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Stromal-derived lactate activates amoeboid motility by engaging the endocannabinoid receptor GPR55 in a prostate cancer cell model

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Abbreviations

12(S)-HPETE	12(S)-hydroperoxy-eicosatetraenoic acid
20-HETE	20-hydroxyeicosatetraenoic acid
2-AcGs	2-acyl-glycerols
2-AG	2-arachidonolylglycerol
2-LG	2-linoleoyl-glycerol
2-OG	2-oleoyl- glycerol
6PGLD	6-phosphogluconate dehydrogenase
7ACC	7 aminocarboxycoumarin
8(S)-HETE	8(S)-hydroxyeicosatetraenoic acid
AA	arachidonic acid
AA-5HT	arachidonoyl serotonin
ABCC1	ATP binding cassette subfamily c member 1
ABDH4	α/β-hydrolase 4
ACC	acetyl-CoA carboxylase
ACEA	arachidonyl-2'-chloroethylanamide
ACF	aberrant crypt foci
ACLY	ATP citrate lyase
ACPA	arachidonylcyclopropylamide
ADT	androgen deprivation therapy
AEA	anandamide
AIF	apoptosis-inducing factor
AMPK	AMP-activated protein kinase
Ang 2	angiotensin 2
AOM	azoxymethane
ATCC	American type culture collection
ATF2/4/…	activating transcription factor
ATM	ataxasia-telangiectasia mutated kinase
ATP	adenosine triphosphate
Bax	Bcl2-associated X protein
BC	bladder capacity
BCA	bicinchoninic acid
BCa	breast cancer
Bcl-2	B-cell leukemia/lymphoma 2 protein
Bcom	compliance
BRCP	breast cancer resistance protein
CAFs	cancer associated fibroblasts
CaMKKβ	calcium/ calmodulin-dependent protein kinase β
cAMP	cyclic adenosine monophosphate
CART	Cocaine-amphetamine-regulated transcript
Cav-1	caveolin-1
CB1R	cannabinoid receptor 1
CB2R	cannabinoid receptor 2
CBC	cannabichromene
CBD	cannabidiol
CBDA	cannabidiolic acid
CBE	cannabielsoin
CBG	cannabigerol
CBL	cannabicyclol
CBN	cannabinol
CBND	cannabinodiol
CBRs	cannabinoid receptors
CBT	cannabitriol
CCL	C-C motif chemokine ligand
CCRCC	clear cell renal cell carcinoma
Cdc25A	cell division cycle 25 A
CDK 2/4/…	cyclin-dependent kinase 2/4/ …

cDNA	complementary deoxyribonucleic acid
CGRP	substance P and calcitonin gene-related peptide
Chk1	checkpoint kinase 1
CHOP	C/EBP homologous protein
ChRCC	chromophobe renal cell carcinoma
CIC	citrate/isocitrate carrier
c-Myc	cellular Myc
CNR1	cannabinoid receptor 1 (gene)
CNR2	cannabinoid receptor 2 (gene)
CNS	central nervous system
COX-2	cyclooxygenase-2
CPRC	castration-resistant PCa
CPT	carnitine palmitoyltransferase
CPT1 C/…	carnitine palmitoyltransferase 1
CRC	colorectal cancer
CREB	cAMP response element-binding protein
CSCs	cancer stem cells
CT	control condition
CV	cardiovascular system
CXCL	C-X-C motif ligand
CXCR4	C-X-C chemokine receptor type 4
CYP	cytochrome P450
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DDR	discoidin domain-containing receptor
DEC1	Differentially expressed in condrocytes-1
DHA	docosahexaenoic acid
DHA-EA	docosahexanoyl ethanolamide
DHG	docosahexanoyl-glycerol
DMBA/TPA	dimethylbenz[a]anthracene and 12-O-tetradecanoyl phorbol-13-acetate
DMEM	Dulbecco's modified eagle medium
DR4/-5	death receptors
DSS	dextran sulfate sodium
eCBs	endocannabinoids
ECM	extracellular matrix 1
ECS	Endocannabinnoid System
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-to mesenchymal transition
EPA	eicosapentaenoic acid
EPA-EA	eicosapentaenoyl ethanolamide
EPG	eicosapentanoyl-glycerol
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
ERα	estrogen receptor α
ET-1	endothelin-1
ETC	electron transport chain
ETS	E26 transformation-specific
ETV4/PEA3	ETS translocation variant 4/polyoma enhancer activator 3
f.c.	fold change
FAAH	Fatty acid amide hydrolase
FADD	Fas-associated death domain
FAK	focal adhesion kinase
FAO	fatty acid oxidation
FASN	fatty acid synthase
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor

FoxP3	Forkhead box P3
G6PD	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde phosphate dehydrogenase
GAPs	GTPase-activating proteins
GBM	glioblastomas
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GI	gastrointestinal
GLS1	glutaminase
GLUD	glutamate dehydrogenase
GLUT1/…	glucose transporters
GM-CSF	granulaocyte-macrophage colony-stimulating factor
GPCRs	G coupled protein receptors
GPR55/-18/-119/…	G coupled protein receptor 55/-18/-119/…
GSCs	GBM strem cells
GSH	glutathione (reduced form)
GSSG	glutathione disulfide (oxidized form)
GST	glutathione S-transferase
GTP	guanosine-5'-triphosphate
H3k18	lysine 18 residue on histone 3
Ham's	named after Richard G. Ham
HBXIP	Hepatitis B virus X-interacting protein
HCAR1	hydroxycarboxylic acid receptors 1
HCC	hepatocellular carcinoma
HER2	human epidermal growth factor receptor 2
HGF	hepatocyte growth factor
HIF-1α	hypoxia-inducible factor-1α
hiPSC-VECs	human-induced pluripotent stem cell-derived vascular endothelial cells
HK2	hexokinase 2
HPFs	healthy prostate fibroblasts
HPRT1	hypoxanthine phosphoribosyltransferase 1
HSF-1	heat shock factor-1
HSP 90/…	heat shock proteins
ICAM-1	intercellular adhesion molecule 1
iCGRP	immunoreactive calcitonin gene-related peptide
ICIs	inter-contraction intervals
Id-1	inhibitor of differentiation 1
IDH1/3α/…	isocitrate dehydrogenase
IFN	interferon
IgA	Immunoglobulin A
IGF-1	insulin-like growth factor-1
IL- 1β/-8/…	interleukin 1β /…
IP3	inositol 1,4,5-triphosphate
IR	insulin receptor (IR),
IUPHAR	Union of Pharmacology
JNK	c-Jun N-terminal kinase
KRAS	Kirsten rat sarcoma virus
LDH-A/B	lactate dehydrogenase
LEA	linoleoyl-ethanolamine
LIF/GP130/IL-6R	leukemia inhibitory factor/glycoprotein 130/interleukin 6 receptor
LOXs	lipoxygenases
LPC	lysophosphatidylcholine
LPG	lyso-phosphatidyl-glycerols
LPI	lysophosphatidylinositol
LTB4	leukotriene B4
MAGL	monoacylglyceride lipase
MAPK	mitogen-activated protein kinase
MAT	mesenchymal-to-amoeboid transition

M-CSF	macrophage colony stimulating factor
MCT1/4/…	monocarboxylic acid transporter
MDR	multiple drug resistant
MDSCs	myeloid-derived suppressor cells
ME1	malic enzyme
MEK	MAPK-ERK kinase
Met-AEA	methanandamide
Met-F-AEA	metfluoroanandamide
MHC	major histocompatibility complex
MICA/B	MHC class I chain-related protein A and B
min	minutes
miRNA	micro ribonucleic acid
MKK3	mitogen-activated protein kinase kinase 3
MLC	myosin light chain
MMPs	matrix metalloproteinases
mRNA	messenger ribonucleic acid
MSCs	mesenchymal stem cells
mTOR	mammalian target of rapamycin
Myc	myelocytomatosis oncogene
n.	number
NAD+	nicotinamide adenine dinucleotide(oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP+	nicotinamide adenine dinucleotide phosphate hydrogen(oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen(reduced form)
NAEs	N-acylethanolamines
NAFLD	Non-alcoholic fatty liver disease
NAFs	normal activated fibroblasts
NAGly	N-arachidonoyl glycine
NAPE	N-arachidonoyl-phosphatidylethanolamine
NAPE-PLD	NAPE- phospholipase D
NASH	Non-alcoholic steatohepatitis
NAT	N-acyltransferase
NEPC	neuroendocrine PCa
NFAT	nuclear factor of acrivated T-cells
NF-κB	nuclear factor-kappaB
NGF	nerve growth factor
NK-1R	neurokinin-1 receptor
NKG2D	Natural killer group 2 member D
NKs	Natural killer cells
nNOS	neuronal Nitric oxide synthase
Noxa	phorbol-12-myristate-13-acetate-induced protein 1
ns.	non-significant
NSCLC	non-small cell lung cancer
OEA	oleoylethanolamide
OXPHOS	oxidative phosphorylation
TKIs	tyrosine kinase inhibitors
P2Y2	purinoceptor 2
p8	protein 8
PAI-1	plasminogen activator inhibitor-1
PAR-2	protease-activated receptor 2
PARP	poly (ADP-ribose) polymerases
PBMCs	peripheral blood mononuclear cells
PC	phosphatidyl choline
PCa	prostate cancer
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PD-1	programmed death-1
PDAC	pancreatic ductal adenocarcinoma

PDGF	platelet derived growth factor
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PDL-1	programmed death-ligand 1
PE	phosphatidylethanolamine
PEA	palmitoylethanolamide
PEP	phosphoenolpyruvate
PGE2	prostaglandin E2
Pgp	P-glycoprotein
PHD	Prolyl-hydroxylase
PI3K	phosphatidylInositol 3-Kinase
PIN	prostatic intraepithelial neoplasia
PKA	protein kinase A
PKC	protein kinase C
PKM2	pyruvate kinase M2
PLC	phospholipase C
PlGF	placental growth factor
p-MLC	phosphorylation of the myosin light chain
PMNs	polymorph nuclear neutrophils
PNS	periferal nervous system
POMC	hypothalamic proopiomelanocortin
PPARs	proliferator–activated receptors
PPP	pentose phosphate pathway
PPREs	PPARs' response elements
pRb	retinoblastoma protein
PSA	prostate-specific antigen
PTEN	phosphatase and tensin homolog
PTP1B	protein tyrosine phosphatase 1B
PUFAs	polyunsaturated fatty acid
PUMA	p53 upregulated modulator of apoptosis
PyMT	polyoma middle T oncoprotein
qRT-PCR	reverse transcription-quantitative PCR
Rac1	Ras-related C3 botulinum toxin substrate 1
RANTES	regulated upon activation, normally T-expressed, and presumably secreted
RCC	renal cell carcinoma
Rho A	Ras homolog family member A
RIP3	receptor-interacting serine-threonine kinase 3
RIPA	radioimmunoprecipitation assay
RO	renal oncocytoma
ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
RRID: CVCL	Research resource identifier: Cellosaurus (controlled vocabulary for cell lines)
RvD2	resolvin D2
SAT	subcutaneous adipose tissue
SCD	stearoyl-CoA desaturase
SCID	severe combined immunodeficiency
SCLC	small cell lung cancer
SDF1	stromal-derived growth factor-1
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEA	stearoylethanolamide
SIRT1/PGC-1α	sirtuin 1/ PGC transcription co-activator 1α
SMAD	suppressor of Mothers against decapentaplegic
STAT3	signal transducer and activator of transcription 3
TAMs	tumor associated macrophages
TANs	tumor associated neutrophils
TCA	tricarboxylic acid cycle
TCGA	The cancer genome atlas

TGF-β2/…	transforming growth factor β2/ …
Th-1/…	T helper cells
THCA	tetrahydrocannabinolic acid
THCV	Δ9-tetrahydro-cannabivarin
TIMP-1	tissue inhibitor matrix metalloproteinases-1
TME	tumor microenvironment
TMPRSS2	transmembrane serine protease 2
TNBC	triple negative breast cancer
TNFα	tumor necrosis factorα
TNM	tumor, node, metastasis
TP53	tumor suppressor protein 53
TRAIL	TNF-related apoptosis-inducing ligand
TRB3	tribbles homolog 3
Tregs	T regulatory cells
TRPMs	transient receptor potential cation channel subfamily M members
TRPVs	transient receptor potential cation channel subfamily V members
TRPVs	transient receptor potential cation channel subfamily V members
UCP	uncoupling proteins
uPA	urokinase-tyoe plasminogen activator
VAT	visceral adipose tissue
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VHL	von Hippel-Lindau
VIM	vimentin
WAT	white adipose tissue
WB	Western blot
Wnt	Wingless-related integration site
XBP	X-box binding protein
Y1068	tyrosine 1068
ZEB1/2	zinc finger E-box binding homeobox 1/ 2
α-KG	α- ketoglutarate
αSMA	α-smooth muscle actin
Δ8-THC	Delta-8 tetrahydrocannabinol
Δ9-THC	Delta-9 tetrahydrocannabinol

1. Introduction

1.1 Cannabinoids

1.1.1 Phytocannabinoids

More than 500 compounds have been identified in Cannabis Sativa, including phytocannabinoids (113), aromatic terpenes (120), and phenols (22) [1-4]. We refer to phytocannabinoids as natural derivatives from the plant, which belong to a larger family of terpenophenolics called cannabinoids. This family also includes synthetic preparations and molecules produced by the body, known as endocannabinoids (eCBs). The concentration of phytocannabinoids can vary based on the growing conditions of the plant (e.g. moisture, soil nutrients, temperature, and UV radiation). These "phyto compounds" are generated differently through the coupling of olivetolic acid and geranyl diphosphate (Fig. 1), and there are 11 distinct types of phytocannabinoids discovered to date [5]: Δ9-THC (Delta-9 tetrahydrocannabinol), Δ8-THC (Delta-8 tetrahydrocannabinol), cannabidiol (CBD), cannabigerol (CBG), cannabichromene (CBC), cannabinodiol (CBND), cannabielsoin (CBE), cannabicyclol (CBL), cannabinol (CBN), cannabitriol (CBT), and miscellaneous cannabinoids [6].

1.1.1.1 Main phytocannabinoids used in medical research

Δ9-THC

It is the main psychoactive product of Cannabis Sativa, responsible for the related psychotic effects. Following its isolation, experiments in animals demonstrated that the compound can induce motor disturbances, redness of the mucous membrane of the eyeball, a reduction in aggression, and a sleepy state. Subsequently, Pertwee and Martin's group concluded that the molecule produced catalepsy, hypokinesia, anti-nociception, and it reduced body temperature. Δ9-THC was shown to provide relief for chronic pain [7-9], especially neuropathic pain, and to eliminate nightmares [10, 11]. Moreover, in advanced cancer patients, it stimulated appetite and reduced the side effects of chemotherapy (nausea and vomiting) [12-14]. In vivo studies highlighted that Δ9-THC reduced inflammation in rat colitis [15-17] and neurological deficits [18]. Finally, it increased survival in amyotrophic lateral sclerosis [19] and it improved hand-eye coordination in animals affected by Parkinson's disease [20].

Δ8-THC

Δ8-THC is particularly used in pediatric chemotherapy, thanks to its anti-emetic effects [21]. Moreover, it is recognized for its potential in promoting weight loss. In fact, low doses of Δ8-THC such as 0.001 mg/kg resulted in a decrease in body weight without side effects [22], also affecting food consumption [23].

CBD

CBD is the primary non-psychoactive cannabinoid, isolated from Mexican marijuana in 1940 and, later, from Lebanese hashish by Mechoulam and Shvo. It possesses anti-psychotic and neuroprotective functions. Additionally, there is evidence supporting its potential use in epilepsy, drug dependence, schizophrenia, social phobia, post-traumatic stress, depression, bipolar disorder, sleep disorders, Parkinson's disease, and Alzheimer's disease [24-29].

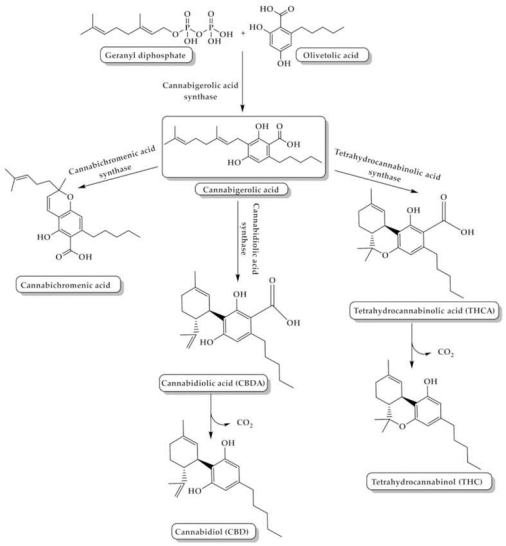


Fig.1 Main biosynthetic pathways for the production of phytocannabinoids

Synthesis of cannabigerolic acid from the alkylation of olivetolic acid in reaction with geranyl diphosphate. Oxidative cyclization of cannabigerolic acid into cannabidiolic acid (CBDA), by the CBDA synthase. Cannabichromenic acid synthase and tetrahydrocannabidinolic acid synthase are responsible for the production of cannabichromenic acid and tetrahydrocannabinolic acid (THCA), respectively. Cannabidiol (CBD) forms from CBDA with the process of decarboxylation by heating cannabis. Decarboxylation of THCA generates tetrahydrocannabinol (THC). Image derived from [5]

1.1.2 Endocannabinoids

eCBs are products of arachidonic acid (AA), conjugated with ethalonamine or glycerol [30]. Arachidonoyl ethanolamide, also known as anandamide (AEA) and 2-arachidonolylglycerol (2-AG) are the first eCBs identified, respectively isolated from porcine brain extracts, and canine intestine [31]. AEA is synthesized through phospholipid membrane cleavage, involving a double step. First, the precursor of the phospholipid membrane, N-arachidonoyl-phosphatidylethanolamine (NAPE), is produced by the N-acyltransferase (NAT) enzyme. This enzyme catalyzes the transfer of AA to the head of the phosphatidylethanolamine group from phosphatidylcholine in presence of Ca^{2+} and cyclic adenosine monophosphate (cAMP). Then, a specific phospholipase D (NAPE-PLD) hydrolyzes AEA from NAPE. Moreover, other enzymes such as phospholipase C (PLC) and α/β-hydrolase 4 (ABDH4) can also be involved in the synthesis of AEA (Fig. 2).

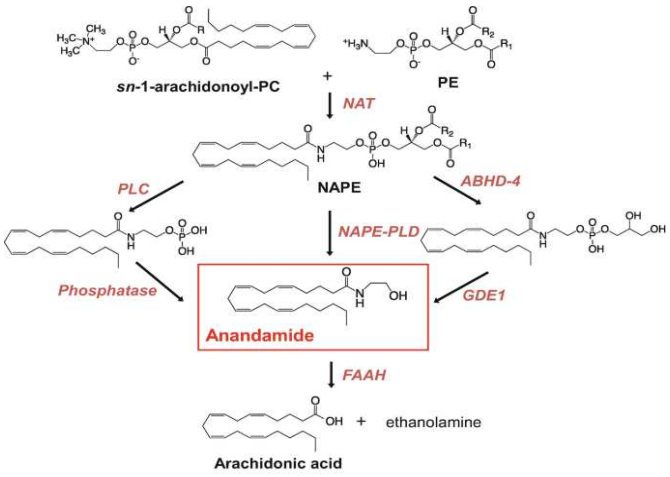


Fig.2 Synthesis and degradation of AEA

Transfer of arachidonic acid (AA) from the sn-1 position of arachidonoyl-phosphatidyl choline (PC) to the nitrogen atom of phosphatidylethanolamine (PE) to generate N-arachidonoyl-phosphatidylethanolamine (NAPE). This reaction is catalized by N-acyltransferase (NAT) enzyme. A specific phospholipase D (NAPE-PLD) hydrolyzes anandamide (AEA) from NAPE. Phospholipase C (PLC), phosphatease, α/β-hydrolase 4 (ABDH4) and Glycerophosphodiester Phosphodiesterase 1 can also be involved in the synthesis of AEA from NAPE. Fatty acid amide hydrolase (FAAH) hydrolyzes AEA into arachidonic acid (AA) and ethanolamine. Image derived from [32]

The production of 2-AG follows a different pathway. It begins with phosphatidylinositol-specific PLC, which generates diacylglycerol, and then diacylglycerol lipase (DAGL) produces 2-AG [33] [34] (Fig. 3). AEA is degraded by fatty acid amide hydrolase (FAAH), while 2-AG is hydrolyzed by monoacylglyceride lipase (MAGL). Both enzymes release AA and ethanolamine (for AEA) or glycerol (for 2-AG) (Fig. 2-3). In some cases, FAAH also degrades 2-AG. Alternatively, AEA and 2-AG can also be oxidized by cyclooxygenase-2 (COX-2), lipoxygenases (LOXs), cytochrome P450 (CYP).

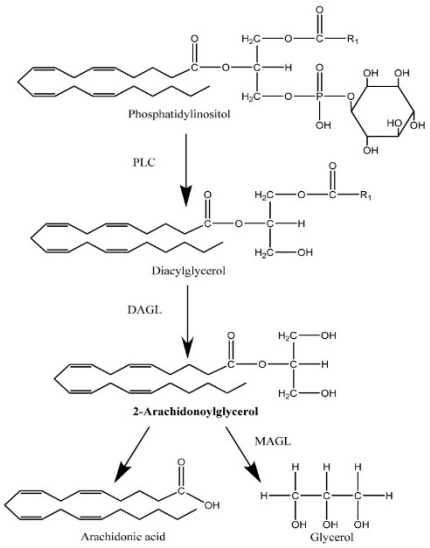


Fig.3 2-AG synthesis and degradation

Phosphatidylinositol-specific phospholipase C (PLC) catalizes the production of diacylglycerol from phosphatidylinositol. Diacylglycerol lipase (DAGL) hydrolyse diacylglycerol to generate 2-arachidonoylglycerol (2-AG). Monoacylglyceride lipase (MAGL) degrades 2-AG into arachidonic acid (AA) and glycerol. Image derived from [35]

eCBs are ubiquitously expressed in the body, carrying out various functions. For example, AEA and 2-AG regulate glucose and lipid metabolism, adipose tissue homeostasis, inflammation, and the function of the respiratory tract. Moreover, their variable levels and different receptor binding in tissues also plays a crucial function in the development and progression of pathologies such as neuromodulatory disorders, metabolic/inflammatory diseases, and cancer [36]. Other eCBs were recognized and characterized later. These include N-acylethanolamines (NAEs) such as N-palmitoyl, N-oleoyl, and N-linoleoyl-ethanolamine (PEA, OEA, and LEA), and 2-acyl-glycerols (2-AcGs) like 2-oleoyl and 2-linoleoyl-glycerol (2-OG and 2-LG), prostaglandin ethanolamides, prostaglandin glycerol esters, and omega-3 eCBs derived from docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). These include docosahexanoyl ethanolamide (DHA-EA), docosahexanoyl-glycerol (DHG), eicosapentaenoyl ethanolamide (EPA-EA), and eicosapentanoyl-glycerol (EPG).

1.1.3 Synthetic cannabinoids

Synthetic cannabinoids are chemical products, developed in 1970 to investigate the mechanisms of action of eCBs'. Since 2000, some products emerged on the "legal highs" market for recreational drug use [37]. In general we can distinguish:

- **equivalents of phytocannabinoids** (e.g Dronabinol that is similar to Δ9-THC)
- **analogs of phytocannabinoids** (e.g Nabilone, HU-210)
- **derivatives of eCBs** (e.g. methanandamide, Met-AEA).

Commonly, the structure of these molecules includes a *principal core* which has an aromatic mono or heterocyclic structure (e.g. 3-indole substituted), a *secondary moiety* (alkyl chain, aromatic cyclic or alicyclic compounds) and a *bridge* (carbon–carbon bond, carbonyl, carboxyl, carboxamide).

In terms of production over time, there are instead 3 generations of compounds:

- **the 1st generation**, includes cyclohexylphenol, dibenzopyrane, and naphthoyl indole derivatives;
- **the 2nd generation**, a class of compounds resulting from a substituted indole core, from pyrrole or indene structures;
- **the 3rd generation**, represented by a carboxylate or carboxamide bridge linked to a substituted indole or indazole core.

In Table 1, we summarize the principal synthetic cannabinoids, referring to their chemical structure.

Group	Subgroup	Examples
First generation derivatives		
3-indole	naphtoyl	JWH-018
Dibenzopyrane		HU-210
Cyclohexylphenol		CP-47,497
Second generation derivatives		
3-indole	benzoyl	AM-679, RCS-4
3-indole	alkoyl	UR-144, XLR-11
3-indole	phenylacetyl	JWH-250
3-indole	naphthylmethyl	JWH-175
Indene	naphthylmethyl	JWH-176
Pyrrole	naphthoyl	JWH-307
Third generation derivatives		
3-indole	carboxylate	PB-22
3-indole	carboxamide	MMB-CHMINACA, STS-135
Indazole	carboxylate	NPB-22
Indazole	carboxamide	AB-CHMINACA, 5F-AKB-48 (5F-APINACA)
Benzimidazole	naphthoyl	FUBIMINA

Table 1. Synthetic cannabinoids

Example of synthetic cannabinoids classified per chemical groups and production over time. Image derived from [38]

1.2 The Endocannabinnoid System

The Endocannabinnoid System (ECS) is a widespread signalling system, expressed by many cells in the human organism. It is composed of eCBs, cannabinoid receptors (CBRs) and enzymes responsible for the synthesis, degradation and transport of eCBs.

1.2.1 Receptors

CBRs are seven class A (Rhodopsin-like) α-helical transmembrane G coupled protein receptors (GPCRs) and channels, distinguished into 2 groups: the *classical* or *canonical* CB1R, CBR2R (cannabinoid receptor 1, -2) and *non-canonical receptors*, mainly GPR55 (G coupled protein receptor 55) and TRPV1 (transient receptor potential cation channel subfamily V member 1) [39].

1.2.1.1 Canonical receptors

CB1R (encoded by CNR1, chromosome 6q15) is predominantly located in the central nervous system (CNS), specifically in the brain and in presynaptic terminals. It mediates retrograde signalling of eCBs and it controls memory, motor coordination and emotional processes [40, 41]. Low expression of CB1R were also found in postsynaptic sites of neurocortical neurons, in astrocytes, oligodendrocytes and microglia, playing a role in the synaptic transmission [42] [43-48]. Other than CNS, CB1R can be expressed in some peripheral tissues (Table 2). In gastrointestinal (GI) system, for example, CB1R is both in enteric nerves and in non-neuronal cells, modulating the motility/ permeability of the GI epithelium, the secretion of gastric acids and the release of neurotrasmitters/ hormones [49]. In heart and in vascular endothelial cells (cardiovascular system, CV), the receptor promotes vasoconstriction and fibrotic processes [50], while in adipose tissue and in the liver, it regulates lipogenesis/ lipolysis, and the insulin resistance, maintaining the energy balance of the body [51, 52]. Other key functions of CB1R involve the control of cell differentiation (e.g. keratinocytes, myocytes and adipocytes), the blood flow/ infiltration rate in the kidney, the micturition of the bladder, the fertility of the human sperm and the embryonic development/ implantation [53-59]. Finally, although there are few articles about it, recently CB1R was detected in some cells and tissues of the immune system such as in memory and naïve B-lymphocytes [36, 60, 61]. However, its functionality has not yet been clarified.

CB2R (encoded by CNR2, chromosome 1p36.11) is principally expressed in lymphoid organs, bone marrow and blood, where it elicits anti-inflammatory and immunosuppressive functions, by modulating reactivity, proliferation and cytokine production of immune cells. B cells, Natural Killer cells (NKs), monocytes, polymorph nuclear neutrophils (PMNs), T- and dendritic cells are the immune cells which mostly present CB2R [62]. However, peripheral tissues such as GI/ adipose tissues, CV system, liver and pancreas also express CB2R (Table 3). In liver, the receptor is required for the organ differentiation, by regulating biliary morphogenesis, hepatocyte proliferation, liver mass and the expression of tissue-specific enzymes [63]. CB2R was found in pre- and mature adipocytes, from visceral and subcutaneous adipose tissue (VAT and SAT, respectively), affecting the regenerative potential of the tissue, fat storage and inflammation. Moreover, recent findings also

demonstrated the involvement of CB2R in the browning process of the white adipose tissue (WAT) [64, 65]. In GI tract and CV system, the canonical CB2R mainly serves protective functions, as it was demonstrated for the CNS. Indeed, although the CNS is not the primary site of localization of the receptor, a low expression of CB2R was observed in different areas of the brain, in neurons, micro- and macroglial tissues [66, 67]. In pancreas, CB2R is responsible for amylase secretion in lobules and acini, while in lungs and bladder it is necessary for the respiratory homeostasis and micturition [68-70]. Moreover, in reproductive tissues, CB2R has some implication on the meiotic stages of germ cells [62].

	EVIDENCE FOR THE PHYSIOLOGICAL ROLE OF CB1R	Ref.
CNS	High CB1R expression was found in brain, particularly in olfactory bulb, hippocampus, basal ganglia, and cerebellum	[42]
	CB1R expression was discovered in cerebral cortex, septum, amygdala, hypothalamus, and parts of the brainstem and the dorsal horn of spinal cord	[42]
	CB1R was discovered in thalamus and ventral horn of spinal cords (low expression)	[42]
	In POMC neurons CB1R regulated feeding behavior, in hippocampus it controlled memory impairment and it had a neuroprotection function	[71, 72]
	In presynaptic terminals CB1R mediated retrograde signalling of endocannabinoids (high expression)	[43, 44]
	In postsynaptic sites of neurocortical neurons CB1R had inhibitory signals after eCBs activation (low expression)	[45]
	In brain CB1R was also found in astrocytes, oligodendrocytes and microglia, mediating synaptic transmission (low expression)	[45]
PNS	CB1R was found mostly expressed in sympathetic nerve terminals	[73]
	CB1R was found to be expressed in trigeminal, dorsal root ganglion, and dermic nerve endings of primary sensory neurons (control of nociception)	[74, 75]
GI TRACT	CB1R was enriched in the enteric nervous system and in non-neuronal cells in the intestinal mucosa (enteroendocrine cells, immune cells, and enterocytes)	[49]
	CB1R modulated the mobility of GI tract, the secretion of gastric acids, neurotransmitter and hormones, as well as the permeability of the intestinal epithelium	[49]
	Regulation of the energy balance and food intake from the GI tract	[49]
	CB1R exerted a protective role in the colon through the regulation of intestinal secretion of IgA and paracellular permeability	[76]
CV SYSTEM	CB1R was up-regulated in the CV system under pathological conditions, contributing to the disease progression and cardiac dysfunction	[50]
	CB1R activation in cardiomyocytes, vascular endothelial cells, and smooth muscle cells promoted inflammation and fibrosis	[50]
	CB1R knockout regulated vasodilatation in vivo	[77]
ADIPOSE TISSUE	Low CB1R expression was found in the adipose tissue	[78]
	Role in adipocytes differentiation, lipogenesis and lipolysis	[79]
SKELETAL MUSKLE	CB1 was recognized in myotubes and tissues of skeletal muscle, impacting on the skeletal muscular activities	[80]
	Role in skeletal muscle cell differentiation	[54]
BONE	CB1R expression was found in osteoblasts, osteoclasts, osteocytes, and chondrocytes	[78]
	Involvement in bone remodeling	[81]
SKIN	CB1R was expressed in sensory neurons of the skin	[78]
	Involvement in keratinocyte differentiation	[55]
	CB1R had a role in pruritus	[82]
EYE	CB1R was expressed in eye tissues (e.g. ciliary body, trabecular meshwork, and Schlemm canal of the eye)	[83]
	Involvement in the intraocular pressure	[83]
LIVER	Low expression of CB1R was found in the liver	[84]
	Hepatic CB1R participated in the regulation of energy balance and metabolism (role in hepatic insulin resistance, fibrosis, and lipogenesis)	[84]
PANCREAS	CB1R expression in β-pancreatic cells	[85]
	CB1R antagonists/genetic ablation enhanced the IR signalling in β-pancreatic cells, leading to an increased β-cell proliferation and mass	[85]
	CB1R antagonism reduced blood glucose in mice	[85]
LUNGS AND AIRWAYS	CB1R activation in CNS reduced respiratory rate	[85]
	CB1R targeting influenced glossopharyngeal and vagal afferents to relay feedback from periphery for the modulation of respiratory rate	[85]
	CB1R targeting influenced bronchial nerve fibers, and bronchiolar smooth muscles to inhibit airway contraction	[85]
	Selective peripheral activation of CB1R mitigated morphine induced respiratory depression	[85]
	CB1R stretches and baroreceptors informed oxygen levels, through lung terminals, structural cells and chemoreceptors on carotid bodies	[85]
	Role in the lung immunity in alveoli and leukocytes of the lung	[85]
	CB1R expression in lobules and in acinar cells	[86]
KIDNEY	CB1R activation in kidney enhanced oxidative and nitrosative stress markers, it increased apoptosis in kidney cells and it contributed to inflammation	[87]
	CB1R activation in juxtamedullary afferent and efferent arterioles incremented vasodilatation	[56]
	CB1R expression in glomerular blood vessels contributeed to increment blood flow in the kidney and to reduce the infiltration rate	[56]

	In thick ascending loop of Henle CB1R activation enhanced NO_2 production and it reduced Na^+ transport	[88]
	In podocytes and mesangial cells, CB1R agonists incremented urinary protein excretion, VEGF expression and it reduced nephrin expression and levels	[89]
URINARY TRACT	CB1R expression in the bladder, mainly in the urothelium and axons/ endings of motor/ sensory neurons	[57]
	CB1R expression in the detrusor muscle	[90]
	Down-regulation of sensory bladder function during urine storage and micturition, under normal physiological conditions	[57]
REPRODUCTIVE SYSTEM	CB1R was expressed in Leydig cells, germ cells, epididymis	[91, 92] [93] [93]
	Role in fertility regulation (capacitation and fertilizing potential of human sperm)	[58]
	Embryonic development and implantation	[59, 94]

Tab.2. In vitro/ in vivo evidence for the physiological role of CB1R.

CB1R expression, phenotypic effects and CB1R-dependent mechanisms of action in different organs of the body : central nervous system (CNS), periferal nervous system (PNS), gastrointestinal (GI) tract, cardiovascolar (CV) system, skeletal muskle, bone, skin, eye, liver, pancreas, lungs and airways, kidney, urinary tract, reproductive system.

Abbreviations: cannabinoid receptor 1 (CB1R), hypothalamic proopiomelanocortin (POMC), Immunoglobulin A (IgA), insulin receptor (IR), vascular endothelial growth factor (VEGF)

	EVIDENCE FOR THE PHYSIOLOGICAL ROLE OF CB2R	**Ref**
CNS	CB2R was found in brain, cerebellum, spinal 5th nucleus, hippocampus, and post-synaptic sites of neurons	[62]
	CB2R was found in microglia where it may be induced by local inflammation, infection or stress	[95]
	CB2R expression was detected in macroglia: e.g. in astrocytes, oligodendroglia progenitors	[96, 97]
	Role in neuroprotection: inhibition of neuroinflammatory signalling pathways	[98]
	Role in the activation of neurogenesis	[62]
GI TRACT	CB2R expression in esophagus, stomach, ileum, colon (in epithelial cells, nerves and immune cells)	[62]
	Role in the inhibition of cholinergic, non-adrenergic, non-cholinergic transmission in the stomach (enteric nerves)	[99, 100]
	CB2R modulated transmitter release and attenuated visceral sensitivity in enteric nerves and epithelial cells of the intestine	[62]
	Gastroprotective effects	[66]
	Role in the induction of gut motility, emesis, diarrhea and colon inflammatory processes	[101]
CV SYSTEM	CB2R was found in myocardium (cardiomyocytes), coronary endothelial cells, smooth muscle cells, and intimal macrophages	[62]
	Pro-inflammatory triggers and/ or mitogens may induce up-regulation of CB2R expression in primary human endothelial and smooth muscle cells	[102, 103]
	CB2R was up-regulated in atherosclerotic plaques, and in the myocardium of chronic heart failure patients, having a protective role against the injuries	[104, 105] [106]
ADIPOSE TISSUE	Expression of CB2R in mature adipocytes and pre-adipocytes (e.g. MSCs) from VAT and SAT express CB2R	[107]
	CB2R affected the regenerative potential of adipose tissue MSCs	[64]
	CB2R expressed in cells from the WAT modulated inflammation, fat storage and the browning process	[65]
	CB2R controlled leptin levels and alters thermogenic mRNA in adipose tissue	[108]
	CB2R modulated obesity-associated inflammation and insulin resistance	[109]
IMMUNE SYSTEM	CB2R expression in spleen, tonsils, thymus, and lymph nodes (B cells; NKs; monocytes; PMNs; T cells; dendritic cells)	[110, 111]
	CB2R modulated reactivity, proliferation, cytokine production of immune cells (e.g. T cells, B cells) and it had a role in retaining B immature cells in bone marrow	[110] [112] [113]
SKELETAL MUSKLE	CB2R activation promoted myogenesis of the skeletal muscle	[114]

SMOOTH MUSCLE	CB2R knockout promoted M1 polarization while inhibited M2 polarization, consequently hindering myoblasts differentiation	[115]
	CB2R expression in smooth muscle cells	[116]
BONE	Presence in osteoblasts, osteocytes, and osteoclasts	[62]
SKIN	CB2R was found in skin tissue (keratinocytes, macrophages, and T-lymphocytes in the epidermis/ dermis of inflamed skin tissues)	[117]
	CB2R regulated production of pro- and anti-inflammatory factors in human keratinocytes and fibroblasts of inflamed skin tissues (treatment with lipopolysaccharides)	[118] [119]
EYE	CB2R expression in cornea (up-regulation after injuries) and retina	[120][121]
LIVER	CBR2 was expressed in biliary epithelial cells, hepatocytes and myofibroblasts of the liver	[62]
	CB2R was required for the liver differentiation	[63]
	CB2R promoted biliary morphogenesis, hepatocyte proliferation and liver mass, as well as upregulation of gene expression of liver-specific enzymes	[66]
	CB2R regulated proliferation and promoted apoptosis of stellate cells and myofibroblasts, thereby serving a hepatic protective function	[66]
	Implication of CB2R in fatty liver diseases, steatosis and liver fibrosis	[66]
PANCREAS	CB2R expression in lobules and in acinar cells	[122]
	Reduction of Ca^{2+} oscillations in acinar cells	[68]
	CB2R agonists inhibited amylase secretion in pancreatic lobules and acini through inhibition of presynaptic acetylcholine release	[122]
LUNGS AND AIRWAYS	Expression in alveoli and lung leukocytes for lung immunity	[69]
	CB2R in microglia inhibited immune cells to respond inflammation necessary for respiratory homeostasis	[69]
	Role in mitigation of morphine induced respiratory depression	[69]
	Role in afferent relay bronchoconstriction and dilation related to inflammation (anti-tussive effect and prevention of bronchoconstriction)	[69, 123, 124]
KIDNEY	Low expression of CB2R in kidney biopsies from patients with advanced kidney nephropathy	[125]
	Role in renal tubular mitochondrial dysfunction and kidney ageing	[126]
URINARY TRACT	CB2R expression in urothelium and detrusor muscle	[90]
	Inhibition of the mechanosensitivity of mucosal afferents in the bladder	[127]
	Role in micturition: control of the bladder ICIs, BC and Bcom	[70]
REPRODUCTIVE SYSTEM	CB2R was found in testis and pre-implantation embryo	[70]
	Implication on the meiotic stages of germ cells	[128]

Tab.3. In vitro/ in vivo evidence for the physiological role of CB2R

CB2R expression, phenotypic effects and CB2R-dependent mechanisms of action in different organs of the body: central nervous system (CNS), gastrointestinal (GI) tract, cardiovascolar (CV) system, adipose tissue, immune system, skeletal muskle, smooth muscle, bone, skin, eye, liver, pancreas, lungs and airways, kidney, urinary tract, reproductive system.

Abbreviations: cannabinoid receptor 2 (CB2R), mesenchymal stem cells (MSCs), messenger RNA (mRNA) visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT), white adipose tissue (WAT), Natural Killer cells (NKs), polymorph nuclear neutrophils (PMNs), inter-contraction intervals (ICIs), bladder capacity (BC), compliance (Bcom)

1.2.1.2 Non canonical receptors

GPR55 (encoded by Gpr55, chromosome 2q37.1) is another class A GPCR, also known as CB3R. The receptor has recently been deorphanized, and as of today lysophosphatidylinositol (LPI) has been recognized as the main endogenous ligand. However, AEA and 2-AG also demonstrated a weak affinity for the receptor. GPR55 was found in brain and spinal cord, principally modulating emotional information, memory and motor coordination [129, 130]. In GI tissue, GPR55 is more present in small intestine, colon, esophagus and stomach, serving for the energy maintenance and physiological functions of the bowel [131-134]. In hematopoietic cells and cardiomyocytes, the receptor controls cardiovascular remodeling and plasma cell proliferation/ maturation [135, 136], while in adipose tissue it contributes to the regulation of energy intake and expenditure, with a positive association between its expression and the onset of obesity and diabetes [137-139]. In bone and skeletal muscle, GPR55 is responsible for the resorption processes of osteoblast and osteoclasts and for the oxidative capacity / fatty acid metabolism of the muscle [140, 141]. Another important function for the control of lipid metabolism was observed in the liver, where GPR55 contributes to the hepatic lipid accumulation through the activation of acetyl-CoA carboxylase (ACC) [142]. In Isles of Langerhans, GPR55 stimulates the insulin release from the β-pancreatic cells, thus impacting on glucose tolerance of blood [143]. In lung, it was found in pulmonary arteries, bronchioles and in some immune cells (e.g. neutrophils), acting for the vasorelaxation, injury resolution, and for the lung inflammation [144]. Although there is little evidence, GPR55 detains a possible role in the regulation of proximal tubular hypertrophy, and in the contractility of the detrusor of the urinary bladder [145, 146]. Moreover, in reproductive tissues, GPR55 is particularly expressed in oocytes, ovaries, uterus, and fallopian tubes and in germinal/ somatic cells of the testis [147]. For a more in-depth analysis about the physiological role of the receptor, we refer to Table 4.

TRPV1 (encoded by Trpv1, chromosome 17p13.2) is member of the six transmembrane cation channels, TRPVs, activated by vanilloids and noxious stimuli (e.g. capsaicin in chili peppers, and high body temperatures). It is mostly expressed in nociceptive primary sensory neurons, spinal cord and brain, regulating nociceptive/ neuropathic pain, thermal hyperalgesia, neural activity and controlling the body temperature (Table 5) [148] [149-154]. In alimentary tract, TRPV1 is located in nerve fibers of esophageal mucosa and in neurons of the myenteric plexi and jejunum. Moreover, it was detected in gastric epithelial cells, in rectum and distal colon, where it principally controls visceral hypersensitivity and motor functions [155-159]. In the heart, the receptor protects the organ perfusion and it is activated with the increment of intraluminal pressure, locating in the nerve endings of the surface of the heart [160-162]. Furthermore, it can be in vascular smooth muscle cells where it leads to vasoconstriction [163]. As demonstrated for other CBRs, TRPV1 may be a marker for the treatment of obesity, being expressed in pre- and adipocytes of the VAT and preventing adipogenesis. Noteworthy, its expression may be a diagnostic indicator for the disease, since it was observed that overweight individuals have lower levels of TRPV1 than lean people [164]. In skeletal muscle and bone, the vanilloid receptor contributes to muscle and bone pain [165, 166], while in the skin it is expressed at different levels such as in sensory nerve fibers, and also in epidermal keratinocytes, epithelial cells of hair follicles, eccrine sweat/ sebaceous glands and blood vessels [167, 168]. The prominently expression of TRPV1 in the skin contributes to the generation of heat shock, to the control of keratinocytes' proliferation/ apoptosis and the secretion of inflammatory factors, as well as to inflammatory responses (e.g. histamine-induced itching) and the maintenance of hair shaft length [169, 170]. In corneal epithelial cells, TRPV1 may constitute a part in the recruitment of the immune cell response, protecting against infections [171]. In pancreas, instead, it innervates the Islets of Langerhans, establishing a feedback mechanism, necessary for the maintenance of blood insulin levels. Indeed, after the release of insulin, TRPV1 enhances the production of neuropeptides, for example substance P and calcitonin gene-related peptide (CGRP), which in turn restores basal insulin levels by blocking insulin production [172] [173]. Finally, TRPV1 has a ubiquitous localization in lungs and airways, where it is responsible for the process of sensitization to bradykinin (for bronchoconstriction) and for mechanisms of cell viability and inflammation [174-177]. Moreover, recent findings supported the presence of the receptor in vestibular organs, where it may evoke the auditory nerve excitability [178, 179]. In kidney, TRPV1 modulates the renal hemodynamics and excretory functions, while in urinary bladder it mediates reflex contraction and pain sensation associated with overfilled bladder [180, 181]. In vulvar epithelial innervations, TRPV1 over-expression may contribute to the sensory symptoms of patients affected by vulvodynia [182].

	EVIDENCE FOR THE PHYSIOLOGICAL ROLE OF GPR55	Ref.
CNS	GPR55 expression in frontal cortex, hippocampus, striatum, hypothalamus, brainstem, cerebellum and spinal cord	[130]
	In frontal cortex: possible involvement in anxiety modulation and emotional information processing	[130, 183]
	In hippocampus: possible involvement in neuropsychiatric and neurodevelopmental diseases; increment of Ca^{2+} levels in presynaptic terminals; increment of neurotransmitter release; modulation of anxiety-like behavior	[184] [183]
	In striatum: modulation of the performance on the T maze task; possible involvement in procedural memory and motor coordination	[185-187]
	In hypothalamus: intra-cerebro-ventricular infusion of GPR55 agonists increased food intake and reduced CART expression	[143, 188]
	In brainstem: pro-nociceptive effects	[189]
	In cerebellum: possible role in motor coordination	[187]
	In spinal cord: increase of intracellular Ca^{2+} in dorsal root ganglion neurons; regulation of nociceptive afferent projections in developing dorsal root ganglion neurons	[190]
GI TRACT	GPR55 expression in small intestine (ileum, jejunum with a mainly localization in the sub mucosa and myenteric plexus), in colon, esophagus and stomach	[131-133]
	Energy maintenance and bowel functions in enteric epithelial cells and enteric neurons (possible involvement in GI motility and secretion)	[134]
CV SYSTEM	Expression in hematopoietic cells and cardiomyocytes	[191]
	Role in cardiovascular remodeling and heart failure (post-myocardial infarction remodeling; atherogenesis, systolic dysfunction etc.)	[191]
	Involvement in plasma cell maturation and proliferation	[136]
	Increase of angiogenesis in primary dermal micro vascular endothelial cells	[192]
ADIPOSE TISSUE	GPR55 expression in WAT: VAT and SAT	[139]
	Role in the regulation of energy intake, expenditure	[137, 138]
	GPR55 expression in VAT was positively associated with obesity and type-2 diabetes	[139]
	LPI plasma levels were higher in obese compared to lean patients. LPI raised intracellular Ca^{2+} levels, in differentiated adipocytes from VAT of obese patients	[139]
	GPR55 increased food intake and adiposity in Sprague-Dawley rats; the increase in food intake is still evident in GPR55 knockout in vivo	[188]
	Link between a GPR55 gene polymorphism and anorexia nervosa	[193]
	WAT CB1R and GPR55 levels were increased after fasting and they were recovered after leptin treatment	[194]
	WAT CB1R and GPR55 levels were modified upon alterations in pituitary functions	[194]
SKELETAL MUSKLE	GPR55 was found expressed in skeletal muscle cells	[140]
	Role in the oxidative capacity and fatty acid metabolism of the skeletal muscle (C2C12 myotubes)	[140]
BONE MARROW	GPR55 was found expressed in bone cells, including osteoblasts and osteoclasts	[141, 143]
	Role in the resorption of the bone	[141]
SKIN	Low expression in healthy skin healthy tissues	[195]
EYE	GPR55 expression in retina during development	[196]
	Role in the regulation of growth and filopodia of cones and in the chemo-attractive effects of the cones	[196]
LIVER	GPR55 expression in hepatic cells	[139, 196]
	Contribution to the hepatic lipid accumulation through activation of ACC (role in NAFLD and NASH)	[142]
PANCREAS	GPR55 expression in islets of Langerhans (insulin-secreting β-cells)	[143]
	Stimulation of insulin release from pancreatic islets via mobilization of Ca^{2+} ions	[197]
	Increase of glucose tolerance in vivo: Reduction of blood glucose in GPR55 knockout in vivo	[143]
LUNGS AND AIRWAYS	Expression in pulmonary arteries, bronchioles and neutrophils of the lung	[144]
	Vasorelaxation in endothelium-intact pulmonary arteries	[144]
	Modulation of neutrophil function (IFN response) and contribution for lung injury resolution	[198]
	GPR55 knockout amplified edema, alveolar neutrophil-ROS production in the lung and it decreases lung tissue inflammatory markers	[198]
KIDNEY	GPR55 was found expressed in proximal tubular cells	[145]
	Possible role in regulating proximal tubular hypertrophy	[145]
	Up-regulation of GPR55 in proximal tubule cells and whole kidney, in response to elevated levels of glucose and albumin (diabetic conditions)	[199]

	GPR55 antagonists attenuated sepsis-induced acute kidney injury	[200]
URINARY TRACT	GPR55 agonists reduced detrusor contractility, oxidative damage in the urinary bladder and neuroinflammation in corticosterone treated mice	[146]
REPRODUCTIVE SYSTEM	GPR55 was found expressed in oocytes, ovaries, uterus, fallopian tubes, and in germinal/ somatic cells of the testis	[201]
	Role in the formation of normal-sized metaphase I and II spindles in oocytes	[201]

Tab.5. In vitro/ in vivo evidence for the physiological role of GPR55

GPR55 expression, phenotypic effects and GPR55-dependent mechanisms of action in different organs of the body: central nervous system (CNS), gastrointestinal (GI) tract, cardiovascolar (CV) system, adipose tissue, skeletal muskle, bone marrow, skin, eye, liver, pancreas, lungs and airways, kidney, urinary tract, reproductive system.

Abbreviations: G coupled protein receptor 55 (GPR55), cannabinoid receptor 1 (CB1R), cocaine-amphetamine-regulated transcript (CART), visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT), white adipose tissue (WAT), acetyl-CoA carboxylase (ACC), Non-alcoholic fatty liver disease (NAFLD), Non-alcoholic steatohepatitis (NASH) , reactive oxygen species (ROS), interferon (IFN)

	EVIDENCE FOR THE PHYSIOLOGICAL ROLE OF TRPV1	**Ref.**
CNS	TRPV1 was mainly expressed in nociceptive primary sensory neurons and also in spinal cord and brain	[148]
	In primary sensory neurons, TRPV1 was expressed by small and medium sized dorsal root ganglion and trigeminal ganglion neurons; and it was localized on the peripheral and central processes	[148]
	TRPV1 was found both pre-synaptically and post-synaptically at the synapse of primary sensory neurons in the spinal cord and it activates noxious evoked activity of dorsal horn neurons	[202-204]
	After sciatic nerve ligation, TRPV1 antagonists counteracted fiber-evoked responses in the dorsal horn neurons	[203]
	After a spinal nerve ligation, TRPV1 antagonists and antisense oligonucleotides against the receptor reduced mechanical hypersensitivity	[165]
	In excitatory terminals of primary afferent fibres,TRPV1 potentiated glutamate release, thereby increasing the activity of spino-thalamic tract neurons of the deep dorsal horn of the rat spinal cord	[205]
	In periaqueductal gray, TRPV1 produced anti-nociceptive effects and it increased the neuronal activity through selective potentiation of glutamatergic synaptic inputs	[150, 151]
	TRPV1 in primary sensory neurons affected neuronal firing patterns to constitute and initiate the transfer to the brain by electrical signalling of an integrated picture of pain-inducing stimuli	[148]
	Acute activation of TRPV1 resulted in an acute burning pain sensation, while prolonged or chronic activation of TRPV1 in peripheral pathological events (e.g. inflammation) resulted in the development of heat	[148]
	TRPV1 had implications in thermal hyperalgesia resulting from noxious stimuli	[206, 207]
	Expression of TRPV1 increased in dorsal root ganglion neurons in conditions of inflammation (e.g. hyperalgesia)	[208]
	TRPV1 reduces cold allodynia	[165]
	Involvement in the development of thirst	[209]
	Role in thermoregulation:TRPV1 antagonists caused an increase in body temperature (hyperthermia)	[210]
GASTRO-INTESTINAL TRACT	TRPV1 was found in the alimentary tract: TRPV1 was expressed in sensory nerve fibers of the human oesophageal mucosa; expression in peripheral terminals of primary and vagal sensory neurons; expression in intrinsic enteric neurons in the myenteric plexi, and gastric epithelial cells; expression in sensory neurons innervating the jejunum; expression in the rectum and distal colon	[156, 159]
	Role in generation and maintenance of persistent visceral hypersensitivity	[157, 158]
	Experimentally induced colonic inflammation results in up-regulation of TRPV1 in dorsal root ganglion neurons with subsequent visceral hyperalgesia to mechanical and chemical stimuli	[211]
	TRPV1 was located in the rectum and distal colon and it appeared to play a major role in the motor function of the large intestine	[159]
CV SYSTEM	TRPV1 was expressed on sensory nerve endings of the surface of the heart	[160]
	Activation of TRPV1 on C-fiber nerve endings, as a result of the elevation of intraluminal pressure associated with generation of 20-HETE, promoted the depolarization of neurons and neuropeptide release	[161]
	Role in protecting organ perfusion: TRPV1 triggered release of substance P and it stimulated the sympathetic axis, thus protecting against endotoxin induced hypotension and mortality	[162]
	Role in protecting organ perfusion during ischemia: 12(S)-HPETE produced during myocardial ischemia activated TRPV1 on sensory C-fibers, thus causing the release of vasoactive peptides (e.g. substance P and CGRP), which protect against further injury during the reperfusion process	[212]
	Increased intracellular Ca^{2+} through TRPV1, was responsible for the stimulation and sensitization of cardiac nociceptors by bradykinin	[213]

	TRPV1 contributed to the ischemic stimulation of cardiac spinal afferent nerves and to the chest pain (hall-mark of cardiac ischemia)	[214]
	TRPV1 was expressed in vascular smooth muscle cells where it led to vasoconstriction in skeletal muscle resistance arterioles	[163]
	Ablation of TRPV1 markedly enhanced post-myocardial infarction fibrosis and impaired myocardial contractile performance, leading to increased propensity to functional heart failure and mortality	[215]
	Over-expression of TRPV1 ion channels may contribute to the endotoxaemic shock, following the increment of CGRP	[216]
ADIPOSE TISSUE	TRPV1 was expressed by pre-adipocytes and visceral adipose tissue from mice and human	[164]
	Overweight individuals as well as obese laboratory animals had lower levels of TRPV1 expression than their lean counterparts	[164]
	TRPV1 prevented adipogenesis and obesity	[164]
SKELETAL MUSKLE	TRPV1 contributed to muscle nociception and hyperalgesia and it was expressed in muscle afferents being involved in the development of pathological muscle pain conditions	[165]
BONE	TRPV1 played an important role in mediating the inflammatory component of arthritic pain. When wild type and TRPV1-null mice were injected in one knee joint with Complete Fruend's Adjuvant, the TRPV1 null mice developed mild joint swelling (evidencing oedema) and reduced mechanical hypersensitivity as compared with wild type animals	[166]
	In mouse models of TNF α-induced bilateral thermal hyperalgesia, unilateral intra-plantar injection of TNFα caused thermal hyperalgesia (within 1 to 4 hours) in the ipsi-lateral inflamed hind-paw and in the contro-lateral uninjured hind-paw which was TRPV1-dependent. TNFα -induced IL- 1β generation in both paws and the presence of local IL-1β in the contralateral paw were essential for the development of bilateral hyperalgesia	[217]
	TRPV1 was shown to mediate cell death in rat synovial fibroblasts through Ca^{2+} entry-dependent ROS production and mitochondrial depolarization	[218]
	TRPV1 was involved in mediating secondary mechanical hyperalgesia/allodynia and spontaneous pain induced by PAR-2 activation in the knee join	[219]
SKIN	Human skin and its appendages prominently expressed TRPV1 in vivo not only on sensory nerve fibers, but also in epidermal keratinocytes, blood vessels, epithelial cells of hair follicles, eccrine sweat glands, and sebaceous glands	[167, 168]
	Activation of epidermal TRPV1 resulted in Ca^{2+} influx, COX-2 expression, and release of PGE2 and IL-8	[220]
	Histamine induced itch by activating TRPV1 ion channels in primary sensory neurons	[169]
	Mice lacking TRPV1 showed markedly reduced histamine-induced scratching compared with wild-type mice	[169]
	Patients with allergic rhinitis featured an increased itch response to TRPV1 stimulation at seasonal allergen exposure	[221]
	TRPV1 was confined to distinct epithelial compartments of the human hair follicle	[170]
	TRPV1 activation resulted in specific inhibition of hair shaft elongation, suppression of proliferation, induction of apoptosis, premature hair follicle regression, and up regulation of intra-follicular TGF-β2	[170]
	Cultured human outer root sheath keratinocytes expressed functional TRPV1, whose stimulation inhibited proliferation, induced apoptosis, elevated intracellular calcium, and up-regulated known endogenous hair growth inhibitors (IL-1β, TGF- β2). Moreover, it down-regulated hair growth promoters (hepatocyte growth factor, insulin-like growth factor-I, and stem cell factor)	[170]
EYE	Corneal epithelial cells expressed TRPV1	[171]
	TRPV1 in corneal epithelial cells may be essential for inducing the release of inflammatory mediators that constitute part of the immune response and protect against infection	[171]
PANCREAS	TRPV1-expressing nerve fibers in Langerhans islets of the pancreas	[148]
	Insulin, by increasing the activity of TRPV1, induced the release of neuropeptides, such as substance P and CGRP, which, in turn, inhibited insulin secretion	[172, 173]
	The reduced TRPV1 responsiveness bringed to decrease in local levels of neuropeptides, thus producing insulin resistance	[222]
	Antagonism on TRPV1 prevented the development of hyperglycemia and increased insulin secretion in Zucker Diabetic fatty rats, which were regarded as a model of human type 2 diabetes mellitus	[223]
	Sensory nerves innervating the pancreas co-expressed TRPV1, substance P, and CGRP in rat models of experimental pancreatitis. Activation of TRPV1 resulted in the release of substance P peripherally which bound to the NK-1R on endothelial cells and led to vascular permeability, plasma extravasation, edema, and neutrophil infiltration in the pancreas	[224-226]
	Antagonism of TRPV1, substance P, and CGRP may suppress pain of pancreatitis	[224-226]
LUNGS AND AIRWAYS	TRVP1 expression in epithelial cells, vascular endothelial cells, submucosal glands and nerves in the human nasal mucosa	[227]
	TRPV1 immunoreactive axons were also found in, and around, sub epithelial regions of the airways, including smooth muscle and blood vessels and within the lower airways, in the vicinity of bronchi and bronchioles, and in, and around, the alveolar tissue	[174]
	TRPV1 controlled acid-induced, and heat-induced, CGRP release and sensitization by bradykinin in the isolated mouse trachea	[175]
	After chronic airway inflammation in rat, there were altered expression of TRPV1 and sensitivity to capsaicin in pulmonary myelinated afferents	[228]
	TRPV1 was found in human laryngeal epithelial cells	[229]
	Cell death of normal human airway epithelial cells and in mouse sensory neurons was occasioned by sustained calcium influx through TRPV1	[176]
	Capsaicinoids found in "pepper sprays" used for self defence, when inhaled by rats, via the nose, produced acute inflammation, moderate epithelial cell dysplasia and necrosis in the upper and lower respiratory tract via TRPV1	[177]

	In creased expression of TRPV1 is found in the airway epithelial nerves of patients with chronic cough	[230]
	Activation of the intracellular sub-population of TRPV1 ion channels caused cell death in human bronchial epithelial and alveolar cells	[231]
	TRPV1 in the guinea pig inner ear is found in the hair cells, in cells of the organ of Corti, spiral ganglion cells of the cochlea, sensory cells of the vestibular end organs; and vestibular ganglion cells	[232, 233]
	TRPV1 expression was up-regulated both in the spiral and vestibular ganglia in the mouse inner ear after kanamycin challenge	[234]
	TRPV1 increased background activity of spiral ganglion cells and it was involved in gating spontaneous and evoked auditory nerve excitability	[179]
KIDNEY	Activation of TRPV1 in primary afferent neurons, innervating the renal pelvis, led to diuresis and natriuresis	[235, 236]
	Vital role of TRPV1 primary sensory neurons in the maintenance of sodium and water homeostasis by segmental regulation of renal function	[236]
	Activation of TRPV1, in the isolated rat kidney, decreased perfusion pressure and increases glomerular filtration rate, suggesting that TRPV1 played an important role in modulating renal haemodynamics and excretory function	[180]
URINARY TRACT	Expression in sensory fibers and urothelial cells of the urinary tract of mammals where it mediated pain sensation as well as the frequency of bladder reflex contraction	[181]
	TRPV1 was responsible for pain sensation associated with overfilled bladder and the hyper-reflex associated with inflammation	[181]
REPRODUCTIVE SYSTEM	Vulvar epithelial innervation, increased inTRPV1 expression, may contribute to the sensory symptoms experienced by vulvodynia patients	[182]

Tab.5. In vitro/ in vivo evidence for the physiological role of TRPV1

TRPV1 expression, phenotypic effects and TRPV1-dependent mechanisms of action in different organs of the body: central nervous system (CNS), gastrointestinal (GI) tract, cardiovascolar (CV) system, adipose tissue, skeletal muskle, bone, skin, eye, pancreas, lungs and airways, kidney, urinary tract, reproductive system.

Abbreviations: transient receptor potential cation channel subfamily V member 1 (TRPV1), 20-hydroxyeicosatetraenoic acid (20-HETE), 12(S)-hydroperoxy-eicosatetraenoic acid (12(S)-HPETE), substance P and calcitonin gene-related peptide (CGRP), tumor necrosis factor α (TNFα), interleukin (IL- 1β/-8/…), protease-activated receptor 2 (PAR-2), cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), transforming growth factor β2 (TGF-β2), neurokinin-1 receptor (NK-1R)

1.2.2 Deregulations of CBRs in cancer

It was seen for quite some decades that deregulations of the ECS can lead to the emergence and development of many diseases, including cancer. In many tumor tissues and cancer cell lines, indeed, alterations of the ECS correlated with the worsening of tumor aggressive traits and with a poor prognosis. However, it is still difficult to be able to make a generalization of which component of the system may most be involved in a pro-tumor action, since there is a high heterogeneity of response, even within the same tumor tissue.

1.1.1.2 Deregulations of canonical receptors

Canonical CBRs were found deregulated in different tumors (Table 6, 7). For instance, high CB1R and CB2R expression are typical of glioma, glioblastomas (GBM), astrocytoma and pituitary tumors [237-239]. In advanced stages of colon carcinogenesis, both CB1R and CB2R are low expressed, disappearing as the disease worsens. The receptors were proposed as tumor suppression markers in colorectal cancer (CRC), due to their ability to counteract tumor cell proliferation, migration, and to induce apoptosis, as demonstrated in DLD-1 and HT29 cell lines. Moreover, CB1R and CB2R agonists suppressed CRC growth in vivo, as reported in azoxymethane (AOM) - and dextran sulfate sodium (DSS)-driven CRC mice. Interestingly, CNR1 was more methylated in CRC tissues compared to healthy samples [240-245]. In skeletal muscle and bone cancers, canonical CBRs have a principal role in attenuating cancer pain, as observed in fibro sarcoma, where CBRs' agonists relieved the related mechanical hyperalgesia [246]. In some bone cancer cells, such as TC-71 and A-673 Ewing sarcoma cells, CB1R and CB2R were detected, although without any information about their function and mechanisms of action [247, 248]. Regarding skin cancers, CB1R and CB2R were observed in papilloma, basal cell/ squamous carcinomas and melanoma [249, 250]. Moreover, their activation induced cycle arrest, apoptosis in vitro and in vivo, in different skin cancer cells [249, 250]. Noteworthy, local administration of CB1R and CB2R agonists impaired tumor vascularization in non-melanoma skin cancers, by altering blood vessel morphology, decreasing the expression of VEGF, platelet derived growth factor (PDGF), angiotensin 2 (Ang 2), and by impairing epidermal growth factor receptor (EGFR) function [249]. Moreover, in addition to the altered assembly of blood vessels, CB2R hampered the migration of melanoma cells through the blood-brain barrier, an important process needed for metastasis [251]. High levels of CB1R and CB2R in hepatocellular carcinoma (HCC) tissues correlated with a better disease-free survival of tumor patients [252]. Conversely, decrease on CB1R expression in pancreatic ductal adenocarcinoma (PDAC) patients was associated with a longer survival rate [253]. Moreover, the activation of CB1R and CB2R delayed tumor cell growth/ invasion and it induced apoptosis in pancreatic cancer cells, through different molecular mechanisms [249, 254-257]. Among these, there is the ability of PDAC cells to recruit immune cells expressing CB2R at the primary site of the tumor [253]. In non-small cell lung cancer (NSCLC), CB1R and CB2R were found low in levels, but their activation led to a reduction in chemotaxis/ chemo-invasion and in the amount of focal adhesion complexes in vitro (e.g. in A549, SW-1573 cell

lines). In vivo, the receptors inhibited tumor growth, vascularization and metastasis of NSCLC cells [258]. Expression and function of CB1R are unclear and contradictory in many renal cancers. Some reports showed that CB1R is high in chromophobe renal cell carcinoma (ChRCC), variable in renal oncocytoma (RO), while in clear cell renal cell carcinoma (CCRCC) CB1R expression is down-regulated with respect to the adjacent non-neoplastic kidney [259]. For what concerns CB2R, it is not expressed in ChRCC, CCRCC, and in non-tumor renal tissues [259, 260], but its activation in certain RCC cell lines was demonstrated to lead cell cycle arrest at the G0/G1 phase [261]. Some bladder cancer cells (e.g. ECV304) express the canonical CBRs [262]. In this context, activation of CB1R was proposed as a trigger for bladder cancer cell proliferation [263], while CB2R was associated to pro-inflammatory states [263]. In breast cancer (BCa), over-expression of CB1R and CB2R was observed in primary human BCa compared to normal breast tissues. Moreover, high levels were detected in triple negative breast cancer (TNBC) cells (e.g. MDA-MB-231, MDA-MB-468 cells) and low amounts in estrogen receptor α (ERα)-positive cells (e.g. MCF-7 and T47D cells). Overall, the targeting of CB1R and CB2R counteracted proliferation and migration of both tumor cell subtypes in vitro/ in vivo, although with more efficiency in highly metastatic MDA-MB-231 cells with respect to less-invasive T47D and MCF-7 cells [264, 265]. Different prostate cancer (PCa) cell lines, such as DU145, PC3 and LNCAP cells, were found to express CB1R and CB2R. Moreover, high CB1R levels in PCa tissues correlated with Gleason score, and metastasis incidence of PCa patients [26, 266]. Generally, CB1R and CB2R exerted anti-proliferative/ -invasive ability on PCa cells, and their activation prevented neuroendocrine differentiation of LNCaP cells [26, 267-269]. Other reports, demonstrated that down-regulation of CB1R and CB2R, may decrease VEGF, prostate-specific antigen (PSA), and pro-inflammatory cytokines (e.g IL-6/IL-8 levels) in LNCaP stem cells, also affecting their stem cell-like traits [26]. Overall, we can conclude that CBRs may elicit various phenotypic effects, depending on tissue and tumor cell line. Therefore, because of the contribution on tumor growth and dissemination, the study of these receptors can be important for new anti-cancer strategies.

The CB1R/CB2R signalling pathways (Fig.4) include *the inhibition of extracellular signal regulated kinase* (ERK) which impacts on cell cycle and proliferation. The *inhibition of adenylate cyclase*, and the subsequent decrease in cAMP/ cAMP-dependent protein kinase A (PKA) activity counteract cell proliferation and sustain apoptosis [270]. The *activation of B-cell leukemia/lymphoma 2 protein* (Bcl-2), the *increase of ceramide* and intracellular *reactive oxygen species* (ROS) may also lead to apoptosis. Ceramide is a sphingolipid which triggers the p38 mitogen-activated protein kinase (MAPK) and the up-regulation of the endoplasmic reticulum (ER) stress regulated protein 8 (p8), culminating in a higher expression of the activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP) and the stress-related pseudo-kinase tribbles homolog 3 (TRB3) [271]. Autophagy, cell migration, angiogenesis, and epithelial-to-mesenchymal transition (EMT) principally occur via *AMP-activated protein kinase* (AMPK), *mammalian target of rapamycin* (mTOR) (autophagy) [272], *Ras homolog family member A* (Rho A*)* (cell migration) and the *Wingless-related integration site*

(Wnt*)/ β-catenin* pathway (EMT) [273]. The inhibition of Rho A is correlated to the release of tissue inhibitor matrix metalloproteinases-1 (TIMP-1) and the decrease of proangiogenic factors (e.g. placental growth factor, PlGF; VEGF and Ang 2) [274].

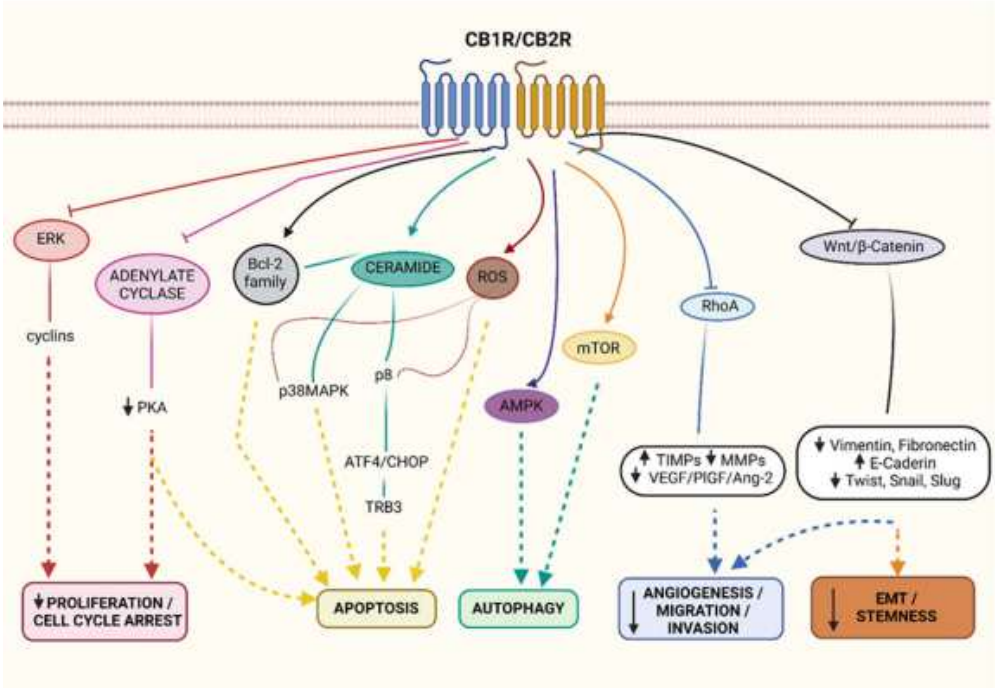


Fig.4. CB1R and CB2R downstream signalling pathways in cancer

Cannabinoids modulations of the canonical CBRs' downstream pathways in cancer: inhibition of proliferation and cell cycle (↓ERK/cyclins; ↓Adenylate cyclase/PKA), induction of apoptosis (↑Bcl-2; ↑p38MAPK; ↑p8/ATF4/CHOP/TRB3; ↑ROS), enhancement of autophagy (↑AMPK; ↑mTOR), reduction of angiogenesis (↓VEGF/PIGF/Ang2), migration and invasion (↓Rho A; ↓MMPs). Inhibition of EMT (↓Vimentin, Fibronectin) and stemness (↓Wnt/β-catenin; ↓Twist, Snail and Slug). Image derived from [274].

Abbreviations: cannabinoid receptor 1, 2 (CB1R/ CB2R), extracellular signal regulated kinase (ERK), protein kinase A (PKA), B-cell leukemia/lymphoma 2 protein (Bcl-2), mitogen-activated protein kinase (MAPK), activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), tribbles homolog 3 (TRB3), reactive oxygen species (ROS), AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), vascular endothelial growth factor (VEGF), placental growth factor (PIGF), angiotensin 2 (Ang2), Ras homolog family member A (Rho A), Matrix metalloproteinases (MMPs), epithelial-to-mesenchymal transition (EMT), Wingless-related integration site (Wnt)

TUMORS	EVIDENCE FOR THE ROLE OF CB1R IN TUMORS	Ref.
CNS	CB1R was found expressed in glioma: conflicting results on whether this protein increases or decreases with tumor grade	[275]
	CB1R was low expressed in GBM than in normal post-mortem brain tissue; no significant differences for astrocytoma and meningioma samples	[238]
	CB1R was expressed in U373 MG and GL-15 astrocytoma cell lines	[276]
	Activation of CB1 R in U373 MG cells inhibited adenylyl cyclase, and it induced the expression of the immediate-early gene krox-24 (via pertussis toxin-sensitive GTP-binding protein)	[276]
	In U251MG glioma cells and primary tumor cell lines derived from glioma patients, CB1R antagonism induced apoptosis via G1 phase stasis and block of TGF-β1 secretion through STAT3 inhibition	[277]
	CB1R was a promising target for NK cell-mediated recognition, through NKG2D in glioma (possible modulation of NK cell anti-tumor reactivity)	[277]
	CB1R gene was highly expressed in pediatric low-grade glioma with or without a spontaneous involution after a subtotal surgical removal, at the time of diagnosis (potential prognostic marker)	[278]
	CB1R expression correlated with hsa-miR-29b-3p levels in pediatric low-grade glioma	[278]
	Estrogen-induced pituitary tumor development was associated with a marked reduction in CB1R expression, accompanied with body weight loss, increase pituitary weight and plasma prolactin levels	[239]
	In estrogen-induced pituitary tumors, CB1R co-localized with lactotroph cells (prolactin- or luteinizing hormone-containing cells) which develop hyperplasia during tumor induction	[239]
	Chronic treatment with dopamine receptor agonists/ antagonists did not alter CB1R expression in pituitary tumors while it changed plasma prolactin levels	[239]
GI TRACT	CB1 R was found low expressed in advanced stages of colon carcinogenesis and disappeared in CRC	[240]
	In stage IV CRC, high vs. low CB1R expression was correlated with a statistically significant poorer overall survival (potential prognostic marker in stage IV CRC)	[279]
	In stage I/II or III CRC following surgery, CB1R expression did not correlate with patient survival	[279]
	CB1R negatively impacted disease-specific survival in stage II microsatellite stable CRC	[280]
	CB1R activation suppressed CRC cell proliferation and migration, and differentiation of M2 macrophages in vitro and in vivo	[241, 242]
	CB1R activation induced apoptosis and increased ceramide levels in the colon cancer DLD-1 and HT29 cells	[244]
	CB1R suppressed tumor growth in AOM- and DSS-driven CRC mouse model, playing an opposing role with respect to GPR55	[245]
	CNR1 was more methylated than GPR55 in CRC tissues compared to control samples (oncosuppressive role)	[245]
	Loss or inhibition of CB1R, accelerated intestinal adenoma growth in ApcMin/+ mice. whereas activation of CB1R attenuated intestinal tumor growth by inducing cell death (down-regulation of survivin)	[281]
	Dietary ω-3 PUFAs inhibited intestinal polyp growth in mice, correlating with the up-regulation of CB1 R expression in intestinal and adipose tissues	[241]
	Dietary ω-3 PUFAs inhibited intestinal polyp growth in mice, correlating with the down-regulation of CB1R expression in colon cancer tissues	[241]
	Down-regulation of CB1R expression in CRC cells was associated with the up-regulation of EGFR	[242]
	17β-estradiol induced CB1R gene expression in colon cancer cells (DLD-1, HT-29 and SW620 cells) through activation of estrogen receptor	[282]
SKELETAL MUSKLE	Intra-plantar injection of CB1R agonists reduced fibrosarcoma-related mechanical hyperalgesia	[246]
	Synergistically action given by the combination of CB1R and CB2R agonists, in reducing fibrosarcoma related mechanical hyperalgesia in vivo	[246]
BONE	CB1R expression in TC-71 and A-673 Ewing sarcoma cell lines (cytotoxic effects mainly induced by non-canonical CBRs' mechanisms)	[247]
	CB1R activation relieved bone cancer pain	[283]
SKIN	CB1R was expressed in papilloma, basal cell and squamous carcinomas, melanoma, in melanocytes and melanoma cell lines	[249, 250]
	CB1R agonists and inverse agonists showed anti-proliferative, pro-apoptotic, anti-angiogenic and anti-migratory effects in melanoma in vitro / in vivo	[250, 284]
	Systemic administration of stable CB1R agonists into SCID mice specifically inhibited liver colonization of melanoma cells	[284]
	CB1R activation induced cycle arrest at the G1-S transition via inhibition of Akt and hypo-phosphorylation of pRb in melanoma cells	[250]
	In non-melanoma skin cancers CB1R activation induced apoptotic death of tumorigenic epidermal cells, unaffecting non transformed epidermal cells	[249] ;
	Local administration of CB1R agonists with or with CB2R agonists inhibited growth of malignant tumors generated by inoculation of epidermal tumor cells into nude mice	[249]
	Local administration of CB1R agonists impaired tumor vascularization in non-melanoma skin cancers: altered blood vessel morphology and decreased expression of VEGF, PDGF, and Ang 2; abrogation of EGFR function	[249]
EYE	Potential involvement in lowering the intraocular pressure in glaucoma patients	[285]
LIVER	CB1R was over-expressed in HCC tissues samples	[252]
	Correlation between CB1R expression, histopathological differentiation and portal vein invasion in HCC (potential marker of good prognosis)	[252]
	High CB1R expression was associated with a better disease-free survival in HCC patients	[252]

PANCREAS	CB1R was demonstrated to be highly expressed in PDAC cell lines compared to the healthy cells	[253]
	CB1R was low expressed in PDAC patients which correlate with a longer survival	[253]
	PDAC tumors recruited various immune cells, which express CB1R	[253]
	CB1R mediated tumor cell growth inhibition in pancreatic cancer	[249, 254]
	Selective agonists of CB1R activated a common ROS mechanism that inhibited Panc1 cell proliferation	[257]
	Selective agonists of CB1R enhanced the ROS-mediated inhibition of pancreatic cancer cell growth of gemcitabine	[286]
	Possible role in the enhancement of PDAC treatment with gemcitabine also in vivo	[287]
LUNGS AND AIRWAYS	CB1R was low expressed in NSCLC patients	[258]
	CB1R agonists decreased random and growth factor-directed chemotaxis, chemoinvasion and focal adhesion complexes, in NSCLC cell lines (A549 and SW-1573 cells)	[258]
	CB1R agonists inhibited *in vivo* tumor growth and lung metastasis	[258]
	CB1R agonists inhibited Akt phosphorylation, MMP-9 expression and activity in NSCLC cells	[258]
	CB1R inhibited the number of metastatic nodes in a model of Lewis lung carcinoma (3LL) C57Bl/6 mice	[288]
KIDNEY	CB1R was expressed in ChRCC and it had a variable expression in RO	[288]
	In clear CCRCC, CB1R expression was down-regulated with respect to adjacent non-neoplastic kidney	[288]
	CB1R expression levels were an independent risk factor for overall survival for RCC patients	[289]
	Over-expression of CBR1 promoted RCC progression	[289]
	CB1R inverse agonists, and in vitro siRNA knockdown of CB1R reduced proliferation, migration, invasion and apoptosis of RCC cell lines (by up-regulating Bax and decreasing Bcl-2)	[289]
URINARY TRACT	CB1R was expressed in ECV304 bladder cancer cell lines	[289]
	CB1R activation induced proliferation of ECV304 cells	[289]
BREAST	CB1R was over-expressed in primary human breast tumors compared with normal breast tissues	[264]
	TNBC cell lines, MDA-MB-231, MDA-MB-231-luc, and MDA-MB-468 cells, expressed CB1R	[264]
	CB1R agonists inhibited proliferation and migration of TNBC cell lines (e.g MDA-MB-231, MDA-MB-468) in vitro / in vivo	[264]
	CB1R agonists modulated COX-2/ PGE2 signalling pathways and apoptosis in MDA-MB-231 cells	[264]
	CB1R antagonists inhibited proliferation of TNBC and ERα-positive cell lines (e.g. MDA-MB-231, MCF-7 and T47D cells) in vitro, and the volume of MDA-MB-231 tumor xenografts in vivo (lipid raft-mediated mechanism)	[265]
	CB1R antagonists were more effective in reduce proliferation in highly metastatic MDA-MB-231 cells with respect to less-invasive T47D and MCF-7 cells	[265]
	CB1R antagonists induced G1/S-phase cell cycle arrest, decreased expression of cyclin D and E, and increased levels of cyclin-dependent kinase inhibitor p27KIP1 in MDA-MB-231, MCF-7 and T47D cells	[265]
PROSTATE	High CB1R expression in comparison to normal prostatic tissues	[26]
	CB1R was over-expressed with the Gleason score, and metastasis incidence, serving as a negative marker for the PCa prognosis	[26]
	High expression in LNCaP, DU145 and PC3 cancer cells	[266]
	Inhibition of the proliferation of LNCaP, DU-145, and PC3, and primary cultures of PCa	[26]
	Decrease of EGFR levels on LNCaP, DU145, and PC3 PCa cells, causing inhibition of the EGF -stimulated growth of PCa cells and apoptosis and/or necrosis	[290]
	Inhibition of the invasive ability of PC3, DU-145, and LNCaP cells, through the inactivation of PKA	[267, 268]
	Possible involvement in the prevention in neuroendocrine differentiation of LNCaP cells by inhibition of PI3K/ Akt/ mTOR activation and stimulation of AMPK	[269]
	Possible involvement in apoptotic induction of PCa cells through the modulation of ERK and Akt signalling pathways	[291]
	CB1R agonists induced apoptosis in LNCaP cells	[266]
	Down-regulation of CB1R after pharmacological treatment, followed by a decrease in spheroid formation, VEGF, PSA, and pro-inflammatory cytokines IL-6/IL-8 in LNCaP stem cells	[26]
	CB1R activation increased NGF production by PC3 cells	[292]

Tab.6. In vitro/ in vivo evidence for the role of CB1R in tumors

CB1R expression in cancer tissues and cell lines. Phenotypic effects and mechanisms of action derived from the deregulation of CB1R expression and activation in cancer tissues and cell lines. Tumor types referring to different organs of the body: central nervous system (CNS), gastrointestinal (GI) tract, skeletal muskle, bone, skin, eye, liver, pancreas, lungs and airways, kidney, urinary tract, reproductive system, breast, prostate.

Abbreviations: cannabinoid receptor 1, 2 (CB1R/ CB2R), G coupled protein receptor 55 (GPR55), glioblastomas (GBM), guanosine-5'-triphosphate (GTP), transforming growth factor β1 (TGF-β1), signal transducer and activator of transcription 3 (STAT3), Natural Killer cells (NKs), Natural killer group 2 member D (NKG2D), colorectal cancer (CRC), azoxymethane (AOM), dextran sulfate sodium (DSS), polyunsaturated fatty acid (PUFAs), epidermal growth factor receptor (EGFR), severe combined immunodeficiency (SCID), retinoblastoma protein (pRb), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), angiotensin 2 (Ang 2), epidermal growth factor receptor (EGFR), hepatocellular carcinoma (HCC), pancreatic ductal adenocarcinoma (PDAC), non-small cell lung cancer (NSCLC), matrix metalloproteinase 9 (MMP-9), chromophobe renal cell carcinoma (ChRCC), renal oncocytoma (RO), clear cell renal cell carcinoma (CCRCC), renal cell carcinoma (RCC), B-cell leukemia/lymphoma 2 protein (Bcl-2), Bcl-2-associated X protein (Bax), triple negative breast cancer (TNBC), cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), prostate cancer (PCa), epidermal growth factor (EGF), phosphatidylInositol 3-Kinase (PI3K), mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), extracellular signal regulated kinase (ERK), prostate-specific antigen (PSA), interleukin (IL-6/8), nerve growth factor (NGF)

TUMORS	EVIDENCE FOR THE ROLE OF CB2R IN TUMORS	Ref.
CNS	CB2R was found expressed in glioma: increased expression with the tumor grade	[275]
	CB2R was highly expressed in membranes of GBM and astrocytoma than in control brain tissues	[238]
	De novo synthesis of ceramide leading to apoptosis in glioma cells	[293]
	CB2R agonists inhibited adhesion, local and distant invasion in induced/ spontaneous metastatic in vitro and in vivo models of glioma	[293]
GI TRACT	CB2R was low expressed in advanced stages of colon carcinogenesis and it disappeared in CRC	[240]
	Suppression of proliferation, migration in CRC cells	[243]
	CB2R activation induced apoptosis and increased ceramide levels in the DLD-1 and HT29 cells in vitro and it reduced the growth of DLD-1 cells in a mouse model of colon cancer	[244]
	Possible involvement in cytotoxic effects in CRC cells treated with pharmacological agonists and drug 5-fluorouracyl	[294]
	Possible involvement in enhance paclitaxel-induced apoptosis, through caspase-3, -8, and -9 activation, in gastric cancer cells; possible involvement in anti-neoplastic actions on xenograft models of gastric cancer	[295, 296]
	Peritumoral treatment with CB2R agonists reduced the growth of xenograft tumors generated by injection of CRC cells to immunodeficient mice	[244]
	CB2R agonists reduced the formation of aberrant crypt foci (pre-neoplastic lesions) induced by AOM	[297]
SKELETAL MUSKLE	Intra-plantar injection of CB2R agonists reduced fibrosarcoma related mechanical hyperalgesia by activation of peripheral CB2R	[246]
	Synergistically action given by the combination of CB2R and CB1R agonists, in reducing fibrosarcoma related mechanical hyperalgesia in vivo	[246]
BONE	CB2R expression in TC-71 and A-673 Ewing sarcoma cell lines (cytotoxic effects mainly induced by non-canonical CBRs' mechanisms)	[247, 283]
SKIN	CB2R was found expressed in papilloma, basal cell and squamous carcinomas, in melanoma, in melanocytes and melanoma cell lines	[249, 250]
	CB2R agonists and inverse agonists showed anti-proliferative, pro-apoptotic, anti-angiogenic and anti-migratory effects in melanoma in vitro / in vivo	[250]
	CB2R activation induced cycle arrest at the G1-S transition via inhibition of Akt and hypo-phosphorylation of pRb in melanoma cells	[250]
	In non-melanoma skin cancers CB2R activation induced apoptotic death of tumorigenic epidermal cells, un-affecting non transformed epidermal cells	[249]
	Local administration of CB2R agonists with or with CB1R agonists inhibited growth of malignant tumors generated by inoculation of epidermal tumor cells into nude mice	[249]
	Local administration of CB2R agonists impaired tumor vascularization in non-melanoma skin cancers: altered blood vessel morphology and decreased expression of VEGF, PDGF, and Ang 2; abrogation of EGFR function	[249]
	CB2R activation inhibits melanoma cell transmigration through the blood-brain barrier	[251]
EYE	Possible/ confirmed involvement in neuroprotection in glioma; reduction of pro-inflammatory cytokines in glioma	[298, 299]
LIVER	CB2R was over-expressed in HCC tissues samples	[252]
	Correlation between CB2R expression, histopathological differentiation and portal vein invasion in HCC (potential marker of good prognosis)	[252]
	High CB2R expression was associated with a better disease-free survival in HCC patients	[252]
	CB2R agonist, exerted anti-tumor effects on the Hep3B and HepG2 HCC cell lines, partly through inactivation of Akt signalling pathway (anti-proliferative, anti-migratory and apoptotic effects)	[300]
	High expression of CB2R in PDAC cell lines compared to the healthy cells	[253]
PANCREAS	PDAC tumors recruited various immune cells, which express CB2R	[253]
	CB2R mediated tumor cell growth inhibition in pancreatic cancer	[249, 254]
	CB2R activation had high anti-tumor effects in pancreatic cancer cell lines via a p8 dependent mechanism, activating ATF4 and leading to expression of the pro-apoptotic proteins CHOP and TRB	[256]

	High efficacy of gemcitabine in PDAC cells over-expressing CB2R	[301]
	Possible role in the enhancement of PDAC treatment with gemcitabine also in vivo	[287]
	Selective agonists of CB2R activated a common ROS mechanism that inhibited Panc1 cell invasion	[257]
LUNGS AND AIRWAYS	CB2R expression in NSCLC patients	[258]
	CB2R agonists attenuated random and growth factor-directed in vitro chemotaxis and chemoinvasion of NSCLC cell lines (A549 and SW-1573 cells)	[258]
	CB2R agonists inhibited tumor growth, proliferation, vascularization and metastasis in NSCLC in vivo	[258]
	CB2R agonists inhibited Akt phosphorylation, MMP-9 expression and activity in NSCLC cells	[258]
	In CB1R- and CB2R-knockout models of mice of NSCLC (CB1-/-; CB2-/-), only deficiency of CB2R resulted in a reduction of tumor burden vs. the wild type littermate	[302]
KIDNEY	CB2R was not expressed in ChRCC, CCRCC and in non-tumor renal tissues	[288]
	CB2R activation led to cell growth inhibition and G0/G1 cell cycle arrest in RCC in vitro	[261]
URINARY TRACT	CB2R was found expressed in ECV304 cell lines	[263]
	In ECV304 cell lines CB2R triggered pro-inflammatory states by enhancing TNF α release, increasing surface exposure of P- and E-selectins and allowing Jurkat T lymphocytes to adhere to treated cancer cells	[289]
BREAST	CB2R was over-expressed in primary human breast tumors compared with normal breast tissues	[264]
	TNBC cell lines, MDA-MB-231, MDA-MB-231-luc, and MDA-MB-468 cells, expressed CB2R	[264]
	CB2R agonists inhibited cell proliferation and migration in vitro and in vivo	[264]
	CB2R agonists delayed and reduced mammary gland tumors in the PyMT transgenic mouse model system	[264]
	CB2R agonists modulated COX-2/ PGE2 signalling pathways and apoptosis in MDA-MB-231 cells	[264]
PROSTATE	High CB2R expression in comparison to normal prostatic tissues	[26]
	High expression in LNCaP, DU145 and PC3 cancer cells	[266]
	Possible involvement in the prevention in neuroendocrine differentiation of LNCaP cells by inhibition of PI3K/Akt/mTOR activation and stimulation of AMPK	[269]
	Possible involvement in apoptotic induction of PCa cells by modulating ERK and Akt signalling pathways	[291]
	Down-regulation of CB2R after pharmacological treatment, followed by a decrease in spheroid formation, VEGF, PSA, and pro-inflammatory cytokines IL-6/IL-8 in LNCaP stem cells	[26]

Tab.7 .In vitro/ in vivo evidence for the role of CB2R in tumors

CB2R expression in cancer tissues and cell lines. Phenotypic effects and mechanisms of action derived from the deregulation of CB2R expression and activation in cancer tissues and cell lines. Tumor types referring to different organs of the body: central nervous system (CNS), gastrointestinal (GI) tract, skeletal muskle, bone, skin, eye, liver, pancreas, lungs and airways, kidney, urinary tract, breast, prostate

Abbreviations: cannabinoid receptor 1, 2 (CB1R/ CB2R), glioblastomas (GBM), colorectal cancer (CRC), azoxymethane (AOM), retinoblastoma protein (pRb), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), angiotensin 2 (Ang 2), epidermal growth factor receptor (EGFR), hepatocellular carcinoma (HCC), pancreatic ductal adenocarcinoma (PDAC), non-small cell lung cancer (NSCLC), activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), tribbles homolog (TRB), matrix metalloproteinase 9 (MMP-9), chromophobe renal cell carcinoma (ChRCC), clear cell renal cell carcinoma (CCRCC), renal cell carcinoma (RCC), tumor necrosis factor α (TNFα), triple negative breast cancer (TNBC), polyoma middle T oncoprotein (PyMT), cyclooxygenase-2 (COX-2), phosphatidylInositol 3-Kinase (PI3K), mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), extracellular signal regulated kinase (ERK), prostate cancer (PCa), vascular endothelial growth factor (VEGF), prostate-specific antigen (PSA), interleukin (IL-6/8)

1.1.1.3 Deregulations of non-canonical receptors

GPR55 has implications in the malignancy process of many tumors (Table 8). In GBM cells and glioma patient-derived tissues samples, GPR55 is highly expressed and it correlates with the GBM higher histological rate [303, 304]. Moreover, recent articles highlighted a strong correlation between GPR55 expression and the presence of different sets of stemness gene markers (e.g. CD15, SOX2, OCT4, and ID19) in GBM [304]. Both in vitro and in vivo, GPR55 promoted migration and adhesion of CRC cells, and also down-regulated the immune cell recruitment, thus hindering the immune-surveillance [305]. Moreover, contrary to CNR1 which, as mentioned before, was found high methylated and with a tumor-suppressor function in CRC, GPR55 gene was observed hypo-methylated, highly expressed and correlated with the development of malignancy in CRC [245]. In skin cancers, GPR55 is often up-regulated, such as in squamous cell carcinomas and in melanoma, giving proliferative advantages on tumor cells, the enhancement of tumor cell anchorage-independent growth, and contributing to invasiveness and tumorigenicity in vivo [195, 306, 307]. On the contrary, evidence attested no difference in GPR55 expression, between malignant and non-malignant cholangiocytes. Some HCC cells (e.g. HepG2 cells) reduced in filopodia formation and invasion when treated with GPR55 antagonists, thus suggesting a role for the receptor in some types of liver cancer [308, 309]. Regarding pancreatic cancers, GPR55 levels were observed to be incremented with the advanced stages of tumor. Moreover, genetic ablation/ knockdown or pharmacologic inhibition of the CBR, elicited anti-tumor effects in PDAC in vitro and in vivo, by reducing tumor cell proliferation, anchorage-dependent/ independent growth, cell cycle progression and by prolonging survival of PDAC mice models [287, 307]. GPR55 expression was attested 79% of squamous cell lung carcinoma and 82% of adenocarcinoma patients, moreover high GPR55 in NSCLC, was seen to be related to the down-regulation of miR-675-5p which contributed to cancer progression [310, 311]. Less evidence discussed about the role of GPR55 in renal and urinary cancers, however few reports found the involvement of the receptor in pheochromocytoma and bladder tumors [312, 313]. Finally, in BCa, GPR55 was detected highly expressed in TNBC cell lines, attending to their increase on proliferative, invasive and chemotactic features and to the acquired resistance to cytotoxic drugs [303, 314-316]. Moreover, GPR55 was found in endometrial cancers, ovary tumors and PCa, in particular in OVACAR-3, A2780, DU145, PC3 and LNCaP cell lines [308, 317, 318].

The GPR55 signalling pathways (Fig. 5) involve the Gαq and $G\alpha_{12/13}$ family proteins. The *Gαq* subunit signals through *PLC*, increasing diacylglycerol and Ca^{2+} from the ER, thus culminating in the activity of protein-kinase C (PKC) [319]. Consequently, PKC catalyzes the phosphorylation of several targets, like the MAPK/ERK proteins, in particular ERK1/2 which activates the cAMP response element-binding protein (CREB) and the nuclear factor kappa- B (NF-kB) [320]. Conversely, *$G\alpha_{12/13}$* up-regulates the *Rho A/ Rho-associated protein kinase (*ROCK*)* pathway, increasing PLC activity, actin cytoskeleton remodeling and p38/ATF2 nuclear translocation [306]. The main phenotypic effects resulting from GPR55 activation are cell proliferation, differentiation, apoptosis and the remodeling of the cytoskeleton.

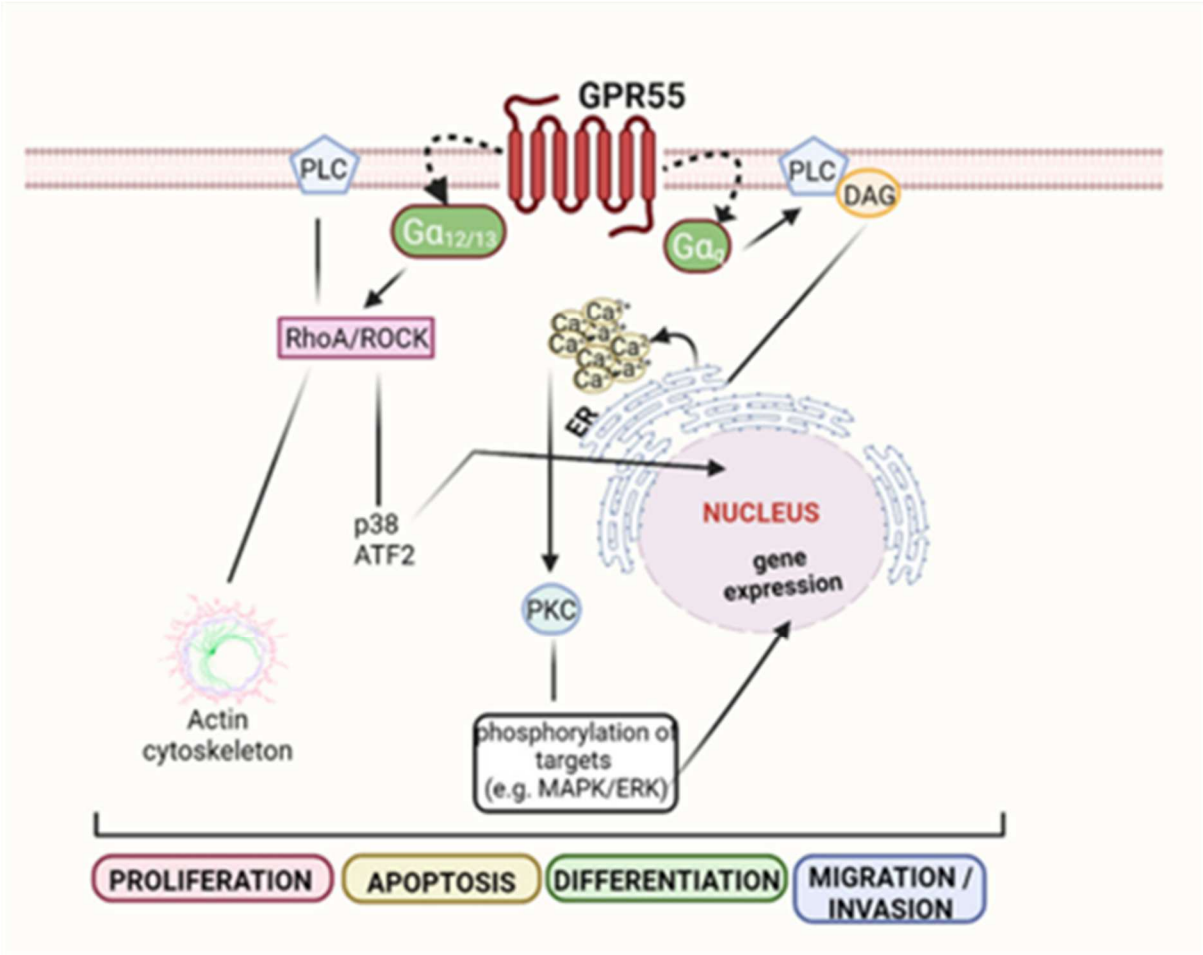


Fig.5. GPR55 downstream signalling pathways

GPR55 elicits proliferation, apoptosis, differentiation, and migration, through different signalling pathways: Rho A/ROCK and PLC pathway. Image from [274].

Abbreviations: G coupled protein receptor 55 (GPR55), Ras homolog family member A (Rho A), Rho-associated protein kinase (ROCK), phospholipase C (PLC). diacylglycerol (DAG) , protein-kinase C (PKC), mitogen-activated protein kinase (MAPK); extracellular signal regulated kinase (ERK), activating transcription factor 2 (ATF2)

TRPV1 (Table 9) was found over-expressed in glioma, GBM and neuroblastoma cell lines, such as U373, U87, DBTRG and N1E-115 cells, where the receptor triggered apoptosis, reduced proliferation and migration. However, levels of TRPV1 were inversely correlated with glioma grading, disappearing in the grade IV GBM [321-323]. Also in primary gastric cancers, RCC and urothelial tumors, TRPV1 was seen down-regulated with respect to the adjacent tissues, positively correlating with histological grade, tumor size, clinical stage, lymphatic metastasis, and poor prognosis of patients [324-326]. Accordingly, low expression of TRPV1 was associated with proliferative/ metastatic and other tumor markers (e.g. Ki67, VEGFR, E-cadherin, von Hippel-Lindau VHL, hypoxia-inducible factor-1α, HIF-1α, mTOR) in gastric cancers and RCC [324] [327]. In urothelial and bladder tumors the activation of TRPV1 elicited anti-cancer effects [328-330]. In CRC, the knockout of TRPV1 elicited tumor cell proliferation, through a constitutively activation of EGFR, whereas agonists for the receptor dephosphorylated EGFR restoring tumor growth [331]. In sarcomas, inhibition of TRPV1 reduced the production of tumor-related pro-inflammatory cytokines, attenuating cancer pain [332] [333]. Similar effects were demonstrated in bone pain in vivo mice models. TRPV1 was detected in some osteosarcoma cells (e.g. G292 and HOS cells), with significant implications in apoptosis and cell invasive reduction [334, 335]. In melanoma and squamous cell carcinomas, TRPV1 activation/up-regulation stimulated anti-tumor properties [336-338]. On the other hand, only little evidence discussed about what function TRPV1 has in HCC. Zhang et al., for instance, reported that TRPV1 induced apoptosis and anti-tumor sensitivity to sorefenib in vivo, through the increase of the phosphorylation of ERK and also the decrease of the levels of STAT3 [339]. In pancreatic PANC-1 cell lines, EGFR was down-regulated upon TRPV1 over-expression, thus leading to a delay in cell proliferation [340]. In nasopharyngheal carcinoma, NSCLC and small cell lung cancer (SCLC) cells, TRPV1 lowered tumor cell proliferation, migration and it elicited apoptotic cell death [341-344]. Major effects on apoptosis were also observed in endometrial and uterine cervix cancers, particularly in C229, Caski and HeLa tumor cells [341, 345, 346]. Regarding BCa, different neoplastic tissues and cell lines, such as MCF7, MDA-MB-231 and SUM149PT, TRPV1 levels were associated to the malignant progression of cancer and related pain; moreover, via a mechanism dependent on ERα, increasing doses of estrogens in BCa cells could induce TRPV1 high expression [347-350]. Finally, in PCa, TRPV1 acted on cell proliferation, apoptosis in PC3/ LNCaP cells and in the modulation of the expression levels of the androgen receptor in LNCaP cells [351, 352].

The TRPV1 signalling pathways mainly affect the balance between cell proliferation and apoptosis, based on Ca^{2+} and Na^{+} influx into the cytosol [353]. Cell proliferation results from the ingress of Ca^{2+} ions, followed by adenosine triphosphate (ATP) release and the activation of the membrane purinoceptor 2 (P2Y2). Moreover, Ca^{2} also trans-activate EGFR. Stimulation of P2Y2 by ATP, triggers the phosphatidylInositol 3-Kinase (PI3K)/Akt pathway and up-regulates, via PLC, inositol 1,4,5-trisphosphate (IP3), which allows the exit of $Ca^{2+}$from the ER. The trans-activation of EGFR, instead, induces Ras/Raf/MAPK-ERK kinase (MEK)/ERK1-2 pathway, favoring cell proliferation together with the PI3K/Akt. On the other hand, mechanisms which regulate apoptosis occur through mitochondria membrane depolarization, and ER stress, in nucleus and cytosol. In mitochondria, after the influx of Ca^{2+} and Na^{+} into the organelle, the pro-apoptotic cytochrome c is released. In ER, instead, oxidative stress lead to c-Jun N-terminal kinase (JNK) activation and to the up-regulation of transcription factors such as ATF4, ATF6, and X-box binding protein (XBP), which decrease the expression of Bcl-2. Finally, the influx of Ca^{2+} in the cytosol, activates calcineurin and ataxia-telangiectasia mutated kinase (ATM) which up-regulate p53, Bcl-2-associated X protein (Bax), p21 and p16INK4A.These last three factors together with cytochrome c, activate caspase 3 and 9, causing apoptotic cell death (Figure 8) [218, 321, 354].

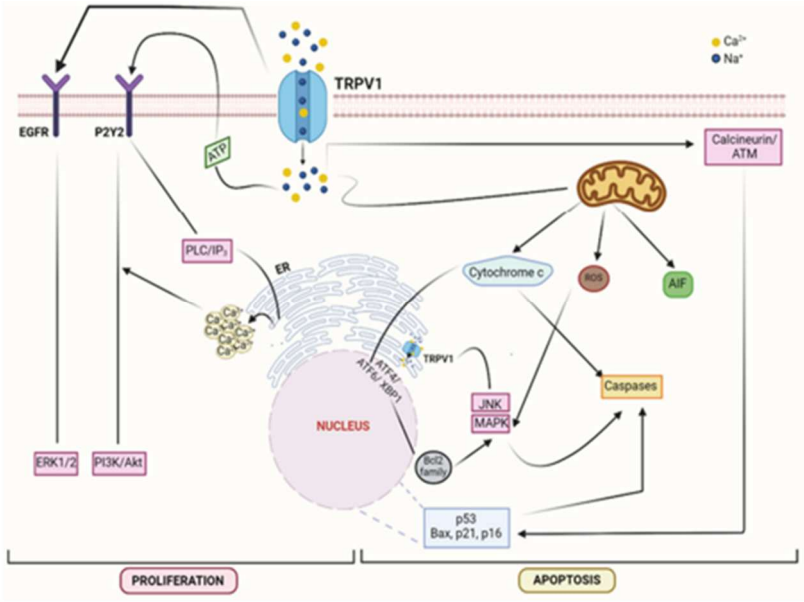


Fig.6. TRPV1 downstream signalling pathways [274]

TRPV1 induces proliferation through ERK1/2 and PI3K/Akt. Moreover it leads apoptosis, by signalling through: cytochrome c/caspases, ROS/JNK/MAPK, AIF, p53/Bax/p21/p16.

Abbreviations: transient receptor potential cation channel subfamily V member 1 (TRPV1), epidermal growth factor receptor (EGFR), purinoceptor 2 (P2Y2), ataxia-telangiectasia mutated kinase (ATM), reactive oxygen species (ROS), apoptosis-inducing factor (AIF), extracellular signal regulated kinase (ERK1/2), phosphatidylInositol 3-Kinase (PI3K), phospholipase C (PLC), inositol 1,4,5-trisphosphate (IP3), c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK), B-cell leukemia/lymphoma 2 (Bcl-2), protein Bcl-2-associated X protein (Bax)

TUMORS	EVIDENCE FOR THE ROLE OF GPR55 IN TUMORS	Ref.
CNS	GPR55 was found highly expressed in GBM cells (e.g. U-87MG), and in glioma patient-derived tissue samples	[304, 355]
	Increased GPR55 expression was correlated with GBM higher histological grade	[307]
	Decrease in the overall survival, increase in GBM cell proliferation and LPI levels in GBM patients	[307]
	Strongly correlation with different sets of stemness gene markers (e.g. CD15, SOX2, OCT4, and ID19) in GBM	[304]
	Higher expression of GPR55 in GSCs with respect to differentiated GBM cells	[304]
	GPR55 antagonists exhibited high cytotoxicity in a panel of patient-derived GSCs	[304]
	GPR55 antagonists reduced chemo resistance in U87MG GBM cells	[303]
	GPR55 antagonists reduced the number of Ki67 immunoreactive nuclei, a cell cycle marker, in GBM cells	[356]
GI	Promotion of migration and adhesion of CRC cells	[357, 358]
	Tumor-promoting role (alterations of leukocyte populations, i.e. myeloid-derived suppressor cells and T lymphocytes, within the tumor tissues) in a AOM- and DSS-driven CRC mouse model	[245]
	In experimental CRC models, GPR55 knockout reduced the presence of tumor-promoting factors (COX-2, STAT3)	[245]
	GPR55 and CB1R expression was differentially regulated in CRC experimental models and in a cohort of CRC patients: methylation of CNR1 and GPR55 was differentially regulated in human CRC tissues compared to controls (differential roles in CRC)	[245]
	Possible role in innate immunity and immunosurveillance by acting on immune cells (T cells and NKs) in colon cancer	[305]
IMMUNE SYSTEM	GPR55 was found over-expressed in B-cell multiple myeloma, lymphomas (Hodgkin and non-Hodgkin) and in B-lymphoblastic cells	[306, 359, 360]
	LPI slightly reduced viability of Hodgkin L428 cells by acting through GPR55	[360]
	GPR55 activation by LPI generated a rapid phosphorylation of p38 MAPK and activation of ATF2 in IM-9 lymphoblastic cells	[306]
SKIN	GPR55 was found up-regulated in human skin tumors and other human squamous cell carcinomas compared with the healthy tissues	[195]
	GPR55 was found expressed in melanoma cells	[306, 307]
	GPR55-deficient mice were more resistant to DMBA/TPA-induced papilloma and skin carcinoma formation than their wild-type littermates	[195]
	Proliferative advantage on skin cancer cell, enhancement of skin cancer cell anchorage-independent growth, invasiveness and tumorigenicity in vivo	[195]
LIVER	Malignant and non-malignant cholangiocytes expressed GPR55 to a similar degree	[308]
	Pre-treatment with GPR55 antagonists inhibited ERK phosphorylation, filopodia formation and invasion induced by GPR55 agonists and fetal bovine serum in HepG2 cells	[361]
	GPR55 silencing inhibited sphingosine 1-phosphate lyase over-expression enhancing cell proliferation in HCC cells	[362]
	Contribution on HCC tumor progression also depending on the correlation with sphingosine 1-phosphate lyase levels	[362]
	Enhancement of HCC tumor progression through activation of p38 and mitochondrial function (LPI, LPG-GPR55 axis)	[362]
PANCREAS	GPR55 was found expressed in pancreatic cancer cells and in advanced stages of cancer	[307]
	Inhibition of GPR55 reduced pancreatic cancer cell growth in vitro and in vivo	[287]
	Reduced proliferation in cholangiocarcinoma cells upon GPR55 activation, in both in vitro and in vivo assays	[363]
	Pre-treatment with GPR55 antagonists inhibited ERK phosphorylation, filopodia formation and invasion induced by GPR55 agonists and fetal bovine serum in PANC-1 cells	[361]
	Genetic ablation of GPR55 in KRAS /TP53 /Pdx1-Cre (KPC) mouse models of PDAC significantly prolonged survival, reduced proliferation of tumor cells, MAPK signalling and ribonucleotide reductase M1 levels in KPC mice	[287]
	Knockdown or pharmacologic inhibition of GPR55 reduces anchorage-dependent and independent growth, cell cycle progression, activation of MAPK and protein levels of ribonucleotide reductases in PDAC cells	[287]
	p53 regulated GPR55 protein expression through modulation of the microRNA miR34b-3p in PDAC	[287]

	GPR55 antagonists reduced glycolysis in PANC-1 cells and tumors through low expression and function at multiple controlling sites in the glycolytic pathway	[364]
	GPR55 antagonists decreased EGFR, PKM2, β-catenin protein levels and nuclear accumulation of HIF-1α, Pgp and BCRP in PDAC cells. They reduced the active forms of PKM2, β-catenin and the amount of MDR proteins. Overall these effects attenuated the MEK/ERK and PI3K-Akt signalling pathways, leading to a reduction in MDR proteins and to an increase in the cytotoxicity of doxorubicin	[355]
LUNGS AND AIRWAYS	Down-regulation of miR-675-5p in NSCLC (correlation with lymph node metastasis, TNM stage, cancer cell proliferation, migration/ invasion) led to up-regulation of GPR55 and its signalling pathways, contributing to cancer progression	[311]
	GPR55 expression GPR55 was enhanced in 79% of squamous cell lung carcinoma and 82% of adenocarcinoma patients	[310]
KIDNEY	GPR55 activation resulted in Ca2+ release, nNOS expression and activity induction, and oxidative stress in pheochromocytoma PC12 cell lines	[312]
URINARY TRACT	GPR55 may promote cell proliferation and apoptosis in bladder cancers (not clarified)	[313]
BREAST	GPR55 was found expressed in BCa cells (e.g. MCFs, MDA-MB-231 cells), particularly in TNBC cells	[365]
	High GPR55 expression was associated with the aggressive basal/triple-negative BCa population, higher probability to develop metastases, and poor patient prognosis	[315]
	Activation of GPR55 by LPI elicited pro-invasive features on BCa cells both in vitro and in vivo. The effect was elicited by coupling to Gq/11 heterotrimeric proteins and the subsequent activation through ERK, of the transcription factor ETV4/PEA3	[315]
	GPR55 induced cell migration of breast cancer cells through the stimulation of LPI and the HBXIP and p-MLC pathways	[315]
	Inducement of proliferation and chemotaxis in MDA-MB-231 cells	[314, 316]
	GPR55 antagonists potentiated doxorubicin cytotoxicity in MDA-MB-231 cells	[355]
	GPR55 antagonists inhibited macrophage populations recruitment to the BCa microenvironment	[366]
ENDOMETRIUM	GPR55 transcript and protein levels were significantly higher in endometrial cancer tissues than in control tissues	[317]
OVARIUM	GPR55 expression in OVCAR-3 and A2780 cell lines	[308, 367]
	Promotion of ERK and Akt pathways in OVACAR-3 and A2780 cells	[308, 367]
	Elevated GPR55 agonists levels (LPI) in blood and ascites fluid of ovarian cancer patients	[367]
	Regulation of the angiogenic potential	[367]
PROSTATE	PC3, DU145and LNCaP cell lines expressed GPR55.	[318, 367, 368]
	The activation of GPR55 by LPI promoted ERK and Akt pathways in PC3 and DU145 cell lines	[318, 367]

Tab.8. In vitro/ in vivo evidence for the role of GPR55 in tumors

GPR55 expression in cancer tissues and cell lines. Phenotypic effects and mechanisms of action derived from the deregulation of GPR55 expression and activation in cancer tissues and cell lines. Tumor types referring to different organs of the body: central nervous system (CNS), gastrointestinal (GI) tract, immune system, skin, liver, pancreas, lungs and airways, kidney, urinary tract, breast, endometrium, ovarium, prostate.

Abbreviations: G coupled protein receptor 55 (GPR55), cannabinoid receptor 1 (CB1R), glioblastomas (GBM), lysophosphatidylinositol (LPI), GBM strem cells (GSCs), colorectal cancer (CRC), azoxymethane (AOM), dextran sulfate sodium (DSS), cyclooxygenase-2 (COX-2), signal transducer and activator of transcription 3 (STAT3), Natural Killer cells (NKs), mitogen-activated protein kinase (MAPK), activating transcription factor 2 (ATF2), dimethylbenz[a]anthracene and 12-O-tetradecanoyl phorbol-13-acetate (DMBA/TPA), extracellular signal regulated kinase (ERK), tumor suppressor protein 53 (TP53), hepatocellular carcinoma (HCC), lyso-phosphatidyl-glycerols (LPG), Kirsten rat sarcoma virus (KRAS), pancreatic ductal adenocarcinoma (PDAC), mitogen-activated protein kinase (MAPK), epidermal growth factor receptor (EGFR), pyruvate kinase M2 (PKM2), hypoxia-inducible factor-1α (HIF-1α), P-glycoprotein (Pgp), breast cancer resistance protein (BCRP), multiple drug resistant (MDR), non-small cell lung cancer (NSCLC), tumor, node, metastasis (TNM), neuronal Nitric oxide synthase (nNOS), triple negative breast cancer (TNBC), breast cancer (BCa), extracellular signal regulated kinase (ERK), Hepatitis B virus X-interacting protein (HBXIP), phosphorylation of the myosin light chain (pMLC2), ETS translocation variant 4/polyoma enhancer activator 3 (ETV4/PEA3)

TUMORS	EVIDENCE FOR THE ROLE OF TRPV1 IN TUMORS	Ref.
CNS	TRPV1 was found expressed in U373, U87, FC1 and FLS glioma cells	[321]
	TRPV1 was highly expressed in GSCs compared with differentiated GBM cells (correlation with stemness gene markers: NOTCH, OLIG2, CD9, TRIM28, and TUFM)	[304]
	TRPV1 inversely correlated with glioma grading, with marked loss of TRPV1 expression in the majority of grade IV GBM	[321]
	TRPV1 agonists triggered apoptosis of U373, involving p38 but not extracellular signal-regulated protein kinase activation	[321]
	Inhibition of TRPV1 modulated GBM cell proliferation and death in DBTRG GBM in vitro cell line model	[369]
	Activation of TRPV1 induced apoptosis of U87-GM glioma cells by up-regulating mitochondrial membrane potential	[370]
	Neural precursor cells migrated to high-grade astrocytomas, reducing glioma expansion and prolonging survival time by releasing endovanilloids that activate TRPV1 in high-grade astrocytoma cells	[371]
	Decrease on cell proliferation via a lipid raft-dependent mechanism in neuroblastoma cell line N1E-115	[323]
	TRPV1 agonists inhibited the activity of NFAT and it decrease MMPs (such as MMP1/2/7) in SK-N-SH cells. Tumor cell migration was reduced	[372]
GI TRACT	TRPV1 was down-regulated in primary gastric cancer tissues compared to their adjacent tissues	[324]
	The decreased expression of TRPV1 in gastric cancer tissues, positively correlated with tumor size, histological grade, lymphatic metastasis, clinical stage, and poor prognosis of patients	[324]
	Low TRPV1 expression in gastric cancer tissues correlated with cancer markers for proliferation and metastasis (e.g Ki67, VEGFR, and E-cadherin)	[324]
	TRPV1 over-expression in gastric cancer cells, blocked cell cycle at G1 phase, thus inhibiting cell proliferation and it attenuated migration and invasion of tumor cells in vitro	[324]
	TRPV1 over-expression in gastric cancer cells increased Ca^{2+}, activated CaMKKβ and AMPK phosphorylation, and decreased expression of cyclin D1 and MMP2	[324]
	TRPV1 significantly reduced gastric tumor size, number and peritoneal dissemination in vivo	[324]
	In gastric cancer AGS cells, TRPV1 agonists disrupted mitochondrial integrity, activated JNK and led to p53 stabilization, impacting on tumor cell apoptosis	[373]
	Over-expression of TRPV1 blocked the phosphorylation of EGFR in HCR116 cells (CRC cells). Cell proliferation was reduced	[331]
	In knockout TRPV1 CRC mice, EGFR Y1068 was constitutively phosphorylated. PCNA, c-Fos, c-Myc expression levels were high, following the increase of tumor cell proliferation	[331]
	TRPV1 activation in HCT116 cells, activated calpain and PTP1B, which dephosphorylated EGFR, decreasing cell proliferation	[331]
	In colon adenocarcinoma Caco-2 cell lines, TRPV1 inhibition reduced the phosphorylation level of Akt	[331]
	Modulation of ROS generation, CHOP expression levels, apoptosis in Caco-2 cells	[374]
	TRPV1 elicited apoptosis of HCA-7 Colony 29 CRC cell lines through oxidative stress and ER-stress	[375]
	The inhibition of TRPV1 inHCT116 increased the oncosuppressive function of TRAIL. Increment on the activity of death receptors (such as DR4/5) through the ROS-JNK-CHOP signaling and on the expression of pro-apoptotic proteins. Cell survival proteins were decreased	[376]
SKELETAL MUSKLE	Inhibition of TRPV1 prevented capsaicin-induced ERK phosphorylation and reduced tumor-related pro-inflammatory cytokine production in sarcomas	[332]
	Rhabdomyosarcoma CRL1598 cells produced high iCGRP release which contributed to cancer pain	[333]
BONE	In osteosarcoma G292 cells, TRPV1 activation induces cytochrome C release, thus impacting on apoptosis	[334]
	Inhibition of TRPV1 degradation reduced invasion of osteosarcoma HOS cells	[377]
	Block of TRPV1 activity in bone cancer pain in in vivo mice models	[335]
SKIN	In A2058 and A375 melanoma cells, activation of the p53 after the up-regulation of TRPV1. Increase in the targets of p53 (p21, PUMA and MDM2). Apoptosis inductions	[336]
	In squamous carcinoma A431 cell lines, over-expression of TRPV1 led to ubiquitination and degradation of EGFR. Decrease of tumor cell death growth and proliferation	[337]
	TRPV1 led to the death of HSC3, SCC25, SCC4 oral cell squamous carcinoma cells	[338]
	TRPV1 activation induced apoptosis of melanoma A375 cells through COX-2, and LOX-derived product synthesis	[378]
LIVER	In HCC cell lines TRPV1 induced apoptosis by increasing the phosphorylation level of ERK and attenuating STAT3 phosphorylation	[339]
	HGF evoked TRPV1 channel activity, causing Ca2+ influx and up-regulating migration of hepatoblastoma HepG2 cells	[379]

	In HCC cell lines TRPV1 agonists up-regulated the anti-tumor activity of sorafenib. Increase in phosphorylation of ERK and down-regulation of the phosphorylation of STAT3	[339]
PANCREAS	In pancreatic PANC-1 cell lines, over-expression of TRPV1 induced ubiquitination and degradation of EGFR. Reduction in cell proliferation	[340]
	TRPV1 disrupts mitochondrial membrane potential and suppresses the synthesis of ATP in pancreatic neuroendocrine tumor BON and QGP-1 cells. Induction of tumor cell apoptosis	[380]
LUNGS AND AIRWAYS	TRPV1 inhibited MKK3-induced p38 activation in nasopharyngeal CNE2 and SUNE1 cells. Reduction in tumor cell proliferation	[381]
	In SCLC (H69, DMS 114, DMS53, and H82 cells) TRPV1 agonists up-regulated the activity of calpain-1/2 and TRPV6. Induction of tumor cell apoptosis	[342]
	In CNE2 and SUNE1 cells, TRPV1 inhibited the phosphorylation of p38 which was driven by MKK3. Impact on tumor cell apoptosis	[381]
	TRPV1 agonists inhibited the MKK3-p38 axis in CNE2 SUNE1cell lines. Reduction in tumor cell migration	[381]
	TRPV1 induced the expression of ICAM-1, via phosphorylation of p42/44. It up-regulated TIMP-1 in lung cancer A549, H358, and H460 cells, reducing cell migration	[343]
	TRPV1 antagonists enhanced cell death induced by γ-rays in A549 cells	[344]
KIDNEY	TRPV1 expression was decreased in RCC tissues vs. normal peritumoral kidney tissues, correlating with RCC histopathological subtype and Fuhrman grade	[325]
	In CCRCC,TRPV1 expression was tightly associated with key molecules of the classical pathways in the cancer, such as VHL, TP53, HIF-1α, mTOR, MAPK1, CTNNB1 (role of tumor suppressor)	[327]
	TRPV1 agonists reduced proliferation and induced apoptosis of RCC cells (up-regulation of the pro-apoptotic genes c-Myc, FADD, Bax, cleaved-caspase-3, -8, and -9, and down-regulation of anti-apoptotic Bcl-2)	[382]
	TRPV1 agonists slowed the growth of 786-O renal cancer xenografts in vivo	[382]
	TRPV1 agonists activated p38 and JNK MAPK pathways to induce apoptosis in renal carcinoma 786-O cells	[75]
URINARY TRACT	Decrease or absence of TRPV1 in urothelial cancer specimens proportionally to differentiation levels of tumor malignancy	[326]
	Activation of TRPV1 in TRPV1-over-expressing urothelial RT4 cancer cells, induced cell cycle arrest in G0/G1 phase and apoptosis by activating p53 to up-regulate Fas/CD95, reducing tumor cell proliferation	[328]
	TRPV1 induced mitochondrial dysfunction of bladder cancer T24 and 5637 cells, causing necrosis	[329]
	In urothelial 5637 cancer cells, TRPV1 agonists changed gene expression (e.g. MMP1, MMP9, and S100A4)	[383]
	TRPV1 agonists increased the anti-tumor efficacy of pirarubicin in bladder transitional cell carcinoma 5637 cells, through the inhibition of PCNA translocation to the nucleus	[330]
OVARIUM AND ENDOMETRIUM	In uterine cervix cancer C299, Caski, and HeLa cells, activation of TRPV1 induced apoptosis	[345]
	TRPV1 activation in endometrial cancer Ishikawa cells induced cell apoptosis through the increase of intracellular calcium levels	[341]
	TRPV1 increased COX-2 expression and activity in HeLa cells, modulating apoptosis	[346]
BREAST	TRPV1 was expressed in different neoplastic tissues and cell lines of BCa and associated with the regulation of tumor growth, tumor neurogenesis, cancer pain, and malignant progression of cancer	[347]
	Increasing doses of estrogens in BCa cells, induced high TRPV1 expression via a mechanism dependent on ERα	[347]
	In breast carcinoma cell MCF7 cell lines TRPV1 activation induced cell proliferation	[350]
	In MBA-MD-231 cells, TRPV1 inhibition up-regulated apoptosis, by elevating ROS species	[349]
	TRPV1 agonists induced the up-regulation of c-Fos and the necrotic marker RIP3 in MCF4 cells	[350]
	TRPV1 stimulated the production of ROS and mitochondrial membrane polarization of MCF7 cells. Induction of tumor cell apoptosis	[384]
	In SUM149PT cells, TRPV1 agonists induced apoptosis and necrosis	[348]
	Positive allosteric modulation of TRPV1 stimulated ROS production and mitochondrial membrane depolarization of MCF7 cells, thus inducing apoptosis	[385]
	TRPV1 activation potentiated the anti-tumor sensitivity of cisplatin in MCF7 cells	[385]
	Down-regulation of apoptosis induced by 5-fluorouracil in MCF7 cells	[386]
PROSTATE	TRPV1 activated PI3K and p44/42 MAPK pathways to suppress ceramide production and it up-regulated the expression of androgen receptor in PCa cells (LNCaP cells)	[351]
	Modulation of the PLC/ PKC/ ERK pathways in PC3 cells, impacting on PCa cell proliferation	[352]

Tab.9. In vitro/ in vivo evidence for the role of TRPV1 in tumors

TRPV1 expression in cancer tissues and cell lines. Phenotypic effects and mechanisms of action derived from the deregulation of TRPV1 expression and activation in cancer tissues and cell lines. Tumor types referring to different organs of the body: central nervous system (CNS), gastrointestinal (GI) tract, skeletal muscle, bone, skin, liver, pancreas, lungs and airways, kidney, urinary tract, ovarium and endometrium, breast, prostate.

Abbreviations: transient receptor potential cation channel subfamily V member 1 (TRPV1), GBM strem cells (GSCs), glioblastomas (GBM), nuclear factor of activated T-cells (NFAT), matrix metalloproteinase (MMPs), vascular endothelial growth factor receptor (VEGFR), calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ), AMP-activated protein kinase (AMPK), c-Jun N-terminal kinase (JNK), epidermal growth factor receptor (EGFR), colorectal cancer (CRC), tyrosine 1068 (Y1068), protein tyrosine phosphatase 1B (PCNA), protein tyrosine phosphatase 1B (PTP1B), reactive oxygen species (ROS), C/EBP homologous protein (CHOP), endoplasmic reticulum (ER), TNF-related apoptosis-inducing ligand (TRAIL), death receptors (DR4/-5), extracellular signal regulated kinase (ERK), immunoreactive calcitonin gene-related peptide (iCGRP), p53 upregulated modulator of apoptosis (PUMA), mouse double minute 2 homolog (MDM2), cyclooxygenase-2 (COX-2), lipoxygenases (LOXs), signal transducer and activator of transcription 3 (STAT3), hepatocyte growth factor (HGF), hepatocellular carcinoma (HCC), adenosine triphosphate (ATP), mitogen-activated protein kinase kinase 3 (MKK3), small cell lung cancer (SCLC), transient receptor potential cation channel subfamily V member 6 (TRPV6), intercellular adhesion molecule 1 (ICAM-1), tissue inhibitor matrix metalloproteinases-1 (TIMP-1), clear cell renal cell carcinoma (CCRCC), renal cell carcinoma (RCC), cellular myelocytomatosis oncogene (c-Myc), von Hippel-Lindau (VHL), tumor suppressor protein 53 (TP53), hypoxia-inducible factor-1α (HIF-1α), mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK), catenin beta-1 (CTNNB1), Fas-associated death domain (FADD), Bcl-2-associated X proteinBcl-2 (Bax), c-Jun N-terminal kinase (JNK), matrix metalloproteinases (MMPs), receptor-interacting serine-threonine kinase 3 (RIP3), phosphatidylInositol 3-Kinase (PI3K), prostate cancer (PCa), phospholipase C (PLC), protein-kinase C (PKC)

1.2.3 Other receptors

Other than the above mentioned receptors, GPR18, GPR119 and TRPV2 represent some new members of the *non-canonical class of CBRs*. However, there is a lack in scientific evidence for what concerns their role/expression in tissues and their regulation by cannabinoid compounds. As GPR55, also GPR18 and GPR119 belong to the family of class A GPCRs.

GPR18 (Gpr18, chromosome 13q 32.3) is present in the CNS, lymphoid tissues and it has a high constitutive activity in cells. Its modulation was associated with various phenotypes, such as cell migration, sperm / cardiac physiology, pain, obesity, intraocular pressure, and cancer [387-391]. Putative endogenous ligands for the receptor are N-arachidonoyl glycine (NAGly), a carboxylic metabolite of AEA [392-394], and the polyunsaturated fatty acid metabolite, resolvin D2 (RvD2), mainly implicated in inflammation [395]. However, due to the lack of consistency amongst reports, the International Union of Pharmacology (IUPHAR) still defines GPR18 as an orphan GPCR [396]. Also the associated downstream pathways of the receptor are still not fully explored [389, 397]. However, it was reported that GPR18 can signal through Gαi/o and Gαq [391, 392]. In the context of cancer, GPR18 was found a potential protective factor in HCC and BCa [398, 399]. Conversely, it was observed over-expressed in human melanoma metastases and its blockade with siRNA enhanced apoptosis [400].

GPR119 (Gpr119, chromosome X q26.1) is predominantly in pancreas (β-cells) and GI tract (enteroendocrine cells). As GPR55, GPR119 has been deorphanized recently, and N-oleoylethanolamide is the most active ligand tested so far [401]. However, other eCBs like N-oleoylethanolamide and stearoylethanolamide (SEA) have been considered endogenous agonists as well. Cells expressing GPR119 were found to have a constitutive increase in cAMP, which followed the coupling with the Gs protein [402]. Moreover, other evidence showed that cells expressing native or recombinant GPR119 increased cAMP lelves, subsequently stimulated adenylyl cyclase and PKA activity [402-404] [405-407]. Furthermore, the ATP-sensitive K^+ and voltage-dependent Ca^{2+} channels also appeared to be activated in GPR119-mediated responses [408]. Although the role of GPR119 in cancer cells has not been deepened yet, few studies demonstrated that the activation of the receptor reduced mitochondrial oxidative phosphorylation (OXPHOS) and stimulated glycolysis in BCa cells, enhancing the responsiveness to tyrosine kinase inhibitors (TKIs), through a lactate-mediated inhibition of autophagy [409]. However, being the receptor mainly implicated in the production of incretins and in the release of insulin, most of the arising interest regards the potential targeting of GPR119 in diabetes and obesity [410].

TRPV2 (Trpv2, chromosome 17 p11.2) is a Ca^{2+}-permeable and non- selective cation channel with an outward rectification. It shares a 50% homology with TRPV1 but with respect to the latter, it does not response to vanilloids, acidic pH and it is activated at temperatures higher than 52°C. Moreover, it can be regulated by lysophospholipids (e.g. lysophosphatidylcholine, LPC, and LPI), hormones like insulin-like growth factor-1 (IGF-1), PDGF and neuropeptide head activator. Many cannabinoids such as CBD, Δ9-THC, CBN, Δ9-tetrahydro-cannabivarin (THCV) also activate the receptor [411]. The

signalling pathways associated to TRPV2 mediate cell survival, proliferation, and invasion. In tumors, alteration of the expression profile of TRPV2 led to major oncogenic features, as observed in leukemia and bladder cancer. In multiple myeloma, TRPV2 correlated with bone tissue damage and poor prognosis, while in PCa, high TRPV2 levels were associated with metastasis and castration-resistant phenotypes. Loss or inactivation of TRPV2 increased cell proliferation and conferred resistance to apoptosis in GBM. Conversely, its over-expression augmented the relapse-free survival in TNBC patients. Instead, patients affected by esophageal squamous cell carcinoma or gastric cancer showed opposite outcomes [411].

The last class of CBRs includes peroxisome proliferator–activated receptors (**PPARs**). Two are the isoforms for these receptors: isoform α and γ. **PPARα** (PPARA, chromosome 22q12-13.1) is distributed in metabolically active tissues (e.g. muscle, liver and heart) where it is responsible for the fatty acid catabolism and the inflammation. Natural ligands for PPARα are saturated and unsaturated fatty acids, and higher affinity were observed for lipoxygenase 8(S)-hydroxyeicosatetraenoic acid (8(S)-HETE) and leukotriene B4 (LTB4) [412]. **PPARγ** (PPARG, chromosome 3p25), instead, has three different isoforms (i.e., -γ1, -γ2-; -γ3) which were found in adipose tissue (-γ2), in macrophages (-γ3) or ubiquitously expressed (-γ1). The principal function for the receptor regards adipocyte formation, insulin sensitivity and inflammation [413, 414]. Fatty acids (mainly polyunsaturated) and eicosanoids activate PPARγ. In particular, essential fatty acids like linoleic, linolenic, AA, and eicosapentaenoic acid were indicated as the major endogenous ligands [415]. Recently, cannabinoids like, PEA, OEA were found to activate PPARα, while Δ9-THC, AEA and 2-AG bind to PPARγ [415]. For what concerns the signalling, PPARs are ligand-dependent transcription factors, which control gene expression by binding to PPARs' response elements (PPREs) within promoters, as heterodimers coupled with a retinoid X receptor. To date, it is still unclear the involvement of these nuclear receptors in cancer, although some tumors like colon, ovarian tumors, BCa and PCa seemed to expressed PPARs with implication in the evolution of the disease [416, 417].

1.2.4 Heteromers

For a long time CBRs were described as members of GPCR heteromers. CB1R, for instance, was demonstrated to interact with:

- **adenosine A_{2A}, dopamine D_2 receptors** (generating a negative modulation on their function upon stimulation with CB1R agonists) [418];
- **opioid μ** and **δ receptors** (negative cross-talk between them) [419];
- **orexin OX1 receptor** (positive cross-talk and cross-antagonism by orexin) [420]
- **angiotensin AT1 receptor** (increase of AT1 receptor signalling) [421];
- **adrenergic β2 receptor** (adrenergic agonists induce CB1R internalization [422];
- **5HT2A serotonin receptor**[423]

Moreover, the CB1R can form heteromers with other CBRs, such as CB2R and GPR55. In neuronal cells in culture and in vivo, for instance, CB1R was found to establish a negative cross-talk and bidirectional cross-antagonism with CB2R, upon the co-activation of both receptors [424]; while the interaction with GPR55 was first observed in HEK-293 cells [425, 426]. Many tissues, in particular the CNS, were seen to express both CB1R and GPR55. In this context, CB1R-mediated signals were enhanced only when CB1R was co-expressed with GPR55, while the GPR55 signalling was inhibited in presence of CB1R [425]. For what regard CB2R, there is not much about the existence of the CB1R-CB2R heteromers formed, however, it is known that CB2R can interact with GPR55 [427], the C-X-C chemokine receptor type 4 (CXCR4) and the human epidermal growth factor receptor 2 (HER2) [428, 429]. CB2R-GPR55 heteromers were found in transfected and in BCa cells, where the coupling contributed to cancer malignity [430]. Moreover, it was speculated that CB2R-GPR55 heteromers exist at other cancer-associated sites, for example in hematopoietic cells or bones [141, 431]. Always in BCa and also in PCa, there is evidence about the interaction between CB2R and CXCR4. Moreover, the coupling of CB2R to CXCR4 was found to inhibit pro-tumor properties mediated by CXCR4, such as cell proliferation [141], invasion and metastatic spread [428]. Finally, CB2R-HER2 heteromers were described in HER2+ BCa cells. Recently, Blasco-Benito proposed that the disruption of CB2R-HER2 heteromers, through the usage of selective agonists or synthetic peptides binding CB2R, inactivated and degraded HER2, generating anti-tumor responses [432]. Therefore, the combination of cannabinoids and anti-HER2 drugs may potentiate the anti-cancer effects of the actual standard therapies in BCa.

1.2.5 Receptor agonists and antagonists

1.2.5.1 Receptor agonists

In terms of chemical structure, the main CBRs' agonists used in the preclinical oncology research can be classified in different groups (Table 10 a,b):

STRUCTURE	EX. of CBRs' AGONISTS
Classical	Δ9-THC, Δ8-THC, HU-210, JWH-133
Non-classical	CP55940, HU-308
Eicosanoid	AEA, 2-AG, noladin ether, Met-AEA, Met-F-AEA, ACPA, ACEA,
Diarylpyrazoles	AM251
N-acylethanolamines	PEA, OEA
Aminoalkylindoles	R-(+)-WIN55212-2, JWH-015, AM1241

EX. of CBR's AGONISTS AND SELECTIVITY TO CBRs					
CB1R	**CB2R**	**GPR55**	**TRPV1**	**PPARα**	**PPARγ**
Δ9-THC, Δ8-THC, HU-210, CP55940, AEA, 2-AG, WIN55212, ACPA, ACEA, Met-AEA, Met-F-AEA, Noladin ether	Δ9-THC, Δ8-THC, HU-210, CP55940, 2-AG, WIN55212, JWH-133, HU-308, JWH-015, AM1241	Δ9-THC, AEA, 2-AG, JWH-015, AM251, O-1602 (analogue of CBD),	AEA 2-AG	PEA, OEA	Δ9-THC, AEA, 2-AG

Tab.10. CBRs agonists.

Chemical classification of CBRs agonists (a) and relative binding to receptors (b)

Abbreviations: cannabinoid receptors (CBRs), G coupled protein receptor 55 (GPR55), transient receptor potential cation channel subfamily V member 1 (TRPV1), proliferator–activated receptors (PPARs), example (EX), Delta-9/ 8 tetrahydrocannabinol (Δ9/8-THC), AEA (anandamide), methanandamide (Met-AEA), metfluoroanandamide (Met-F-AEA), 2-AG (2-arachidonolylglycerol), arachidonylcyclopropylamide (ACPA), arachidonyl-2'-chloroethylanamide (ACEA), palmitoylethanolamide (PEA), oleoylethanolamide (OEA), cannabidiol (CBD)

The classical group includes *dibenzopyran derivates* which are: Δ9-THC, Δ8-THC and their synthetic analogues HU-210, JWH-133, respectively [433, 434]. Both Δ9-THC and Δ8-THC are partial agonists of CB1R and CB2R, even though with low affinity to CB2R. Moreover, Δ8-THC is an isomer of the Δ9- form, which works with a similar pharmacodynamics, but with a lower efficacy to CBRs [435]. HU-210 displays a better affinity/ efficacy than THC and it also agonizes GPR55 and PPARγ [274, 433, 436]. Instead, JWH-133 binds exclusively to CB2R, acting as an agonist [433]. **Non-classical agonists** are *bicyclic and tricyclic derivates of THC*, which lack the pyran ring [433]. Among these, CP55940 has great affinity and potency for CBR1/ CB2R, thus working at low nanomolar concentrations [433] [434]. HU-308 agonizes only CB2R, overcoming the psychotropic effects resulting from CB1R activation [433, 434]. Members of **the eicosanoid group** are *mainly eCBs* [433, 434]. AEA and 2-AG bind CB1R/ CB2R, even though 2-AG is a partial agonist for CB2R [437, 438]. As mentioned, both the eCBs activate GPR55, but weaker than the endogenous ligand LPI and the selective agonist AM251 (class of **diarylpyrazoles**). TRPV1 and PPARγ are targets for AEA and 2-AG, and AEA is more potent on TRPV1 compared to 2-AG [274]. Other eicosanoids, considered as CB1R selective agonists, are analogs of 2-AG (e.g noladin ether) and AEA. Examples of AEA analogues are arachidonyl-2'-chloroethylanamide (ACEA), arachidonylcyclopropylamide (ACPA) and the two non-hydrolyzable compounds by the AEA metabolic enzymes, R-(+)-

methanandamide (Met-AEA) and Metfluoroanandamide (Met-F-AEA) [433, 434, 439, 440]. The group of **N-acylethanolamines**, instead, includes palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), which are eCBs agonists for PPARα. Finally, R-(+)- WIN55212-2, JWH-015 and AM1241 are examples of **aminoalkylindoles**. R-(+)- WIN55212-2 binds both CB1R and CB2R, but more powerfully on CB2R, while JWH-015 and AM1241 exclusively agonize CB2R [433]. Noteworthy, it has been demonstrated that JWH-015 can activate GPR55 as well [441].

In vitro studies on cancer cells, demonstrated that all these compounds are able to inhibit tumor cell growth, induce cell cycle arrest, reduce proliferation and migration in many cancers such as lung, prostate, breast, brain and endometrial cancer cells [442, 443]. In Table 11, we illustrate some of the anti-tumor effects supported by these compounds in vitro.

COMPOUND	TUMOR	ACTION	Ref.
WIN 55,212-2	GI cancer	▪ The compound inhibited cell proliferation and it induced apoptosis ▪ Akt was inhibited and VEGF-A, MMP-2 decreased ▪ Cell migration and invasion were inhibited ▪ Cell migration and invasion were inhibited. Down-regulation of COX-2 which led to low expression of EMT markers	[444-446]
	PCa	▪ Cell proliferation was inhibited and apoptosis induced. Androgen receptor, PSA, PCNA and VEGF were down-regulated in LNCaP cells ▪ Downregulation of neuroendocrine differentiation in LNCaP cells thanks to the inhibition of PI3K/Akt/mTOR axis and the activation of AMPK pathway	[266, 269]
	Renal carcinoma	Cell survival and proliferation (even in 3D) were reduced. Cell cycle arrest at the G0/G1 phase. Induction of tumor cell apoptosis	[261]
	Osteosarcoma	Down-regulation of MMP-2/-9 which led to inhibition of tumor cell migratory abilities	[447]
	Lung and testicular cancer	Induction of tumor cell apoptosis	[448]
AEA	GI cancer	▪ Block of the cell cycle at the G0/G1 phase. Induction of apoptosis ▪ Reduce in tumor cell proliferation through the non-canonical pathway of Wnt5a ▪ Translocation of the Fas-death receptor in lipid rafts. Inhibition of cancer cell proliferation	[449-451]
	Lung cancer	Down-regulation of tumor cell spreading, resembling the anti-invasive effect of the inhibitors of FAAH	[452]
	BCa	Activation of MAPK which led to inhibition of tumor cell proliferation	[453-455]
	PCa	Inhibition of cell proliferation through the down-regulation of EGF. Down-regulation of EGFR which led to induction of cell apoptosis and necrosis. Activation of ERK and inhibition of Akt drove apoptosis induction	[290, 291]
	Non melanoma skin cancer	Induction of oxidative stress which led to apoptosis (no CBRs involvement)	[375]
	Lymphoma	Inhibition of cancer cell survival	[456]
R(+)-Met-AEA	PCa	Inhibition of PC3 cell growth	[457]
	Cervical cancer	Synthesis of COX-2 and prostaglandin which led to tumor cell apoptosis	[346]
	GI cancer	G0/G1 cell cycle arrest and necrosis	[449]
Met-F-AEA	BCa	▪ Cell cycle arrest, activation of Chk1, degradation of Cdc25A and decrease of CDK2 activity ▪ Inhibition of migration and adhesion of cancer cell through RhoA/ ROCK and FAK phosphorylation	[458-460]
	Melanoma	Tumor cell proliferation was inhibited	[284]
	Lung cancer	Cell cycle arrest at the G0/G1 phase and induction of apoptosis (when given in combination with UR597)	[461]
	GI cancer	Up-regulation of AEA availability. Up-regulation of the estrogen receptor β. Reduction of cell proliferation after the up-regulation of CB1R	[462]
	Thyroid cancer	Apoptosis was induced via p53, p21	[463]
PEA	Brain cancer	Induction of cancer cell death	[371]
	Melanoma	Down-regulation of cell viability after the treatment with URB597	[464]
	BCa	Increase of the cytotoxic effects of AEA	[465]
ACEA	GI cancer	TNFα-mediated de novo ceramide synthesis. Induction of apoptosis	[244]

	HCC	Reduction of cell survival, invasion and on the levels of MMP-2/-9	[466]
	BCa	Cancer stem cells were inhibited in invasion abilities	[467]
	Pancreatic cancer	ROS-mediated autophagy via AMPK. Inhibition of cell metabolism. Down-regulation of GAPDH, PKM2. Increase in the oncosuppressive effects of gemcitabine	[286]
JWH-015	PCa	Induction of apoptosis via ceramide de novo synthesis. Inhibition of cell growth	[457]
	BCa	▪ Down-regulation of tumor cell growth, chemotaxis and wound healing ▪ In ERα tumor cells, inhibition of EGFR	[468, 469]
	Lung cancer	▪ Reduction of growth factor-mediated in vitro chemotaxis and chemo-invasion. Decrease of focal adhesion complexes. ▪ Reduction of Akt phosphorylation and MMP-9 expression/ activity	[258]
JWH-133	Brain cancer	Reduction of tumor cell viability in glioma	[470]
	BCa	Down-regulation of cell proliferation. Induction of apoptosis, and inhibition of migration of BCa cells	[264]
	Melanoma	Block of tumor cell trans-endothelial migration	[251]

Tab. 11. In vitro evidence for the role of the CBRs' agonists in tumors

Evidence of the principal CBRs' agonists in various tumor subtypes. Image derived from [274].

Abbreviations: cannabinoid receptors (CBRs), gastrointestinal (GI), vascular endothelial growth factor α (VEGF-α), matrix metalloproteinases (MMPs), epithelial-to-mesenchymal transition (EMT), cyclooxygenase-2 (COX-2), prostate-specific antigen (PSA), proliferating cell nuclear antigen (PSA), vascular endothelial growth factor (VEGF), phosphatidylInositol 3-Kinase (PI3K), mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), prostate cancer (PCa), breast cancer (BCa), Wingless-related integration site 5a (Wnt5a), fatty acid amide hydrolase (FAAH), mitogen-activated protein kinase (MAPK), epidermal growth factor (EGF), extracellular signal regulated kinase (ERK), checkpoint kinase 1 (Chk1), cell division cycle 25 A (Cdc25A), cyclin-dependent kinase 2 (CDK2), Rho-associated protein kinase (ROCK), Ras homolog family member A (Rho A), focal adhesion kinase (FAK), tumor necrosis factorα (TNFα), hepatocellular carcinoma (HCC), reactive oxygen species (ROS), glyceraldehyde phosphate dehydrogenase (GAPDH), C-X-C chemokine receptor type 4 (CXCR4), pyruvate kinase M2 (PKM2), estrogen receptor α (ERα), epidermal growth factor receptor (EGFR), anandamide (AEA), Methanandamide (Met-AEA), palmitoylethanolamide (PEA), arachidonyl-2'-chloroethylanamide (ACEA)

1.2.5.2 Receptor antagonists/ inverse agonists and allosteric modulators

On the basis of the receptor bound, CBRs' antagonists/ inverse agonists are summarized in Table 12 .

EX. OF CBR's ANTAGONISTS/ INVERSE AGONISTS/ ALLOSTERIC MODULATORS		
CB1R	**CB2R**	**GPR55**
Rimonabant, CBD, AM251, AM281 LYS320135	AM630	CBD, ML193, CID16020046

Tab. 12. CBRs' antagonists/ inverse agonists/ allosteric modulators

Chemical classification of CBRs antagonists/ inverse agonists/ allosteric modulators (a) and relative binding to receptors (b)

Abbreviations: cannabinoid receptors (CBRs), G coupled protein receptor 55(GPR55), example (EX), cannabidiol (CBD)

Rimonabant (SR141716A), AM281 and LYS320135 belong to the group of CB1R selective antagonists. AM281 and LY320135 are Rimonabant structure analogues, with a slightly better affinity for CB1R. However, preclinical studies have not yet fully explored their activity. AM251 is another Rimonabant analogue. Moreover, it can activate GPR55 [433]. As already introduced, CBD is a natural derived from Cannabis Sativa, devoid from psychoactive effects. It is an allosteric modulator of CB1R and an antagonist for GPR55 [471-473]. For what concerns CB2R, AM630 is an inverse agonist. CID1261822 (ML193) and CID16020046 are the principal selective antagonists for GPR55 [474]. TRPV1 and PPARγ have not cannabinoid antagonists already discovered. Non-cannabinoid antagonists for PPARγ include T0070907 and GW9662; while SB366791 and capsazepine (synthetic analogue of capsaicin) are non-cannabinoid antagonists for TRPV1.

Evidence for the implication of the CBRs' antagonists/ inverse agonists in tumor cell malignancy are indicated in Tab.13, as follow.

COMPOUND	TUMOR	ACTION	Ref.
Rimonabant	GI cancer	▪ Cell cycle arrest at the G2/M phase. Induction of mitotic catastrophe ▪ Synergic effect when given in combination with oxaliplatin ▪ Regulation of chemoresistance and cancer stemness. Integrity of human healthy organoids in ex vivo cultures through the inhibition of Wnt/β-catenin pathway	[475-478]
	Brain cancer	Reduction of tumor cell proliferation. Induction of caspase-dependent apoptosis. Up-regulation of NKG2D ligand (i.e MHC class I chain-related protein A and B, MICA/B)	[277]
	BCa	Inhibition of tumor cell proliferation via CB1R	[265]
CBD	GI cancer	▪ Reduction in the CDK2-cyclin E pathway and cell cycle arrest at the G0/G1 phase ▪ Increase of ROS levels and mitochondria-driven apoptosis ▪ Inhibition of tumor cell migration ▪ Protection from the oxidative damages to the DNA. High levels of eCBs. Inhibition of tumor cell proliferation via CB1R, TRPV1, PPARγ ▪ Reduction on tumor cell invasion/ migration ▪ ROS production and apoptosis induction. Inducement of ER stress and Noxa acitivity	[357, 479-481]
	Lung cancer	▪ Induction of PPARγ-dependent apoptosis through up-regulation of COX-2-dependent prostaglandins ▪ Decrease of cell migration and PAI-1 ▪ Cytotoxic effects mediated by lymphokine-activated killer (LAK) and ICAM-1 ▪ ICAM-1/ TIMP-1 increase. Down-regulation of cancer cell migration through CBRs, TRPV1, p42/-44 MAPK	[343, 482-484]
	BCa	▪ Induction of cancer cell death via apoptosis and autophagy ▪ Tumor cell proliferation and apoptosis were induced through ER stress in MDA-MB-231 ▪ Induction of G1/S cycle arrest in MCF-7 cells, via CBRs ▪ Down-regulation of mTOR, cyclin D1. Up-regulation of PPARγ. Induction of apoptosis in T47 and MDA-BB-231 cell lines ▪ Reduction in the EGF signalling which led to inhibition of colony formation, invasive abilities of cancer cells. Redcution in cytokine production ▪ Down-regulation of cell proliferation and invasion through inhibition of the Id-1 ▪ Induction of the uptake of doxorubicin and apoptosis, via TRPV2 in TNBC cells	[349, 366, 485-488]
	PCa	▪ Induction of cytotoxic effects. Down-regulation of CB1R, CB2R, VEGF, PSA, IL-6/-8 in LNCaP cell lines ▪ Block of spheroid formation in LNCaP stem cells ▪ Reduction in cell proliferation and induction of apoptosis in LNCaP cells, partially caused by the antagonism on TRPM8, the down-regulation of androgen receptor, p53, and elevated ROS. ▪ Anti-proliferative effect when give with docetaxel or bicalutamide in DU-145 and LNCaP cells	[489, 490]
	Brain cancer	▪ Reduction of tumor cell proliferation. ROS production followed by apoptosis together to Δ9-THC treatment ▪ ROS production reached through the over-expression of HSP super family genes ▪ Increase of cytotoxicity of CBD when given together with HSP inhibitors ▪ Down-regulation of Id-1 which led to inhibition of cell invasion ▪ Inhibition of tumor cell proliferation and invasion. Inhibition of ERK, Akt and HIF-1α pathways ▪ Reduction of tumor cell proliferation and increased apoptosis. Increase of chemosensitivity to temozolomide, bis-chloroethylnitrosourea- carmustin (BCNU), and doxorubicin through TRPV2 activation	[491-495]
AM251	Pancreatic cancer	Cell apoptosis in a receptor-independent mechanism	[496]
	GI cancer	Pro-proliferative effects when given with Met-F-AEA	[462]
	BCa	Up-regulation of invasiveness in BCa stem cells when given together with ACEA	[467]
	RCC	Cell proliferation was reduced and apoptosis induced (up-regulation of Bax and down-regulation of Bcl-2). Inhibition of cell migration.	[289]
6-	RCC	Proliferation and migration were reduced.	[497]

iodopravadoline (AM-630)		Cell cycle arrest at the G2/M phase.	
CID16020046	GI cancer	Block of the migration and adhesion to endothelial cells in GI cancer cells	[357]
	BCa	▪ Inhibition of tumor cell proliferation and ERK1/2 phosphorylation ▪ Inhibition of filopodia formation and migratory abilities ▪ Down-regulation of MDR (e.g. BCRP) which led to inhibition of chemoresistance	[245, 303, 498]

Tab.13 In vitro evidence for the role of the CBRs' antagonists and allosteric modulators in tumors

Evidence of the principal CBRs' antagonists in various tumor subtypes. Image derived from [274].

Abbreviations: cannabinoid receptors (CBRs), endocannabinoids (eCBs), gastrointestinal (GI), cannabidiol (CBD), breast cancer (BCa), Wingless-related integration site (Wnt), Natural killer group 2 member D (NKG2D), major histocompatibility complex (MHC), MHC class I chain-related protein A and B (MICA/B), cyclin-dependent kinase 2 (CDK2), proliferator–activated receptors (PPARs), reactive oxygen species(ROS), endoplasmic reticulum (ER), phorbol-12-myristate-13-acetate-induced protein 1 (Noxa), plasminogen activator inhibitor-1 (PAI-1), intercellular adhesion molecule 1 (ICAM-1), tissue inhibitor matrix metalloproteinases-1 (TIMP-1), mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK), inhibitor of differentiation 1 (Id-1), prostate-specific antigen (PSA), interleukin (IL-6/-8 /…),transient receptor potential cation channel subfamily M member 8 (TRPM8), transient receptor potential cation channel subfamily V member 1 and 2 (TRPV1, TRPV2), reactive oxygen species (ROS), vascular endothelial growth factor (VEGF), Delta-9 tetrahydrocannabinol (Δ9-THC), heat shock proteins (HSPs), cannabidiol (CBD), extracellular signal regulated kinase (ERK), hypoxia-inducible factor-1α (HIF-1α), metfluoroanandamide (Met-F-AEA), arachidonyl-2'-chloroethylanamide (ACEA), B-cell leukemia/lymphoma 2 protein(Bcl-2), Bcl-2-associated X protein (Bax), extracellular signal regulated kinase (ERK), multiple drug resistant (MDR), breast cancer resistance protein (BCRP), renal cell carcinoma (RCC), prostate cancer (PCa)

1.2.6 Deregulations of enzymes involved in endocannabinoid metabolism in cancer

Together with CBRs, synthesis and degradation of eCBs impact on the worsening of several cancer tissues. In some forms of colorectal, endometrial, hepatocellular cancers, for example, NAPE-PLD**,** was found highly expressed [499-501], while it lowered in GBM and astrocytomas [502]. Concerning MAGL, it was observed up-regulated in aggressive tumors (e.g. melanoma, pancreatic, colon, ovarian cancers and in androgen-independent PCa cells) when compared to the healthy tissue/ cell lines. Remarkably, the high MAGL activity was associated with high proliferation, invasiveness, and tumor growth in vivo [503, 504]. In HCC, MAGL was high in patients with a poor outcome [505]. According to this, a positive correlation between the expression of the enzyme and negative tumor prognostic factors was observed, such as body mass index in colon cancer [506]. In glioma, MAGL was low expressed, in keeping with the increase in 2-AG. Finally, in BCa the enzyme was lowered in ductal forms with respect to the less malignant tumors [507].

FAAH, increased in pancreatic cancer and PCa, whereas in glioma and endometrial carcinoma it declined, resulting in an increase of AEA [253, 500, 507, 508].

Overall, given the increasing evidence regarding the involvement of cannabinoid enzymes in the cancer setting, new pharmacologic inhibitors have been formulated and tested at the preclinical phase.

1.2.6.1 MAGL inhibitors

Two examples of MAGL inhibitors are JZL184 and URB-602. In androgen- independent PC3 cell lines, JZL184 reduced cell migration, invasion, and survival [508] while in CRC it lowered proliferation, migration and increased apoptosis in vitro. Interestingly, the compound also improved CRC cell sensitivity to 5-fluorouracil [509]. In HCC and orthotropic BCa and PCa cells, JZL184 suppressed tumor cell proliferation, migration and invasion. [510]. Moreover, JZL184 had a protective action in mouse models of advanced osteotropic BCa, PCa and osteosarcoma, by inhibiting skeletal tumor growth, cancer-related bone damage, cachexia and by prolonging survival. However, in the absence of tumor, JZL184 exerted a paradoxical decrease of bone volume through CBRs [511]. In a syngeneic tumor model with KP cells (KrasLSL-G12D/p53fl/fl; from mouse lung adenocarcinoma), JZL184 as well as MAGL knock-out showed a decrease in tumor burden, correlating with increased number of CD8+ T cells and eosinophils [512]. In keeping in a same KP cells' tumor model, the increase of CD8+ cells and eosinophils in MAGL knockout mice was accompanied with a large increase in 2-AG content and a specific lipid profile in KP cells [513].

Regarding URB-602, in colon cancer in vivo, it inhibited tumor growth and angiogenesis and it attenuated azoxymethane-induced pre-neoplastic lesions and polyps [514]. Other evidence also demonstrated that the compound, given with phytocannabinoids (e.g. CBG) and O-1602, reduced angiogenesis, induced apoptosis, and reduced tumor volume, and the generation of aberrant crypt foci (ACF) on CRC [515].

1.2.6.2 FAAH inhibitors

Arachidonoyl serotonin (AA-5HT) and URB597 are FAAH inhibitors. In lung cancer, both compounds were able to reduce metastasis in vivo, and to prompt A549 cell invasion in vitro, by up-regulating TIMP-1 [452]. Moreover, in lung cancer, URB597 also blocked the cell cycle (by down-regulating cyclin D1 and cyclin-dependent kinase 4, CDK4), it activated apoptosis (via caspase-9 and poly (ADP-ribose) polymerases, PARP), and it inhibited MMP-2 and stress fiber formation [461]. In NSCLC, URB597 given in combination with Met-F-AEA significantly reduced in vitro EGF-induced proliferative and chemotactic activities. Moreover, in melanoma, the combination of URB597 with PEA reduced B16 cancer cell viability [461].

1.2.7 Cannabinoids in medicine and advances in cancer therapy

Cannabinoid compounds approved by the Food and Drug Administration (FDA) are mainly used for palliative care, in particular in managing cancer pain and chemotherapy-related disturbances [516, 517]. Nabilone (Cesamet®) and Dronabinol (Marinol®) are two synthetic analogs of Δ9-THC, which were employed for the treatment of vomiting and nausea, together with chemotherapy. However, Dronabinol is also indicated for AIDS-related anorexia [517]. Another cannabinoid related drug, is Syndros® whose formulation includes the active ingredient Dronabinol. Differently from the others, Nabiximols (Sativex®) has a balanced ratio of Δ9-THC and CBD (1:1) and it reached the phase III clinical trial, as care for cancer associated pain. Moreover, it was also approved as an add-on therapy for symptomatic relief of spasticity in people with multiple sclerosis and for epilepsy [518, 519]. Finally, Epidiolex® contains the purified form of CBD derived from Cannabis Sativa, and it is indicated for the treatment of seizures associated with Lennox-Gastaut syndrome or Dravet syndrome in patients 2 years of age and older.

With regard to the progress in the directly anti-cancer therapy, advanced preclinical studies indicated that synthetic cannabinoids such as JWH-133, WIN 55,212-2, HU-210, hold significant potential in glioma treatment by reducing tumor size and angiogenesis [470, 520-522]. THC in combination with the chemotherapy drug temozolomide was proven to reduce tumor growth [523]. In colon cancer models, both phytocannabinoids (CBG) and a synthetic hydroxylquinone derived from CBD (HU-331) down-regulated tumor growth and angiogenesis with less toxicity compared to doxorubicin [374, 499]. CBD demonstrated efficacy in reducing colon tumor size and promoting apoptosis [480]. Cannabinoids also exhibit positive effects in BCa and melanoma models, reducing metastasis, and improving survival rates [432, 489, 524].

Inhibitors of FAAH and MAGL have shown promising effects in preclinical studies [462, 464] [525], and they reached the phase II clinical trials to evaluate potential synergies with traditional cancer therapies [526]. Overall, further research is needed to fully understand the potential benefits and limitations of cannabinoids and eCBs in tumor therapy.

1.3 Prostate cancer

PCa is the most prevalent non-cutaneous cancer in men worldwide [527]. Its development starts with prostatic intraepithelial neoplasia (PIN), progressing to localize and advanced prostate adenocarcinoma which may ultimately culminate in metastasis. Key genetic alterations which contribute to the development of PCa include fusion of transmembrane serine protease 2 (TMPRSS2) with E26 transformation-specific (ETS) family genes, amplification of the myelocytomatosis oncogene (Myc), and deletion/ mutation of phosphatase and tensin homolog (PTEN) and tumor suppressor protein 53 (TP53) genes. Moreover, advanced stages are characterized by amplification and/or mutation to the genes associated to the androgen receptor. Other risk factors regard family risk, ethnicity, age and obesity.

PCa is usually diagnosed by tumor biopsy after evaluation of altered levels of PSA and a digital rectal exam. To date, the treatment for the localize tumor regards a radical prostatectomy or ablative radiotherapy. However, patients with local relapses after the surgery are treated with radiotherapy and / or androgen deprivation therapy (ADT). In systemic relapses, instead, treatments principally include ADT combined with chemotherapy.

Advanced PCa progressing despite androgen ablation is considered a castration-resistant PCa (CPRC). This cancer is hard to treat; however therapies may include androgen receptor-targeted agents, radionuclides and the poly (ADP-ribose) inhibitor, chemotherapy, olaparib. Another aggressive variant of PCa is neuroendocrine PCa (NEPC) which commonly occurs in the latter stages of CPRC as a mechanism of treatment resistance [528]. To date there is a poor molecular description of NEPC and a lack in specific curative treatments.

1.4 The Tumor microenvironment

The tumor microenvironment (TME) is a heterogeneity environment found in tumors, composed of infiltrating and resident cells, secreted factors and the extracellular matrix (ECM). On the basis of the tumor type, the composition of the TME varies; however, hallmark features include stromal cells, immune cells, blood vessels and the ECM. Within the host tissue, tumor cells stimulate molecular, cellular and physical changes to support survival, local invasion and metastasis. Indeed, it is now recognized that the TME is not just a silent bystander, but rather an active promoter of tumor progression [529]. Among the different cells types which are included in the TME, there are T- / B-lymphocytes, NKs, macrophages, neutrophils, endothelial cells, dendritic cells, adipocytes, stellate cells and cancer associated fibroblasts (CAFs). While, exosomes, signalling molecules, cytokines and growth factors are some of the non-cellular components.

1.4.1 Non cellular components of the TME

The ECM

The ECM is a portion of a tissue which does not consist of cells. It is composed of different types of glycoproteins, proteoglycans and hyaluronic acid such as fibronectin, collagen, elastin and laminin

[530]. Functionally, the ECM is a solid scaffold for cancer cells which gives support and soundness to the tumor growth. Moreover, it should also be considered that the ECM is not just a static component, but it constantly evolves, remodeling for the tumor spreading. Indeed, stromal cells, in particular CAFs, are able to secrete components of the ECM, making it a deposit for cytokines, growth factors, collagen and MMPs [531].

Exosomes

They are microvesiscles of 30-100 nm in size which deliver proteins, RNA, DNA and lipids [532-534]. Their functionality is to facilitate the cross-talk between cancer and stromal cells, thus contributing to tumor inflammation, angiogenesis and metastasis within the TME. Noteworthy, hypoxia can exacerbate the production of exosomes by tumor cells, leading to the transition of stromal cells into CAFs [531].

1.4.2 Cellular components of the TME

Immune cell components

Immune cells have a critical role within the TME, since they can either suppress or promote tumor growth. Broadly, immune cells fall into two categories: adaptive cells and innate cells. The adaptive immunity is activated by the exposure to specific antigens and it uses a specific immunological memory to respond to an insult. Innate immunity, instead, is a non-specific defense which acts within hours. T-/B- and NKs constitute the adaptive immunity, while macrophages, neutrophils and dendritic cells belong to the innate immunity. On the basis of the immune landscape within the TME, tumors can be categorized into three classes: the immune infiltrated, immune excluded and the immune silent. In the first scenario, immune cells are homogeneously distributed through the tumor, serving for an active immune response. On the other hand, immune excluded tumors have T cells only located at the periphery of the tumor, while immune silent tumors completely lose the immune cell infiltration [531].

T cells

Different T cell populations can influence tumorigenesis. Cytotoxic T cells (CD8+), after recognizing abnormal tumor antigens, kill tumor cells; moreover they suppress angiogenesis via the release of INF-γ [535]. Among CD4+ cells, T helper (Th-1) cells are pro-inflammatory and they support the activity of CD8+ cells through the secretion of interleukin-2 (IL-2) and INF-γ, [531, 536, 537]. T regulatory cells (Tregs) are other CD4+ cell types, which control autoimmunity and suppress inflammatory response. Moreover, they can also directly support tumor cells by releasing secretory factors and interacting with stromal cells [531, 538].

B cells

They are responsible for antigen presentation to T cells, secretion of cytokines (e.g. IL-10, TGF-β) and antibody production [539, 540]. Typically, B cells reside outside the tumor, in lymph nodes;

however, certain cancers such as BCa, melanoma and ovarian cancer demonstrated to have ectopic lymph node structures within the TME [541-543]. Other than have an anti-tumorigenic role, B cells can be predictive of a poor outcome, especially in bladder cancer, PCa and RCC [544-546].

NKs

They control the bloodstream to check for virally infected host cells or tumor cells. Typically they can be directly involved in the cell-mediated killing of cancer cells or in the secretion of cytokines to block metastasis. However, within the TME they are less active compared to the bloodstream [531, 547].

Macrophages

They derived from monocytes' differentiation [548]. We distinguish 2 types of macrophages: M1 macrophages which present the antigen, phagocytize and kill cells; and the M2 subtype which, instead, takes part to the tissue repair [549]. Although both species can be present in the TME, the M2 phenotype is much more expressed in condition of hypoxia and tumor progression (tumor associated macrophages, TAMs). Indeed, in response to low oxygen, macrophages usually surround blood vessels to secrete VEGF and thus induce new blood vessel formation [550, 551]. In keeping, it was found quite common in many tumors, like BCa, lung and gastric cancers, that high macrophage infiltration correlate with a poor patient prognosis [552-554].

Neutrophils

They represent the 70% of circulating leukocytes and constitute the first means of defense as soon as the tumor begins to grow. However they can act either as anti-tumorigenic or pro-tumor factors [555]. Indeed, other than principally promote tumor cell apoptosis, they support angiogenesis and local invasion through modification of the ECM, the release of VEGF and MMPs [555-557].

Myeloid-derived suppressor cells (MDSCs)

They are neutrophils/ monocytes pathologically activated that possess strong immunosuppressive capabilities with unfavorable clinical outcomes in cancer [558].

Dendritic cells

They derived from monocytes. They are dedicated to the recognition and presentation of tumor-associated antigens to T cells at secondary lymphoid organs in order to begin an adaptive immune response. However, on the basis of the composition of the TME and on the secreted factors, dendritic cells can be reprogrammed toward an anti-tumor or immunogenic tolerance phenotype [531, 559].

1.4.3 Stromal cell components

Depending on tumor type, the stromal cell composition change, although the following cell classes are generally distinguished [531]

Endothelial cells

They constitute the thin layer of the endothelium of blood vessels, participating to the formation of new blood vessels and to the supply of nutrients/ water to circulating cells. During the first stages of cancer progression, tumor cells adopt passive diffusion for their primitive sustenance. However, once tumor reaches 1-2 mm^3 in volume, oxygen demand and waste products of tumor cells increase, thus contributing to the generation of an acidic and hypoxic environment. This leads tumor to generate an own blood supply, and endothelial cells participate to this process through the release of pro-angiogenic factors such as EGF, PDGF and VEGF [531]. Besides their role in angiogenesis, endothelial cells play important functions in tumor cell migration, invasion and metastasis [560]. Indeed, during intravasation, endothelial cells allow tumor cells to enter the blood barrier, through the binding interactions established between these two cell populations and through the conformational changes that endothelial cells undergo to enable this process [560-562]. Moreover, intravasation is also allowed by an immature blood vasculature, which is typical of the TME and it is constituted by the loss of cell-to-cell connections [563].

Adipocytes

They are responsible for the maintenance of energy balance and the storage of fatty acids. In the context of tumor, these cells support tumor progression, mutually interacting with cancer cells through the secretion of metabolites, enzymes, hormones, growth factors and cytokines [531],[564]. Another product of adipocytes is leptin, a protein hormone which controls the energetic homeostasis, by acting as anti-obesity, anti-diabetic and anti-atherosclerotic adiponectin. In BCa, leptin was shown to sustain tumor growth by stimulating tumor cell proliferation, and by indirectly activating macrophages. Moreover, through the secretion of MMPs adipocytes can also modify the ECM, making the metastatisation process easier for cancer cells [565, 566].

Stellate cells

They are quiescent cells of mesenchymal origin which are located in pancreas and liver. Upon a tissue injury, or in cancer, these cells become activated and induced to transform into myoblasts, thus producing new ECM and proangiogenic factors, necessary for tumor evolution [531, 567].

CAFs

The major cellular component of the TME is represented by CAFs, a group of activated fibroblasts with a mesenchymal cell lineage and heterogeneity in the TME [568]. Remarkably, CAFs are the major producer of the ECM [569] and they also secrete a series of pro-inflammatory factors, such as cytokines, interleukins, growth factors and pro-angiogenic factors, which contribute to the generation and maintenance of immune regulation, cancer cell stemness, angiogenesis, metabolic response, ECM remodeling, therapeutic resistance and other biological processes [570-574]. To date fibroblasts are defined as interstitial cells of mesenchymal lineage that are not epithelial, not endothelial and not immune cells [575]. However, although a specific definition is difficult to find due to their unclear origin and function, some well accepted features and biomarkers can be used for defining CAFs [569]. Among them, there are the elongated spindle morphology, the absence of non-mesenchymal biomarkers (epithelial, endothelial and leukocyte), the positivity for mesenchymal markers such as vimentin (VIM), α-smooth muscle actin (αSMA), PDGF-α, and lack of genetic mutations [576, 577]. Nevertheless, many views supported that some epigenetic regulation such as micro RNA (miRNA)-mediated post-transcriptional modification, DNA or promoter methylation and regional chromosomal reorganization, play an important role in the activation of CAFs, rather than gene mutations or mutation in copy number [578, 579].

1.4.4 Origin and activation of CAFs

Although clarifying the origin of CAFs remains a challenge, putative generation of activated fibroblasts may include mesenchymal stem cells (MSCs), bone marrow- derived precursors, hematopoietic stem cells, endothelial cells, adipocytes, pericytes, liver/pancreatic stellar cells, cancer stem cells (CSCs) and some types of epithelial cells [580, 581].

Under physiological conditions, fibroblasts are usually quiescent and they become activated during a wound healing response in order to repair and regenerate the tissue [569]; they are also known as normal activated fibroblasts (NAFs). However, two are the profiles of activated fibroblasts which have been identified: the “reversible” and “irreversible” ones [582]. The “reversible” phenotype is the one of normal activated fibroblasts (NAFs), while the “irreversible” form is typical of a chronic would healing, which is similar to what there is in fibrosis and cancer [583]. Once that the wound is repaired, NAFs return to quiescence [584], otherwise the repair process continues [569].

The activation of fibroblasts can be regulated by different active factors, such as growth factors (e.g. TGFβ, PDGF and fibroblast growth factor, FGF), heat shock factor-1 (HSF-1), transcription factors (nuclear factor-kappaB, NF-κB) and MMPs secreted by tumor cells or immune cells. Moreover, it is noted that signalling pathways like the leukemia inhibitory factor/glycoprotein 130/interleukin 6 receptor (LIF/GP130/IL-6R) can activate a signalling cascade which promotes CAFs’ activation [585]. Other to the direct secretion modalities, many fibroblast-activating factors can be delivered via microvesicles. In bladder cancer, for example, exosomes containing TGFβ and suppressor of mothers against decapentaplegic (SMAD) proteins were released by tumor cells and uploaded by fibroblasts, thus favoring their switch into CAFs [586]. Moreover, melanoma-derived exosomes led endothelial

cells to differentiate into CAFs through the EMT. In metastatic liver cancer, instead, exosomal miR-1247-3p delivered by tumor cells, led to the activation of integrin β1-NF-κB pathway in normal fibroblasts to make them activated [587]. Another key feature for the process of differentiation and function of CAFs, is the increase in stromal stiffness and mechanical force within the TME [588, 589].

1.4.5 Role of CAFs in cancer progression

CAFs exert mainly a tumor-promoting activity in cancer tissues, however few studies suggested their tumor-suppressive function, especially in the early stages of tumorigenesis [590, 591]. CAFs are the main sources of growth factors, cytokines, chemokines, proteins and enzymes with broad-spectrum activity on tumor cells and the stromal components of the TME. The cross-talk between cancer cells and CAFs, enhances cancer cell growth [569], invasion, EMT [592], the induction of stem-like features [593] and the metabolic reprogramming between CAFs and tumor cells [530]. Moreover, CAFs are the main source responsible for the ECM remodeling, thus modifying the stiffness and mechanical force of the TME, through the deposition of collagen and the secretion of proteases, such as MMPs [594]. In a BCa model, for example, CAFs produced the collagen receptor (discoidin domain-containing receptor (DDR)) reshaping collagen fibers of the ECM, thereby rendering the TME more suitable for tumor cell invasion and lung metastasis [595]. Indeed, the remodeling of the ECM, brought changes in cell-cell adhesion, and in the regeneration of malformed and more permeable blood vessels, which facilitate the intra- and extravasation of tumor cells [596]. In a constantly changing environment, where there is creation but also destruction of blood circulation, the establishment of a hypoxic environment allows the appearance of tumor stem-like phenotypes, and to the resistance of the anti-cancer therapy [569]. The acquisition of a more aggressive cell phenotype induced by CAFs in tumor cells is a derivation in changes of tumor cell metabolism, which encompasse glucose, lipid and amino acid metabolism. This metabolic reshaping is allowed by CAFs through directly and/ indirectly exporting nutrients, providing mitochondria and by modulating the activity and oxidative properties of metabolic enzymes. [530]. Moreover, CAFs, also through the modeling of the ECM, may re-educate cancer cells toward different metabolic processes [530]. In the context of the immune microenvironment, instead, CAFs have a complex interaction mechanisms with T lymphocytes, NKs, dendritic cells, MDSCs, tumor-associated neutrophils (TANs) and TAMs [597] (Fig.7).

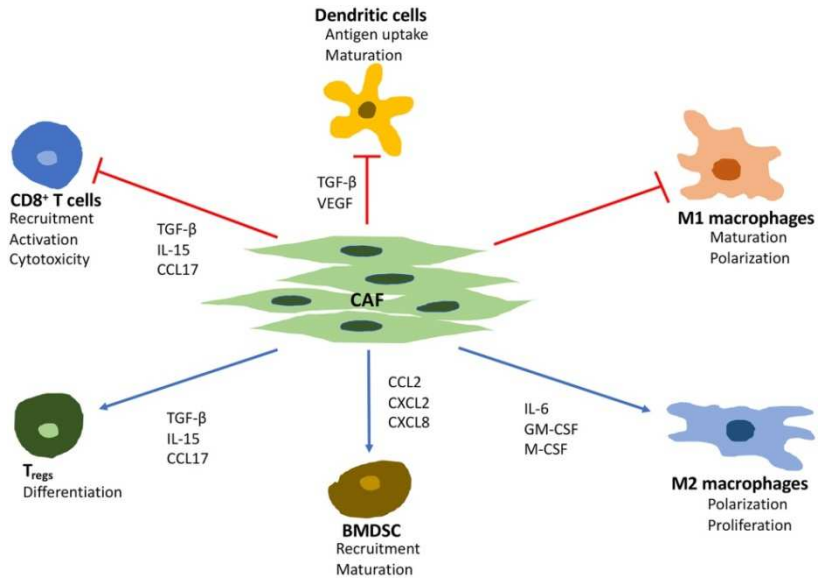


Fig. 7 Principal effects of CAFs on the immune microenvironment

Abbreviations: Transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), interleukin 6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), C-C motif chemokine ligand (CCL), C-X-C motif ligand (CXCL). Image derived from [598]

In relation to dendritic cells, IL-6 secreted by CAFs was implicated in restricting cell maturation, and consequently the antigen presenting ability of dendritic cells which activate T cells [599]. However, other findings have proven that the cross-talk between CAFs and mature dendritic cells exerts anti-tumor effects. Indeed, co-culture of CAFs with mature dendritic cells, derived from liver and bone marrow respectively, produced an inflammatory environment constituted of TNF-α, IL-1, IL-6 and IL-12p70 which enhanced the cytotoxic effect of T lymphocytes [600]. With respect to macrophages, CAFs lead to the trans-differentiation toward the M2 phenotype. In a PCa model, for example, CAFs induced monocyte recruitment towards tumor cells, mainly acting through stromal-derived growth factor-1 (SDF1), and then allowing the activation of M1 macrophages towards the M2 type. Remarkably, the relationship between M2 macrophages and CAFs was reciprocal, as M2 macrophages were able to enhance fibroblasts' reactivity. Moreover, CAFs and M2 macrophages fostered cancer cells to escape from the primary tumor, and endothelial cells to drive de novo angiogenesis [601]. The cooperation between CAFs and M2 macrophages can also inhibit NKs' function and NKs' infiltration in tumor tissues [602]. On the other hand, Cheng Y et al. showed that CAFs regulate the activation, survival and function of tumor associated neutrophils (TANs) through the IL6/STAT3/ programmed death-ligand 1 (PDL-1) signal axis [603], providing a new mechanism to regulate the immune-TME. Leukocyte infiltration plays an active role in tumor development. CAFs enhance the Th2 /Th1 ratio, thus inducing an immunosuppressive environment. According to this, prostate CAFs exposure to naïve CD4+ cells, reduced the percentage of the Th1 anti-tumor subset and it increased Treg cells, driving naive T cells polarization, through a CAFs' derived lactate-based NF-kB activation and Forkhead box P3 (FoxP3) expression [604]. In conclusion, the action exerted by CAFs in TME is mainly immunosuppressive, and a key role is determined by the secretion factors of

CAFs, such as TGFβ which influences many immune cells, including T cells, macrophages and neutrophils [605].

1.4.6 Metabolic reprogramming of tumor cells and CAFs

The metabolic reprogramming is a complex adaptive change in cell metabolism, which is considered a hallmark of cancer, responsible for the tumorigenesis in tissues [606].

Under conditions of nutrient deficiency, indeed, tumor cells rewire their metabolism, by directing limited biomolecules and intermediate metabolites toward biosynthetic pathways which support the energy demand required for keeping the high proliferation rate and to survive [530, 607]. Besides serving as a nutritional support, the reshape in cell metabolism is closely linked to the regulation of the epigenetic signature. Indeed, intermediate metabolites can be exploited for post-transcriptional modifications, by serving as substrates or cofactors of chromatin-modifying enzymes [608]. Moreover, epigenetic modifications, in turn, can control the expression of metabolic genes and key controllers of the metabolism, thus impacting on the energy homeostasis and redox balance of cancer cells [609]. The process of metabolic rewiring comes from both cancer cell-driver mutations and the crosstalk between tumor and stromal cells within the TME [606, 610]. In fact, in addition to tumor cells, accessory cells within the TME could be reshaped to support the metabolic needs of cancer cells [530]. In this context, the role of CAFs is crucial, as well as their metabolic coupling with tumor cells.

1.4.6.1 Metabolic reprogramming in cancer cells

The metabolic reprogramming in cancer is distinguished by enhanced glycolysis, pentose phosphate pathway (PPP), lipid metabolism, increased glutaminolysis, and up-regulation of mitochondrial biogenesis, among other metabolic changes (Fig.8). These pathways provide precursors for a rapid proliferation, metastasis, survival and resistance to anti-tumor therapies.

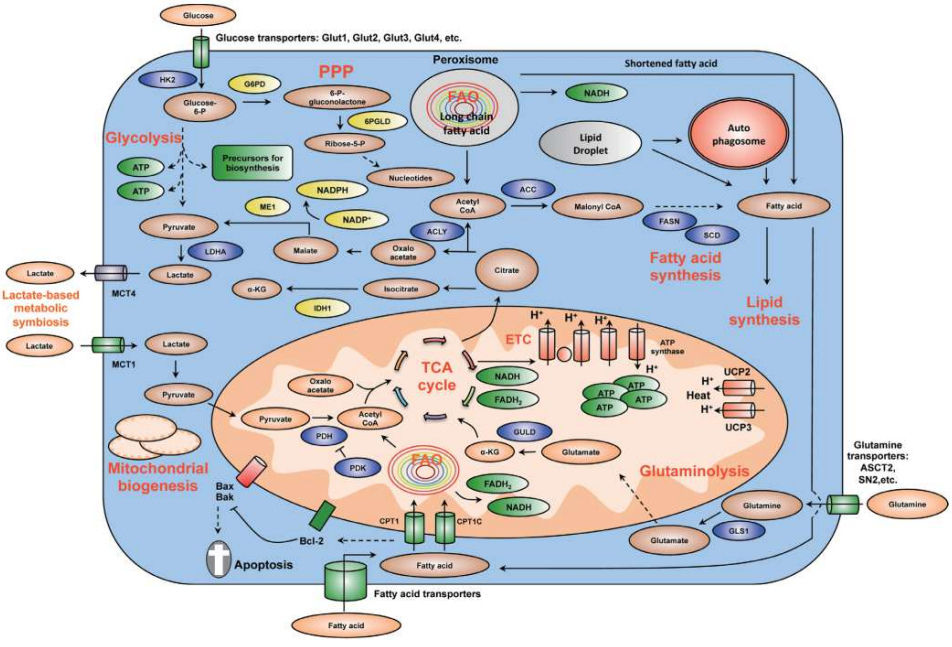


Fig. 8 Representation of the metabolic reprogramming in cancer cells.

Abbreviations: Hexokinase 2 (HK2), lactate dehydrogenase A (LDH-A), glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGLD), acetyl-CoA carboxylase (ACC), ATP citrate lyase (ACLY),: fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD), carnitine palmitoyltransferase (CPT), carnitine palmitoyltransferase 1C (CPT1C), pyruvate dehydrogenase (PDH), pyruvate dehydrogenase kinase (PDK), uncoupling proteins (UCP), monocarboxylic acid transporter (MCT), malic enzyme (ME1), isocitrate dehydrogenase1 (IDH1), glutaminase;(GLS1), glutamate dehydrogenase (GLUD), fatty acid oxidation (FAO), electron transport chain (ETC), pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA), α- ketoglutarate (α-KG). Image derived from [611]

Enhanced glycolysis has been observed even in normoxic conditions, referring to the term of Warburg effect, and it is characterized by a high glucose uptake and fermentation of glucose into lactate [612]. Regarding energy production, glycolysis is low efficient in generating high amounts of ATP compared to the mitochondrial OXPHOS. Indeed, the OXPHOS produces 38 ATP from 1 molecule of glucose, whereas aerobic glycolysis generates only 2 ATP. Important aspects for which cancer cells prefer to undergo the Warburg effect lie in the limited nutrient availability and in the production of carbon source [613, 614]. During expansion, oxygen levels change and tumor requires a constant supply of blood and nutrients to sustain the growing rate. In this scenario, the reprogramming toward a non-oxygen-dependent metabolism becomes an adaptive strategy for the spread, and the aerobic glycolysis supplies metabolic intermediates that can be used for lipid, protein and nucleotide synthesis [613, 615]. Tumor cells accelerate glucose uptake by over-expressing glucose transporters (GLUTs); moreover the glycolytic rate is maintained high thank to the restoring of the oxidized form of nicotinamide adenine dinucleotide (NAD+). This reaction is catalyzed by the enzyme lactate dehydrogenase (LDH), utilizing the reduced form of nicotinamide adenine dinucleotide (NADH) produced by the glyceraldehyde phosphate dehydrogenase (GAPDH). As already introduced, the Warburg effect provides glycolytic intermediates for other biosynthetic programs, such as glucose-6-phosphate which is employed in the PPP. This pathway generates ribose-5-phosphate and nicotinamide adenine dinucleotide phosphate hydrogen (reduced form) (NADPH). The first serves for nucleotide synthesis (used in DNA replication) RNA synthesis, and DNA damage repair, while NADPH is a defense against oxidative stress [616]. Indeed, NADPH is a resource for glutathione reductase, which converts oxidized glutathione (GSSG) into the reduced form (GSH), a principal cellular anti-oxidant used by cancer cells [617].

Although originally it was believed that cancer cells depend on the “inefficient” metabolism of aerobic glycolysis due to a mitochondrial “respiratory injury”, it was later demonstrated that mitochondrial OXPHOS defects are not common in spontaneous tumors [618, 619]. Nevertheless, mitochondrial metabolism of neoplastic cells differs from that of healthy cells. The main characteristics of cancer cell mitochondria are: I) accumulation of ROS, II) mutations at the level of tricarboxylic acid cycle (TCA) enzymes, III) accumulation of metabolites at the TCA level useful for anabolism, and III) altered mitochondrial membrane properties that allows to avoid programmed cell death. High ROS levels promote the generation of tumor activating mutations and oncogenic signalling which sustain the transformation process. Moreover, accumulation of intermediates from the TCA such as fumarate and succinate (considered as oncometabolites) can be responsible for the alteration of the epigenetic profile of tumor cells.

Lipids are one of the main sources for biomass formation in cells, being the building blocks for cellular membranes (e.g. phospholipids and cholesterol), and an energy storage employed as ATP when the cellular energy need is elevated (e.g. triglycerides). Tumor cells adopted various strategies for up-regulating lipid synthesis: through I) the de novo synthesis of fatty acids, II) the uptake of fatty acids from the extracellular milieu, III) the up-regulation of genes implicated in lipid metabolism. Fatty acids are imported into mitochondria by carnitine palmitoyltransferase 1 (CPT1) and then oxidized into acetyl-CoA, which fuels the TCA. The initiation of the lipid anabolic pathway can also directly start from citrate provided by the TCA and then transported from the mitochondria into the cytosol by a citrate/isocitrate carrier (CIC) [620, 621]. In the cytoplasm, ATP-citrate lyase (ACLY) catalyzes the conversion of citrate into oxaloacetate and acetyl-CoA. The first is carboxylated into malate and then oxidized into pyruvate, which feeds the TCA, generating reducing power for lipid synthesis. On the other hand, acetyl-CoA can be precursor of fatty acid biosynthesis, through the reactions of ACC, fatty acid synthase (FASN) and stearoyl-CoA desaturase (SCD). Remarkably, ACC, ACLY and FASN are often up-regulated or activated by oncogenes, as for example cellular Myc (c-Myc), HIF-1α and Akt in cancer cells [622, 623].

Glutaminolysis is another metabolic pathway that is altered in tumor cells. Glutamine is uploaded by cancer cells from the TME and then hydrolyzed into glutamate (to replenish the TCA) by the enzyme glutaminase [624]. Moreover, glutamine also provides nitrogen for biosynthesis of purine and pyrimidine nucleotides and it contributes to glutathione production [625]. Remarkably, glutaminolysis can also produce lactate. After the entry of glutamine in the TCA, α-ketoglutarate (α-KG) is converted into malate, exported in the cytosol for the production of pyruvate from which derives lactate [626].

1.4.6.2 Reverse Warburg effect and tumor cell metabolic coupling

Under influence of tumor cells, CAFs exhibit a similar aerobic glycolysis (reverse Warburg effect) [627]. It was demonstrated that the metabolic switch from the OXPHOS to glycolysis may be led by the down-regulation of α subunit of the isocitrate dehydrogenase 3 α (IDH3α), whose increased expression prevented the activation of NAFs into CAFs [628]. Other than IDH3α, further insights concerning the up-regulation of glycolytic genes (e.g. pyruvate kinase M2, PKM2 and LDH-A) in CAFs provided evidence in favor of the reverse Warburg effect [629]. The activation of TGFβ signalling in CAFs often correlates with down-regulation of caveolin-1 (Cav-1) [630, 631]. In keeping, Bonuccelli et al. demonstrated that the absence of Cav-1 in stromal fibroblasts up-regulated PKM2, LDH-B and it was sufficient to promote tumor growth and angiogenesis in vivo [632]. In the TME, CAFs are a resource for the production of energy-rich metabolites (e.g. lactate, ketone bodies, fatty acids and glutamine) absorbed by cancer cells in a paracrine fashion and then utilized for a metabolic reshaping that promotes rapid growth [632, 633]. The exposure of CAFs-derived lactate to tumor cells, for instance, stimulates mitochondrial biogenesis and OXPHOS, as observed in BCa, pancreatic cancer, and oral squamous cell carcinoma [634-636]. Consistently, regarding PCa, Fiaschi et al. demonstrated that CAFs reprogrammed by PCa cells toward glycolysis expressed high levels of GLUT1 and the lactate exporter, monocarboxylate transporter 4 (MCT4), to allow lactate upload from PCa cells via monocarboxylate transporter 1 (MCT1). The up-regulation of MCT1 with respect to low GLUT1 in PCa cells, led to a preferential usage of lactate over glucose, for fueling the TCA, along with the anabolic processes needed for cell proliferation. In keeping, the inhibition of MCT1 in PCa cells prevented tumor survival and outgrowth [637]. The consequence of the lactate-driven metabolic coupling between CAFs and PCa cells consisted in an altered NAD+/NADH ratio, resulting from oxidation of lactate into pyruvate by LDH-B, over-expressed in PCa cells (Fig.9). The accumulation of NAD+ in PCa cells was demonstrated to lead the activation of the deacetylase sirtuin 1/ PGC transcription co-activator 1α (SIRT1/PGC-1α) axis, the main regulator of mitochondrial biogenesis. As a result, oxygen consumption and intermediates of the TCA (e.g. citrate, succinate and fumarate) increased, causing stress and dysfunction of the electron transport chain, with the accumulation of mitochondrial ROS [638].

Accumulation of ROS within the TME is one of the central factors responsible for maintaining the cross-talk between CAFs and tumor cells. The oxidative stress produced by ROS leads to the stabilization of HIF-1 [639]. In CAFs, HIF-1 is an activator for the transcription of genes encoding for: I) GLUTs, II) glycolytic enzymes and III) pyruvate dehydrogenase kinase (PDK), which inhibits the pyruvate dehydrogenase (PDH) complex that converts pyruvate into acetyl-CoA. The inhibition of PDH consequently brings to pyruvate accumulation and conversion into lactate (by LDH-A), then secreted in the tumor milieu through MCT4 (also regulated by HIF-1) [640, 641]. On the other hand, in tumor cells, HIF-1 up-regulates MCT1 and down-regulates GLUT 1, thus favoring the OXPHOS [637].

Enhanced mitochondrial OXPHOS in tumor cells triggers the oxidation of the redox-sensitive Src protein kinase, which phosphorylates PKM2. This enzyme is over-expressed in cancer cells where it catalyzes the last rate-limiting step of glycolysis from phosphoenolpyruvate (PEP) to pyruvate, promoting a Warburg metabolism [642, 643]. When phosphorylated, PKM2 loses its catalytic activity and it translocates to the nucleus, forming heterotrimeric complexes with HIF-1 and the Differentially expressed in condrocytes-1 (DEC1) transcriptional repressor. The PKM2/HIF-1/DEC1 heteromers down-regulate miR205, which normally inhibits the expression of the transcription factor zinc finger E-box binding homeobox 1/2 (ZEB1 / 2). Hence, ultimately, EMT is facilitated [644]. According to this, the targeting of PKM2/OXPHOS was proposed as a therapeutic approach for PCa metastatic spread [644]. The cross-relationship between CAFs and tumor cells can also include amino acids and lipids. CAFs can synthetize amino acids such as glutamine, for tumor cells, as well lipids [624]. Moreover, it was demonstrated that the uptake of CAFs-derived lactate in PCa cells increased the concentrations of citrate, malate, succinate and fumarate, (but not α-KG) favoring lipid biosynthesis [645].

Finally, CAFs participate to the horizontal passage of exosomes and functional organelles [530]. Ippolito et al. reported that CAFs unidirectionally transfer their mitochondria to PCa cells via cytoplasmic bridges [638].

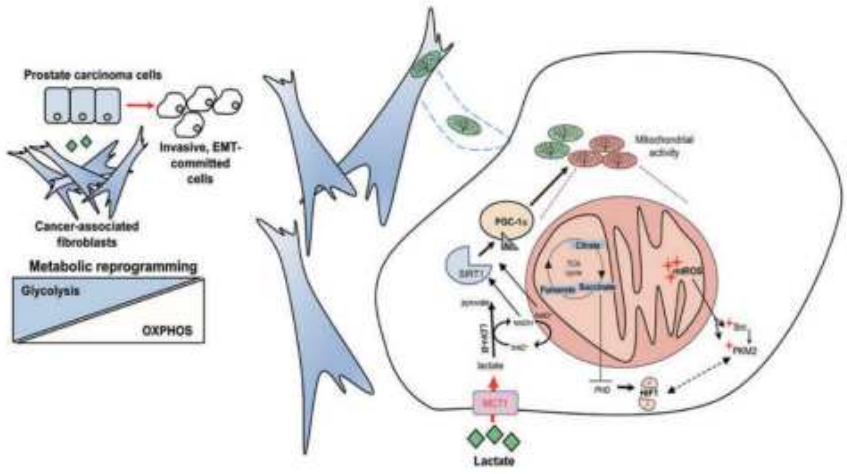


Fig. 9. Metabolic symbiosis between PCa cells and CAFs.

CAFs enhance mitochondrial activity of PCa cells: I) by activating the lactate/SIRT1/PGC1-α axis; (II) by accumulating the TCA intermediates (i.e. citrate, succinate and fumarate) and III) by incrementing mitochondrial ROS (mtROS) following Src and PKM2 oxidation. CAFs also create cytoplasmic bridges for supplying mitochondria to PCa cells. Overall the contact of PCa cells with CAFs stimulates an OXPHOS metabolism which is drives a more invasive phenotype. Image derived from [638].

Abbreviations: sirtuin 1 (SIRT1), PGC transcription co-activator 1α (PGC1-α), tricarboxylic acid cycle (TCA), pyruvate kinase M2 (PKM2), cancer associated fibroblasts (CAFs), prostate cancer (PCa), oxidative phosphorylation (OXPHOS), monocarboxylic acid transporter (MCT1), hypoxia-inducible factor-1 (HIF-1), prolyl-hydroxylase (PHD), lactate dehydrogenase B (LDH-B), nicotinamide adenine dinucleotide, oxidized form (NAD+), nicotinamide adenine dinucleotide, reduced form (NADH)

1.5 Acidification of the TME

Tumor acidification consists in the decrease of pH levels within the TME, derived from lactate accumulation and hypoxia [646]. Today this process is not considered just a mere passive side effect of cancer growth, rather a distinctive trait of tumors [647]. Tumor vasculature contributes to the generation of the hypoxic state, and it makes difficult to eliminate protons in the TME due to a disorganized and abnormal structure [648]. Extracellular acidification is also a protective mechanism employed by tumor cells against the intracellular pH changes, which may be detrimental for the cell. Therefore, cancer cells reprogram the systems implicated in the acid-base balance, redirecting protons to the extracellular space [649].

The physiological blood concentration of lactate is typically 1-2 mM at rest, and 10/20 mM after an intense physical exertion. However, in tumors and chronically inflammatory tissues lactate levels reach up to 40 mM concentration [650, 651]. As already said, the transport of lactate through cell membrane happens thanks to MCTs. MCT1 favors lactate (but also pyruvate) upload, and its expression is mainly regulated by c-Myc. On the other hand, MCT4 is the major exporter of lactate which is under control of HIF-1 [652, 653]. Both HIF-1α and c-Myc are responsible for keeping the high glycolytic rate [653]. Prolyl-hydroxylases (PHDs) act on the stability of HIF-1. In normoxia, they are active and hydroxylase HIF-1, making it recognizable to VHL protein, which ubiquinates HIF-1, thereby favoring the proteasome degradation. Contrarily, in hypoxia, PHDs do not work, and HIF is stabilized [654]. Other than oxygen, lactate can lead to the inhibition of PHDs [655]. Moreover, ROS, pro-inflammatory cytokines and growth factors also contribute to HIF-1 stabilization [654, 656, 657].

1.5.1 Role of lactate in the immune microenvironment

Regarding the immune microenvironment, lactate displays an immunosuppressive role acting on T cells, NK cells, macrophages and dendritic cells [653, 658] (Fig.10).

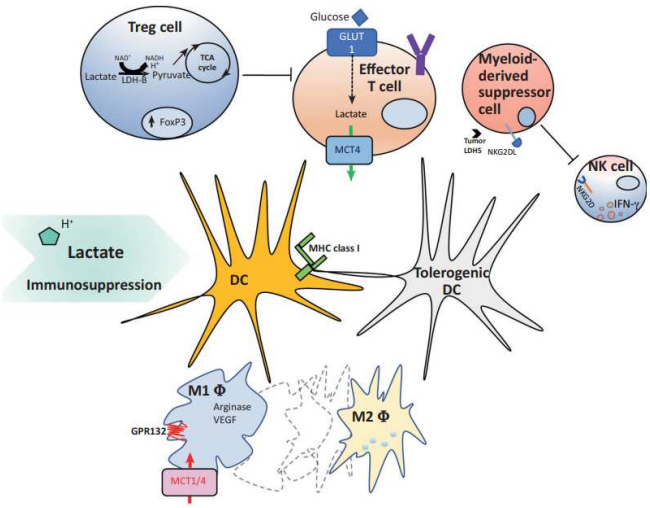


Fig. 10 Effects of lactate in the TME

In the TME lactate inhibits immune surveillance by affecting T cells, NKs and by enhancing the immunosuppressive function of Tregs. Soluble LDH-A secreted by tumor cells impairs the NKG2D signalling in NKs interacting with myeloid-derived suppressor cells. In macrophages lactate favors the M2 pro-tumorigenic phenotype, while in dendritic cells it enhances the tolerogenic phenotype. I macrophages

lactate also promotes the transcription of ARG1 and VEGF. Abbreviations: dendritic cells (DCs), interferon-g (IFN-γ), lactate dehydrogenase (LDH), type M1/M2 macrophages (M1/M2 F), natural killer cells (NKs), Natural killer group 2 member D (NKG2D), and regulatory T cells (Tregs). Image derived from [658]

The metabolite was shown to inhibit the activation of CD4+/ CD8+ cells [659], and it prevented the cytotoxicity of NKs and the recruitment of MDSCs [653, 660]. Accordingly, tumor-secreted soluble LDH-A impaired NKG2D signalling in NKs by interacting with MDSCs [661]. Lactate was also demonstrated to enhance the differentiation of dendritic cells toward a tolerogenic phenotype, the M2 polarization and to suppress the activation of T helper cells [662]. In Tregs, instead, the transcriptional factor Foxp3 was shown to stimulate the oxidation of extracellular lactate and mitochondrial functionality [663]. Lactate also induces pro-angiogenic effects. Indeed, it was demonstrated to stimulate the signalling of NF-kB in endothelial cells, supporting the autocrine release of IL-8 and subsequently cell activation [664].

1.5.2 Role of lactate in the epigenetic reprogramming

In the TME, nutrient availability regulates gene expression epigenetically, through changes on DNA and histones, such as histone methylation/ acetylation and DNA methylation. In PCa cells, lactate from CAFs stimulated DNA methylation necessary for EMT [665] and it provided acetyl-CoA required for histone acetylation [666].

Lactylation is a post-transcriptional modification induced by lactate demonstrated to enrich genes implicated in cellular metabolism and phenotypic aggressive traits [667]. For instance, lactylation of lysine 18 residue on histone 3 (H3k18) was found associated with aggressive proliferation, migration in melanoma cells and poor prognosis in melanoma affected patients [668]. Additionally, histone lactylation may also affect tumor-infiltrating immune cells [669].

1.5.3 Role of lactate as a binding receptor

Besides the canonical role as a nutrient and the role as epigenetic reprogrammer, lactate can act as a soluble hormone which binds membrane receptors; indeed, it is often referred to the term of "lactormone" [658]. The **hydroxycarboxylic acid receptors 1** (HCAR1), better known as **GPR81**, is the main recognized receptor for lactate. It signals through Gαi subunit lowering cAMP levels, and subsequently decreasing PKA activity [651]. Adipose tissue is the primary tissue where GPR81 is most highly expressed and involved in the processes of maintaining energy homeostasis, such as the inhibition of lipolysis [651, 670]. However, although in lower levels, the receptor is expressed in other tissues (e.g. brain, immune system) with a potent anti-inflammatory action [671]. Several cancers demonstrated up-regulation in GPR81 expression with implication in tumor growth and immunosurveillance [672, 673]. In BCa, the receptor regulated glycolysis, migration and invasion mechanisms of tumor cells [672, 674]; whereas in pancreatic cancer GPR81 provided survival and growth under conditions of low glucose supplemented with lactate in vitro. In pancreatic models in vivo, the silencing of the GPR81 was proven to consistently decrease tumor growth and metastasis [672]. In human lung cancer, lactate autocrine produced by tumor cells induced TAZ-dependent up-

regulation of PDL-1 via GPR81 [673]. Moreover, lactate sensor GPR81 decreased the secretion of pro-inflammatory cytokines, the expression of major histocompatibility complex (MHC) in dendritic cells and antigen presentation of T cells, thus eluding the immune response [675, 676]. **GPR132** is the second functional receptor for lactate which actively modulates cell cycle, proliferation, and immunity, principally signalling through cAMP, PKA, and ERK [677]. In acute myeloid leukemia pharmacological activation of GPR132 also triggered tumor cell differentiation [678]. Many reports showed GPR132 implication in BCa, particularly referring to the induction of macrophage M2 like phenotype, in turn facilitating cancer cell adhesion, migration, and invasion [677, 679]. In keeping, in BCa, GPR132 was proven to be implicated in the development of lung metastasis in vivo and to clinically correlate with poor prognosis of tumor patients [679].

1.5.4 Targeting of lactate transporters and production

The targeting of lactate transporters and the enzymes responsible for its production has become a new anti-tumor strategy to investigate. Targeting MCTs can block cross-talk mechanisms that develop between tumor cells themselves and accessory cells within the TME [658]. Currently, no MCT inhibitor is in clinical practice. A small molecule developed by AstraZeneca, AZD3965, is a MCT1-selective inhibitor which has undergone the phase I clinical trial for advanced solid tumors and lymphomas [680]. Nonetheless, the major disadvantage of MCT1-specific inhibition is its ineffectiveness in the presence of MCT4, due to compensatory mechanisms provided by MCT4 when MCT1 is inhibited. Similar to MCT1 inhibitors, the specific MCT4-inhibitor, AZ93 (from AstraZeneca as well) was found ineffective in cells co-expressing MCT1 and MCT4 [681]. On the other hand, the dual (MCT1/MCT4) inhibitor, 7 aminocarboxycoumarin (7ACC) inhibited lactate influx but not efflux [682]. Syrosingopine also blocks both MCT1/ MCT4 and it induced synthetic lethality in cancer cells in combination with metformin, an inhibitor of mitochondrial NADH dehydrogenase necessary for the regeneration of NAD+ during glycolysis [683].

Another proposed targeting for lactate is LDH-A. However, due the heterogeneity of cell population for LDH-A expression the use of this targeting strategy results difficult [683].

1.6 The role of the ECS within the TME

Besides the extensively studied impact of the ECS on cancer cells there is now emerging evidence regarding the direct influence of the ECS on accessory cells within the TME. Various immune cells, such as T- / B-cells, macrophages, and NKs release eCBs and express CBRs in response to the inflammatory milieu [274]. In turn, activation of the ECS can influence the pro-inflammatory activity of immune cells [684]. According to this, it was found that cytokines' stimulation increased CBRs expression in peripheral blood mononuclear cells (PBMCs) and that CB1R and CB2R in macrophages inhibited the release of VEGF-A [684, 685].

In immune cells, the ECS modulates several biological functions including proliferation, differentiation, migration, cytotoxic activity, antibody formation and apoptosis [67, 686]. Among immune cells, **B cells** have the highest expression of CB2R [687], that is responsible for cell differentiation, and for the isotype switch of immunoglobulines [688]. CB1R, instead, is not detected in B cells. **T cells** express both CB1R and CB2R whose targeting was proven to affect cell proliferation, migration, apoptosis and cytolytic activity [689, 690]. Activation of CB2R with AEA and selective ligands was demonstrated to suppress the production of inflammatory cytokines (e.g. IL-2, TNFα and IFN-γ) from activated human peripheral T cells [691]. Contrary, CB2R antagonists prevented these effects [692]. In mice models of NSCLC, the knockout of CB2R increased accumulation and tumoricidal activity of CD8+ cells (and NKs), together with an increased expression of programmed death-1 (PD-1) and the related ligand on myeloid and lymphoid cells. In keeping, CB2R knockout mice responded in a better significantly way to anti-PD-1 therapy with respect to the wild type littermate [302].

Macrophages produce several eCBs, including AEA, 2-AG and PEA [693]. Shaping of the ECS is crucial for the recruitment, polarization and chemotaxis abilities of TAMs, as observed in peritoneal murine macrophages where THC treatment inhibited CB2R-dependent cell migration through the RANTES/CCL5 (regulated upon activation, normally T-expressed, and presumably secreted /C-C motif chemokine ligand) signalling [694]. Antigen presentation, phagocytosis and macrophages-assisted vascular remodeling are other key features which can be modulated by eCBs in macrophages [695, 696]. **Dendritic cells** express both canonical CBRs [696]. AEA inhibited cytokines production in myeloid and plasmacytoid dendritic cells [697]. While 2-AG was seen to be chemoattractive for these cells, and responsible for their phenotypic maturation [698, 699]. Cannabinoids like AEA and Met-F-AEA affected **endothelial cells**' function, proliferation and production of pro-inflammatory mediators [699, 700]. Accordingly, CBD inhibited primary vascular activities HUVEC cells, related to invasion, sprouting, and the secretion of factors such as MMP-9, TIMP-1, plasminogen activator inhibitor-1 (PAI-1), urokinase-type plasminogen activator (uPA), endothelin-1 (ET-1), and PDGF [701]. The contribution of the ECS was also valuated in relation to the crosstalk between cancer cells and vascular endothelial cells. A report showed that CB2R activation significantly interfered with human melanoma cells adhesion to brain endothelial cells, thereby decreasing trans-endothelial migration and metastatic potential of tumor cells [251]. Moreover, in HCC, THC-treated tumor cells

were able to promote migration and tube formation of human-induced pluripotent stem cell-derived vascular endothelial cells(hiPSC-VECs) [702]. 2-AG was demonstrated to promote **monocytes**' migration and to increase the immunosuppressive population of **MDSCs** in pancreatic ductal adenocarcinoma murine models [687, 699]. **NKs** produce AEA/ 2-AG, and they express CB1R, CB2R and GPR55 [688]. Moreover, 2-AG treatment was demonstrated to stimulate NK cells' migration via CB2R [703]. GPR55 was also detected in **neutrophils**, and its activation increased migration and decreased degranulation/ ROS production [431].

Within the TME, the surrounding adipose tissue release molecules and signals that influence cancer progression, as observed in several solid cancers such as BCa [704]. In co-cultured conditions of **adipocytes** and BCa cells, alteration of the catabolic activity of adipocytes led to BCa progression through down-regulation of PPARγ [705].

Finally, in **CAFs**, CB1R and CB2R were found highly expressed with respect to healthy prostate fibroblasts (HPFs). Moreover, cytokines stimulation (e.g. IL-6 and TGFβ) which turned HFPs into CAFs, concomitantly induced canonical CBRs' expression. CBRs agonists, in particular WIN552512-2 mesylate prevented CAF reactivity and tumor-induced HPFs' activation, by down-regulating αSMA, MMP-2 expression and by inhibiting CAFs' migration and CAFs-mediated cancer cell invasiveness [706].

2. Purpose of the thesis

Considering the role of environmental lactate and the ECS in the etiopathogenesis of tumors, this study aimed to investigate a potential functionality of lactate in the modulation mechanisms of gene expression and activity of the ECS, taking into account an experimental model of PCa.

3. Materials and methods

3.1 Cell lines

Human PCa cells, DU145 (Research resource identifier: Cellosaurus, RRID: CVCL_0105) and PC3 (RRID: CVCL_0035) were purchased from the American type culture collection (ATCC). Cells were grown at 37°C and 5% CO2. DU145 cells were maintained in Dulbecco's modified eagle medium (DMEM, #ECB7501L; Euroclone), whereas PC3 cell line was in Nutrient Mixture F-12 Ham's (named after Richard G. Ham) (#N4888; Merck Millipore). Media were supplemented with 10% fetal bovine serum (FBS) (#ECB4004L; Euroclone), 2 mmol/L L-glutamine and 1% penicillin/streptomycin. All cells were tested for Mycoplasma contamination with the MycoAlert Mycoplasma Detection kit (#LT07–318; Lonza) every 2 weeks, and kept in culture for 20/ 12 passages, after thawing.

3.2 Cell treatments

Cell treatments were carried out with lactate at 2.5 mM / 20 mM concentration, to a time of 15 minutes / 48 hours depending on the experiment. Alternatively cells were treated in serum-free culture medium (control condition, CT). Unless otherwise stated, all reagents were purchased from Merck Millipore. The GPR55 inhibitors, ML193 (SML1340, Merck Life Science) and CID 16020046 (HY-16697 D.B.A s.r.l.) were used at 5 μM and 10 μM concentration respectively, for a time of 30 minutes or 48 hours before/ together with lactate stimulation. The treatment with Rho inhibitor (CT04, Societa' Italiana ChimDNAici Divisione Scientifica S.R.L) and the broad spectrum MMPs inhibitor Marimastast (HY-12169 D.B.A s.r.l.) were administered to 1μg/ml and 10μM concentration, respectively. CT04 was incubated 5/6 hours prior to the preparation of cellular invasion assay, while Marimastat was given 24 hours before.

3.3 RNA extraction and qRT-PCR

The RNeasy Kit (#74104; Qiagen) was used for the extraction of total RNA. Complementary DNA (cDNA) synthesis was carried out with the iScript cDNA Synthesis Kit (#1708891; Bio-Rad). CFX96 Touch Real-Time polymerase chain reaction (PCR) Detection System (Bio-Rad) was considered for the reverse transcription-quantitative (qRT)- PCR, using TaqMan assays (Applied Biosystems). In the study the following probes were used: CNR1 (#Hs01038522_s1 Life Technologies), CNR2 (#Hs05019229_s1, Life Technologies), GPR55 (#Hs00271662_s1, Life Technologies) and TRPV1 (#Hs00218912_m1, Life Technologies). Hypoxanthine phosphoribosyltransferase 1 (HPRT1, #Hs02800695_m1, Life Technologies) was used for normalizing data.

3.4 Western blot (WB) analysis

Protein samples were collected in Laemmli sample buffer and denatured at 95°C for 10 minutes. Afterwards, protein quantification was performed by using the bicinchoninic acid (BCA) Kit. Samples in a range of 20–25 mg concentration were loaded on 4% to 20% acrylamide precast sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad, #456–8096). Gels were then

transferred on polyvinylidene difluoride membrane by Trans-Blot Turbo Transfer Pack (Bio-Rad, #1704157). Membranes were activated in methanol, and successively incubated with the antibody of interest (diluted in 5% milk), overnight at 4°C. In the study, the following primary antibodies were considered: rabbit anti-phosphorylation of the myosin light chain 2 (pMLC2, 1:1,000; 3671S; Cell Signaling Technology), rabbit anti- myosin light chain 2 (MLC2, 1:1,000;3672S Cell Signaling Technology), rabbit anti-Rho A (1:1,000; sc-418 Santa Cruz) and mouse anti- heat shock protein 90 (HSP90, 1:1,000; 11818 Santa Cruz).

3.5 Immunoprecipitation (Rhotekin assay)

PCa cells were washed with phosphate buffered saline and lysed in radioimmunoprecipitation assay (RIPA) buffer (implemented with 0.1% sodium dodecyl sulfate, SDS) on ice. Cell lysates were centrifuged at 12 000 rpm at 4°C for 20 min, and then incubated with 25 µg glutathione S-transferase (GST)–Rhotekin beads (14-383 Merck Life Science) at 4°C for 45 min. Rhotekin-bound Rho proteins, guanosine-5'-triphosphate (GTP)-bound Rho proteins, were detected by Western blot analysis. The Rho A monoclonal antibody was used in this respect. The amount of Rhotekin-bound Rho was normalized to the total of Rho proteins in cell lysates.

3.6 Transwell migration and invasion assay

The assays were performed in Boyden chambers with 8-µm pore size filters (CC3422, Costar™, Corning, New York, USA). PCa cells were seeded ($3 \cdot 10^4$ cell per well) in serum-free medium or 2.5 mM lactate, in absence or presence of ML193 and CID 16020046. For a time of 16-20 hours, cells were allowed to migrate toward 10% FBS-supplemented DMEM. For the invasion assay, Boyden chambers were pre-coated with Matrigel (Corning® Matrigel®, VWR International Srl) overnight at room temperature and left to hydrate the following day for 2 hours with a serum free medium at 4°C, before the cell seeding. Migrating cells were fixed, stained with Hematoxylin and Eosin. Cell counting was performed by the Image J software and considering five chosen fields (10X) in bright field.

3.7 Statistical analysis

Statistical analysis was carried out with Graph Pad software (Prism). Data show means ± SEM from at least three independent experiments. Statistical comparisons: Unpaired t-test (two-tailed), ordinary one-way ANOVA followed by Turkey or Dunnett's post-hoc test. Statistical significance was considered when $p < 0.05$.

4. Results

4.1 Contribution of lactate in the modulation of GPR55 expression and activity in PCa cells

Considering the crucial role of lactate in the progression of PCa, we delved into examining the potential modulations induced by the metabolite on the components of the ECS in PCa cells. Our investigation particularly focused on CBRs. PCa cells were treated with lactate at 20 mM concentration (48 hours). The dosage of lactate was determined based on measurements obtained from patient-collected cancer biopsies [637]. The treatment with serum-free medium (48 hours) was considered as control condition (CT). Gene expression of CBRs was assessed trough qRT-PCR analysis (Fig.11). From the results we noticed that the treatment did not change the expression of *canonical* CB1R and CB2R in PCa cells. On the other hand, *non-canonical* CBRs were modulated by lactate. Specifically, GPR55 was up-regulated, while TRPV1 was down-regulated.

Overall these data indicated an involvement of lactate in reshaping the *non-canonical* CBRs' expression in PCa cells, in particular, with a positive modulation of GPR55.

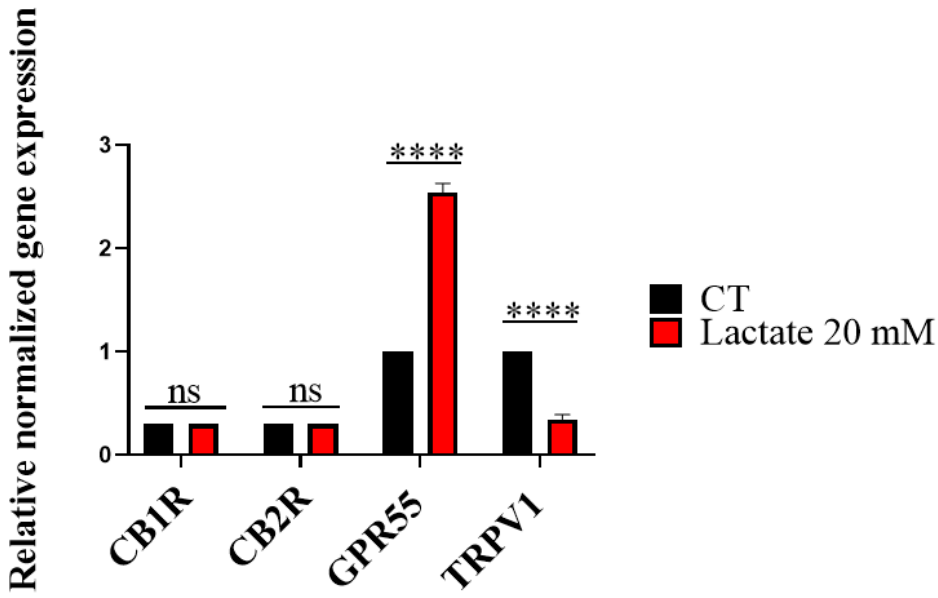


Fig.11. CBRs' expression in lactate-treated PCa cells

qRT-PCR analyses for the evaluation of gene expression of *classical* (CB1R, CB2R) and *non-canonical* CBRs (GPR55, TRPV1) on DU145 cells. Treatment was performed with 20 mM concentration of lactate for 48h, while CT was obtained in serum-free medium. Lactate induced no changes in the expression of CB1R and CB2R and negative modulations in the levels of TRPV1. GPR55 was, instead, up-regulated by lactate in DU145 cells. Data are represented as mean ± SEM of three independent experiments. Unpaired t-test, two-tailed; *p < 0.05; **p < 0.01; ***p < 0.001

Abbreviations: cannabinoid receptor 1 (CB1R), cannabinoid receptor 2 (CB2R), G coupled protein receptor 55 (GPR55), transient receptor potential cation channel subfamily V member 1 (TRPV1), reverse transcription-quantitative PCR (qRT-PCR), control condition (CT), non-significant (ns)

GPR55 was found over-expressed across different tumors, with characteristics associated to malignancy, such as cell migration and invasiveness. Notably, these key aggressive features are enhanced by lactate in PCa, contributing to the promotion of the metastatic potential of tumor cells [638, 707]. The phosphorylation of MLC was demonstrated to be downstream of GPR55, resulting in an increase in cellular invasiveness [498].

Exploring likely involvements of lactate in the mechanisms of motility associated with GPR55, we assessed the phosphorylation status of MLC2 in DU145 and PC3 cells, treated with lactate (20 mM) and/ or with a GPR55 selective inhibitor, ML193 (5 μM). Under a short stimulation time (15 min), lactate induced the activation of MLC2 in PCa cells. Moreover, the inhibition of GPR55 with ML193 prevented this effect (Fig. 12 a-b).

These findings suggested that the activation of MLC2 signalling derived from lactate was dependent on the functioning of GPR55. Furthermore, the brief activation time of a GPR55- downstream signalling excluded that lactate behaved as metabolic reprogrammer for prompting GPR55 functionality. Hence, the data led us to hypothesize that lactate might work as a binding molecule towards GPR55, mirroring the established behavior already known for GPR81 and GPR132 [670], [679].

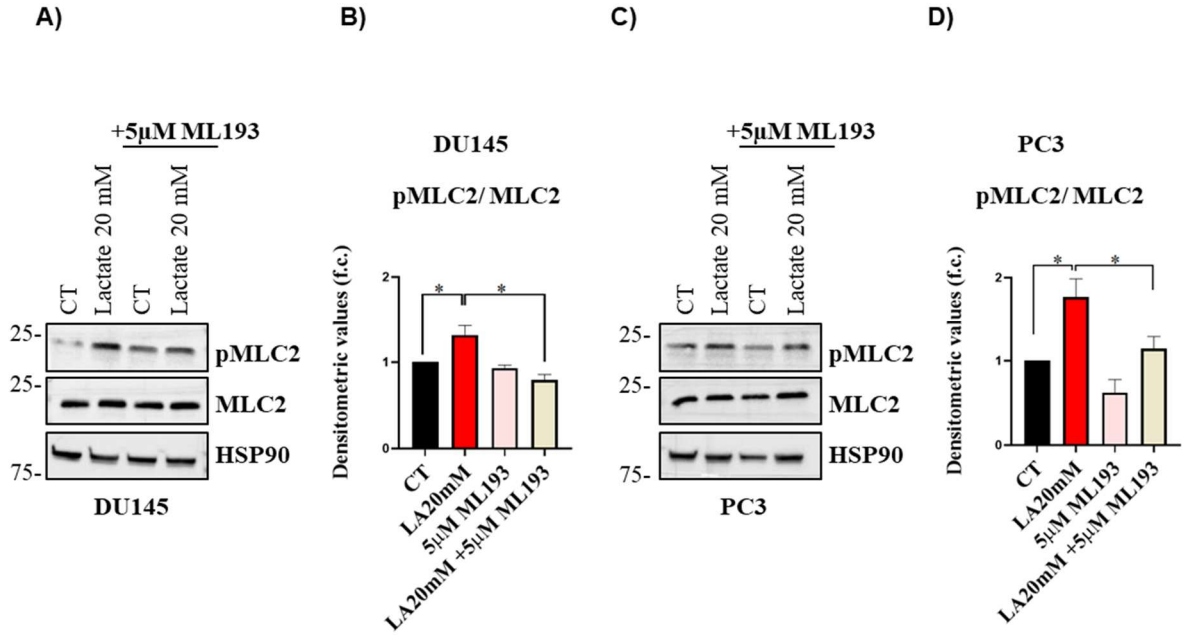


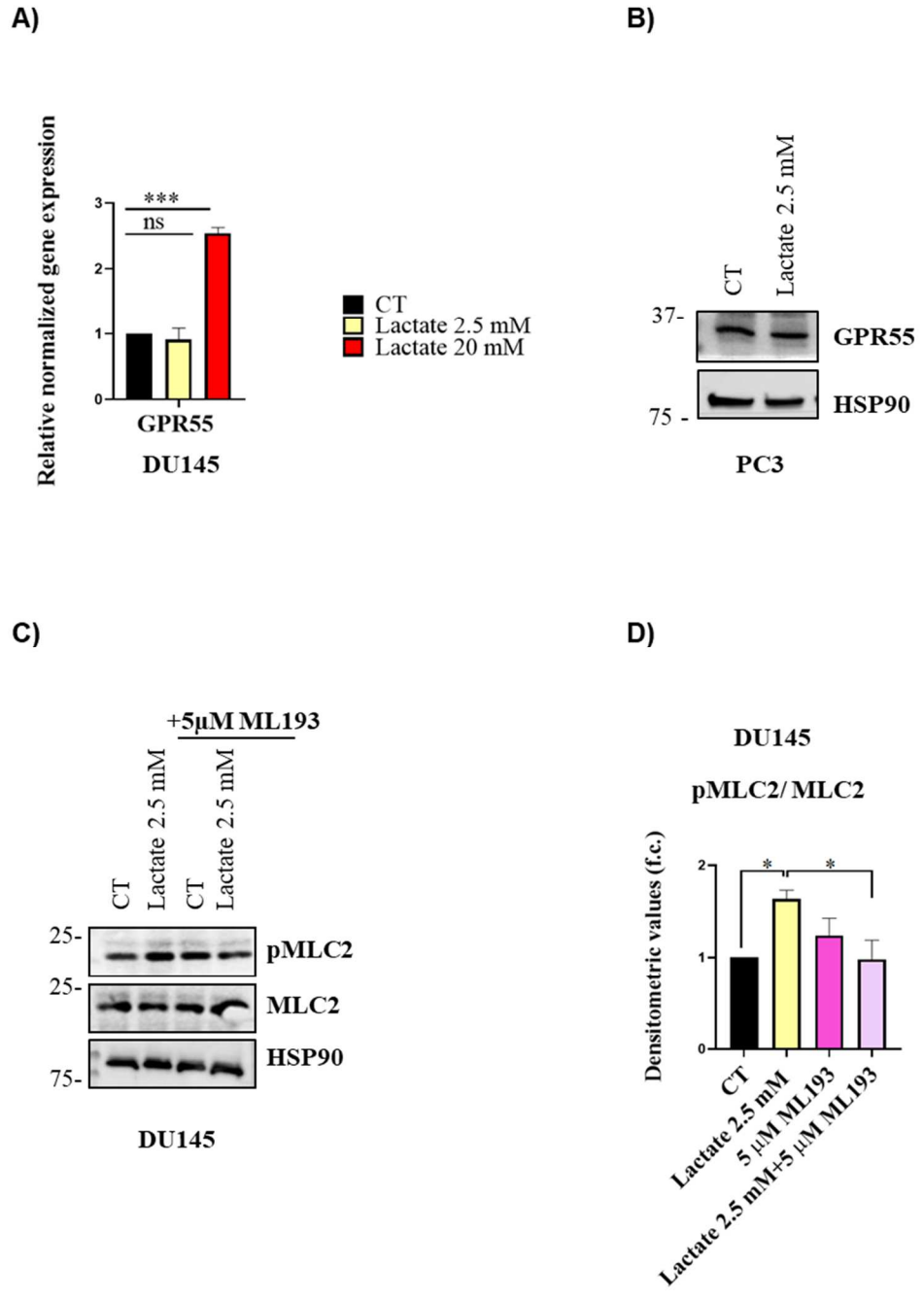
Fig.12. Effects of high doses of lactate on GPR55 activity in PCa cells

WB representing the pMLC2 status, normalized to the total of MLC2 expression. HSP90 was used as a housekeeping gene. DU145 cells (A-B) and PC3 cells (C-D) were treated with lactate (20 mM) for 15 minutes (15') in presence/ absence of the GPR55 inhibitor, ML193 (5 μM). The CT condition was obtained maintaining cells in serum-free medium. B-D) Densitometric analyses of 3 independent WB experiments referred to the pMLC2/MLC2 expression in DU145 cells (B) and PC3 cells (D). Values are expressed in f.c. All data are represented as mean ± SEM of three independent experiments. One-way ANOVA; Dunnett's corrected; *p < 0.05; **p < 0.01; ***p < 0.001.

Abbreviations: Western blot (WB), control condition (CT), phosphorylation of the myosin light chain 2 (pMLC2), myosin light chain 2 (MLC2), heat shock protein 90 (HSP90), G coupled protein receptor 55 (GPR55), fold change (f.c.)

4.2 Lactate activates GPR55 in PCa cells

The activation of GPR81 by lactate was documented for concentrations within the range of 1 to 5 mM [708]. DU145 cells and PC3 cells were treated with a low dose of lactate (2.5 mM) and then evaluated for the expression of GPR55. With respect to the CT condition, GPR55 expression was not changed in PCa cells treated with the low dose of lactate, contrary to what was observed with the high stimulation (Fig.13 a-b). To delve deeper in the proposed ligand functionality of lactate towards GPR55, we assessed lactate's capacity to induce rapid phosphorylation of MLC2, employing this instance the 2.5 mM concentration, more suitable for a ligand activity. Similarly to the high dose, the lower concentration of lactate triggered the phosphorylation of MLC2 in PCa cells at 15-minute time point, and this effect was hindered by the block of GPR55 with ML193 (Fig. 13 c-d).



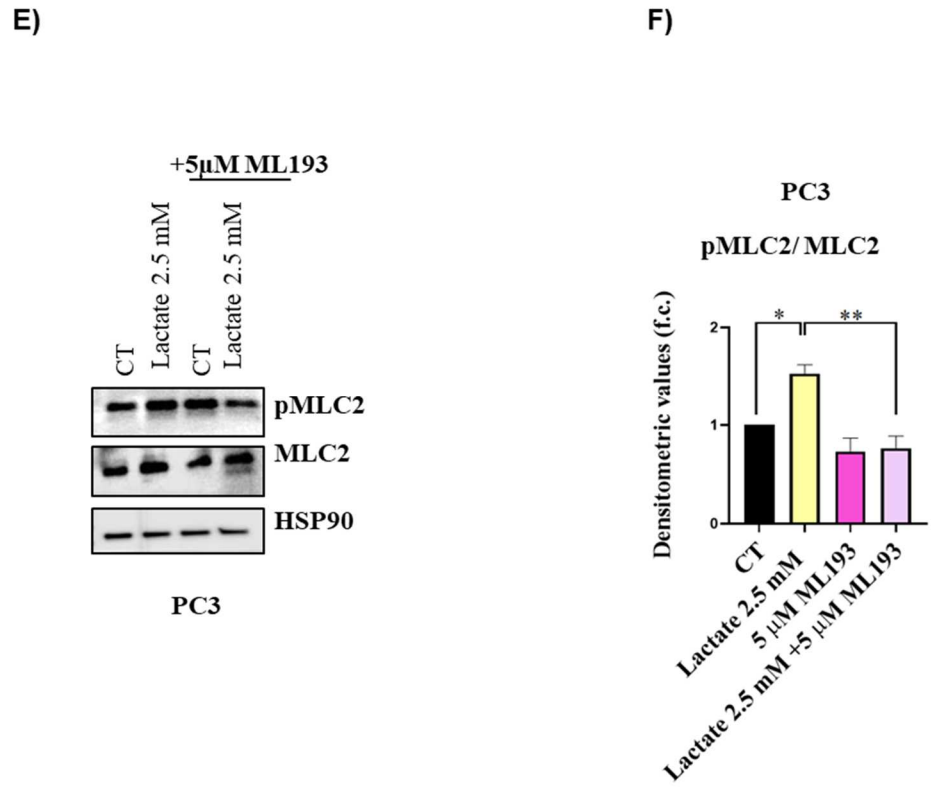


Fig.13 Effects of low doses of lactate on GPR55 expression and activity in PCa cells

A) qRT-PCR for the evaluation of GPR55 gene expression in DU145 cells treated with 2.5 mM or 20 mM lactate (48 hours). B) Immunoblot representation of GPR55 expression in PC3 cells stimulated with 2.5 mM lactate (48 hours). C-E) Representative WB showing pMLC2 expression in DU145 cells (C) and PC3 cells (E), upon 15-minute lactate conditioning (2.5 mM) in presence/ absence of GPR55 inhibition with ML193 (5 µM). pMLC2 was normalized on the total of MLC2. HSP90 was chosen as housekeeping gene. D-F) Densitometric analyses of 3 independent WB experiments referred to the pMLC2/MLC2 expression in DU145 cells (D) and PC3 cells (F) treated with 2.5 mM ± 5 µM ML193. Values are expressed in f.c. All data are represented as mean ± SEM of three independent experiments. One-way ANOVA; Tukey's/Dunnett's corrected; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

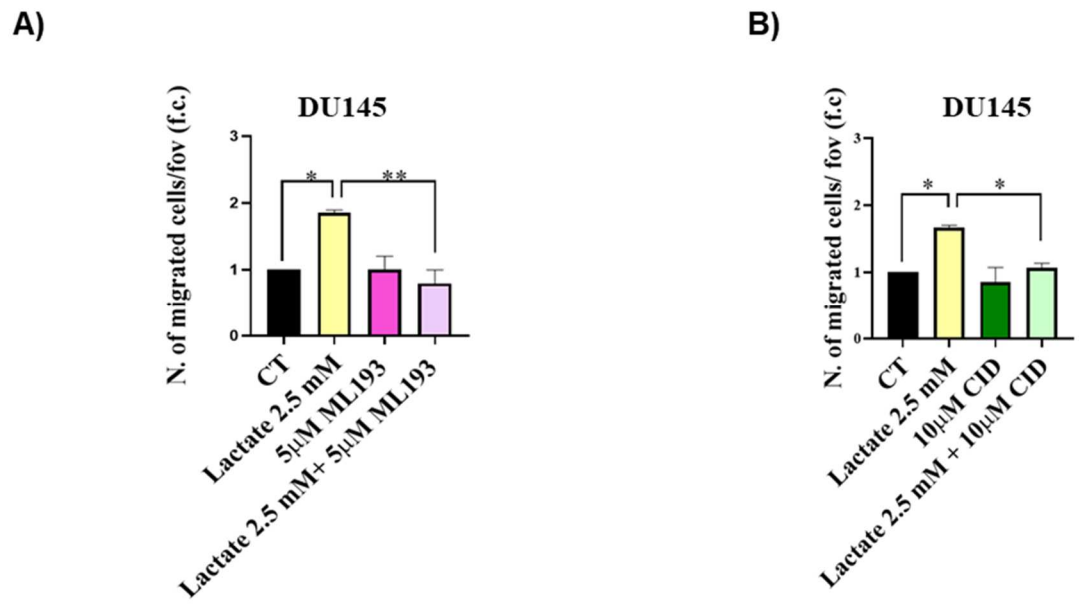
Abbreviations: reverse transcription-quantitative polymerase chain reaction (qRT-PCR), Western blot (WB), control condition (CT), phosphorylation of the myosin light chain 2 (pMLC2), myosin light chain 2 (MLC2), heat shock protein 90 (HSP90), G coupled protein receptor 55 (GPR55), non-significant (ns), fold change (f.c.)

4.3 Lactate orchestrates cellular motility processes by activating GPR55

Given the previous insights, we investigated the ability of lactate to elicit motility of PCa cells, under the influence of GPR55. PCa cells were stimulated with the low lactate dose in presence/ absence of ML193 (5 µM) and a second selective inhibitor of GPR55, CID16020046 (10 µM). Upon lactate conditioning, PCa cells exhibited a higher tendency to migrate in contrast to the effects given by ML193 and CID16020046 (Fig. 14 a-b). Moreover, stimulation with the GPR55 endogenous ligand LPI (10 µM) confirmed the involvement of GPR55 in the acquisition of migratory traits elicited in PCa (Fig.14 c).

GPR55 promotes migration and invasion of cancer cells, likely associated to the Rho A/ ROCK pathway [315]. Remarkably, the treatment with lactate of DU145 cells activated Rho A, favoring its GTP-bound form (Rho A-GTP). Whereas, ML193 partially blocked the lactate-mediated Rho A signalling (Fig 14 d).

Taken together, these data shown that lactate can induce molecular changes related to the GPR55-associated Rho A/ pMLC2 pathways, thereby contributing to the establishment of migratory phenotypes in PCa cells.



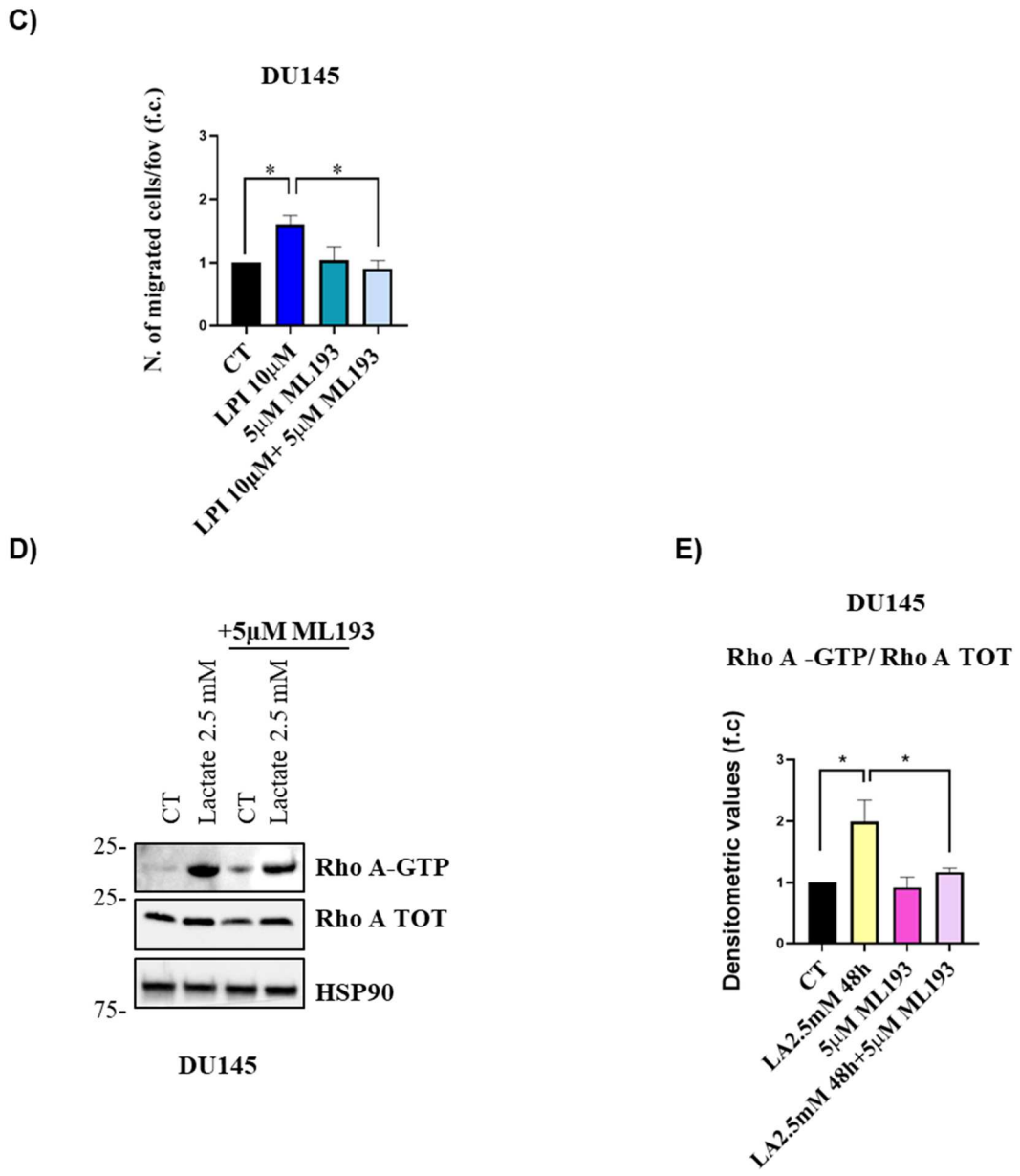


Fig.14. Lactate-derived GPR55 activation of migratory phenotypes in PCa cells

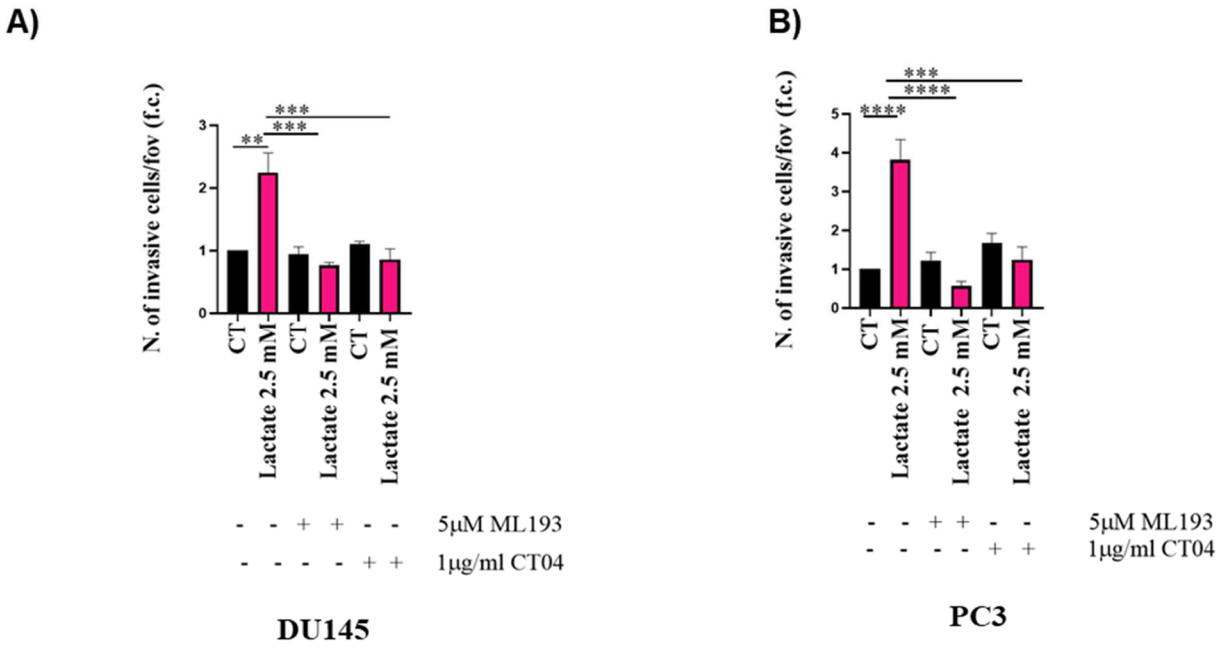
A-B) Migration assay of DU145 cells following 2.5 mM lactate stimulation (48 hours) ± ML193 (5 µM) (A) or CID 16020046 (10 µM) (B). C) LPI stimulation (10 µM) of DU145 cells (48 hours) ± ML193 (5 µM) for the evaluation of cancer cells' migratory abilities. D) Immunoprecipitation assay of Rho A-GTP and WB analyses representing the levels of Rho A-GTP over the totality of Rho A (Rho A TOT) in DU145 cells treated with 2.5 mM lactate (48 hours) ± ML193 (5 µM). HSP90 was chosen as housekeeping. E) Densitometric analyses of 3 independent WB experiments referred to the Rho A- GTP/Rho A TOT expression in DU145 cells treated as indicated in D)

All data are represented as mean ± SEM of three independent experiments. One-way ANOVA; Tukey's/Dunnett's corrected; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Abbreviations: Western blot (WB), fold change (f.c.), control condition (CT), CID 16020046 (CID), lysophosphatidylinositol (LPI), heat shock protein 90 (HSP90), Ras homolog family member A -guanosine-5'-triphosphate (Rho A- GTP), totality of Rho A (Rho A- tot), number (n.), G coupled protein receptor 55 (GPR55)

4.4 GPR55 activation by lactate triggers the acquisition of amoeboid-like phenotypes in PCa cells

The phosphorylation of MLC2 and activation of Rho A represent typical events underlying amoeboid motility, a specific type of cell movement which does not necessarily depend on matrix proteolytic activity, unlikely mesenchymal migration [709]. Once noted lactate activated pMLC2 and Rho signalling, we endeavored to study amoeboid-like traits associated to lactate stimulation, utilizing two inhibitors: i) Rho inhibitor (CT04), which acts on the Rho/ ROCK pathway and it is descriptive of amoeboid-like status; and ii) a broad-spectrum of MMPs' inhibitor, Marimastat, which discriminates a mesenchymal-like cell movement. Upon lactate stimulation, PCa cells were allowed to invade in the presence/ absence of ML193 (5 μM) and/ or CT04 (1μg/ml) / Marimastat (10 μM). Remarkably, as illustrated in Fig. 15 a-b, we observed that CT04 hindered the cellular movement induced by lactate in PCa cells, similarly to what was observed with ML193. However, Marimastat did not alter the effect of lactate in tumor cells, unlike the inhibition of GPR55 (Fig. 15 c). In conclusion, the inability of Marimastat to counteract lactate-induced invasiveness, conversely to ML193, and the comparable reduction of cell migration achieved with CT04 and ML193 after lactate treatment, suggested that lactate may be a promoter of an amoeboid–like phenotype in PCa cells through the activation of GPR55.



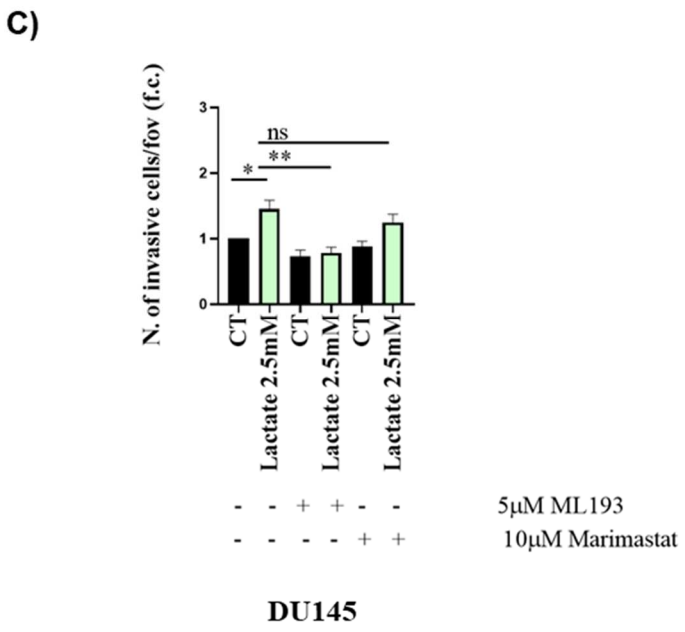


Fig15. Effects of lactate on the amoeboid-like motility characteristics of PCa cells through the involvement of GPR55

A-B) Invasion assay of DU145cells (A) and PC3 cells (B) upon 2.5 mM lactate treatment (48 hours) ± ML193 (5 μM) or the Rho inhibitor, CT04 (1μg/ml). C) Invasion assay of DU145 cells treated 2.5 mM lactate (48 hours) ± ML193 (5 μM) or Marimastat (10 μM). All data are represented as mean ± SEM of three independent experiments. One-way ANOVA; Tukey's corrected; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Abbreviations: number (n.), control condition (CT), fold change (f.c.), G coupled protein receptor 55 (GPR55)

5. Discussion

The accumulation of lactate in solid tumors and extracellular environment is considered early evidence of malignant development, associated with a poor prognosis [710]. As the tumor grows, the rapid increase of tumor mass generates a greater demand for oxygen and energy. To overcome the establishment of a pseudo-hypoxic and nutrient- poor environment, cancer cells and accessory cells within the TME activate adaptive processes, such as neovascularization or metabolic reprogramming, aimed to re-oxygenate and nutritive feed the tumor tissue. Lactate production by tumor cells and stromal cells is a strategic choice to prevent intracellular acidity derived from the high cellular glycolytic rate in response to low oxygen levels [658]. Moreover, even under normoxic conditions, the production of lactate may be favored to support cellular anabolic processes that ensure tumor progression [613, 614]. Within the TME, lactate thus represents a cellular energetic source [645]. However, it is to date known that the metabolite fulfills broader functions that implicate the regulatory mechanisms of cellular epigenetic signature, immunosuppression, and signaling functionalities through receptors [653, 658],[666],[667].

The ECS was discovered in the late '90s by Prof. Mechoulam, as a system structured by eCBs (e.g. AEA, 2-AG), receptors, and enzymes responsible for the synthesis / hydrolysis of eCBs [711]. The implications that the ECS has in the context of tumor pathology, have been documented for various tumor subtypes, as indicated in Tab. 6-9 and in Tab. 11-13. In particular, in PCa, CB1R and CB2R were found expressed in androgen-resistant/ -sensitive cell lines [26, 266], with a reported involvement in cell proliferation, apoptosis/ necrosis, neuroendocrine differentiation and spheroid formation [26, 266, 269, 290, 291]. On the other hand, TRPV1 was detected in PC3 cells and LNCaP cells, mainly regulating proliferation, ceramide production and the levels of the androgen receptor [351, 352]. Regarding GPR55, DU145 cells and PC3 cells expressed the receptor, whose activation influenced tumor cell growth [308, 317, 318, 367]. Moreover, also LNCaP cells were found to express GPR55 [368].

Deregulations adopted by environmental lactate on the ECS elements of cancer cells have not been addressed in the literature before. Considering a PCa cell model, our study thus focused on investigating potential changes induced by lactate in the expression/ activity of the receptor components of the ECS in tumor cells; then examining regulatory mechanisms and downstream phenomena resulting from the lactate derived- ECS modulation.

Lactate concentration within the TME ranges from 10 to 40 mM [650, 651]. Moreover, measurements performed on PCa biopsies derived from patients, shown lactate dosages at values of 20 mM [637]. From our experiments, the stimulation with lactate (20 mM) in DU145 cells pointed out a lack of variation in the expression of CB1R and CB2R, a down-regulation of TRPV1, contrary to a significant over-expression of GPR55 with respect to the CT condition (Fig.11).

GPR55 is a GPCR highly present in many cancers (Tab. 8) and with a predominantly pro-tumorigenic role [304]; [305]; [287, 362].One of the phenotypes closely linked to the functioning of GPR55 concerns the induction of cell migratory and invasive capabilities which trigger the process of tumor

metastasis. It is indeed reported that GPR55 is involved in the promotion of migration and adhesion abilities of CRC cells, in the anchorage-independent growth of skin cancer cells, and in the formation of filopodia which accompanies invasiveness of liver and pancreatic cancer cells [357, 358]; [195]; [361]. In keeping, GPR55 expression correlated with lymph node metastasis in NSCLC and with the reduced metastasis-free survival of BCa patients [315].

Within the TME, lactate strongly sustains the metastatic potential of tumor cells, as demonstrated in head/ neck carcinomas, BCa, NSCLC, and particularly in PCa [712]; [713]; [714, 715]. Accordingly, in vivo evidence highlighted that the neutralization of the external acidity of tumors is an effective strategy for the prevention and inhibition of metastasis [716]. Lactate elicited cells' spread with different mechanisms of action. In PCa, for instance, lactate produced by CAFs boosted the formation of anchorage-independent tumor clusters, transendothelial migration in vitro and in vivo (via a collagen dependent mechanism) and it enhanced tumor cells' invasive skills by engaging the SIRT1/PGC-1α axis [638, 707].

Exploring a GPR55's role in the phenomena of lactate-induced invasiveness within PCa cells, we treated DU145 cells and PC3 cells with lactate in presence/ absence of GPR55 inhibition. The pMLC-MLC signalling is one of the molecular events downstream of GPR55 and closely associated with invasiveness [498]. This pathway triggers the myosin/ actin interaction, necessary for the cytoskeleton contraction during cell movement. Moreover, in BCa, the inhibition of GPR55 activity/ expression in MCF-7/ MDA-MB-231 cell lines prompted phosphorylation of MLC with a substantial reduction in cell motility in vitro, and lung metastasis in vivo [498]. In our experimental model, lactate stimulation at brief time point (15 minutes) triggered the pMLC2-MLC2 pathway, by activating GPR55 (Fig. 12 a-d). The short activation time of GPR55, suggested a role for lactate as a direct signalling molecule for GPR55, as already demonstrated by the literature for GPR81 and GPR132 [670]; [679].

GPR81 is the main recognized sensor receptor of lactate, principally expressed in adipose tissue, but also in brain, immune system, intestine, kidney, and many other tissues [671, 717]. The receptor works in maintaining energy balance, and in adipose tissue it inhibits lipolysis thus supporting the anti-lipolytic action of insulin [651, 670]. The involvement of GPR81 in tumors is not very clear to date. However it is known that it can control survival, growth and metastatic features of tumor cells, as demonstrated in BCa, pancreatic and lung cancers [587; 589]. Moreover, other functions related to GPR81 concern the immunosurveillance [590, 591]. As GPR81, also GPR132 has not been in-deep investigated in tumors. It actively modulates cell cycle, proliferation, differentiation and immunity, as shown in leukemia and BCa [677, 679].

GPR81 can sense lactate at low concentrations ranging from 1 to 5 mM [708]. At a dose of 2.5 mM lactate, we observed a rapid activation of GPR55, which was sustained through the signaling of pMLC2-MLC2 in DU145 and PC3 cells (Fig.13 c-f). Hence, as the high dose, also the lower dose of lactate was able to activate GPR55. Moreover, no variations in the expression of GPR55 were observed following the treatment with 2.5 mM lactate, in contrast to what was obtained with the high dose stimulation (Fig. 13 a-b).

Upon lactate treatment, DU145 cells were promoted in migration, which was instead hindered by the blockage of GPR55 (Fig.14 a-b). Lastly, the involvement of GPR55 in the migration phenomena of PCa cells was confirmed by the usage of the endogenous ligand of GPR55, LPI (Fig. 14 c).

In HEK-293 cell lines engineered to stably express recombinant human GPR55, the activation of GPR55 led oscillations in Ca^{2+} currents which were dependent on the Gα13 coupled protein and that required Rho A activity [718]. The Rho A pathway is thus today a well-known downstream signaling of GPR55 [273]. In TNBC, the GPR55-dependent Rho A signalling mediated the metastatic potential of MDA-MB-231 cells [315]. Moreover, Rho activity in human neutrophils culminated in efficient migratory skills through the engagement of GPR55 [431]. Rho A belongs to the family of small GTPases, including Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homolog (Cdc42). Activation of GTPases involves GTPase-activating proteins (GAPs) which hydrolize the GTP bound to GTPases, rending them inactive, until a guanine nucleotide exchange factor (GEF) makes a guanosine diphosphate (GDP) -GTP exchange. The stimulation with low concentration of lactate of DU145 cells allowed the switch from the GDP-bound form of Rho A into its GTP-bound active form, dependently on the functioning of GPR55 (Fig. 14 d-e).

Abnormal cell migration is characteristic of cancer cells. Cells can move through different migratory strategies: a **collective migration** which required coordinated cell-cell connections, and **individual types of cell movement**, typical of mesenchymal and amoeboid cells, which rely on dynamics junctions with the ECM [719] Mesenchymal motility is characterized by elongated cell morphology, and it uses proteases to degrade the ECM (e.g. MMPs). Moreover, mesenchymal cells need a high degree of adhesion to bind the ECM. Conversely, in the amoeboid phenotype, cells squeeze through the mesh of the ECM, without necessary matrix degradation, acquiring a round shape and forming protrusions known as “blebs” [720, 721]. Mesenchymal cells can acquire amoeboid features in a process called mesenchymal-to-amoeboid transition (MAT). During MAT, cell–cell and cell–ECM interaction decrease, and molecular signals, like Rho/ ROCK and MLC2, are promoted [709]. Rho–ROCK pathway directly controls MLC2 activity and it is now considered an epicenter of the amoeboid state [709]. Amoeboid cells show enhanced ability to metastasize in different tumor subtypes, as observed in HCC, BCa, sarcoma, chronic lymphocytic leukemia, head/ neck squamous cell carcinoma and, in particular in PCa [709]. Moreover, multiple stimuli within the TME, such as external soluble factors and receptor-driven signalling, were demonstrated to converge in the regulation of amoeboid cancer cell migration. Indeed, the amoeboid behavior can be considered an adaptation to the tumor environmental conditions, such as of the hypoxia which occurs during the increase of tumor mass [722].

While the activation of Rho A and pMLC2-MLC2 suggested a new role for lactate in promoting amoeboid-like motility by engaging GPR55 in PCa cells, we deepened the study of the amoeboid phenotype by using a Rho inhibitor (CT04) and a broad spectrum MMPs inhibitor (Marimastat). The inhibition of Rho activity, a pivotal event in amoeboid motility, effectively countered the lactate-induced invasive skills developed by PCa cells, similarly to what was observed with GPR55 inhibition

(Fig. 15 a-b). Conversely, the inhibition of protease activity with Marimastat, a central figure in mesenchymal movement, did not reduce the effects of lactate (Fig. 15 c).

Overall, these data propose a novel contribution of lactate to PCa malignancy. Particularly, we suggested a potential role for lactate in regulating the expression and activity of GPR55 in PCa cells and in driving invasiveness. The invasive skills acquired by PCa cells seemed to be due to the GPR55 downstream Rho A- pMLC2 signalling, which endows tumor cells with amoeboid-like motility traits. Although results are promising, further studies will be needed to better characterize the role of lactate in amoeboid invasiveness mechanisms of PCa cells.

In the presented research work, we focused our attention in two androgen-resistant PCa cell lines, which are otherwise most dealt within the literature for the study of the ECS in PCa.

As already mentioned, there are no many information about androgen-sensitive cells, although it is known that they can express GPR55 and the selective inhibition of the LPI transporter (ATP binding cassette subfamily c member 1, ABCC1) reduces tumor cell growth and sensitizes to chemotherapy [368]. Unfortunately, the relationship between GPR55 and androgen response, as well as the androgen receptor expression/ activity have never yet been addressed.

Beyond physiological functions, mainly related to the maturation of sexual organs and traits during puberty, the androgen receptor plays a key role in the development of PCa. During the early stages, PCa is dependent on androgens, which positive influence growth and progression. Although hormonal therapies (consisting in a reduction of androgen levels) led a recovery of these initial phases of PCa, often relapses are observed due to the development of drug resistance mechanisms and receptor mutations which higher receptor sensibility to low amounts of hormones [723, 724].

Androgen receptor activity in PCa enhances phenomena of metabolic reprogramming, interesting metabolic pathways such as aerobic glycolysis, mitochondrial respiration, fatty acid and de novo lipid synthesis [725]. A link between androgen receptor and glycolysis has been well established and it was observed that androgen stimulation can higher glucose compsumption, as well as lactate production/ exploitation by cancer cells through MCT4 [726]. Having previously mentioned that LNCaP cells express GPR55, we could assume an association between androgenic stimulation, lactate production and the activation of GPR55 in those cells. This could help in understanding potential events involving GPR55 to the responses to the hormonal therapies, and we could use anti-androgens such as enzalutamide, bicalutamide and flutamide to investigate how the functioning of GPR55 varies in this context.

The involvement of GPR55 in tumor invasiveness phenomena should also be investigated in androgen- sensitive cells, since how the ECS influences such phenotype in these models has not been fully elucidated yet. Nithipatikom et al. reported that LNCaP cells did not respond to anti-invasiveness changes given by high endogenous 2-AG, contrary to what DU145 and PC3 cells did [727]. Moreover, Pietrovito et al. demonstrated that LNCaP cells had an increased expression of CB1R and CB2R than DU145 cells and PC3 cells, while TRPV1 was lower [706]. Given these considerations, it might be that canonical CBRs and related ligands play a fundamental role in the migratory ability of

androgen-responsive cells, although the involvement of other receptors, such as GPR55, cannot be excluded.

A limitation of this project is certainly that of not investigating the role of the ECS in the cross-talk mechanisms between stromal and epithelial cells. As before discussed, CAFs are the stromal cells mostly present in the TME of PCa and the main productive resource of lactate. Experiments performed by using fibroblasts, demonstrated that CAFs actively support aggressiveness of both androgen resistance and sensitivity PCa cell lines [638, 728]. Therefore, the knowledge of a possible contribution of GPR55 to these processes would be very important for the future.

In relation to androgen-sensitive cells, Thalmann et al. demonstrated that fibroblasts deriving from different zones of radical prostatectomy in PCa, when co-cultured with LNCaP cells, sped up growth capability in an anchorage-dependent or independent modality and up-regulated PSA in tumor cells. Moreover, CM derived from peripheral fibroblasts increased sensitivity to dihydrotestosterone stimulation in LNCaP cells. Finally, fibroblasts affected the progression of LNCaP cells to a castration resistance phenotype, with a strong impact on cell metastatic potential [728].

In conclusion, it is definitely desirable to extend the study of GPR55 to both androgen responsive and stromal cells (such as CAFs) in order to have more insights on androgenic impact, response to therapies, and tumor-stroma interactions with reference to the receptor.

Moreover, having found no correlation between GPR55 and PCa patient survival by using databases such as The cancer genome atlas (TCGA) (maybe because GPR55 is low express in PCa), we might conduce immunohistochemistry analyses on tissue microarrays (TMAs) to delve into this conclusion.

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