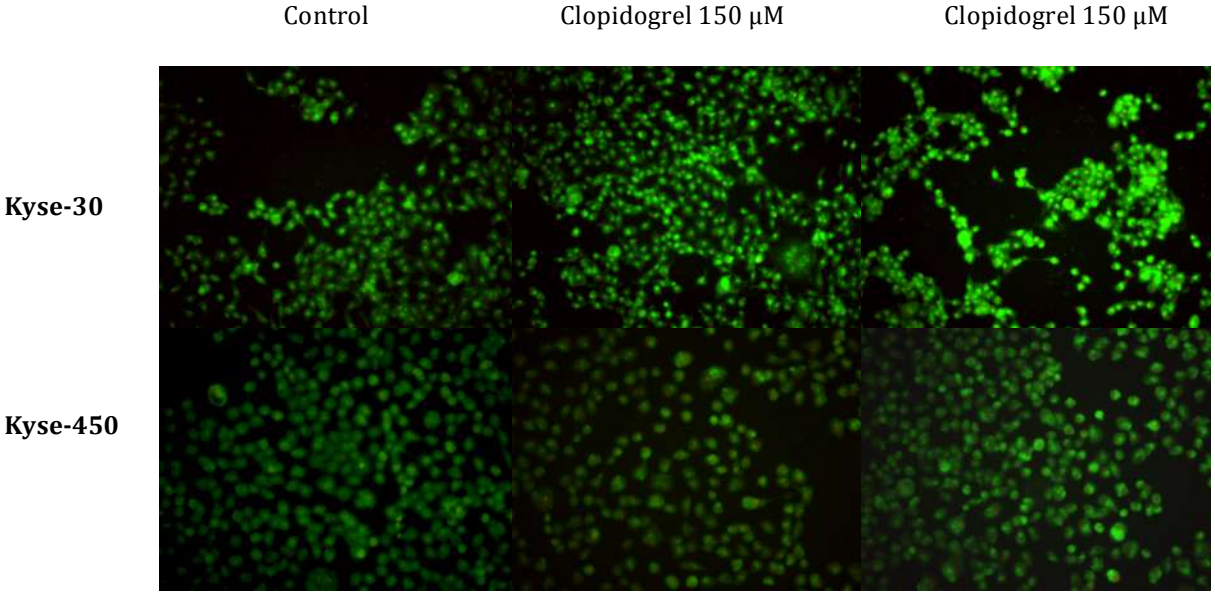

C
Control
ADP
Clopidogrel
Clopidogrel + ADP
Kyse-30
Kyse-450

Figure 5


A
Kyse-30
*
% of staining cells
25
20
15
10
5
0
Control
150
300
Clopidogrel μM


B
Kyse-450
***
% of staining cells
120
90
60
30
0
Control
150
300
Clopidogrel μM


Control
Clopidogrel 150 μM
Clopidogrel 150 μM
Kyse-30
Kyse-450

5 CONSIDERAÇÕES FINAIS

O desenvolvimento do câncer de esôfago é multifatorial, sendo dividido em dois tipos histológicos principais: carcinoma de células escamosas (ESCC) e adenocarcinoma (EAC). As contribuições das alterações genéticas e epigenéticas para o câncer de esôfago são muito heterogêneas, ou seja, dentro do mesmo tipo tumoral pode haver diferenças de mutações, na ativação ou supressão de genes (Gonzaga *et al.*, 2017). Esses fatores terão influência direta na eficácia ou resistência ao tratamento empregado (Lagergren *et al.*, 2017).

O tratamento mais empregado para essa patologia é a quimio e a radioterapia, a qual irá gerar danos nas células e promover a liberação de nucleotídeos, como o ATP e o UTP para o meio extracelular. Esses nucleotídeos irão atuar nos receptores P2 das células, exercendo funções inflamatórias e pró-metastáticas, podendo estimular a quimiotaxia em células tumorais (Ratajczak *et al.*, 2013; Gunjal *et al.*, 2015). Além disso, o conteúdo intracelular de ATP também pode aumentar devido à internalização dessa molécula pelas células tumorais através de macropinocitose, e evento já foi relacionado com o aumento da resistência a fármacos (Qian *et al.*, 2014).

Já foi verificada a expressão de $P2Y_2R$ em células tumorais de diferentes órgãos, tais como: cânceres hepáticos (promovendo o metabolismo celular, proliferação e migração) (Espelt *et al.*, 2013; Xie *et al.*, 2014) e câncer pancreático (promovendo proliferação) (Choi *et al.*, 2013). No câncer colorretal a função do $P2Y_2R$ varia conforme o tipo celular estudado, podendo estar envolvido com metabolismo celular, indução de apoptose e outros eventos antiproliferativos (células HT-29) (Hopfner *et al.*, 2001; Coutinho-Silva *et al.*, 2005) ou envolvido com a indução da proliferação (células Caco2) (Buzzi *et al.*, 2009; Buzzi *et al.*, 2010). Apesar dos receptores purinérgicos serem

expressos por diversas células ao longo do sistema gastrointestinal (Burnstock, 2014), há escassez de estudos que relacionem o sistema purinérgico, especialmente os receptores P2Y com câncer de esôfago.

A primeira parte deste estudo foi de caráter retrospectivo, utilizando dados obtidos por meio da revisão de prontuários e análise de material proveniente de biópsias de pacientes com câncer esofágico do tipo escamoso e adenocarcinoma. Com a análise dos prontuários foi possível observar que os pacientes são diagnosticados quando o tumor apresenta-se em estadio avançado. Isso se deve ao fato da doença não apresentar sintomas específicos, o que resulta em prejuízos no tratamento e altas taxas de mortalidade (Crew e Neugut, 2006; Zhang, 2013; Backemar *et al.*, 2016).

Um estudo prévio realizado por Maaser e colaboradores (2002) identificou a presença de receptores P2 (P2X4, P2X5, $P2Y_2$ e $P2Y_4$) no tecido tumoral esofágico e a ação da modulação farmacológica destes receptores *in vitro*, levou a eventos relacionados com a diminuição da proliferação e morte celular. Na análise imunohistoquímica das biópsias em nosso estudo, observamos de forma qualitativa que há expressão do receptor $P2Y_2$ em tecidos neoplásicos (ESCC) e (EAC), mas também nos tecidos normais. É necessário quantificarmos a expressão para identificarmos se há diferença nos padrões de expressão do $P2Y_2R$ nos tecidos tumorais e não neoplásicos. Recentemente, foi elucidado que a expressão dos receptores $P2Y_2$ e $P2Y_4$ é maior em células representativas de tumor de estômago em comparação com a expressão encontrada em células epiteliais gástricas normais (Wan *et al.*, 2017).

Devido a ausência de achados científicos que abordem o ESCC e o EAC e sua relação com o sistema purinérgico, decidimos utilizar três linhagens celulares humanas de tumores esofágicos, a Kyse-30 e a Kyse-450 que são representativas de ESCC e a OE-33 de EAC. Na segunda parte deste estudo foi avaliada a expressão do $P2Y_2R$ nas linhagens

celulares de câncer de esôfago. Nossos achados demonstraram que as linhagens utilizadas apresentam expressão dos receptores P2Y, de modo particular os $P2Y_2R$ e $P2Y_{12}R$, os quais foram o foco deste estudo. De modo interessante, observou-se que a linhagem celular de EAC (OE-33) apresentou uma maior expressão do $P2Y_2R$ do que as linhagens ESCC, Kyse-30 e Kyse-450. Posteriormente, buscou-se avaliar a ação dos receptores $P2Y_2$ na proliferação, migração e adesão das células, bem como estudar as vias de sinalização envolvidas.

O receptor $P2Y_2$ encontra-se amplamente envolvido nos processos de proliferação, angiogênese e migração em diversos tipos tumorais (Burnstock e Di Virgilio, 2013; Burnstock, 2014; Di Virgilio e Adinolfi, 2017). Nossos achados sugerem que o $P2Y_2R$ possui papel importante na proliferação e sobrevivência celular, pois quando foi utilizado o antagonista seletivo para $P2Y_2R$, as células tiveram a proliferação inibida. Além disso, os dados deste estudo mostraram que o UTP apresentou maior capacidade de induzir a formação de colônias pelas células Kyse-450 e OE-33, e a inibição do $P2Y_2R$ levou a diminuição dessa capacidade. Esses resultados sugerem que a atividade do $P2Y_2R$ sofre variações entre as células estudadas.

Estudos prévios evidenciam a função de nucleotídeos extracelulares na regulação da proliferação de várias linhagens de células cancerígenas via $P2Y_2R$ (Wagstaff *et al.*, 2000; Schafer *et al.*, 2003). Schumacher e colaboradores (2013) reportaram que a formação de metástases envolve a ativação purinérgica endotelial dos $P2Y_2R$ pelo ATP, através da secreção de grânulos de nucleotídeos de adenina pelas plaquetas no microambiente tumoral.

O trabalho realizado por Jin et al. (2014), comparou uma linhagem pouco metastática de câncer de mama (MCF-7) com uma linhagem altamente metastática (MDA-MB-231), essas células não apresentaram diferença na expressão do $P2Y_2R$, mas

mostraram respostas diferentes quando estimuladas com ATP e UTP. A linhagem MDA-MB-231 apresentou resposta imediata e mais rápida no aumento dos níveis de cálcio do que e também apresentou maiores quantidades de ATP liberado no meio extracelular. O mesmo trabalho ainda mostrou que a ativação do $P2Y_2R$ foi capaz de induzir proliferação, migração e aumento da expressão de moléculas de adesão nas células MDA-MB-231 (Jin et al. (2014).

Ainda em câncer de mama, a ativação do $P2Y_2R$ pelo ATP liberado das células MDA-MB-231 foi capaz de gerar a formação de um nicho pré-metastático, através do aumento da expressão do fator induzido por hipóxia 1α (HIF-1α), da secreção de lisil oxidase e aumento de *cross-linking* de colágeno (fator contribuinte para o enrijecimento de matriz que favorece estabelecimento do tumor), o ATP também exerceu uma função pró-inflamatória por ativar o $P2Y_2R$ presente nos monócitos (Joo *et al.*, 2014).

Nossos resultados mostram que o tratamento com UTP (100 μM) aumentou a adesão celular na linhagem Kyse-30 e a inibição do $P2Y_2R$ pelo antagonista seletivo resultou em menor adesão celular. Em um estudo conduzido por Qiu *et al*., (2018), foi evidenciado que a expressão do $P2Y_2R$ é abundante na borda invasiva tumoral, no infiltrado de células tumorais, nódulos linfáticos e no tecido adiposo de mama em comparação ao centro do tumor. Esses achados indicam que a ativação do $P2Y_2R$ promove a migração e invasão tumoral através da regulação dos genes relacionados com a EMT como a E-caderina e Snail (Qiu *et al*., (2018).

A fim de explicar as ações desempenhadas pelo receptor $P2Y_2$, as células tumorais foram estimuladas com os nucleotídeos (ATP e UTP) que levaram à ativação da fosforilação de ERK1/2 e Akt. A ativação de vias de sinalização como ERK1/2 podem induzir à proliferação celular, diferenciação, invasão e sobrevivência (Muscella *et al.*, 2003; Chen *et al.*, 2004; Czajkowski *et al.*, 2004; Eun *et al.*, 2015). Vários estudos

demonstraram a capacidade dos nucleotídeos em ativar várias vias de sinalização celular em diferentes tumores. Em células MCF-7 o UTP ativa a fosforilação de ERK1/2 (Chadet *et al.*, 2014). Nas células HeLa o ATP e UTP promovem a ativação de $P2Y_2$ levando a fosforilação de ERK1/2 e PI3K (Muscella *et al.*, 2003)

Adicionalmente, nossos dados demonstraram que a linhagem Kyse-30 exibiu a participação do receptor $P2Y_2$ na sinalização mediada por cálcio, sendo que os nucleotídeos ATP e UTP foram capazes de aumentar a concentração de cálcio intracelular. Esse efeito foi em parte suprimido quando houve o bloqueio farmacológico do $P2Y_2R$ com o antagonista seletivo.

Na terceira parte deste estudo avaliou-se a ação do receptor $P2Y_{12}$ na proliferação das células. Observamos que o ADP foi capaz de induzir proliferação na linhagem Kyse-450, da mesma forma quando estimulamos $P2Y_{12}R$ houve aumento da capacidade das células em formar colônias.

Outro dado interessante refere-se ao fato de que as linhagens apresentaram diferentes perfis de atividade das ectonucleotidases, e esse resultado tem relação direta de como as células irão se comportar na presença de nucleotídeos. De fato, a linhagem Kyse-30 degrada o ATP mais lentamente do que o ADP, com isso podemos inferir que o ATP ficará mais tempo em contato com as células, ativando os receptores. A Kyse-450 apresenta uma atividade muito inferior, porém o perfil semelhante de hidrólise é semelhante ao da Kyse-30.

Já está bem descrito que plaquetas expressam os receptores $P2Y_1$ e $P2Y_{12}$ em sua superfície, tendo importante função na mudança de forma nas plaquetas e na agregação. As plaquetas são ativadas quando o ADP se liga a esses receptores, e através deste e de outros sinais levam o recrutamento das plaquetas para o local de dano (Jackson *et al.*, 2003; Kunapuli *et al.*, 2003). O $P2Y_{12}R$ tornou-se um alvo farmacológico, pois ao ser

antagonizado possui importante efeito terapêutico na prevenção da formação de trombos e seus eventos relacionados como isquemia do miocárdio e cerebral (Dorsam e Kunapuli, 2004). Além disso, a expressão do $P2Y_{12}R$ tem sido observada em diferentes linhagens celulares de tumores, como de gliomas, astrocitomas, renal, pulmão, mama e cólon (White e Burnstock, 2006; Sarangi *et al.*, 2013; Schneider *et al.*, 2015).

Camundongos fêmeas com tumor de ovário e *knockout* para $P2Y_{12}R$ tratadas com aspirina, tiveram redução significativa no tamanho tumoral em comparação com camundongos selvagens que receberam o mesmo tratamento (Cho *et al.*, 2017). Em células de câncer pancreático, o bloqueio farmacológico do $P2Y_{12}R$ através do inibidor tricagrelor inibiu a sobrevivência das células após o estímulo com ADP, e os resultados também demostraram que as plaquetas atribuem resistência à quimioterapia com gemcitabina (Elaskalani *et al.*, 2017) .

Nossos dados mostraram que o tratamento das células com o antagonista clopidogrel foi capaz de diminuir a viabilidade celular, e a concentração que mais induziu esse resultado foi a de 500 μM. Para melhor avaliar o papel do $P2Y_{12}R$, escolhemos concentrações menos tóxicas para as células, porém até mesmo a concentração de 300 μM não permitiu que as células tivessem capacidade de formar colônias. Quanto à avaliação da possibilidade dessas células entrarem em autofagia, percebemos que após tratamento com clopidogrel um maior número células marcadas com laranja de acridina foram observadas, sugerindo que essas células podem tornar-se autofágicas mediante o bloqueio do $P2Y_{12}R$.

Em ensaios clínicos, a modulação farmacológica do $P2Y_{12}R$ com antagonistas, como o clopidogrel já foi relacionada com o aumento da citotoxicidade da cisplatina, mostrando um efeito sinérgico entre o bloqueio desse receptor e o tratamento quimioterápico

(Sarangi *et al.*, 2013). Bem como já foram observados resultados favoráveis do bloqueio do $P2Y_{12}R$ em pacientes com câncer de próstata durante o tratamento com radioterapia (Choe *et al.*, 2010).

Frente aos obstáculos impostos pelo câncer de esôfago, a pesquisa por novos alvos farmacológicos, incluindo a modulação de receptores purinérgicos $P2Y_2$ e $P2Y_{12}$, torna-se uma ferramenta promissora para alcançar eficácia no tratamento. Em suma, os achados deste estudo demonstram que o $P2Y_2R$ quando ativado por ATP e UTP, tem importante contribuição nos eventos de proliferação, ativando vias de sinalização específicas para cada tipo celular. Em contrapartida, o bloqueio do $P2Y_2R$ leva a prejuízos na capacidade proliferativa das células, menor adesão, menor migração e redução na liberação de cálcio intracelular após o estímulo com nucleotídeos.

Do mesmo modo, sugere-se que o $P2Y_{12}R$ desenvolve uma importante ação na proliferação e o seu bloqueio pelo clopidogrel leva a prejuízos na capacidade proliferativa das células de câncer de esôfago, bem como o aumento de um indicador da etapa final de autofagia, mecanismo este que precisa ser melhor investigado.

Em decorrência diagnóstico tardio, o câncer de esôfago torna-se uma doença com baixa taxa de responsividade ao tratamento e alta taxa de mortalidade. Com a identificação de novos alvos farmacológicos, entre eles a os receptores purinérgicos surge a possibilidade de ampliar as estratégias de tratamento. Nesse contexto, o presente trabalho sugere que os receptores P2Y2 e P2Y12 são importantes para a proliferação e a manutenção da sobrevivência da células de câncer de esôfago. Dessa forma, o bloqueio farmacológico desses receptores pode constituir uma importante ferramenta que corrobora com diminuição dos eventos que conferem malignidade ao tumor.

6. PERSPECTIVAS

1- Quantificar a expressão nas amostras já marcadas com imunohistoquímica para $P2Y_2R$.

2- Quantificar a expressão dos receptores $P2Y_1$, $P2Y_2$ e $P2Y_{12}$ por Western Blot nas linhagens de esôfago.

3- Verificar a se há ativação das vias de sinalização intracelular ERK1/2 e Akt após o estímulo com ADP nas células tumorais de esôfago.

4- Realizar a marcação com Ki67 para verificar a proliferação induzida pelo tratamento com nucleotídeos.

5- Analisar se o bloqueio dos receptores $P2Y_2$ e $P2Y_{12}$ são capazes de induzir morte celular e quais os mecanismos relacionados.

6- Realizar a modulação farmacológica do $P2Y_1$ com antagonista seletivo e específico, e a partir disso verificar sua interferência na proliferação, migração e adesão celular.

7. REFERÊNCIAS

ABBRACCHIO, M. P. et al. Purinergic signalling in the nervous system: an overview. **Trends Neurosci,** v. 32, n. 1, p. 19-29, Jan 2009.

AJANI, J. A. et al. Esophageal and esophagogastric junction cancers. **J Natl Compr Canc Netw,** v. 9, n. 8, p. 830-87, Aug 1 2011.

BURNSTOCK, G. Purinergic nerves. **Pharmacol Rev,** v. 24, n. 3, p. 509-81, Sep 1972.

_____. Introduction: P2 receptors. **Curr Top Med Chem,** v. 4, n. 8, p. 793-803, 2004.

_____. Purine and pyrimidine receptors. **Cell Mol Life Sci,** v. 64, n. 12, p. 1471-83, Jun 2007.

CHUNG, C. S. et al. Secondary prevention of esophageal squamous cell carcinoma in areas where smoking, alcohol, and betel quid chewing are prevalent. **J Formos Med Assoc,** v. 109, n. 6, p. 408-21, Jun 2010.

COUTINHO-SILVA, R. et al. P2X and P2Y purinergic receptors on human intestinal epithelial carcinoma cells: effects of extracellular nucleotides on apoptosis and cell proliferation. **Am J Physiol Gastrointest Liver Physiol,** v. 288, n. 5, p. G1024-35, May 2005.

DANDARA, C. et al. Gene-environment interaction: the role of SULT1A1 and CYP3A5 polymorphisms as risk modifiers for squamous cell carcinoma of the oesophagus. **Carcinogenesis,** v. 27, n. 4, p. 791-7, Apr 2006.

DUBYAK, G. R.; EL-MOATASSIM, C. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. **Am J Physiol,** v. 265, n. 3 Pt 1, p. C577-606, Sep 1993.

GEHRING, M. P. et al. P2X7 receptor activation leads to increased cell death in a radiosensitive human glioma cell line. **Purinergic Signal,** v. 8, n. 4, p. 729-39, Dec 2012.

JIN, H. et al. P2Y2 receptor activation by nucleotides released from highly metastatic breast cancer cells increases tumor growth and invasion via crosstalk with endothelial cells. **Breast Cancer Res,** v. 16, n. 5, p. R77, 2014.

JOO, Y. N. et al. P2Y2R activation by nucleotides released from the highly metastatic breast cancer cell MDA-MB-231 contributes to pre-metastatic niche formation by mediating lysyl oxidase secretion, collagen crosslinking, and monocyte recruitment. **Oncotarget,** v. 5, n. 19, p. 9322-34, Oct 15 2014.

KO, H. et al. Synthesis and potency of novel uracil nucleotides and derivatives as P2Y2 and P2Y6 receptor agonists. **Bioorg Med Chem,** v. 16, n. 12, p. 6319-32, Jun 15 2008.

KUNZLI, B. M. et al. Upregulation of CD39/NTPDases and P2 receptors in human pancreatic disease. **Am J Physiol Gastrointest Liver Physiol,** v. 292, n. 1, p. G223-30, Jan 2007.

MAASER, K. et al. Extracellular nucleotides inhibit growth of human oesophageal cancer cells via P2Y(2)-receptors. **Br J Cancer,** v. 86, n. 4, p. 636-44, Feb 12 2002.

MALUF, F. et al. Accuracy of 30 MHz radial EUS for superficial esophageal and gastric cancer: Can submucosal invasion be reliably detected? **Gastrointestinal Endoscopy,** v. 55, n. 5, p. Ab233-Ab233, Apr 2002.

MORITA, M. et al. Alcohol drinking, cigarette smoking, and the development of squamous cell carcinoma of the esophagus: epidemiology, clinical findings, and prevention. **Int J Clin Oncol,** v. 15, n. 2, p. 126-34, Apr 2010.

MORRONE, F. B. et al. Extracellular nucleotides and nucleosides induce proliferation and increase nucleoside transport in human glioma cell lines. **J Neurooncol,** v. 64, n. 3, p. 211-8, Sep 2003.

MORRONE, F. B. et al. In vivo glioblastoma growth is reduced by apyrase activity in a rat glioma model. **BMC Cancer,** v. 6, p. 226, 2006.

NYLUND, G. et al. P2Y2- and P2Y4 purinergic receptors are over-expressed in human colon cancer. **Auton Autacoid Pharmacol,** v. 27, n. 2, p. 79-84, Apr 2007.

RAPAPORT, E.; FONTAINE, J. Anticancer activities of adenine nucleotides in mice are mediated through expansion of erythrocyte ATP pools. **Proc Natl Acad Sci U S A,** v. 86, n. 5, p. 1662-6, Mar 1989.

ROMAN, R. M.; FITZ, J. G. Emerging roles of purinergic signaling in gastrointestinal epithelial secretion and hepatobiliary function. **Gastroenterology,** v. 116, n. 4, p. 964-79, Apr 1999.

SCHAFER, R. et al. ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. **Am J Physiol Lung Cell Mol Physiol,** v. 285, n. 2, p. L376-85, Aug 2003.

SCHUMACHER, D. et al. Platelet-derived nucleotides promote tumor-cell transendothelial migration and metastasis via P2Y2 receptor. **Cancer Cell,** v. 24, n. 1, p. 130-7, Jul 8 2013.

SKACEL, M. et al. The diagnosis of low-grade dysplasia in Barrett's esophagus and its implications for disease progression. **Am J Gastroenterol,** v. 95, n. 12, p. 3383-7, Dec 2000.

SPYCHALA, J. Tumor-promoting functions of adenosine. **Pharmacol Ther,** v. 87, n. 2-3, p. 161-73, Aug-Sep 2000.

TERRY, P. et al. Antioxidants and cancers of the esophagus and gastric cardia. **Int J Cancer,** v. 87, n. 5, p. 750-4, Sep 1 2000.

WAGSTAFF, S. C. et al. Extracellular ATP activates multiple signalling pathways and potentiates growth factor-induced c-fos gene expression in MCF-7 breast cancer cells. **Carcinogenesis,** v. 21, n. 12, p. 2175-81, Dec 2000.

WHITE, N.; BUTLER, P. E.; BURNSTOCK, G. Human melanomas express functional P2 X(7) receptors. **Cell Tissue Res,** v. 321, n. 3, p. 411-8, Sep 2005.

WHITE, N. et al. P2Y purinergic receptors regulate the growth of human melanomas. **Cancer Lett,** v. 224, n. 1, p. 81-91, Jun 16 2005.

WIJNHOVEN, B. P.; TILANUS, H. W.; DINJENS, W. N. Molecular biology of Barrett's adenocarcinoma. **Ann Surg,** v. 233, n. 3, p. 322-37, Mar 2001.

WINK, M. R. et al. Altered extracellular ATP, ADP and AMP catabolism in glioma cell lines. **Cancer Lett,** v. 198, n. 2, p. 211-8, Aug 20 2003.

WOLF, M. C. et al. Curative treatment of oesophageal carcinoma: current options and future developments. **Radiat Oncol,** v. 6, p. 55, 2011.

ZHANG, X. M.; GUO, M. Z. The value of epigenetic markers in esophageal cancer. **Front Med China,** v. 4, n. 4, p. 378-84, Dec 2010.

ABBRACCHIO, M. P.; BURNSTOCK, G. Purinoceptors: are there families of P2X and P2Y purinoceptors? **Pharmacology & therapeutics,** v. 64, n. 3, p. 445-475, 1994.

ABBRACCHIO, M. P. et al. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. **Pharmacol Rev,** v. 58, n. 3, p. 281-341, Sep 2006.

ABBRACCHIO, M. P. et al. Purinergic signalling in the nervous system: an overview. **Trends Neurosci,** v. 32, n. 1, p. 19-29, Jan 2009.

AJANI, J. A. et al. Esophageal and esophagogastric junction cancers. **J Natl Compr Canc Netw,** v. 9, n. 8, p. 830-87, Aug 1 2011.

ANTONIOLI, L. et al. Immunity, inflammation and cancer: a leading role for adenosine. **Nat Rev Cancer,** v. 13, n. 12, p. 842-57, Dec 2013.

ANTONIOLI, L. et al. CD39 and CD73 in immunity and inflammation. **Trends Mol Med,** v. 19, n. 6, p. 355-67, Jun 2013.

ARNOLD, M. et al. Global incidence of oesophageal cancer by histological subtype in 2012. **Gut,** v. 64, n. 3, p. 381-7, Mar 2015.

ASLAN, J. E. et al. S6K1 and mTOR regulate Rac1-driven platelet activation and aggregation. **Blood,** v. 118, n. 11, p. 3129-36, Sep 15 2011.

BACKEMAR, L. et al. Co-morbidity after oesophageal cancer surgery and recovery of health-related quality of life. **Br J Surg,** v. 103, n. 12, p. 1665-1675, Nov 2016.

BALDWIN, S. A. et al. The equilibrative nucleoside transporter family, SLC29. **Pflugers Arch,** v. 447, n. 5, p. 735-43, Feb 2004.

BALLERINI, P. et al. P2Y12 Receptors in Tumorigenesis and Metastasis. **Front Pharmacol,** v. 9, p. 66, 2018.

BLAYDON, D. C. et al. RHBDF2 mutations are associated with tylosis, a familial esophageal cancer syndrome. **Am J Hum Genet,** v. 90, n. 2, p. 340-6, Feb 10 2012.

BOTTSFORD-MILLER, J. et al. Differential platelet levels affect response to taxane-based therapy in ovarian cancer. **Clin Cancer Res,** v. 21, n. 3, p. 602-10, Feb 1 2015.

BOURS, M. J. et al. P2 receptors and extracellular ATP: a novel homeostatic pathway in inflammation. **Front Biosci (Schol Ed),** v. 3, p. 1443-56, Jun 1 2011.

BOYA, P.; REGGIORI, F.; CODOGNO, P. Emerging regulation and functions of autophagy. **Nat Cell Biol,** v. 15, n. 7, p. 713-20, Jul 2013.

BRAGANHOL, E. et al. Selective NTPDase2 expression modulates in vivo rat glioma growth. **Cancer Sci,** v. 100, n. 8, p. 1434-42, Aug 2009.

BURGERING, B. M.; COFFER, P. J. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. **Nature,** v. 376, n. 6541, p. 599-602, Aug 17 1995.

BURNSTOCK, G. Purinergic nerves. **Pharmacol Rev,** v. 24, n. 3, p. 509-81, Sep 1972.

_____. A basis for distinguishing two types of purinergic receptor. In: STRAUB, R. e BOLIS, L. (Ed.). **Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach.** New York: Raven Press, 1978. p.107–118.

_____. Introduction: P2 receptors. **Curr Top Med Chem,** v. 4, n. 8, p. 793-803, 2004.

_____. Pathophysiology and therapeutic potential of purinergic signaling. **Pharmacol Rev,** v. 58, n. 1, p. 58-86, Mar 2006a.

_____. Purinergic P2 receptors as targets for novel analgesics. **Pharmacol Ther,** v. 110, n. 3, p. 433-54, Jun 2006b.

_____. Purine and pyrimidine receptors. **Cell Mol Life Sci,** v. 64, n. 12, p. 1471-83, Jun 2007.

_____. Purinergic signalling: pathophysiology and therapeutic potential. **Keio J Med,** v. 62, n. 3, p. 63-73, 2013.

_____. Purinergic signalling in the gastrointestinal tract and related organs in health and disease. **Purinergic Signal,** v. 10, n. 1, p. 3-50, Mar 2014.

_____. Purinergic Signalling: Therapeutic Developments. **Front Pharmacol,** v. 8, p. 661, 2017.

BURNSTOCK, G.; DI VIRGILIO, F. Purinergic signalling and cancer. **Purinergic Signal,** v. 9, n. 4, p. 491-540, Dec 2013.

BURNSTOCK, G.; EVANS, L. C.; BAILEY, M. A. Purinergic signalling in the kidney in health and disease. **Purinergic Signal,** v. 10, n. 1, p. 71-101, Mar 2014.

BURNSTOCK, G.; JACOBSON, K. A.; CHRISTOFI, F. L. Purinergic drug targets for gastrointestinal disorders. **Curr Opin Pharmacol,** v. 37, p. 131-141, Dec 2017.

BUSTIN, S. A. et al. The need for transparency and good practices in the qPCR literature. **Nat Methods,** v. 10, n. 11, p. 1063-7, Nov 2013.

BUZZI, N. et al. Extracellular ATP activates MAP kinase cascades through a P2Y purinergic receptor in the human intestinal Caco-2 cell line. **Biochim Biophys Acta,** v. 1790, n. 12, p. 1651-9, Dec 2009.

BUZZI, N.; BOLAND, R.; RUSSO DE BOLAND, A. Signal transduction pathways associated with ATP-induced proliferation of colon adenocarcinoma cells. **Biochim Biophys Acta,** v. 1800, n. 9, p. 946-55, Sep 2010.

CAMERER, E. et al. Platelets, protease-activated receptors, and fibrinogen in hematogenous metastasis. **Blood,** v. 104, n. 2, p. 397-401, Jul 15 2004.

CEKIC, C.; LINDEN, J. Purinergic regulation of the immune system. **Nat Rev Immunol,** v. 16, n. 3, p. 177-92, Mar 2016.

CHADET, S. et al. The activation of P2Y2 receptors increases MCF-7 breast cancer cells migration through the MEK-ERK1/2 signalling pathway. **Carcinogenesis,** v. 35, n. 6, p. 1238-47, Jun 2014.

CHAI, S. et al. Ca2+/calmodulin-dependent protein kinase IIgamma enhances stem-like traits and tumorigenicity of lung cancer cells. **Oncotarget,** v. 6, n. 18, p. 16069-83, Jun 30 2015.

CHEKENI, F. B. et al. Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. **Nature,** v. 467, n. 7317, p. 863-7, Oct 14 2010.

CHEN, C. et al. Myosin light chain kinase (MLCK) regulates cell migration in a myosin regulatory light chain phosphorylation-independent mechanism. **J Biol Chem,** v. 289, n. 41, p. 28478-88, Oct 10 2014.

CHEN, L. et al. ERK1/2 and p38 pathways are required for P2Y receptor-mediated prostate cancer invasion. **Cancer Lett,** v. 215, n. 2, p. 239-47, Nov 25 2004.

CHO, M. S. et al. Role of ADP receptors on platelets in the growth of ovarian cancer. **Blood,** v. 130, n. 10, p. 1235-1242, Sep 7 2017.

CHOE, K. S. et al. The use of anticoagulants improves biochemical control of localized prostate cancer treated with radiotherapy. **Cancer,** v. 116, n. 7, p. 1820-6, Apr 01 2010.

CHOI, J. H.; JI, Y. G.; LEE, D. H. Uridine triphosphate increases proliferation of human cancerous pancreatic duct epithelial cells by activating P2Y2 receptor. **Pancreas,** v. 42, n. 4, p. 680-6, May 2013.

CHUNG, C. S. et al. Secondary prevention of esophageal squamous cell carcinoma in areas where smoking, alcohol, and betel quid chewing are prevalent. **J Formos Med Assoc,** v. 109, n. 6, p. 408-21, Jun 2010.

COUCH, G. et al. The Discovery and Validation of Biomarkers for the Diagnosis of Esophageal Squamous Dysplasia and Squamous Cell Carcinoma. **Cancer Prev Res (Phila),** v. 9, n. 7, p. 558-66, Jul 2016.

COUTINHO-SILVA, R. et al. P2X and P2Y purinergic receptors on human intestinal epithelial carcinoma cells: effects of extracellular nucleotides on apoptosis and cell proliferation. **Am J Physiol Gastrointest Liver Physiol,** v. 288, n. 5, p. G1024-35, May 2005.

CREW, K. D.; NEUGUT, A. I. Epidemiology of gastric cancer. **World J Gastroenterol,** v. 12, n. 3, p. 354-62, Jan 21 2006.

CZAJKOWSKI, R. et al. Differential effects of P2Y1 and P2Y12 nucleotide receptors on ERK1/ERK2 and phosphatidylinositol 3-kinase signalling and cell proliferation in serum-deprived and nonstarved glioma C6 cells. **Br J Pharmacol,** v. 141, n. 3, p. 497-507, Feb 2004.

DI VIRGILIO, F.; ADINOLFI, E. Extracellular purines, purinergic receptors and tumor growth. **Oncogene,** v. 36, n. 3, p. 293-303, Jan 19 2017.

DIXON, C. J. et al. Regulation of human hepatocytes by P2Y receptors: control of glycogen phosphorylase, Ca2+, and mitogen-activated protein kinases. **J Pharmacol Exp Ther,** v. 313, n. 3, p. 1305-13, Jun 2005.

DONG, J.; THRIFT, A. P. Alcohol, smoking and risk of oesophago-gastric cancer. **Best Pract Res Clin Gastroenterol,** v. 31, n. 5, p. 509-517, Oct 2017.

DORSAM, R. T.; KUNAPULI, S. P. Central role of the P2Y12 receptor in platelet activation. **J Clin Invest,** v. 113, n. 3, p. 340-5, Feb 2004.

DULAK, A. M. et al. Gastrointestinal adenocarcinomas of the esophagus, stomach, and colon exhibit distinct patterns of genome instability and oncogenesis. **Cancer Res,** v. 72, n. 17, p. 4383-93, Sep 1 2012.

DULAK, A. M. et al. Exome and whole-genome sequencing of esophageal adenocarcinoma identifies recurrent driver events and mutational complexity. **Nat Genet,** v. 45, n. 5, p. 478-86, May 2013.

EL-SERAG, H. B. et al. Epidemiological differences between adenocarcinoma of the oesophagus and adenocarcinoma of the gastric cardia in the USA. **Gut,** v. 50, n. 3, p. 368-72, Mar 2002.

ELASKALANI, O. et al. The Role of Platelet-Derived ADP and ATP in Promoting Pancreatic Cancer Cell Survival and Gemcitabine Resistance. **Cancers (Basel),** v. 9, n. 10, Oct 24 2017.

ERB, L.; WEISMAN, G. A. Coupling of P2Y receptors to G proteins and other signaling pathways. **Wiley Interdiscip Rev Membr Transp Signal,** v. 1, n. 6, p. 789-803, Nov-Dec 2012.

ESPELT, M. V. et al. On the role of ATP release, ectoATPase activity, and extracellular ADP in the regulatory volume decrease of Huh-7 human hepatoma cells. **Am J Physiol Cell Physiol,** v. 304, n. 10, p. C1013-26, May 15 2013.

EUN, S. Y. et al. P2Y2 nucleotide receptor-mediated extracellular signal-regulated kinases and protein kinase C activation induces the invasion of highly metastatic breast cancer cells. **Oncol Rep,** v. 34, n. 1, p. 195-202, Jul 2015.

FAGUNDES, R. B. et al. p53 immunoexpression: an aid to conventional methods in the screening of precursor lesions of squamous esophageal cancer in patients at high-risk? **Cancer Detect Prev,** v. 29, n. 3, p. 227-32, 2005.

FANG, J. et al. P2X7R suppression promotes glioma growth through epidermal growth factor receptor signal pathway. **Int J Biochem Cell Biol,** v. 45, n. 6, p. 1109-20, Jun 2013.

FELS ELLIOTT, D. R. et al. Impact of mutations in Toll-like receptor pathway genes on esophageal carcinogenesis. **PLoS Genet,** v. 13, n. 5, p. e1006808, May 22 2017.

FERLAY, J. et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. **Int J Cancer,** v. 136, n. 5, p. E359-86, Mar 1 2015.

FITZ, J. G. Regulation of cellular ATP release. **Trans Am Clin Climatol Assoc,** v. 118, p. 199-208, 2007.

FRANKE, H. et al. Pathophysiology of astroglial purinergic signalling. **Purinergic Signal,** v. 8, n. 3, p. 629-57, Sep 2012.

FREDHOLM, B. B. et al. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. **Pharmacol Rev,** v. 53, n. 4, p. 527-52, Dec 2001.

FREDHOLM, B. B. et al. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. **Pharmacological reviews,** v. 53, n. 4, p. 527-552, 2001.

GARAY, T. et al. Cell migration or cytokinesis and proliferation?--revisiting the "go or grow" hypothesis in cancer cells in vitro. **Exp Cell Res,** v. 319, n. 20, p. 3094-103, Dec 10 2013.

GARCIA, A. et al. Role of phosphoinositide 3-kinase beta in platelet aggregation and thromboxane A2 generation mediated by Gi signalling pathways. **Biochem J,** v. 429, n. 2, p. 369-77, Jul 15 2010.

GAY, L. J.; FELDING-HABERMANN, B. Contribution of platelets to tumour metastasis. **Nat Rev Cancer,** v. 11, n. 2, p. 123-34, Feb 2011.

GEBREMESKEL, S. et al. The reversible P2Y12 inhibitor ticagrelor inhibits metastasis and improves survival in mouse models of cancer. **Int J Cancer,** v. 136, n. 1, p. 234-40, Jan 1 2015.

GEHRING, M. P. et al. P2X7 receptor activation leads to increased cell death in a radiosensitive human glioma cell line. **Purinergic Signal,** v. 8, n. 4, p. 729-39, Dec 2012.

GONZAGA, I. M. et al. TFF1 hypermethylation and decreased expression in esophageal squamous cell carcinoma and histologically normal tumor surrounding esophageal cells. **Clin Epigenetics,** v. 9, p. 130, 2017.

GOODE, E. F.; SMYTH, E. C. Immunotherapy for Gastroesophageal Cancer. **J Clin Med,** v. 5, n. 10, Sep 22 2016.

GRAY, J. H.; OWEN, R. P.; GIACOMINI, K. M. The concentrative nucleoside transporter family, SLC28. **Pflugers Arch,** v. 447, n. 5, p. 728-34, Feb 2004.

GREENSTEIN, A. J. et al. Racial disparities in esophageal cancer treatment and outcomes. **Ann Surg Oncol,** v. 15, n. 3, p. 881-8, Mar 2008.

GUIDETTI, G. F. et al. The Gi-coupled P2Y12 receptor regulates diacylglycerol-mediated signaling in human platelets. **J Biol Chem,** v. 283, n. 43, p. 28795-805, Oct 24 2008.

GUNJAL, P. M. et al. Evidence for induction of a tumor metastasis-receptive microenvironment for ovarian cancer cells in bone marrow and other organs as an unwanted and underestimated side effect of chemotherapy/radiotherapy. **J Ovarian Res,** v. 8, p. 20, Mar 28 2015.

HAM, J.; EVANS, B. A. An emerging role for adenosine and its receptors in bone homeostasis. **Front Endocrinol (Lausanne),** v. 3, p. 113, 2012.

HECHLER, B.; GACHET, C. P2 receptors and platelet function. **Purinergic Signal,** v. 7, n. 3, p. 293-303, Sep 2011.

HOFFMANN, C. et al. Agonist-selective, receptor-specific interaction of human P2Y receptors with beta-arrestin-1 and -2. **J Biol Chem,** v. 283, n. 45, p. 30933-41, Nov 7 2008.

HOPFNER, M. et al. Growth inhibition and apoptosis induced by P2Y2 receptors in human colorectal carcinoma cells: involvement of intracellular calcium and cyclic adenosine monophosphate. **Int J Colorectal Dis,** v. 16, n. 3, p. 154-66, Jun 2001.

HUANG, F. L.; YU, S. J. Esophageal cancer: Risk factors, genetic association, and treatment. **Asian J Surg**, Dec 13 2016.

HVID-JENSEN, F. et al. Incidence of adenocarcinoma among patients with Barrett's esophagus. **N Engl J Med,** v. 365, n. 15, p. 1375-83, Oct 13 2011.

IDZKO, M. et al. Extracellular nucleotide and nucleoside signaling in vascular and blood disease. **Blood,** v. 124, n. 7, p. 1029-37, Aug 14 2014.

INSTITUTO NACIONAL DO CÂNCER, I. **Incidência de Câncer no Brasil.** 2018.

ISOMOTO, H. et al. Elevated levels of chemokines in esophageal mucosa of patients with reflux esophagitis. **Am J Gastroenterol,** v. 98, n. 3, p. 551-6, Mar 2003.

JACKSON, S. P.; NESBITT, W. S.; KULKARNI, S. Signaling events underlying thrombus formation. **J Thromb Haemost,** v. 1, n. 7, p. 1602-12, Jul 2003.

JACOB, F. et al. Purinergic signaling in inflammatory cells: P2 receptor expression, functional effects, and modulation of inflammatory responses. **Purinergic Signal,** v. 9, n. 3, p. 285-306, Sep 2013.

JACOBSON, K. A. et al. Nucleotides Acting at P2Y Receptors: Connecting Structure and Function. **Molecular Pharmacology,** v. 88, n. 2, p. 220-230, 2015.

JACQUES-SILVA, M. C. et al. ERK, PKC and PI3K/Akt Pathways Mediate Extracellular ATP and Adenosine-Induced Proliferation of U138-MG Human Glioma Cell Line. **Oncology,** v. 67, n. 5-6, p. 450-459, 2004.

JIN, H. et al. P2Y2 receptor activation by nucleotides released from highly metastatic breast cancer cells increases tumor growth and invasion via crosstalk with endothelial cells. **Breast Cancer Res,** v. 16, n. 5, p. R77, Aug 26 2014.

JOO, Y. N. et al. P2Y2R activation by nucleotides released from the highly metastatic breast cancer cell MDA-MB-231 contributes to pre-metastatic niche formation by mediating lysyl oxidase secretion, collagen crosslinking, and monocyte recruitment. **Oncotarget,** v. 5, n. 19, p. 9322-34, Oct 15 2014.

KAHNER, B. N. et al. Nucleotide receptor signaling in platelets. **J Thromb Haemost,** v. 4, n. 11, p. 2317-26, Nov 2006.

KAMANGAR, F. et al. Environmental causes of esophageal cancer. **Gastroenterol Clin North Am,** v. 38, n. 1, p. 27-57, vii, Mar 2009.

KANTHI, Y. M.; SUTTON, N. R.; PINSKY, D. J. CD39: Interface between vascular thrombosis and inflammation. **Curr Atheroscler Rep,** v. 16, n. 7, p. 425, Jul 2014.

KANTHI, Y. M.; SUTTON, N. R.; PINSKY, D. J. CD39: Interface between vascular thrombosis and inflammation. **Current atherosclerosis reports,** v. 16, n. 7, p. 425, 2014/07// 2014.

KESHTELI, A. H. et al. The relationship between fruit and vegetable intake with gastroesophageal reflux disease in Iranian adults. **J Res Med Sci,** v. 22, p. 125, 2017.

KHAKH, B. S. et al. Neuronal P2X transmitter-gated cation channels change their ion selectivity in seconds. **Nature Neuroscience,** v. 2, p. 322, 04/01/online 1999.

KHAKH, B. S. et al. Neuronal P2X transmitter-gated cation channels change their ion selectivity in seconds. **Nat Neurosci,** v. 2, n. 4, p. 322-30, Apr 1999.

KO, H. et al. Synthesis and potency of novel uracil nucleotides and derivatives as P2Y2 and P2Y6 receptor agonists. **Bioorg Med Chem,** v. 16, n. 12, p. 6319-32, Jun 15 2008.

KUNAPULI, S. P. et al. ADP receptors--targets for developing antithrombotic agents. **Curr Pharm Des,** v. 9, n. 28, p. 2303-16, 2003.

LABELLE, M.; BEGUM, S.; HYNES, R. O. Platelets guide the formation of early metastatic niches. **Proc Natl Acad Sci U S A,** v. 111, n. 30, p. E3053-61, Jul 29 2014.

LAGERGREN, J. et al. Oesophageal cancer. **Lancet**, Jun 22 2017.

LAVOIE, E. G. et al. Ectonucleotidases in the digestive system: focus on NTPDase3 localization. **Am J Physiol Gastrointest Liver Physiol,** v. 300, n. 4, p. G608-20, Apr 2011.

LAZAROWSKI, E. R. Vesicular and conductive mechanisms of nucleotide release. **Purinergic Signal,** v. 8, n. 3, p. 359-73, Sep 2012.

LECEA, B. et al. Regional functional specialization and inhibitory nitrergic and nonnitrergic coneurotransmission in the human esophagus. **Am J Physiol Gastrointest Liver Physiol,** v. 300, n. 5, p. G782-94, May 2011.

LEE, S. H. et al. beta-arrestin 2-dependent activation of ERK1/2 is required for ADP-induced paxillin phosphorylation at Ser(83) and microglia chemotaxis. **Glia,** v. 60, n. 9, p. 1366-77, Sep 2012.

LEON, C. et al. Differential involvement of the P2Y1 and P2Y12 receptors in platelet procoagulant activity. **Arterioscler Thromb Vasc Biol,** v. 23, n. 10, p. 1941-7, Oct 1 2003.

LEVIN, R.; BRAIMAN, A.; PRIEL, Z. Protein kinase C induced calcium influx and sustained enhancement of ciliary beating by extracellular ATP. **Cell Calcium,** v. 21, n. 2, p. 103-13, Feb 1997.

LI, W. H. et al. P2Y2 receptor promotes cell invasion and metastasis in prostate cancer cells. **Br J Cancer,** v. 109, n. 6, p. 1666-75, Sep 17 2013.

LI, W. H. et al. P2Y2 Receptor and EGFR Cooperate to Promote Prostate Cancer Cell Invasion via ERK1/2 Pathway. **PLoS One,** v. 10, n. 7, p. e0133165, 2015.

LIN, E. W. et al. The tumor microenvironment in esophageal cancer. **Oncogene,** v. 35, n. 41, p. 5337-5349, Oct 13 2016.

LIU, X. et al. Genetic Alterations in Esophageal Tissues From Squamous Dysplasia to Carcinoma. **Gastroenterology,** v. 153, n. 1, p. 166-177, Jul 2017.

LORDICK, F. et al. Oesophageal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. **Ann Oncol,** v. 27, n. suppl 5, p. v50-v57, Sep 2016.

MA, J. et al. HCl-induced and ATP-dependent upregulation of TRPV1 receptor expression and cytokine production by human esophageal epithelial cells. **Am J Physiol Gastrointest Liver Physiol,** v. 303, n. 5, p. G635-45, Sep 01 2012.

MA, J. et al. ATP: a mediator for HCl-induced TRPV1 activation in esophageal mucosa. **Am J Physiol Gastrointest Liver Physiol,** v. 301, n. 6, p. G1075-82, Dec 2011.

MAASER, K. et al. Extracellular nucleotides inhibit growth of human oesophageal cancer cells via P2Y(2)-receptors. **Br J Cancer,** v. 86, n. 4, p. 636-44, Feb 12 2002.

MAGNI, G.; RICCIO, D.; CERUTI, S. Tackling chronic pain and inflammation through the purinergic system. **Curr Med Chem**, Jul 10 2017.

MALUF, F. et al. Accuracy of 30 MHz radial EUS for superficial esophageal and gastric cancer: Can submucosal invasion be reliably detected? **Gastrointestinal Endoscopy,** v. 55, n. 5, p. Ab233-Ab233, Apr 2002.

MELCHIONDA, M. et al. Ca2+/H+ exchange by acidic organelles regulates cell migration in vivo. **J Cell Biol,** v. 212, n. 7, p. 803-13, Mar 28 2016.

MENZIES, R. I. et al. Purinergic signaling in kidney disease. **Kidney Int,** v. 91, n. 2, p. 315-323, Feb 2017.

MIYASHITA, T. et al. Metastasis-promoting role of extravasated platelet activation in tumor. **J Surg Res,** v. 193, n. 1, p. 289-94, Jan 2015.

MOORE, S. F.; HUNTER, R. W.; HERS, I. Protein kinase C and P2Y12 take center stage in thrombin-mediated activation of mammalian target of rapamycin complex 1 in human platelets. **J Thromb Haemost,** v. 12, n. 5, p. 748-60, May 2014.

MORITA, M. et al. Alcohol drinking, cigarette smoking, and the development of squamous cell carcinoma of the esophagus: epidemiology, clinical findings, and prevention. **Int J Clin Oncol,** v. 15, n. 2, p. 126-34, Apr 2010.

MORRONE, F. B. et al. Extracellular nucleotides and nucleosides induce proliferation and increase nucleoside transport in human glioma cell lines. **J Neurooncol,** v. 64, n. 3, p. 211-8, Sep 2003.

MORRONE, F. B. et al. In vivo glioblastoma growth is reduced by apyrase activity in a rat glioma model. **BMC Cancer,** v. 6, p. 226, 2006.

MOSMANN, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. **J Immunol Methods,** v. 65, n. 1-2, p. 55-63, Dec 16 1983.

MULLER, L. B. et al. Stepwise expression of CDKN2A and RB1 proteins in esophageal mucosa from patients at high risk for squamous cell carcinoma. **Appl Immunohistochem Mol Morphol,** v. 22, n. 9, p. 669-73, Oct 2014.

MURPHY, G. et al. International cancer seminars: a focus on esophageal squamous cell carcinoma. **Annals of Oncology,** v. 28, n. 9, p. 2086-2093, 2017.

MUSCELLA, A. et al. Activation of P2Y2 receptor induces c-FOS protein through a pathway involving mitogen-activated protein kinases and phosphoinositide 3-kinases in HeLa cells. **J Cell Physiol,** v. 195, n. 2, p. 234-40, May 2003.

NAPIER, K. J.; SCHEERER, M.; MISRA, S. Esophageal cancer: A Review of epidemiology, pathogenesis, staging workup and treatment modalities. **World J Gastrointest Oncol,** v. 6, n. 5, p. 112-20, May 15 2014.

NATIONAL COMPREHENSIVE CANCER NETWORK, N. C. C. N. **NCCN guidelines for patients — esophageal cancer.** 2016.

NG, S. W. et al. Cysteinyl leukotriene type I receptor desensitization sustains Ca2+-dependent gene expression. **Nature,** v. 482, n. 7383, p. 111-5, Jan 9 2012.

NIESWANDT, B. et al. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. **Cancer Res,** v. 59, n. 6, p. 1295-300, Mar 15 1999.

NYLUND, G. et al. P2Y2- and P2Y4 purinergic receptors are over-expressed in human colon cancer. **Auton Autacoid Pharmacol,** v. 27, n. 2, p. 79-84, Apr 2007.

PALUMBO, J. S. et al. Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. **Blood,** v. 105, n. 1, p. 178-85, Jan 1 2005.

PANDOLFINO, J. E.; KWIATEK, M. A.; KAHRILAS, P. J. The Pathophysiologic Basis for Epidemiologic Trends in Gastroesophageal Reflux Disease. **Gastroenterology Clinics of North America,** v. 37, n. 4, p. 827-843, 2008/12/01/ 2008.

PEDATA, F. et al. Purinergic signalling in brain ischemia. **Neuropharmacology,** v. 104, p. 105-30, May 2016.

PELLEGATTI, P. et al. Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. **PLoS One,** v. 3, n. 7, p. e2599, Jul 09 2008.

PENNATHUR, A. et al. Oesophageal carcinoma. **Lancet,** v. 381, n. 9864, p. 400-12, Feb 2 2013.

PETTIT, M. Gastroesophageal reflux disease: clinical features. **Pharm World Sci,** v. 27, n. 6, p. 417-20, Dec 2005.

PILLAT, M. M. et al. Glioblastoma-mesenchymal stem cell communication modulates expression patterns of kinin receptors: Possible involvement of bradykinin in information flow. **Cytometry A,** v. 89, n. 4, p. 365-75, Apr 2016.

QIAN, Y. et al. Extracellular ATP is internalized by macropinocytosis and induces intracellular ATP increase and drug resistance in cancer cells. **Cancer Lett,** v. 351, n. 2, p. 242-51, Sep 1 2014.

QIU, Y. et al. P2Y2 receptor promotes the migration and invasion of breast cancer cells via EMT-related genes Snail and E-cadherin. **Oncol Rep,** v. 39, n. 1, p. 138-150, Jan 2018.

QU, X.; BEN, Q.; JIANG, Y. Consumption of red and processed meat and risk for esophageal squamous cell carcinoma based on a meta-analysis. **Ann Epidemiol,** v. 23, n. 12, p. 762-770 e1, Dec 2013.

QU, X. et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. **J Clin Invest,** v. 112, n. 12, p. 1809-20, Dec 2003.

RAFEHI, H. et al. Clonogenic assay: adherent cells. **J Vis Exp**, n. 49, Mar 13 2011.

RAJENDRA, S.; SHARMA, P. Barrett Esophagus and Intramucosal Esophageal Adenocarcinoma. **Hematol Oncol Clin North Am,** v. 31, n. 3, p. 409-426, Jun 2017.

RALEVIC, V.; BURNSTOCK, G. Receptors for purines and pyrimidines. **Pharmacol Rev,** v. 50, n. 3, p. 413-92, Sep 1998.

RAPAPORT, E.; FONTAINE, J. Anticancer activities of adenine nucleotides in mice are mediated through expansion of erythrocyte ATP pools. **Proc Natl Acad Sci U S A,** v. 86, n. 5, p. 1662-6, Mar 1989.

RATAJCZAK, M. Z. et al. Induction of a tumor-metastasis-receptive microenvironment as an unwanted and underestimated side effect of treatment by chemotherapy or radiotherapy. **J Ovarian Res,** v. 6, n. 1, p. 95, Dec 27 2013.

RHO, H. W. et al. Identification of valid reference genes for gene expression studies of human stomach cancer by reverse transcription-qPCR. **BMC Cancer,** v. 10, p. 240, May 28 2010.

RILEY, C. A. et al. Association of Gastroesophageal Reflux With Malignancy of the Upper Aerodigestive Tract in Elderly Patients. **JAMA Otolaryngol Head Neck Surg**, Dec 21 2017.

ROBSON, S. C.; SEVIGNY, J.; ZIMMERMANN, H. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. **Purinergic Signal,** v. 2, n. 2, p. 409-30, Jun 2006.

RONG, Y.; DISTELHORST, C. W. Bcl-2 protein family members: versatile regulators of calcium signaling in cell survival and apoptosis. **Annu Rev Physiol,** v. 70, p. 73-91, 2008.

SAMATAR, A. A.; POULIKAKOS, P. I. Targeting RAS-ERK signalling in cancer: promises and challenges. **Nat Rev Drug Discov,** v. 13, n. 12, p. 928-42, Dec 2014.

SANTOS, A. A. et al. Potential role of P2X7R in esophageal squamous cell carcinoma proliferation. **Purinergic Signalling**, p. 1-14, 2017.

SANTOS, A. A., JR. et al. Potential role of P2X7R in esophageal squamous cell carcinoma proliferation. **Purinergic Signal**, Apr 11 2017.

SARANGI, S. et al. P2Y12 receptor inhibition augments cytotoxic effects of cisplatin in breast cancer. **Med Oncol,** v. 30, n. 2, p. 567, 2013.

SCHAFER, R. et al. ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. **Am J Physiol Lung Cell Mol Physiol,** v. 285, n. 2, p. L376-85, Aug 2003.

SCHNEIDER, G. et al. Extracellular nucleotides as novel, underappreciated pro-metastatic factors that stimulate purinergic signaling in human lung cancer cells. **Molecular Cancer,** v. 14, p. 201, 11/24
05/31/received
11/10/accepted 2015.

SCHUMACHER, D. et al. Platelet-derived nucleotides promote tumor-cell transendothelial migration and metastasis via P2Y2 receptor. **Cancer Cell,** v. 24, n. 1, p. 130-7, Jul 8 2013.

SECRIER, M. et al. Mutational signatures in esophageal adenocarcinoma define etiologically distinct subgroups with therapeutic relevance. **Nat Genet,** v. 48, n. 10, p. 1131-41, Oct 2016.

SHANKAR, H. et al. P2Y12 receptor-mediated potentiation of thrombin-induced thromboxane A2 generation in platelets occurs through regulation of Erk1/2 activation. **J Thromb Haemost,** v. 4, n. 3, p. 638-47, Mar 2006.

SHENOY, S. K.; LEFKOWITZ, R. J. beta-Arrestin-mediated receptor trafficking and signal transduction. **Trends Pharmacol Sci,** v. 32, n. 9, p. 521-33, Sep 2011.

SHIEH, K. R. et al. Evidence for up-regulation of purinergic receptor genes associating with TRPV1 receptors and neurotrophic factors in the inflamed human esophagus. **Curr Mol Med,** v. 14, n. 9, p. 1205-14, 2014.

SIEGEL, R. L.; MILLER, K. D.; JEMAL, A. Cancer statistics, 2016. **CA Cancer J Clin,** v. 66, n. 1, p. 7-30, Jan-Feb 2016.

SMYTH, E. C. et al. Oesophageal cancer. **Nat Rev Dis Primers,** v. 3, p. 17048, Jul 27 2017.

SONG, S. et al. ATP promotes cell survival via regulation of cytosolic [Ca2+] and Bcl-2/Bax ratio in lung cancer cells. **Am J Physiol Cell Physiol,** v. 310, n. 2, p. C99-114, Jan 15 2016.

SOUZA, R. F. et al. A new paradigm for GERD pathogenesis. Not acid injury, but cytokine-mediated inflammation driven by HIF-2alpha: a potential role for targeting HIF-2alpha to prevent and treat reflux esophagitis. **Curr Opin Pharmacol,** v. 37, p. 93-99, Dec 2017.

SPECHLER, S. J.; SOUZA, R. F. Barrett's esophagus. **N Engl J Med,** v. 371, n. 9, p. 836-45, Aug 28 2014.

SPYCHALA, J. Tumor-promoting functions of adenosine. **Pharmacol Ther,** v. 87, n. 2-3, p. 161-73, Aug-Sep 2000.

STACHLER, M. D. et al. Paired exome analysis of Barrett's esophagus and adenocarcinoma. **Nat Genet,** v. 47, n. 9, p. 1047-55, Sep 2015.

STAGG, J.; SMYTH, M. J. Extracellular adenosine triphosphate and adenosine in cancer. **Oncogene,** v. 29, n. 39, p. 5346-58, Sep 30 2010.

STALLER, K.; KUO, B. Development, Anatomy, and Physiology of the Esophagus. In: SHAKER, R.;BELAFSKY, P. C.*, et al* (Ed.). **Principles of Deglutition: A Multidisciplinary Text for Swallowing and its Disorders**. New York, NY: Springer New York, 2013. p.269-286. ISBN 978-1-4614-3794-9.

STONE, R. L. et al. Paraneoplastic thrombocytosis in ovarian cancer. **N Engl J Med,** v. 366, n. 7, p. 610-8, Feb 16 2012.

TAKAMURA, A. et al. Autophagy-deficient mice develop multiple liver tumors. **Genes Dev,** v. 25, n. 8, p. 795-800, Apr 15 2011.

TAMAGAWA, T. et al. Involvement of Microglial P2Y12 Signaling in Tongue Cancer Pain. **J Dent Res,** v. 95, n. 10, p. 1176-82, Sep 2016.

TAN, W. K.; DI PIETRO, M.; FITZGERALD, R. C. Past, present and future of Barrett's oesophagus. **Eur J Surg Oncol,** v. 43, n. 7, p. 1148-1160, Jul 2017.

THE CANCER GENOME ATLAS RESEARCH, N. Integrated genomic characterization of oesophageal carcinoma. **Nature,** v. 541, p. 169, 01/04/online 2017.

THOME, M. P. et al. Ratiometric analysis of Acridine Orange staining in the study of acidic organelles and autophagy. **J Cell Sci,** v. 129, n. 24, p. 4622-4632, Dec 15 2016.

TORRE, L. A. et al. Global cancer statistics, 2012. **CA Cancer J Clin,** v. 65, n. 2, p. 87-108, Mar 2015.

TOTSUKAWA, G. et al. Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts. **J Cell Biol,** v. 164, n. 3, p. 427-39, Feb 2 2004.

TSAI, F. C.; MEYER, T. Ca2+ pulses control local cycles of lamellipodia retraction and adhesion along the front of migrating cells. **Curr Biol,** v. 22, n. 9, p. 837-42, May 8 2012.

VAKIL, N. et al. The Montreal Definition and Classification of Gastroesophageal Reflux Disease: A Global Evidence-Based Consensus. **The American Journal Of Gastroenterology,** v. 101, p. 1900, 08/01/online 2006.

VALSTER, A. et al. Cell migration and invasion assays. **Methods,** v. 37, n. 2, p. 208-15, Oct 2005.

VAN HAGEN, P. et al. Preoperative chemoradiotherapy for esophageal or junctional cancer. **N Engl J Med,** v. 366, n. 22, p. 2074-84, May 31 2012.

VAN LAETHEM, J. L. et al. The multidisciplinary management of gastro-oesophageal junction tumours: European Society of Digestive Oncology (ESDO): Expert discussion and report from the 16th ESMO World Congress on Gastrointestinal Cancer, Barcelona. **Dig Liver Dis,** v. 48, n. 11, p. 1283-1289, Nov 2016.

VANHAESEBROECK, B. et al. The emerging mechanisms of isoform-specific PI3K signalling. **Nat Rev Mol Cell Biol,** v. 11, n. 5, p. 329-41, May 2010.

VANINETTI, N. M. et al. Inducible nitric oxide synthase, nitrotyrosine and p53 mutations in the molecular pathogenesis of Barrett's esophagus and esophageal adenocarcinoma. **Mol Carcinog,** v. 47, n. 4, p. 275-85, Apr 2008.

VELAZQUEZ, B. et al. Differential agonist-induced desensitization of P2Y2 nucleotide receptors by ATP and UTP. **Mol Cell Biochem,** v. 206, n. 1-2, p. 75-89, Mar 2000.

VERKHRATSKY, A.; BURNSTOCK, G. Biology of purinergic signalling: its ancient evolutionary roots, its omnipresence and its multiple functional significance. **Bioessays,** v. 36, n. 7, p. 697-705, Jul 2014.

VERKHRATSKY, A.; KRISHTAL, O. A.; BURNSTOCK, G. Purinoceptors on neuroglia. **Mol Neurobiol,** v. 39, n. 3, p. 190-208, Jun 2009.

VERSCHUUR, E. M.; SIERSEMA, P. D. [Diagnostics and treatment of esophageal cancers]. **Ned Tijdschr Tandheelkd,** v. 117, n. 9, p. 427-31, Sep 2010.

VOLONTE, C. et al. P2X7 receptors: channels, pores and more. **CNS Neurol Disord Drug Targets,** v. 11, n. 6, p. 705-21, Sep 2012.

VON KUGELGEN, I.; HARDEN, T. K. Molecular pharmacology, physiology, and structure of the P2Y receptors. **Adv Pharmacol,** v. 61, p. 373-415, 2011.

WAGSTAFF, S. C. et al. Extracellular ATP activates multiple signalling pathways and potentiates growth factor-induced c-fos gene expression in MCF-7 breast cancer cells. **Carcinogenesis,** v. 21, n. 12, p. 2175-81, Dec 2000.

WAN, H. et al. Anti-proliferative Effects of Nucleotides on Gastric Cancer via a Novel P2Y6/SOCE/Ca(2+)/beta-catenin Pathway. **Sci Rep,** v. 7, n. 1, p. 2459, May 26 2017.

WAN, H. X. et al. Important roles of P2Y receptors in the inflammation and cancer of digestive system. **Oncotarget,** v. 7, n. 19, p. 28736-47, May 10 2016.

WANG, L. et al. Ecto-5'-nucleotidase promotes invasion, migration and adhesion of human breast cancer cells. **J Cancer Res Clin Oncol,** v. 134, n. 3, p. 365-72, Mar 2008.

WANG, M. X.; REN, L. M.; SHAN, B. E. Inhibitory effects of extracellular adenosine triphosphate on growth of esophageal carcinoma cells. **World J Gastroenterol,** v. 11, n. 38, p. 5915-9, Oct 14 2005.

WANG, Q. et al. P2X7 receptor-mediated apoptosis of human cervical epithelial cells. **Am J Physiol Cell Physiol,** v. 287, n. 5, p. C1349-58, Nov 2004.

WEAVER, J. M. J. et al. Ordering of mutations in preinvasive disease stages of esophageal carcinogenesis. **Nat Genet,** v. 46, n. 8, p. 837-843, Aug 2014.

WEI, C. et al. Calcium gradients underlying cell migration. **Curr Opin Cell Biol,** v. 24, n. 2, p. 254-61, Apr 2012.

WEI, Y. et al. EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance. **Cell,** v. 154, n. 6, p. 1269-84, Sep 12 2013.

WHITE, N.; BURNSTOCK, G. P2 receptors and cancer. **Trends Pharmacol Sci,** v. 27, n. 4, p. 211-7, Apr 2006.

WHITE, N.; BUTLER, P. E.; BURNSTOCK, G. Human melanomas express functional P2 X(7) receptors. **Cell Tissue Res,** v. 321, n. 3, p. 411-8, Sep 2005.

WHITE, N. et al. P2Y purinergic receptors regulate the growth of human melanomas. **Cancer Lett,** v. 224, n. 1, p. 81-91, Jun 16 2005.

WIJARNPREECHA, K. et al. Association between gastroesophageal reflux disease and nonalcoholic fatty liver disease: A meta-analysis. **Saudi J Gastroenterol,** v. 23, n. 6, p. 311-317, Nov-Dec 2017.

WILHELM, K. et al. Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. **Nat Med,** v. 16, n. 12, p. 1434-8, Dec 2010.

WINK, M. R. et al. Altered extracellular ATP, ADP and AMP catabolism in glioma cell lines. **Cancer Lett,** v. 198, n. 2, p. 211-8, Aug 20 2003.

WU, C. C. et al. The roles and mechanisms of PAR4 and P2Y12/phosphatidylinositol 3-kinase pathway in maintaining thrombin-induced platelet aggregation. **Br J Pharmacol,** v. 161, n. 3, p. 643-58, Oct 2010.

WU, L. et al. Adenosine triphosphate induces P2Y2 activation and interleukin-8 release in human esophageal epithelial cells. **J Gastroenterol Hepatol,** v. 32, n. 7, p. 1341-1347, Jul 2017.

WU, L. et al. PAR-2 activation enhances weak acid-induced ATP release through TRPV1 and ASIC sensitization in human esophageal epithelial cells. **Am J Physiol Gastrointest Liver Physiol,** v. 309, n. 8, p. G695-702, Oct 15 2015.

XIE, R. et al. The P2Y2 nucleotide receptor mediates the proliferation and migration of human hepatocellular carcinoma cells induced by ATP. **J Biol Chem,** v. 289, n. 27, p. 19137-49, Jul 4 2014.

YAGUCHI, T. et al. Higher concentrations of extracellular ATP suppress proliferation of Caco-2 human colonic cancer cells via an unknown receptor involving PKC inhibition. **Cell Physiol Biochem,** v. 26, n. 2, p. 125-34, 2010.

ZHANG, H. Z.; JIN, G. F.; SHEN, H. B. Epidemiologic differences in esophageal cancer between Asian and Western populations. **Chin J Cancer,** v. 31, n. 6, p. 281-6, Jun 2012.

ZHANG, Y. Epidemiology of esophageal cancer. **World J Gastroenterol,** v. 19, n. 34, p. 5598-606, Sep 14 2013.

ZIMMERMANN, H. Extracellular ATP and other nucleotides-ubiquitous triggers of intercellular messenger release. **Purinergic Signal,** v. 12, n. 1, p. 25-57, Mar 2016.

ZIMMERMANN, H.; ZEBISCH, M.; STRATER, N. Cellular function and molecular structure of ecto-nucleotidases. **Purinergic Signal,** v. 8, n. 3, p. 437-502, Sep 2012.

ANEXO I

De: Current Medicinal Chemistry [mailto:onbehalfof@manuscriptcentral.com]
Enviada em: quinta-feira, 25 de janeiro de 2018 12:15
Para: Fernanda Bueno Morrone <fernanda.morrone@pucrs.br>
Assunto: Current Medicinal Chemistry - Manuscript ID CMC-2018-0030

25-Jan-2018

Dear Dr. Morrone:

Your manuscript entitled "P2 Purinergic Receptors Involvement in Esophageal Diseases" has been successfully submitted online and is presently being given full consideration for publication in the Current Medicinal Chemistry.

Your manuscript ID is CMC-2018-0030.

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Sincerely,
Current Medicinal Chemistry Editorial Office

ANEXO II

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL - PUC/RS

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: CARACTERIZAÇÃO DOS RECEPTORES PURINÉRGICOS P2Y EM TUMORES DE ESÔFAGO E ESTÔMAGO

Pesquisador: Fernanda Bueno Morrone

Área Temática:

Versão: 3

CAAE: 49696115.0.0000.5336

Instituição Proponente: UNIAO BRASILEIRA DE EDUCACAO E ASSISTENCIA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.645.764

Apresentação do Projeto:

O pesquisador principal do estudo: "CARACTERIZAÇÃO DOS RECEPTORES PURINÉRGICOS P2Y EM TUMORES DE ESÔFAGO E ESTÔMAGO" encaminhou ao CEP-PUCRS, em 28/06/2016, emenda solicitando a inclusão do biomarcador P2X7R (receptor purinérgico P2X7) no projeto.

Objetivo da Pesquisa:

IO pesquisador principal do estudo: "CARACTERIZAÇÃO DOS RECEPTORES PURINÉRGICOS P2Y EM TUMORES DE ESÔFAGO E ESTÔMAGO" encaminhou ao CEP-PUCRS, em 28/06/2016, emenda solicitando a inclusão do biomarcador P2X7R (receptor purinérgico P2X7) no projeto.

Avaliação dos Riscos e Benefícios:

O pesquisador principal do estudo: "CARACTERIZAÇÃO DOS RECEPTORES PURINÉRGICOS P2Y EM TUMORES DE ESÔFAGO E ESTÔMAGO" encaminhou ao CEP-PUCRS, em 28/06/2016, emenda solicitando a inclusão do biomarcador P2X7R (receptor purinérgico P2X7) no projeto.

Comentários e Considerações sobre a Pesquisa:

O pesquisador principal do estudo: "CARACTERIZAÇÃO DOS RECEPTORES PURINÉRGICOS P2Y EM TUMORES DE ESÔFAGO E ESTÔMAGO" encaminhou ao CEP-PUCRS, em 28/06/2016, emenda solicitando a inclusão do biomarcador P2X7R (receptor purinérgico P2X7) no projeto.

Endereço: Av.Ipiranga, 6681, prédio 50, sala 703
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UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3320-3345 **Fax:** (51)3320-3345 **E-mail:** cep@pucrs.br

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos foram apresentados.

Conclusões ou Pendências e Lista de Inadequações:

Não há pendências.

Considerações Finais a critério do CEP:

O CEP-PUCRS, de acordo com suas atribuições definidas na Resolução CNS n° 466 de 2012 e da Norma Operacional n° 001 de 2013 do CNS, manifesta-se pela aprovação da emenda que solicita a inclusão do do biomarcador P2X7R (receptor purinérgico P2X7) no projeto.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_748929_E1.pdf	28/06/2016 16:00:46		Aceito
Outros	Emenda.pdf	28/06/2016 15:58:52	Fernanda Bueno Morrone	Aceito
Declaração de Instituição e Infraestrutura	anuencia_patologia.pdf	03/11/2015 14:56:13	Fernanda Bueno Morrone	Aceito
Outros	carta_resposta_material_biologico.pdf	03/11/2015 14:54:48	Fernanda Bueno Morrone	Aceito
Projeto Detalhado / Brochura Investigador	projetodoutorado.pdf	20/10/2015 14:34:02	Fernanda Bueno Morrone	Aceito
Outros	Links_lattes.pdf	21/09/2015 11:29:24	Fernanda Bueno Morrone	Aceito
Outros	intox.pdf	21/09/2015 11:28:48	Fernanda Bueno Morrone	Aceito
Outros	sipesq.pdf	21/09/2015 11:26:49	Fernanda Bueno Morrone	Aceito
Folha de Rosto	folhaderosto.pdf	03/09/2015 11:07:18	Fernanda Bueno Morrone	Aceito
Cronograma	Cronograma.pdf	03/09/2015 10:47:47	Fernanda Bueno Morrone	Aceito
Orçamento	orcamento.pdf	03/09/2015 10:47:15	Fernanda Bueno Morrone	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	termo.pdf	03/09/2015 10:35:15	Fernanda Bueno Morrone	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 23 de Julho de 2016

Assinado por:
Denise Cantarelli Machado
(Coordenador)

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