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Targeting Unique Metabolic Signatures in Gastrointestinal Cancers with Selective Full Agonists of Cannabinoid Receptors

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Dedication

To my mother,
For giving me life, and her mitochondria!
Her resilience shall forever be reflected in all of my endeavors.

Contents

Abstract

External stimuli significantly influence the gastrointestinal (GI) tract and employ various metabolic pathways to maintain physiological homeostasis. Consequently, gastric and colon cancers, shaped by their differing tissue environments, genetic profiles, and biological behaviors, exhibit unique metabolic characteristics that result in distinct metabolomic signatures, affecting their growth, spread, and responses to treatment. Reprogrammed metabolism and rewiring metabolic networks are widely recognized hallmarks of oncogenic progression, and targeting these alterations is increasingly considered a therapeutic strategy to enhance current chemotherapy and immunotherapy approaches.

Cannabinoids have garnered attention for their potential antitumor properties in various cancers, including gastric and colon cancer. Research on the effects of cannabinoids in these contexts has highlighted their therapeutic role in inhibiting tumor growth and viability through both cannabinoid receptor-mediated and non-cannabinoid receptor-mediated mechanisms. However, a comprehensive study investigating the effects of cannabinoids on the metabolism of gastric and colon cancers has yet to be conducted.

The study began with the profilation of the metabolomes of various gastric (AGS and MKN45) and colon (HCT116 and HT29) cancer cells. NMR-based OPLS-DA was applied to distinguish the metabolic profiles between gastric and colon cancer cells. The model exhibited a significant class separation, indicating that these two types of cancer rely on a distinct array of metabolites to meet their high energy demands. When we examined the effect of two cannabinoids (ACEA, as a selective CB1 full agonist, and JWH133 as a selective CB2 full agonist) on the main metabolic pathways in AGS and HCT116 cells, we noted differences in how the two cancer cells responded metabolically to the treatments. [redacted] Additional opposing effects upon treatments in

AGS and HCT116 cells were observed on the mRNA expression of the mitochondrial isoform enzyme regulating the glucose-alanine cycle GPT1 and the serine-glutamine transporter ASCT2. Notably, ACEA and JWH133 reduced cell growth – although with a different extent – migration, and stemness marker gene expression, irrespective of the cancer type. Finally, there was a difference in how treatments impacted homotypic tumor spheroid formation. In AGS and HCT116 cells, JWH133 strongly impacted both the size and the number of spheroids, while ACEA was able to reduce spheroids diameter only in HCT116 cells.

The results suggest that cannabinoids can interfere with the distinct metabolic strategies used by GI tumors to evade control over cell fate. Further analyses are needed to address the therapeutic potential of these molecules as specific metabolic inhibitors for GI cancer therapy.

Keywords: gastrointestinal cancer, colorectal cancer, gastric cancer, endocannabinoid system, metabolomics.

List of Abbreviations

2-AG	2-Arachidonoylglycerol
2-AGE	2-Arachidonyl glyceryl ether
2-DG	2-Deoxy glucose
AA	Arachidonoyl acid
ABHD	Alpha/beta-Hydrolase domain
ACEA	Arachidonyl-2'-chloroethyl amide
ACPA	Arachidonylcyclopropylamide
ADP	Adenosine diphosphate
AEA	Anandamide
AFP	Alpha-fetoprotein
Akt	Protein kinase B or PKB
AMP	Adenosine monophosphate
AMPK	5' AMP-activated protein kinase
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CARD9	Caspase recruitment domain-containing protein 9
CB1R	Cannabinoid receptor type 1
CB2R	Cannabinoid receptor type 2
CB	Cannabinoid
CBR	Cannabinoid receptor
CD36	Colorectal cancer
CEA	Carcinoembryonic antigen
CEACAM5	Carcinoembryonic antigen-related cell adhesion molecule 5
CNS	Central nervous system
COX	Cyclooxygenase
CRC	Colorectal cancer
CXCR4	C-X-C chemokine receptor type 4
DA	Dopamine
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DNA	Deoxyribonucleic acid
EA	Cis-11-eicosenoic acid

EC	Esophageal adenocarcinoma
ECAR	Extracellular acidification rate
eCB	Endocannabinoid
ECS	Endogenous cannabinoid system
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FA	Fatty acid
FAAH	Fatty-acid amide hydrolase 1
FACS	Fluorescence-assorted cell sorting/ Flow-cytometry
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
GABA	Gamma aminobutyric acid
GC	Gastrointestinal cancer
GI	Gastrointestinal
GPCR	G protein-coupled receptor
GPR55	G protein-coupled receptor 55
HCC	Hepatocellular carcinoma
HK	Hexokinase
HFD	High-fat diet
HU210	1,1-dimethylheptyl- 11-hydroxytetrahydrocannabinol
IBD	Inflammatory bowel disease
IFN-γ	Interferon-gamma
IL	Interleukin
JNK	c-Jun N-terminal kinase
JWH-133	Dimethylbutyl-deoxy-delta-8-THC
LC-MS	Liquid chromatography-mass spectrometry
LOX	Lipooxygenase
LPS	Lipopolysaccharide
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MSEA	Metabolite Set Enrichment Analysis

mTOR	Mammalian target of Rapamycin
mtROS	Mitochondrial reactive oxygen species
NADA	N-Arachidonoyl dopamine
NAM	Negative allosteric modulator
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
NAT	N-acetyltransferase
NMR	Nuclear magnetic resonance
OA	Oleamide
OCR	Oxygen consumption rate
OEA	Oleoylethanolamide
OPLS-DA	Orthogonal Partial Least Squares Discriminant Analysis
OXPHOS	Oxidative phosphorylation
p53	Tumor suppressor protein 53
PC	Pancreatic cancer
PCA	Principal Component Analysis
PD	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PEA	Palmitoylethanolamide
PEP	Phosphoenol pyruvate
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGE2	Prostaglandin E2
PI3K	Phosphatidyl inositol 3-kinase
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptor
PTEN	Phosphatase and tensin homolog
PUFA	Polyunsaturated fatty acid
qPCR	Quantitative polymerase chain reaction
ROS	Reactive Oxygen Species
SCD	Stearoyl-CoA desaturase
SPM	Specialized pro-resolving mediator
STAT3	Signal transducer and activator of transcription 3
SYK	Tyrosine-protein kinase
TCA	Tri-carboxylic acid cycle
THC	Delta-9-tetrahydrocannabinol
TNFα	Tumor necrosis factor-alpha

TRP	Transient receptor potential channel
TRPA1	Transient receptor potential cation channel subfamily A, member 1
TRPM8	Transient receptor potential cation channel subfamily M (melastatin), member 8
TRPV1	Transient receptor potential cation channel subfamily V, member 1
VEGF	Vascular endothelial growth factor
VTA	Ventral tegmental area
WB	Western Blot
WHO	World Health Organization

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Introduction

1

Gastrointestinal carcinoma: molecular basis and metabolic features

Gastrointestinal (GI) cancers represent a group of malignancies affecting the digestive system, including esophageal adenocarcinoma (EC), gastric cancer (GC), colorectal cancer (CRC), hepatocellular carcinoma (HCC), and pancreatic cancer (PC)[1]. In 2018, the World Health Organization (WHO) reported approximately 3.5 million new cases of GI cancer worldwide[2]. GC ranks as the fifth most common cancer and the third leading cause of cancer-related deaths, while CRC is the third most prevalent cancer globally and the second leading cause of cancer death[3].

The etiology and clinical management of gastrointestinal cancers are varied, leading to significant differences in their origin, progress, prognosis, and treatment response. Given their clinical importance, this thesis specifically focuses on gastric and colorectal cancer.

Gastric tumors can develop in any part of the stomach and tend to spread rapidly throughout the stomach, impacting nearby organs, such as the small intestines, adjacent lymph nodes, liver, and pancreas, and can invade the colon[4]. The tumor progression often involves, and usually develops through a series of changes, starting from potentially reversible precancerous metaplasia (arising from zymogen-secreting chief cell plasticity in response to severe gastric injury)[5–7] followed by dysplasia and, ultimately, adenocarcinoma[8]. These cellular alterations are characterized by increased rapidly dividing cells and alterations in cell adhesion and tight junction molecules, like carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5) and claudin 3, which indicate a loss of cell polarity and asymmetric cell division. Such changes emerge as significant markers of progression from metaplasia to dysplasia[9], the latter representing localized neoplastic (abnormal tissue growth) lesions that pose the highest risk for the development of GI cancer. Therefore, early surgical resection becomes critical for treating

GC. However, because many cases are diagnosed in advanced stages, systemic therapy, often involving a combination of multiple cytotoxic chemotherapeutic agents, is typically required[4].

Shifting the focus to colorectal tumors, this encompasses cancers of the colon, rectal, and anus[10]. Patients with long-standing ulcerative colitis are at elevated risk of developing CRC[11], with detection estimated to take about 14.2 years from its onset[11]. Increasing evidence suggests that most cases of CRC start from precancerous lesions or adenomatous polyps. At the cellular level, CRC demonstrates considerable biological diversity, with dysplastic lesions known as aberrant crypt foci, appearing as early indicators. While early-stage CRC grows slowly and is confined to the mucosa, its growth rate accelerates as the tumor invades the submucosa, typically taking 5-15 years for a small adenoma to become malignant[11]. These lesions can progress to adenomatous polyps and eventually develop into invasive cancer. The liver is the predominant site for CRC metastasis, primarily due to the direct anatomical connection between the intestine and the liver via the portal vein. This connection facilitates the development of CRC liver metastasis, supported by the liver's unique metabolic and immune microenvironment of the liver[12]. In the case of a fatty liver, the production of hepatocyte-derived extracellular vesicles is enhanced, further promoting the progression of CRC liver metastasis by augmenting Yes-associated protein signaling and the immunosuppressive tumor microenvironment[13]. Notably, approximately 70% of CRC patients will experience progression to develop liver metastasis, representing a significant factor contributing to mortality[14].

Several risk factors are associated with gastric cancer, including hyposecretion of gastric acid and corpus-predominant gastritis. The risk for GC is significantly heightened by chronic infection with *Helicobacter pylori*, which progresses through atrophic gastritis and intestinal metaplasia over approximately ten years[15]. In *H. pylori*-induced infection, the Hippo pathway signaling plays a critical role in regulating tissue homeostasis, often dysregulated by accelerating GC development through the Yes-associated protein 1, a key effector of the Hippo pathway[16]. Dietary factors are additional important factors that can significantly promote the occurrence and development of gastrointestinal tumors. Particularly, a high-fat diet (HFD) contributes to an imbalance in the stomach's microbial population (marked by an increase in *Lactobacillus*), leading to intestinal metaplasia and elevated production of leptin, phosphorylated leptin receptor, and signal transducer and activator of transcription 3 (STAT3)[17]. Overactivation of leptin signaling induces precancerous gastric lesions by inhibiting cytokine signaling 3 suppressors in GI epithelial

cells[18]. Moreover, HFD increases the level of O-Glc-N-acylation, which promotes the transcriptional activation of cluster of differentiation 36 (CD36), leading to increased fat uptake in GC cells, and fostering a vicious cycle that ultimately promotes metastasis[19].

For colorectal cancer, major risk factors include tobacco[20] and alcohol consumption[21], high consumption of red and processed meat[22,23] accompanied by a sedentary lifestyle[24], and metabolic abnormalities such as diabetes[10,25]. Traditional screening methods, including fecal occult blood tests and endoscopic procedures like colonoscopy, can effectively detect and remove precursor lesions[10]. Despite these efforts, prognostic outcomes remain poor, suggesting a 5-year survival rate of <15%[26]. This highlights the significant clinical challenge of early detection as a major clinical challenge, particularly for the advanced and metastasized forms of the disease. A substantial proportion of CRC patients present with unresectable tumors and nearly 50% of all diagnosed cases likely develop metastases[27,28]. Treatment options for such advanced cases often involve preoperative radiotherapy, local ablative therapies for metastases, palliative chemotherapy, and immunotherapy[15].

Tumor markers are crucial biochemical substances that can be detected in the blood, urine, or tissues of individuals. Their presence or elevated levels often indicate the presence of cancer or certain tumor-related conditions. These markers play a vital role in cancer diagnosis, prognosis, and treatment monitoring, as they provide valuable insights into tumor biology and assist clinicians in making informed decisions about patient management. By facilitating early detection, tracking disease progression, and evaluating therapeutic responses, tumor markers significantly contribute to improving patient outcomes and personalizing treatment strategies in oncology. In the context of gastric and colorectal cancers, specific tumor markers such as alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) are notably elevated in patients[11]. These markers serve as important biomarkers for diagnosis, monitoring disease progression, and assessing treatment response.

The metabolic processes in GI cells are complex and diverse, with cancer cells mainly relying on aerobic glycolysis - commonly referred to as the Warburg effect – and increasing lipogenesis to fuel cell growth (as indicated by **Figure 1**). The carcinogenic process in the GI tract typically involves the activation of oncogenic genes, which initiates signaling pathways, including the RAS signaling pathway[29]. Different metabolites are present in gastric mucosa at different cancer stages. Glycolytic metabolites are prevalent in metaplastic glands, whereas fatty acid (FA) metabolites are concentrated in dysplastic

glands. Notably, high levels of long-chain monounsaturated FAs, such as cis-11-Eicosenoic acid (EA), are observed in the transitioning cell zone of dysplastic glands. This shift from glycolysis to fatty acid metabolism signals the progression to metaplasia (as indicated by **Figure 1**). FAs can be desaturated by different enzymes such as the stearoyl-CoA desaturases (SCDs). The increased activity of SCDs in GC contributes to metabolic changes as metaplasia progresses to dysplasia. Additionally, EA, produced through SCD-dependent FA desaturation, supports rapid GC cell proliferation and survival in dysplastic cells[9]. Meanwhile, colorectal tumorigenesis is associated with genetic alterations arising from two potential oncogenic pathways: the adenoma-carcinoma sequence pathway and the *de novo* carcinogenesis[11]. At the molecular level, the progressive accumulation of genetic changes and epigenetic alterations may induce the pathological transformation of normal colonic epithelium into malignant tumors[30–32] (as indicated by **Figure 1**). The colon hosts a significant amount of GI microflora, crucial in forming feces and synthesizing essential vitamins[33,34]. However, changes to the gut microbiota are commonly observed in CRC, where reduced diversity and altered community structure are prevalent. A decrease in beneficial bacteria and an increase in potentially pro-cancer bacteria are typical in CRC (as indicated by **Figure 2**). Specific bacterial groups are more frequently detected in premalignant adenomas and CRC. Specific bacterial groups are more frequently detected in premalignant adenomas and CRC. Intestinal microbes such as *Fusobacterium nucleatum*[35,36], *Escherichia coli*, and *Bacteroides fragilis*[37,38] are recognized as inducers of adenoma, an important precancerous lesion in CRC. For instance, *E. coli* infection can promote the occurrence and development of CRC by activating the P38 mitogen-activated protein kinase (MAPK) pathway in macrophages to up-regulate cyclooxygenase-2 (COX-2) expression[39]. In terms of intestinal microbial functions, the production of lipopolysaccharide (LPS) (*Mucispirillum schaedleri*), butyrate metabolism (*Lachnospiraceae bacterium A4*), and heightened oxidative metabolites (*Helicobacter hepaticus*) are related to the activation of carcinogenic pathways, changes of tumor immune microenvironment, deoxyribonucleic acid (DNA) damage[40] (as indicated by **Figures 1** and **2**). On the other hand, Conversely, commensal gut fungi activate immune responses through C-type lectin receptors, triggering downstream tyrosine-protein kinase (SYK) and caspase recruitment domain-containing protein 9 (CARD9) pathways that promote inflammasome activation, interleukin-18 (IL-18) maturation, and anti-tumor T-cell responses, thereby limiting CRC progression[41]. An imbalance in intestinal microbes can lead to colorectal inflammation, which may ultimately result in CRC[42] (as indicated by **Figure 2**). This shift in the microbial landscape is associated with a diminished host antibacterial defense mediated by Paneth cells. This leads to reduced recruitment of

dendritic cells and decreased expression of major histocompatibility complex (MHC) class II molecules in intestinal-associated lymphoid tissues[43].

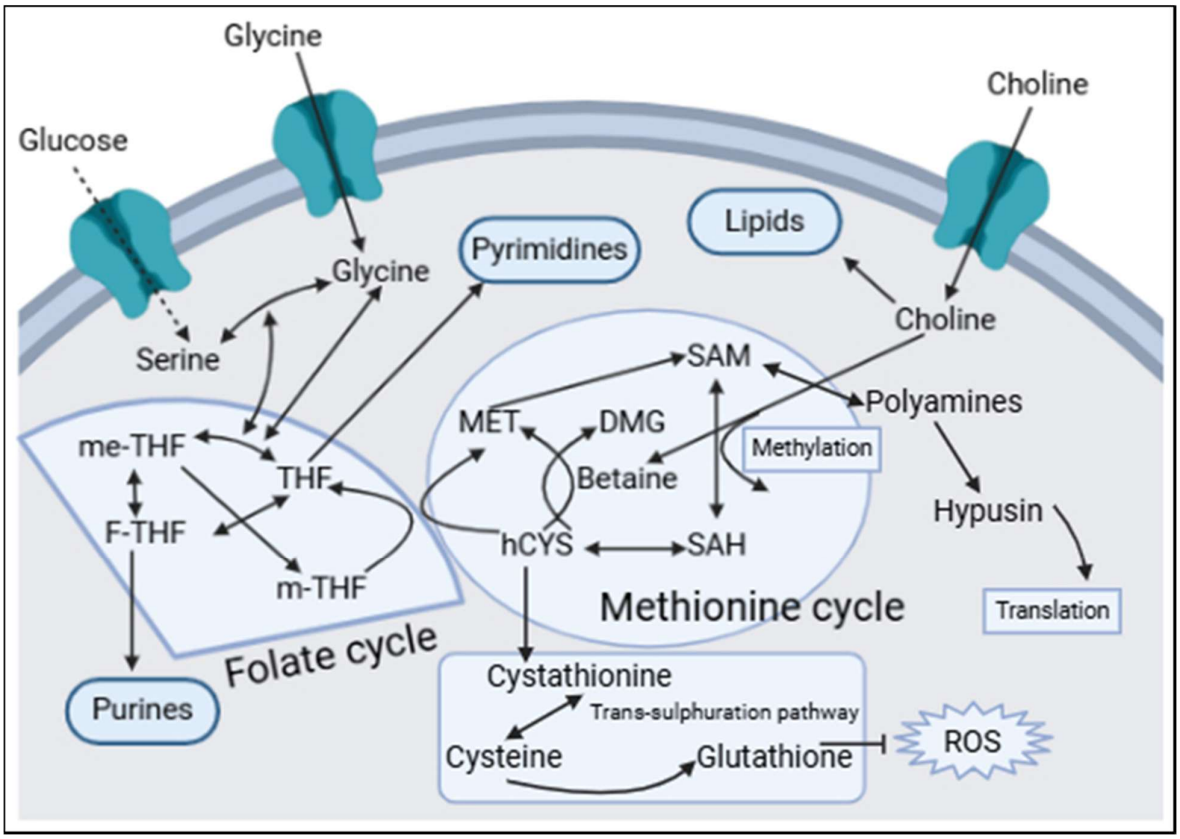


Figure 1: Schematic representation of the main metabolic pathways potentially targetable in gastrointestinal cancer therapies. *In the folate cycle, glycine and serine fuel mitochondrial enzymes SHMT2, MTHFD2, and ALDH1L2, which play critical roles in purine/pyrimidine production. Dysregulation of the production of purines and pyrimidines promotes gastrointestinal cancer survival and proliferation. In the methionine cycle, S-adenosyl methionine serves hydrocarbons and polyamines. Dysregulation critically affects the epigenetic control of DNA and histones. The trans-sulfuration pathway is a critical component in the synthesis of glutathione, whose dysregulation is involved in the promotion of reactive oxygen species in gastrointestinal cancer stem cells. THF, tetrahydrofolic acid; me-THF, N5N10-methylene-tetrahydrofolic acid; m-THF, N5-methyl-tetrahydrofolic acid; F-THF, N10-formyl-tetrahydrofolic acid; MET, methionine; SAM, S-adenosylmethionine; SAH, S-adenosyl homocysteine; hCYS, homocysteine; DMG, dimethylglycine; ROS, reactive oxygen species. (Adapted from* ***Konno et al, 2017***[44]*)*

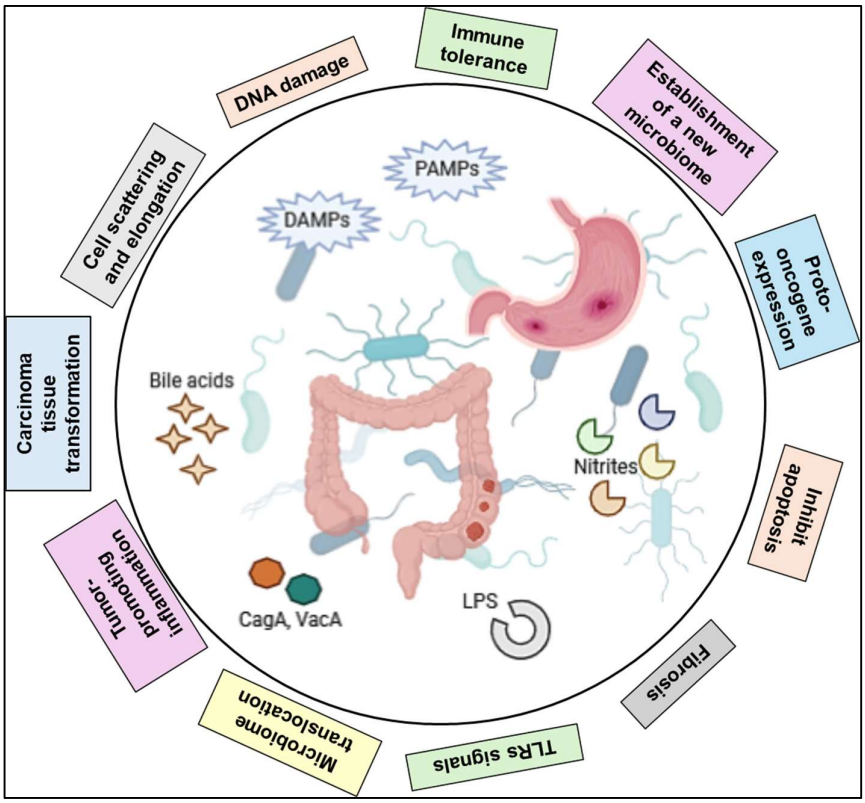


Figure 2: Main consequences associated with an unbalanced intestinal microbiome that can lead to gastrointestinal cancer development. *The virulence factor cytotoxin-associated gene A (CagA) is a known oncoprotein that contributes to the development of gastric cancer. The other major virulence factor vacuolating cytotoxin A (VacA), disrupts endolysosomal vesicular trafficking and impairs the autophagy pathway. Chronic inflammation mediated by damage-associated molecular patterns (DAMPs) accelerates immunosenescence and influences both tumor progression and anti-tumor immunity. Severe infections such as H. pylori infection are important risk factors for gastrointestinal cancers, causing inflammation and damage. Macrophages detect the presence of pathogen-associated molecular patterns (PAMPs) through a series of pattern recognition receptors. (Adapted from* ***Tong et al, 2017***[43]*)*

Overall, gastrointestinal cancers present a substantial global health burden, intricately linked to chronic low-grade inflammation, which contributes to poor patient survival rates. Enhancing diagnostic approaches to include personalized therapy strategies could potentially improve prognoses and reduce side effects for GI cancer patients.

2

Cannabinoids and the Endocannabinoid system

The endogenous cannabinoid system (ECS) is a fundamentally ancient lipid signaling network that has evolved over millennia and is expressed throughout the human body. Discovered nearly three decades ago, the ECS plays an essential role in regulating adaptive responses to both internal and environmental stressors[45]. This system includes at least two G protein-coupled receptors (GPCR) – the cannabinoid receptor type 1 (CB1)[46] and the cannabinoid receptor type 2 (CB2)[47], along with their endogenous ligands, and the enzymes needed for their synthesis and degradation (**Figure 3**).

The primary endogenous ligands discovered to activate cannabinoid receptors (CBRs) were N-arachidonoylethanolamine (AEA, commonly referred to as anandamide) and 2-arachidonoyl-glycerol (2-AG) (**Figure 3**). AEA is an amide derivative of arachidonic acid (AA), a polyunsaturated fatty acid (20:4) that serves as a precursor for bioactive lipids like prostaglandins. In contrast, 2-AG is an ester derivative of AA[48,49]. Both AEA and 2-AG act on CBRs, with 2-AG functioning as a full agonist and AEA as a partial agonist. Their effects include analgesia, catalepsy, hypolocomotion, hypothermia, bradycardia, and reductions in blood and intraocular pressures. Additionally, other N-acyl-ethanolamides like palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) are classified as eCB-like compounds, as by interacting with alternate targets, they are capable of influencing eCB signaling without directly activating CBRs[50]. Altogether, initially recognized as neuromodulators/neurotransmitters[51,52], these bioactive lipids also exhibit diverse and significant peripheral and immunomodulatory properties[53]. Additional identified molecules like 2-arachidonoyl glyceryl ether (noladin ether, 2-AGE), O-arachidonoylethanolamine (virodhamine), N-arachidonoyldopamine (NADA), and oleic acid amide (oleamide, OA) have further expanded the classification of endocannabinoids[54]. However, the presence of 2-AG ether in appreciable amounts in the brain and its role as an endogenous cannabinoid receptor ligand has been questioned.

The regulation of eCB tone is operated via a combination of a cellular uptake process and subsequent breakdown by specific enzymes (**Figure 3**). The primary enzyme responsible for breaking down AEA is the serine hydrolase fatty acid amide hydrolase (FAAH), which releases AA and ethanolamine[55]. Meanwhile, 2-AG is broken down into arachidonic acid and glycerol primarily by the monoacylglycerol lipase (MAGL)[56], and to a lesser extent by other enzymes such as FAAH[57], ABHD2 (identified as progesterone (P4)-dependent 2-AG hydrolase)[58], ABHD6, and ABHD12 (they may regulate different pools of 2-AG in the nervous system)[59]. Additionally, AEA and 2-AG can be oxidized by cyclooxygenase (COX), lipoxygenase (LOX), and by several cytochrome P450 monooxygenases into various derivatives such as PG-ethanolamides and glyceryl esters, hydroxy-AEAs, and hydroxyeicosatetraenoyl-glycerols, respectively[60,61], which although do not bind well to CBRs, have demonstrated new biological activities mediated by non-cannabinoid receptors thus adding complexity to the signaling pathways through which eCBs and related compounds may regulate cellular functions[45–53,60,62–64].

The two major endocannabinoids are synthesized by cells in the human body in response to diverse (patho)physiological stimuli, utilizing membrane lipid precursors as substrates for multiple biosynthetic pathways (**Figure 3**). They are predominantly derived from omega-3 and omega-6 polyunsaturated fatty acids (PUFA). 2-AG serves as a metabolic intermediary in the lipid breakdown process, whereas AEA is a byproduct of phospholipid membrane cleavage. The primary pathway for AEA biosynthesis begins with the remodeling of multifunctional membrane phospholipids. Phosphatidylethanolamine and arachidonic acid (AA) are converted into N-arachidonoylphosphatidylethanolamine (NAPE) by the enzyme N-acyltransferase (NAT). Subsequently, NAPE is hydrolyzed by the NAPE-selective phospholipase D (NAPE-PLD), producing anandamide (AEA) and phosphatic acid[47]. Alternatively, cells can utilize two additional parallel pathways. The first pathway involves the sequential deacylation of N-arachidonoylphosphatidylethanolamine (NAPE) by α,β-hydrolase 4 (ABHD4), which can act on either NAPE or lyso-NAPE to produce glycerophospho-arachidonoyl ethanolamide (GpAEA). This compound is then cleaved by a metal-dependent phosphodiesterase to yield anandamide (AEA). The second pathway proceeds through the phospholipase C-mediated hydrolysis of NAPE to generate phospho-anandamide (phospho-AEA), primarily catalyzed by the enzyme N-acyltransferase (NAT). Subsequently, phospho-AEA is dephosphorylated to AEA by phosphatases, including the tyrosine phosphatase PTPN22 and the inositol 5′ phosphatase SHIP1[60,62]. The biosynthetic pathway of 2-AG begins with the hydrolysis of its precursor, phosphatidylinositol (PI). This process converts PI into 1,2-diacylglycerol (1,2-DAG) via

the action of phospholipase C (PLC), which is then further transformed into 2-AG by sn-1-DAG lipases (DAGLs) α and β. An alternative pathway involves the conversion of PI into 2-arachidonoyl-lysophospholipid (lyso-PI) by phospholipase A1 (PLA1). Lyso-PI is subsequently hydrolyzed by lyso-phospholipase C (lyso-PLC) to generate 2-AG[63].

Endocannabinoid endogenous levels show significant fluctuations. AEA accumulation in the brain is 200 times lower compared to the concentration of 2-AG. Upon receptor stimulation or depolarization, levels of AEA can transiently increase by 5 to 12 times[65,66].

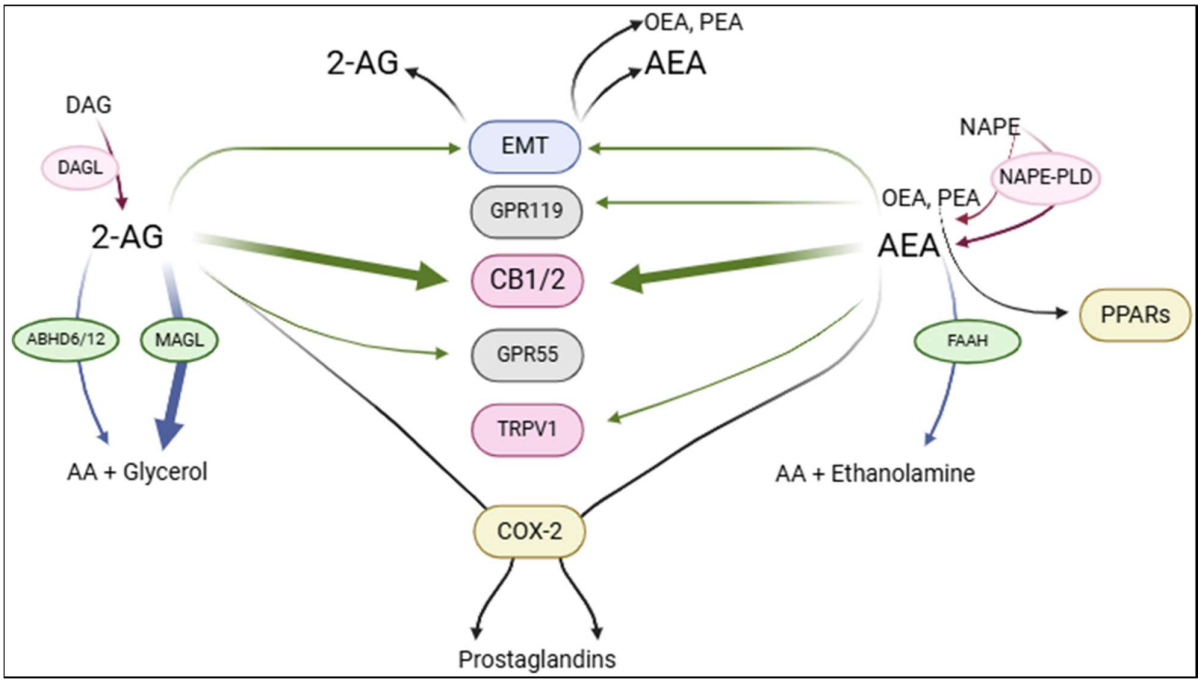


Figure 3: A schematic representation of the key components of the endocannabinoid system. *The main biosynthetic and catalytic pathways of the two main endocannabinoids, AEA and 2-AG, along with their major molecular targets. The oxidative metabolism occurring via the COX-2 pathway is also reported. EMT: endocannabinoid membrane transporter (Adapted from* ***Aran et al, 2019***[67]*)*

The discovery of the endocannabinoid system has also catalyzed interest in non-endogenous cannabinoids, which encompass a wide variety of compounds that target the same cell surface receptors (but not only) as eCBs (**Figure 3**). Such compounds, whether extracted from plants or synthetically produced, target the same cell surface receptors as endogenous cannabinoids (eCBs), specifically the cannabinoid receptors CB1 and CB2. Notably, plant-derived cannabinoids are well-known for their polypharmacology, as they can activate additional molecular targets including TRP channels, nuclear receptors like PPARs, and orphan GPCRs. The most known plant-derived cannabinoid, Δ9-tetrahydrocannabinol (THC) (**Figure 4**), is the primary psychoactive constituent of *Cannabis sativa* and *Cannabis indica*, which can mimic some actions of eCBs such as

anandamide and 2-arachidonoylglycerol (2-AG). Extensive research has classified cannabinoid compounds into five structural categories: classical cannabinoids (e.g., THC and Δ8-THC-dimethylheptyl or HU210), bicyclic cannabinoids (such as CP-55,940), indole-derived cannabinoids (like WIN 55,212), eicosanoids (the endogenous ligands), and synthetic antagonists and inverse agonists (e.g., SR141716A for CB1 and SR145528 for CB2). Regardless of their origin, these molecules were primarily studied for their ability to activate $G_{i/o}$ proteins linked to presynaptic CB1 and CB2 receptors while modulating postsynaptic transmission through interactions with dopamine and other neurotransmitters.

Currently, there are several hundred different synthetic cannabinoids, which are categorized into classical, nonclassical, aminoalkyl indoles, eicosanoids, and others based on their structure. Many such synthetic cannabinoids are used in research to study their effects and mechanisms of action in the body. The most commonly used synthetic cannabinoids in laboratory research as CB1 and CB2 receptor agonists are HU-210, CP-55,940, and WIN-55,212-2, though they exhibit high affinity at CB2 but lack selectivity for CB2 over CB1[68].

So far, the most potent CB1-selective agonists developed are arachidonyl-2´-chloroethylamide (ACEA)[69] and arachidonylcyclopropylamide (ACPA), both of which exhibit high CB1 potency. ACEA is highly selective for CB1 (nanomolar affinity at CB1 and >1000 fold selectivity for CB1 vs. CB2)[70] while ACPA displays >300-fold selectivity over CB2[71]. The CB1-selective and potent antagonist rimonabant (SR141716), CB1 inverse agonist taranabant (MK-0364) and otenabant (CP-945,598) were developed as anti-obesity drugs (**Figure 4**). Due to their crippling CNS side effects, they were, as a consequence, either withdrawn from the market or dropped in clinical trials. Other notable CB1 selective antagonists include analogs of rimonabant, AM251, and AM281. However, AM251 is often used as a selective CB1 receptor antagonist, although it is also a GPR55 agonist[72,73]. The first allosteric modulator described for the CB1 receptor was ORG27569 (ORG). This 1H-indole-2-carboxamide analog was first classified as a negative allosteric modulator (NAM) for CB1[74]. ORG inhibits G-protein signaling and stimulates β-arrestin signaling, whether alone or in the presence of an orthosteric ligand, generating β-arrestin-biased signaling. Pregnenolone, 3α-hydroxy-5β-pregnan-20-one, a steroid hormone, was then found to be a biased NAM for CB1 β-arrestin signaling[75]. It inhibits β-arrestin signaling in the presence of an orthosteric ligand, generating G-protein-biased signaling. GAT211, a compound derived from 2-phenylindole, was described as a positive

allosteric ligand for the CB1 receptor[76]. The compound stimulates G-protein signaling and β-arrestin-2 recruitment to a smaller extent but not β-arrestin-1 recruitment, generating G-protein-biased signaling.

Many compounds have been developed to produce CB2-selective agonists. JWH015 was one of the first potent CB2 selective agonists[77] but it also acts as agonist at GPR55[78]. Other early examples include HU308[79] and GW405833 (L-768242)[80,81]. AM1241[82] and JWH133[83] are two of the most commonly used potent and selective CB2 agonists. More recently synthesized compounds include GW833972A[84], MDA7[85], A-796260[86], and A-836339[87]. SR144528[88] and AM630[89] are widely used as CB2 selective antagonists, frequently demonstrating the specificity of many of these other CB2 selective agonists (**Figure 4**).

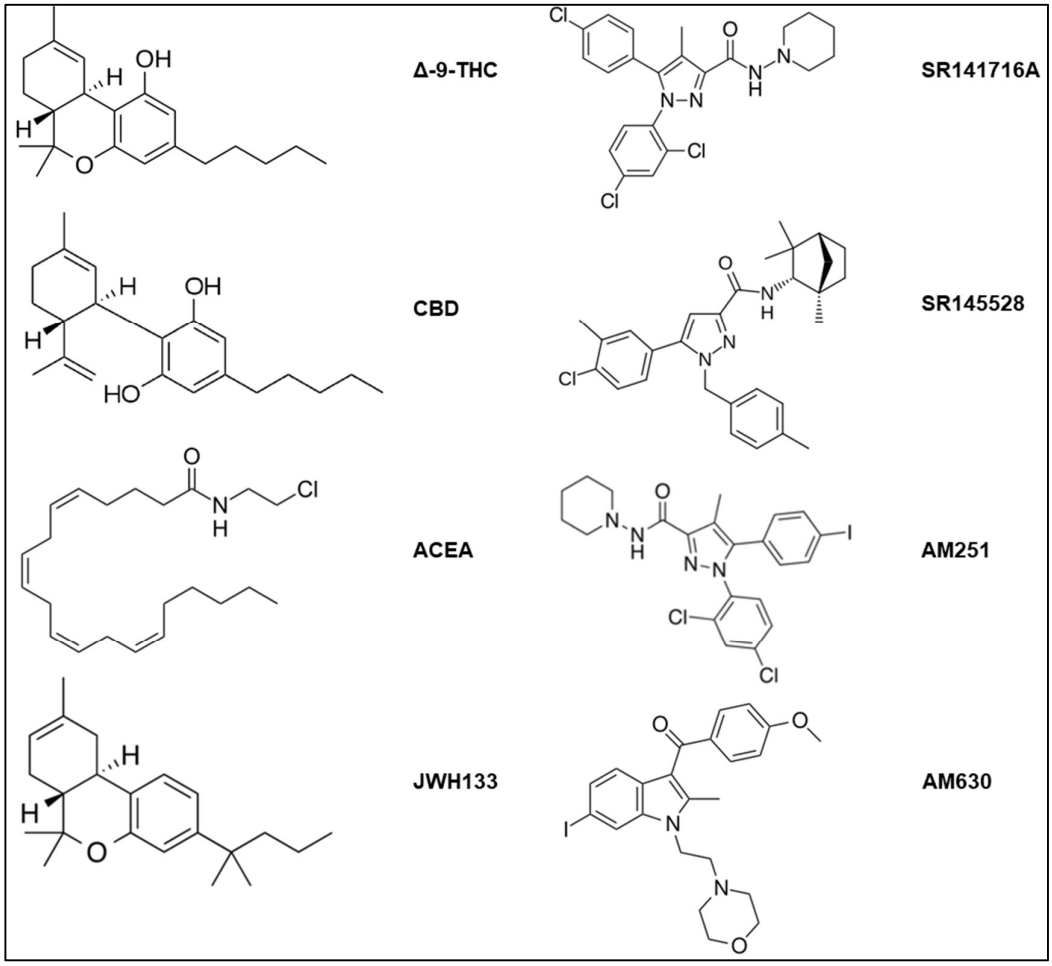


Figure 4: Chemical structures of key synthetic and plant-derived cannabinoids.

Concerning CBR signaling, the activation of these receptors induces a decrease in neurotransmitter release. Notably, CB1 and CB2 receptors trigger distinct signaling pathways, including the mammalian target of Rapamycin (mTOR), and the mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) pathways. This signaling is mediated through the activation of p38 MAPK, ERK1 and ERK2, c-Jun N-terminal kinases (JNK), and phosphatidyl inositol 3-kinases (PI3K/Akt) pathways. Additionally, the activation of these receptors inhibits the activation of adenylyl cyclase and cyclic AMP-dependent protein kinase A (PKA), resulting in the diminished expression of cyclic adenosine monophosphate (cAMP). Of note, the regulatory context of cannabinoid signaling is further strengthened by the ability of CB1 receptors to form homodimers and heterodimers with other GPCRs, including CB2Rs, as well as dopamine, opioid, serotoninergic, and orexin receptors. Furthermore, eCBs exert their biological effects through additional non-cannabinoid receptors, such as the transient receptor potential vanilloid type-1 (TRPV1) ion channel, the nuclear peroxisome proliferator-activated receptors (PPAR) α and γ, and the orphan receptors GPR55 and GPR119[51,52] (**Figure 3**). Shedding light on the pharmacological behavior of cannabinoid ligands on CBRs, their structure is such that the agonist binding site is not readily available extracellularly, and the CBR ligands need to enter into the orthosteric site laterally, via narrow openings within close transmembrane helical domains in the lipid bilayer on the cells[90–95]. Exosites have also been described to which agonist ligands of GPCRs can interact and may regulate CBR functionality[96]. The CB1Rs mediate an inhibition of Q-type calcium channels and activation of inward rectifying potassium channels. In contrast, the CB2Rs do not modulate the activity of either channel[97]. Therefore, CB1 and CB2 receptors display similar pharmacological and biochemical properties that differ significantly in their signaling[94].

In conclusion, it is widely accepted that the endocannabinoid system plays a crucial role in maintaining homeostasis within the body. Dysregulation of this system is often associated with the onset and progression of various diseases. Alterations resulting from fluctuations in the endogenous tone of endocannabinoids or modulation in the expression and function of cannabinoid receptors and their associated enzymes have been linked to several conditions, such as acute stress, autism, Alzheimer's disease, mastocytosis, and cancer[70,98–101]. The accumulated knowledge of the endocannabinoid system has sparked interest in the search for targeted cannabinoid-based therapeutics[45].

3

Physio-pathological role of endocannabinoids

The endocannabinoid system (ECS) is intricately involved in vital homeostatic processes such as cell cycle regulation, autophagy, and apoptosis[102–104]. The main endogenous ligands of this system, the endocannabinoids (eCBs), play a complex role in various physiological and pathological processes. One relevant example is given by the fact that endocannabinoids act as retrograde signaling molecules at synapses in the central nervous system (CNS), particularly at GABAergic and glutamatergic synapses (**Figure 5**). By acting via this signaling, they can mediate short- and long-term forms of plasticity at both excitatory and inhibitory synapses. They influence dopamine (DA) transmission and related behaviors by modulating inputs to DA neurons. By regulating synaptic plasticity in the ventral tegmental area (VTA), endocannabinoids play a key role in controlling DA output[105], affecting motivated behaviors such as pain, mood, appetite, and memory, mainly through CB1 and CB2 receptors[106–110]. Interestingly, these effects appear to be direct rather than mediated via inflammatory signal induction. They can reduce pain perception, making them relevant in conditions like chronic pain and neuropathic pain, highlighting their role in pain modulation[111–113]. Moreover, endocannabinoids modulate immune responses[114–116]. CB2 receptors are particularly important in controlling inflammation, suggesting a potential therapeutic target for autoimmune diseases. They play a role in neuroprotection and can influence the progression of diseases like Alzheimer's, Parkinson's, and Multiple Sclerosis. Another point of interest is that the dysregulation of the ECS is linked to mood disorders, such as anxiety and depression[117–119], highlighting their potential in mental health treatments. Governing both central and peripheral CB sites, eCBs may play a significant role in managing nausea by delaying gastric emptying[120–122]. This promptly connects eCBs to be involved in regulating appetite and energy balance, which is significant in obesity and metabolic syndrome[123].

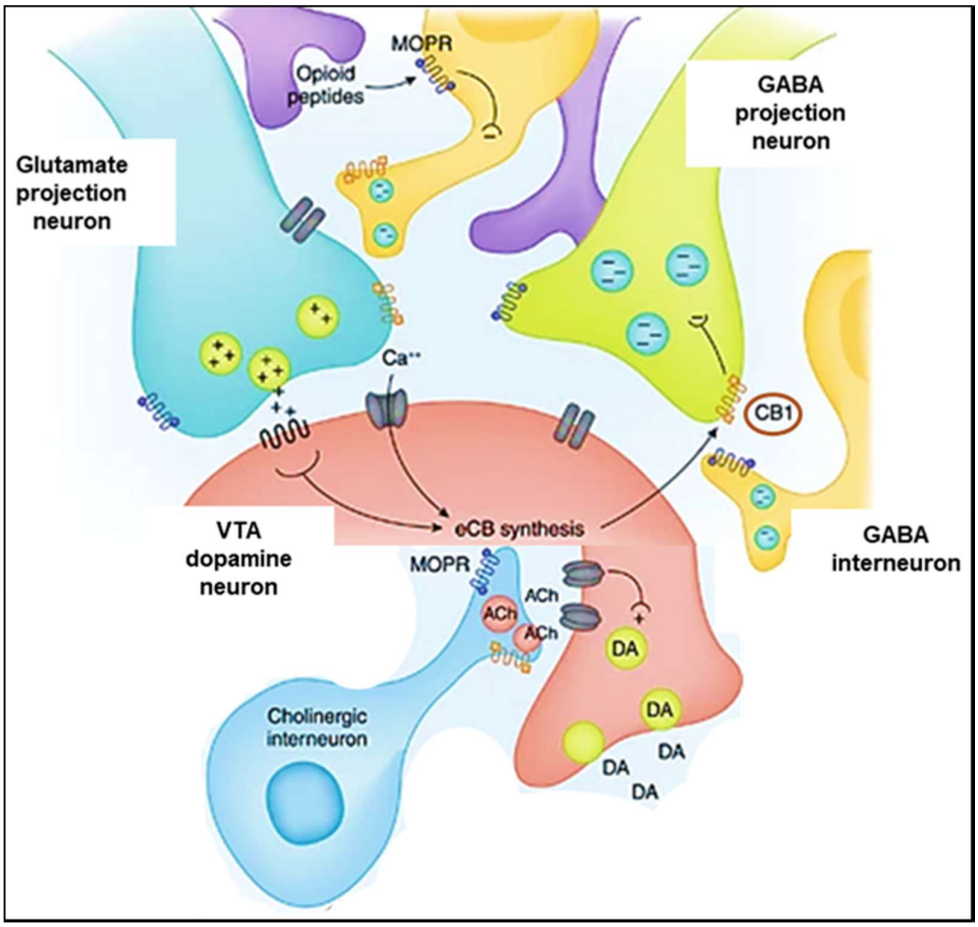


Figure 5: Retrograde signaling mediated by endocannabinoids in the central nervous system. *The ventral tegmental area (VTA) contains nerve endings that use two main signals: glutamatergic and GABAergic. These endings have mu-opioid receptors (MOPR) and cannabinoid type-1 receptors (CB1). When glutamatergic neurons activate VTA dopamine (DA) neurons, they release endocannabinoids (eCBs) that bind to CB1 receptors, increasing DA release by blocking GABA signals. MOPR agonists also enhance VTA DA cells by blocking GABA neurons. The VTA sends GABA signals to cholinergic interneurons, which usually inhibit acetylcholine (ACh) signals to DA nerve endings. When CB1 or MOPR reduces GABA activity, more ACh is released, stimulating DA terminals. (Adapted from* ***Wenzel and Cheer, 2018***[124]*)*

The connection between ECS and the gastrointestinal (GI) system goes back to 1995, when 2-AG was extracted from a canine's intestine, followed sometime later by obtaining AEA from the tissues of mice's small intestine with the simultaneous presence of the FAAH enzyme[125]. Since then, it has been subsequently established that the ECS in the GI tract regulates inflammation, motility, sensation, and secretion. ECS activation is implicated in various GI pathogeneses[126,127], where the components of this system are significantly upregulated and bring upon a beneficial action[128–130]. As an example, research on inflammatory bowel disorder (IBD) has shown that activating the eCB signaling reduces chronic inflammation[131], proposed to be achieved by modulating the gut ECS

pharmacologically through three primary mechanisms, distinctively, (i) attenuation of the release of neurotransmitters regulating intestinal motility and secretion; (ii) reduction in the production of proinflammatory mediators, such as tumor necrosis factor α (TNFα) and IL-1β; and (iii) facilitation of intestinal barrier wound healing.

In tune with the mechanisms of gut ECS activation, the activation of CB1 receptors in the GI tract is crucial for regulating functions such as gastric juice secretion, intestinal motility, and gastric emptying[132]. These receptors work alongside cholinergic neurons to inhibit intestinal motility and gastric secretions, indicating their active role in maintaining gut function[133]. Cannabinoids' impact on CB1 receptors helps manage conditions like paralytic ileus, where increased anandamide and CB1 receptors reduce intestinal motility[134]. In agreement, the inhibition of DAGLα – a major biosynthetic enzyme for 2-AG production, reverses slow motility, intestinal contractility, and constipation through decreased 2-AG signaling at CB1[135]. Both cases highlight the potential treatment for gastrointestinal motility disorders. Selective interaction via CB1R with the capsaicin-sensitive sensory terminals, can modulate food intake and thereby present an unexpected role of eCBs in the control and monitoring of diet, and can regulate feeding[136,137]. Although, under physiological conditions, CB2 receptors do not seem to play a major role in GI function, their activation in pathological states limits abnormal GI tract motility and gastric emptying. Multiple research inferences confirm the statement. Lipopolysaccharide (LPS) treatment increases gastrointestinal motility, which can be regulated by CB2 agonists[138]. FAAH inhibition also dampens intestinal contractility in pathophysiological states[139]. Using a selective inhibitor of MAGL, 2-AG protects against gastric damage induced by nonsteroidal anti-inflammatory drugs[140].

The ECS is often interconnected with other systems governed by distinct bioactive lipids, the most impactful players being the specialized pro-resolving mediators (SPMs)[141]. Interestingly, CB2 and GPR55 (known to interact with both eCBs and SPMs) are notably overexpressed in various cancer cell lines and human malignant tumors[142]. Functional CB2R-GPR55 heteromers were reported to have a major impact on the anti-tumor cannabinoid action in human glioblastoma cancer cells depending on their dose[143,144]. Moreover, CB2R-GPR55 heteromers were found to be involved in the cancer cell fate of different cancers where its targeting reduces tumor growth[144]. C-X-C chemokine receptor type 4 (CXCR4)-CB2R heteromers regulate proliferation, adhesion, and invasion, thus metastatic potential. In this case, CB agonists of the CB2R inhibit the effect of CXCR4 agonist, thus indirectly affecting invasion[144,145].

Overall, the endocannabinoid system's multifaceted roles present both opportunities for therapeutic intervention and challenges in understanding its complexities in health and disease. Importantly, the close interaction between GI diseases and disruptions in ECS homeostasis suggests that the ECS is a promising target for future pharmacological interventions, which could be harnessed with a deeper understanding.

4

Cannabinoids and cancer: focus on GI tract cancers

Cannabinoids have gained attention for their potential therapeutic effects in various cancers[104,146,147] – breast cancer[148], glioma[149], leukemia[150], lung cancer[151], melanoma[152], myeloma, endometrial and cervical cancers, oral cancer, and prostate cancer[153] including those of the gastrointestinal (GI) tract, such as hepatocellular carcinoma (HCC)[154], pancreatic cancer (PC)[155], and colorectal cancer (CRC)[156].

Numerous studies support the endocannabinoid system (ECS) to have demonstrated anti-proliferative, anti-angiogenic, and anti-metastatic effects in pancreatic tumors[157], breast cancer cell lines[158], and glioma growth[159] (as illustrated in **Figure 6**). Similarly, synthetic or plant-derived cannabinoids are reported to exhibit an anticancer potential by modulating several pathways involved in cell growth, differentiation, migration, and angiogenesis[160,161]. The modulation of the ECS governs the epithelial-mesenchymal transition (EMT)[162]. Notably, the pro-apoptotic effect of CBR agonists on pancreatic adenocarcinoma[163], melanoma[164], rhabdomyosarcoma[165], glioma[166–168], breast cancer[169–172] and hepatocellular carcinoma[173] is mediated by an increased ceramide biosynthesis, which modulates p38/MAPK, induces endoplasmic reticulum (ER) stress, and leads to a COX-2-dependent synthesis of pro-apoptotic PGE2[160,174]. A possible involvement of caspase activation and reactive oxygen species (ROS) induction is indicated in the apoptotic effect brought about by CBD[175]. The ECS, furthermore, exhibits anti-angiogenic action in several types of carcinomas, such as in lung, breast, skin, and brain tumorigenesis[176]. The signaling of CBRs has also been associated with the regulation of cell cycle checkpoints by inhibiting the Akt pathway or calcium signaling, ultimately leading to the apoptotic demise of such tumor cells[160,174,177,178].

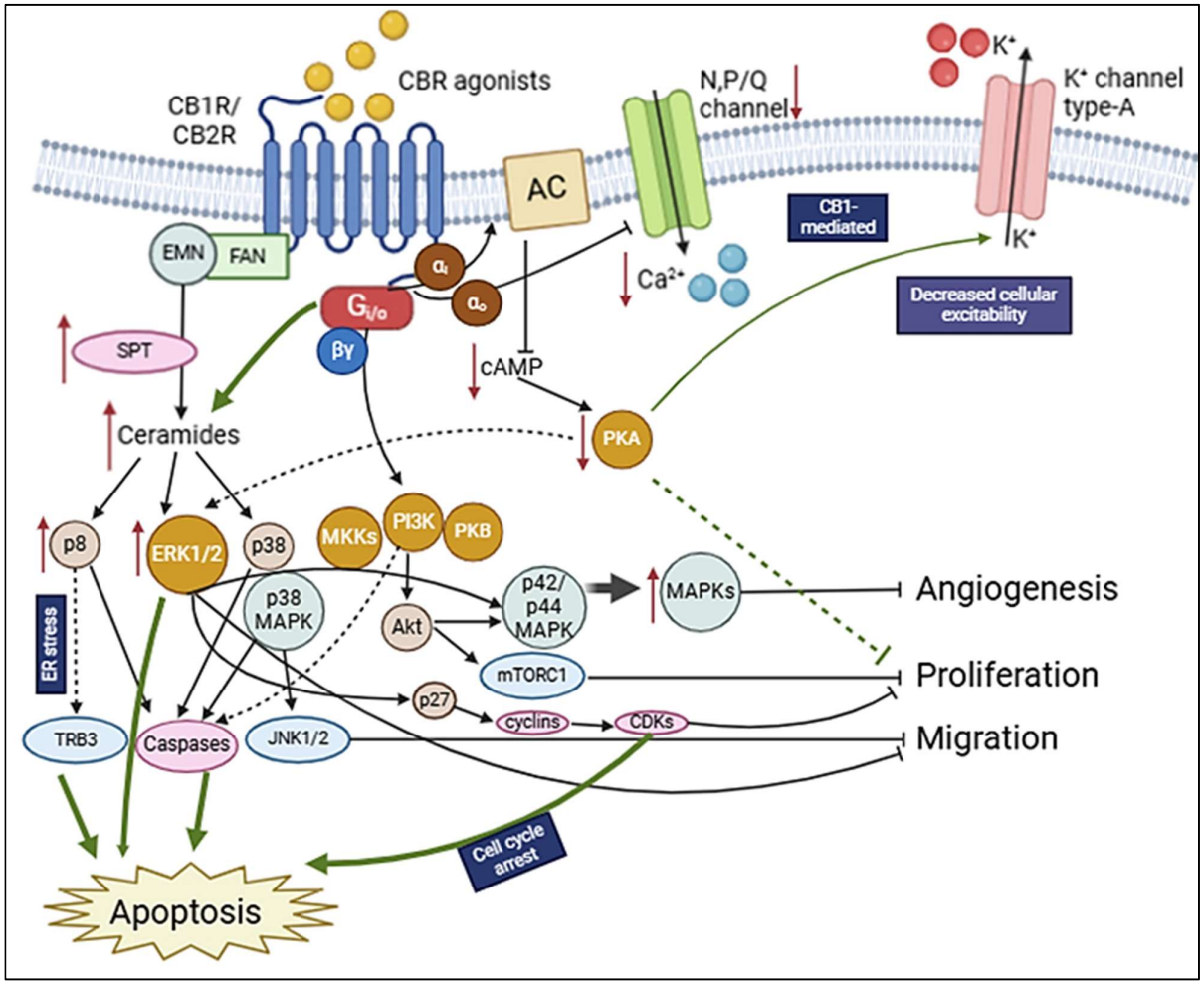


Figure 6: Antitumor properties mediated by cannabinoid receptor activation. *When cannabinoids bind to their receptors, they decisively activate the $G_{i/o}$ α subunit, which effectively inhibits adenylate cyclase (AC) and reduces cyclic AMP (cAMP) levels, thus significantly lowering protein kinase A (PKA) activity. The βγ dimer robustly stimulates phosphoinositide 3-kinase (PI3K) and initiates the mitogen-activated protein kinase (MAPK) pathways, while Akt directly activates mTORC1. This cascade unequivocally suppresses cell proliferation through diminished cAMP-dependent protein kinase activity. Furthermore, serine palmitoyltransferase (SPT) activation drives ceramide synthesis, leading to heightened p8 expression and subsequent apoptosis. Prolonged activation of ERK1/2 unequivocally induces the cyclin-dependent kinase inhibitor p27, resulting in G1 phase arrest and apoptosis. AC: adenylate cyclase; PKA: protein kinase A; PI3K: phosphatidylinositol-3 kinase; mTORC1: mammalian target of rapamycin complex 1; MKK: mitogen-activated protein kinase kinases; MAPK: mitogen-activated protein kinase; JNK: Jun kinases; ERK: extracellular signal-regulated kinases. (Adapted from* ***Ligresti et al, 2016; Fernández-López et al, 2013; Sarfaraz et al, 2008***[179–181]*)*

Cannabinoids' action in GI cancers has been demonstrated *in vitro* and *in vivo*, indicating their antiproliferative, proapoptotic, and antimetastatic properties[182]. Supported inferences from the literature have been highlighted for specific GI cancers – gastric and colorectal adenocarcinoma.

Studies have reported higher levels of endogenous CBs – anandamide (AEA) and 2-arachidonoylglycerol (2-AG), as well as increased expression of AEA-synthesizing enzymes and degrading enzymes in CRC compared to normal mucosa[183,184]. Both AEA and 2-AG potently inhibit CRC cell proliferation[185]. Conversely, colon cancer tissues have shown down-regulated CB1 receptor expression[186,187]. The selective CB1 antagonist AM251 at 3μM reversed the antiproliferative effect of AEA analog Met-F-AEA, demonstrating that the control on CRC cell line proliferation was mediated by increased expression of the CB1 receptor through transcriptional activation of the CNR1 promoter. While CB1 has been reported to suppress intestinal tumor growth, the role of GPR55 is tumor-promoting[188]. Additionally, the GPR55-lysophosphatidylinositol axis has been implicated in CRC progression, with CBD reducing the adhesion and migration of cancer cells to a cell monolayer in vitro[189]. Furthermore, the pro-apoptotic effect in CRC cells has been linked to excessive ROS production by mitochondria and ER stress induction upon CBD treatment[190]. Finally, Δ9-THC, through CB1 activation, induced apoptosis in colorectal cancer cells by inhibiting the phosphoinositide 3-kinases-Akt (PI3K-Akt) survival cascade[191]. Pharmacological inhibition of 2-AG degradation reduces angiogenesis in colon tumours[192]. In a murine model of CT26 cell line-induced colon cancer, CBD at a dose of 1 mg/kg was found to exhibit anti-angiogenetic and antimetastatic effects associated with vascular endothelial growth factor (VEGF) downregulation[190].

The anticancer role of cannabinoids in gastric cancer is an area of active research, and several studies have highlighted their potential therapeutic effects. Cannabinoid agonists have been shown to reduce cell viability and proliferation in human gastric adenocarcinoma cell lines. AEA and its synthetic analog methanandamide (Meth-AEA) at concentrations of 0.5–5μM reduced gastric carcinoma cell volume and density, inducing apoptosis and necrosis[193]. This occurs through G1 phase cell cycle arrest, which is mediated by the activation of the MAPK pathway and inhibition of pAKT[194]. Additionally, the cannabinoid receptor agonist WIN 55,212-2 has exhibited antineoplastic effects on gastric cancer in xenograft models[195], indicating a potential protective role against gastrointestinal malignancies. Furthermore, anandamide (AEA) has been established to augment paclitaxel-induced apoptosis in gastric cancer cells, potentially via caspases 3/8/9 activation[196].

In addition to their potential anti-cancer effects, cannabinoids can be effective in managing symptoms associated with GI tract cancers, such as nausea, vomiting, and appetite loss, especially in patients undergoing chemotherapy[197]. Ongoing clinical trials are exploring the use of CBs in treating chemotherapy-induced vomiting and nausea, as well as in managing emesis associated with GI-related cancers and pathologies[198,199].

In summary, cannabinoids present a versatile approach to addressing and alleviating symptoms of GI tract cancers. While the preclinical data are encouraging, continued research is vital to gain a comprehensive understanding of their potential and to optimize the application of cannabinoids in treating GI cancers in clinical settings. Further investigations into dosing, delivery methods, and individual patient responses are crucial for advancing our knowledge.

Aim

This thesis aims to elucidate the effects of external stimuli on the metabolic pathways associated with gastrointestinal (GI) cancers, with a particular focus on gastric and colon cancers, two of the most prevalent malignancies worldwide. The increasing occurrence of GI cancers highlights the urgent need to understand the metabolic processes involved in their development.

Within this framework, cannabinoids have emerged as potential therapeutic agents due to their documented effects on various tumor processes, including those related to GI cancers. Prior studies indicate that cannabinoids may inhibit tumor growth and migration, suggesting their possible role in cancer therapy. Additionally, an expanding body of evidence points to cannabinoids' ability to modulate metabolic processes that are essential for tumor survival and proliferation.

To accomplish the objectives of this research, we profiled the metabolomes of diverse gastric (AGS and MKN45) and colon cancer cell lines (HCT116 and HT29) using NMR-based OPLS-DA to identify distinct metabolic signatures that differentiate these two types of cancer. We investigated how the activation of cannabinoid receptors—specifically through the selective CB1 agonist ACEA and the selective CB2 agonist JWH133—affects critical metabolic pathways and alters metabolic responses in AGS and HCT116 cells.

Our key objectives include evaluating the effects of cannabinoid treatments on mitochondrial respiratory capacity, glycolytic activity, and the expression of enzymes involved in essential metabolic cycles. Furthermore, the study examines the impact of cannabinoid treatments on tumor cell growth, migration, and spheroid formation, based on the hypothesis that cannabinoids may disrupt the unique metabolic adaptations employed by GI tumors.

The findings aim to underscore the therapeutic potential of cannabinoids as metabolic inhibitors and to pave the way for innovative strategies that could enhance the effectiveness of existing chemotherapy and immunotherapy regimens in the treatment of gastric and colon cancers.

Materials and Methods

Chemical reagents for treatments

ACEA and JWH133 (Cayman Chemical, Ann Arbor, Michigan USA) were used as synthetic exogenous CB1 and CB2 agonists, respectively, in the study.

Cell culture

GC models – AGS, MKN45 cells, and CRC models – HCT116, HT29 cells were studied. Cells were cultured as monolayers in plastic plates in DMEM (Dulbecco's Modified Eagle Medium (Invitrogen, Grand Island, NY, USA) supplemented with 100U/mL penicillin G sodium, 100µg/mL streptomycin and 10% FBS (Fetal Bovine Serum), and maintained at 37°C in a humidified atmosphere of 5% carbon dioxide. To execute a starvation procedure, the cells were cultured in 0.5% FBS media for 12 hours before the treatments. Cell treatments with ACEA and JWH133 to verify mRNA fold changes were performed in serum-free media following 12h serum starvation. Treatment regimen: ACEA (1 and 10µM) and JHW133 (1 and 10µM) were prepared in 0.5% FBS media and treated for up to 24 hours. Analysis was performed on data obtained from distinct independent experimental groups (Mean ± SEM).

RNA purification and quantitative RT-PCR analyses

Total RNA was extracted from cell pellets in 1.0 mL of Trizol® (Invitrogen) following the manufacturer's instructions, and dissolved in an adequate volume of RNA-DNA-free water. RNA aliquots were digested by RNase-free DNase I (Invitrogen) in a 10µL final volume reaction mixture to remove residual contaminating genomic DNA. The concentration and purity of RNA samples were evaluated by UV-quantified by a Bio-Photometer® (Eppendorf, Hamburg, Germany) following the manufacturer's instructions and stored at -80°C until use. 1µg of total RNA was reverse-transcribed in cDNA (Invitrogen, SuperScript™ III). qPCR was done using SYBR green master mix in CFX384 Real-Time detection system (Bio-Rad™). Relative gene expression calculation, corrected for PCR efficiency and normalized to the reference gene, was performed by the Bio-Rad CFX™ manager software.

β-Actin FOR	5'-TGC GTG ACA TTA AGG AGA AG-3'
β-Actin REV	5'-GCT CGT AGC TCT TCT CCA-3'
Human CB1R FOR	5'-GCT GCT TGG CAA GTG ATC AA -3'
Human CB1R REV	5'-TGG TAT CTG CAA GGc CAT CT – 3'
Human CB2R FOR	5'-CTG CGC TAT CCA CCT TCC TA -3'
Human CB2R REV	5' – CGG AAA AGA GGA AGG CGA TG -3'
Human ASCT2 FOR	5'-TCC TCT TCA CCC GCA AAA ACC C-3'
Human ASCT2 REV	5'-CCA CGC CAT TAT TCT CCT CCA C-3'
Human GPT1 FOR	5'-CAA GAA GGT GCT CAT GGA GAT GG-3'
Human GPT1 REV	5'-TGT TCA CCA CCT CCA CAT AGC C-3'
Human GLUT1 FOR	5'-TTG CAG GCT TCT CCA ACT GGA C-3
Human GLUT1 REV	5'-CAG AAC CAG GAG CAC AGT GAA G-3'
Human PPARγ FOR	5'-AGC CTG CGA AAG CCT TTT GGT G-3'
Human PPARγ REV	5'-GGC TTC ACA TTC AGC AAA CCT GG-3'

Table summarizing the specific primer sequences used for the study.

NMR sample extraction and spectra acquisition

Cells were seeded (3 million per dish, n=4/experimental group) one day before harvesting in 10cm Petri dishes, and treated with 10μM concentration of cannabinoids for 24 hours. After incubation, cells were harvested. The growth medium was removed and cells were rapidly washed with Dulbecco's phosphate-buffered saline (DPBS, Gibco™) and detached using Trypsin-EDTA (0.05%), phenol red (Gibco™). Then, cells were centrifuged to remove the traces of trypsin and obtain cell pellets. The cell pellets were washed 3X using sterile DPBS, to remove any traces of the growth media. Sample harvesting was performed following the 2X Methanol:2X Water:4X Chloroform protocol (as described by Bligh and Dyer[200]) to obtain polar and non-polar extracts by ultracentrifugation at 1000g for 15min, followed by rapid evaporation of volatile substances in vacuum condition. The procedures were executed on ice and the dried sample extracts were stored at -80°C until metabolites' acquisition.

NMR spectra were recorded at 600.13MHz on a Bruker Avance-600 spectrometer, equipped with a TCI CryoProbe™ fitted with a gradient along the Z-axis, at a probe temperature of 27°C. 1D proton spectra of cells were acquired using the excitation sculpting sequence. A double-pulsed field gradient echo was used, with a soft square pulse of 4ms at the water resonance frequency, with the gradient pulses of 1ms each in duration, adding 256 transients of 16384 points with a spectral width of 8389.3Hz. Time-domain data were all zero-filled to 32768 points, and before the Fourier transformation, an exponential multiplication of 0.6Hz was applied. Moreover, two-dimensional (2D) experiments were recorded to assist signal identification of discriminant metabolites. To

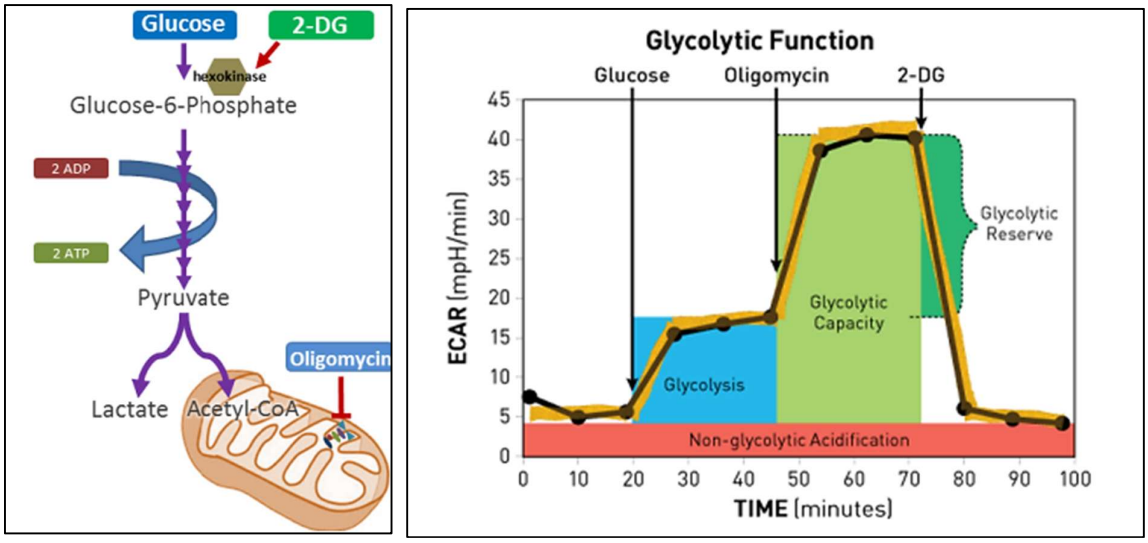
that purpose, clean total correlation spectroscopy (TOCSY) spectra were recorded using a standard pulse sequence and incorporating the excitation sculpting sequence for water suppression (details in supporting information). In general, 320 equally-spaced evolution-period t_1 values were acquired, averaging 4 transient of 2048 points, with 7002.8Hz of spectral width. Time-domain data matrices were all zero-filled to 4096 points in both dimensions, thus yielding a digital resolution of 3.42Hz/pt. Before Fourier transformation, a Lorentz-to-Gauss window with different parameters was applied for both t1 and t2 dimensions for all the experiments. TOCSY experiments were recorded with a spin-lock period of 64ms, achieved with the MLEV-17 pulse sequence. Spectra were referred to 0.1mM sodium trimethylsilylpropionate (TSP), assumed to resonate at δ=0.00ppm. In addition, natural abundance 2D $^{1}H-^{13}C$ heteronuclear single quantum coherence (HSQC) spectra were recorded at 150.90MHz for ^{13}C, using an echo–anti echo phase sensitive pulse sequence with adiabatic pulses for decoupling and water suppression using presaturation. 128 equally spaced evolution-time period t_1 values were acquired, averaging 240 transient of 2048 points and using GARP4 for decoupling. The final data matrix was zero–filled to 4096 in both dimensions and apodized before Fourier transformation by a shifted cosine window function in t_2 and t_1. Linear prediction was also applied to extend the data to twice its length in t1. HSQC spectra are referred to the lactate doublet (βCH3) resonating at 1.33ppm for ^{1}H and 20.76ppm for ^{13}C.

Data analysis: The 0.60–9.60 ppm proton region of cell spectra was automatically data reduced to 450 integrated regions (buckets) of 0.02ppm width using the AMIX 3.9.7 package (Bruker Biospin GmbH, Rheinstetten, Germany). The residual water resonance region (5.16–4.50ppm) was excluded, and each integrated region was normalized to the total spectrum area to avoid possible signal variation due to dilution of samples, which could result in an artificial increase of the signals. To discriminate the different classes of samples through NMR spectra according to the condition/treatment of mice, we carried out a multivariate statistical data analysis using projection methods. The integrated data reduced format of the spectra was imported into the SIMCA 14 package (Umetrics, Umeå, Sweden), and Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS–DA) were performed. Mean–centering and Pareto scaling were used as data pre-treatment for both PCA and OPLS–DA. PCA was first applied to extract and display the systematic variation in the data matrix to identify trends and clustering in an unsupervised manner. However, to better define grouping and select molecular markers related to specific experimental treatments/conditions, supervised OPLS–DA analysis was used.

Pathway Analysis: Metabolite Set Enrichment Analysis (MSEA) to identify biologically meaningful patterns that are significantly enriched in selected and more representative metabolites in class separation was applied to the pathway topology search tool in Metaboanalyst 4.0. In this analysis, the metabolic "pathway-associated metabolite sets" (currently containing 25 entries) from the human library were selected. Over Representation Analysis was implemented using the hypergeometric test to evaluate whether a particular metabolite set is represented more than expected by chance within the selected compound list. Metabolites were selected by evaluating both VIP values > 1 in class discrimination and correlation values |p(corr)|> 0.7.

Agilent Seahorse XF Glycolysis Stress Test

The Glycolysis Stress test assessed the glycolytic flux under various conditions using key substrates and inhibitors for glycolysis, and the protocol was followed as described by the Agilent company[201,202]. The impact of cannabinoids on the glycolytic pathway was evaluated by measuring the Extracellular Acidification Rate (ECAR). The glycolytic rate was measured following the exogenous feeding with 100mM D(+)-glucose, and was followed by the addition of 100µM oligomycin, which is responsible for blocking the ATP-coupled mitochondrial respiration via inhibition of ATP Synthase Complex V. The end-point of the assay was marked by the addition of 500mM of glucose analog 2-deoxyglucose (2-DG), which accumulates within functional cells (via GLUT1 on the plasma membrane) but not acted upon by Hexokinase (the pivotal enzyme in glycolysis, which phosphorylates glucose into glucose-6-phosphate to facilitate downstream glycolytic processes), therefore halting the glycolytic flux[203].

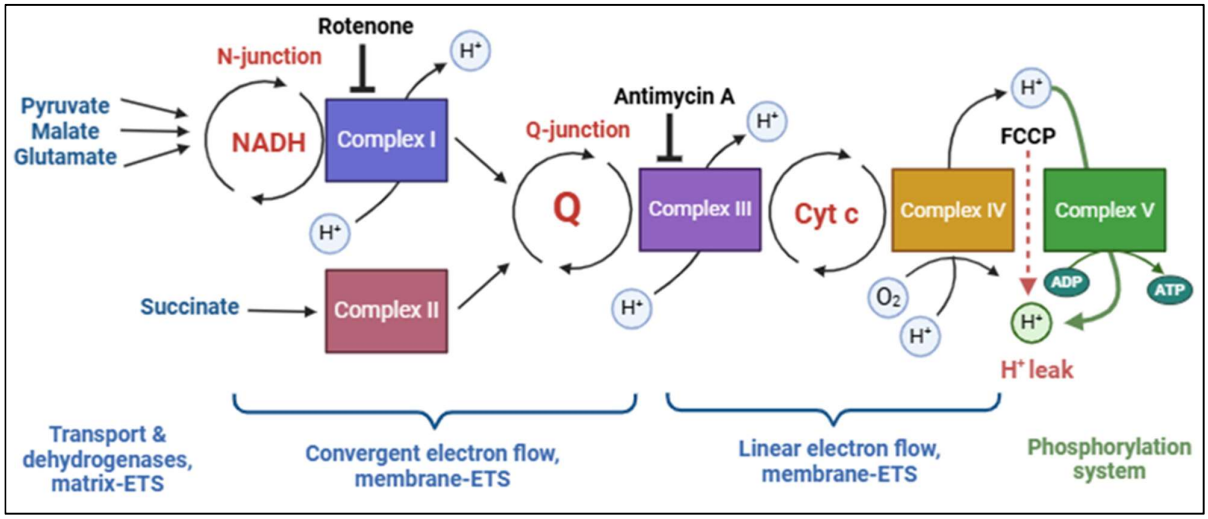


A concise illustration of the glycolytic pathway adapted from the Seahorse XF Glycolytic Stress test guide, whereupon the evaluation scheme of the test has been represented. Alongside, a typical experimental tracing obtained from the test, defines the potential of the study to determine the glycolytic function of the samples to be studied.

Substrate-uncoupler-inhibitor titration (SUIT) protocol for analysis of oxidative phosphorylation (OXPHOS)

To comprehensively quantify mitochondrial pathways, substrate-uncoupler-inhibitor-titration (SUIT) protocols are implemented[204,205]. The recording of basal respiration (ROUTINE) reflects the physiological metabolic capacity (net cytosolic+mitochondrial metabolic outputs) in intact cells. Following plasma membrane disintegration with 10mg/mL Digitonin, the oxygen (O_2) flux change due to mitochondrial respiration can be exclusively quantified, marking the start point of the experiment. The three coupling states, LEAK (intrinsic or inducible uncoupled O_2 consumption), OXPHOS (phosphorylating respiration), and ET (ie. electron transfer, experimental noncoupled respiration) are, therefore, representatively obtained by following the SUIT protocol. Electron flow from feeding 2M pyruvate + 0.4M malate + 2M glutamate (PMG, denotes LEAK I) converges at the N-junction (ie. NADH-cycle), leading to the induction of Complex I (CI). Subsequently, exogenous injection of 0.5M ADP+Mg^{2+} preparation (denotes OXPHOS I) pushes the phosphorylation system (ADP->ATP) in Complex V (CV), which is marked by a positive O_2 flux change. Upon addition of 1M succinate (denotes OXPHOS I+II), the Complex II (CII, succinate-ubiquinone oxidoreductase) activity is induced. These electrons from CI and CII converge at the coenzyme Q-junction, followed by a linear downstream segment through Complexes III (CIII, ubiquinol-cytochrome c oxidoreductase) and CIV (cytochrome c oxidase), to the final electron acceptor oxygen. CI, CIII, and CIV are proton pumps that

generate an electrochemical potential difference across the inner mitochondrial membrane. Coupling of the phosphorylation system with the electron transport chain/system (ETS) allows the proton (H^+) potential to drive the phosphorylation of ADP to ATP. Protonophores such as Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) uncouple the ETS from ATP production, artificially boosting the ATP production within mitochondria, which helps in implicating the maximal respiratory capacity of the mitochondria. To achieve the mentioned phenomenon, FCCP is injected in a consecutive manner of 0.5-1.0mM concentrations, which promotes O_2 consumption (O_2->H_2O). Specific inhibitors of CI (1mM Rotenone, which denotes OXPHOS II) and CIII (5mM Antimycin A, AmA) are sequentially added at saturating concentrations. The residual O_2 consumption (denotes ROX) reading indicates either non-mitochondrial O_2 reduction or O_2-consuming reactions within the mitochondria that are not related to electron transfer. Therefore, ROX values are the rationale for background correction. Since CIV is a proton pump of the ETS, measurement of CIV activity requires uncoupler titrations to eliminate any potential control by the phosphorylation system. Therefore, electron flow through Complex IV (CIV, cytochrome c oxidase) is measured in intact mitochondria after inhibition of CIII by AmA, with the addition of 0.2M tetramethyl-p-phenylenediamine (TMPD) and 0.8M ascorbate (Asc) to sustain CIV in a reduced state – this establishes the end-point of the SUIT protocol[206–209].



A schematic representation of the electron transfer system (ETS) coupled to the phosphorylation system (ATP synthase, adenylate translocator, and inorganic phosphate transporter), to elucidate the SUIT protocol.

Data analysis: Manual selections were determined on the tracings, which were obtained following the substrates' addition. Calculations were performed on the oxygen flux readings in pmol/(s*10^6 cells) based on ETS' normalization. Background calculation was performed using ROX values.

Migration assay

The 48-well micro chemotaxis Boyden chamber (NeuroProbe, Gaithersburg, MD, USA) and 8μm pore polycarbonate membranes (Nucleopore, Pleasanton, CA, USA) coated with 10μg/ml fibronectin (Merck) were used to assess chemotaxis, as previously described[210].

Sphere-forming assay

To assess the ability of cells to form spheroids, gastric and colon cancer cells were plated at 15 cells/well in 96-well ultra-low attachment plates (Corning, Corning, NY) in 1:1 serum-free DMEM/F12 (LifeTechnologies, Waltham, MA), supplemented with 2% B27 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Subsequently, the number and diameter of the spheroids formed were evaluated by analyzing each well[211].

Statistical analyses

For normally distributed data, ANOVA analysis followed by Dunnett's post-hoc test procedures was used to evaluate differences among multiple groups in *in vitro* experiments. Unpaired t-tests with Bonferroni's correction were used to make comparisons between two groups evaluating whether fold changes are different from one, respectively. For data sets not following a Gaussian distribution, non-parametric analysis like the Kolgomorov-Smirnov test was performed. Metabolite Set Enrichment Analysis (MSEA) was used to identify biologically meaningful patterns that are significantly enriched in selected and more representative metabolites using Metaboanalyst 4.0. Different levels of statistical significance are indicated as follows: n.s., non-significant, (∗p <0.05, ∗∗p <0.005, ∗∗∗p <0.0005, ∗∗∗∗p <0.00005). The analysis was performed using GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla, CA, USA.

1

Antiproliferative effect of cannabinoids in gastric and colon cancer

The basal cannabinoid receptor expression level on different gastrointestinal cancer cell lines was measured by a quantitative PCR experiment. As displayed in **Figures 7A** and **7B**, AGS and MKN45 (representing gastric cancer models) and HCT116 and HT29 (representing colon cancer models) displayed basal expression of mRNA levels for both CB1R and CB2R.

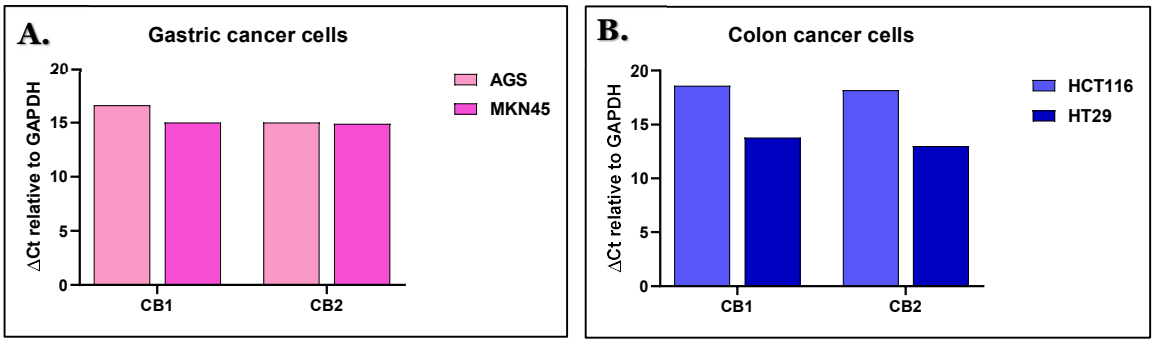


Figure 7: mRNA expression of cannabinoid receptors on select gastrointestinal cancer cell lines. *qPCR analyses of* ***(A)*** *GC and* ***(B)*** *CRC cells (represented as cycle threshold or Δct value relative to the housekeeping gene GAPDH) to detect mRNA expression levels of CB1 and CB2 receptors.*

Cannabinoids are reported to inhibit cell growth in GI cancer cells[182,212–216]. To confirm the action of a cannabinoid receptor activation on GI cancer cell growth, we tested the effect of selective full agonists for CB1 and CB2 receptor types (ACEA and JWH133, respectively) on two different human-derived cell lines. Specifically, AGS cells were used as a model of gastric cancer, and HCT116 cells as a model of colon cancer. In agreement with the evidence from the literature (based on other *in vitro* models)[217,218], both ACEA (10μM) and JWH133 (10μM) significantly reduced the number of AGS (**Figure 8A**) and HCT116 (**Figure 8B**) cells, as illustrated by the representative microscopic determination of cell viability by the Hoechst 33342 dye's staining.

Based on the quantification of the effect shown in the corresponding histograms on AGS (**Figure 8C**) and HCT116 (**Figure 8D**), we established 10µM as the effective concentration to be utilized for the study.

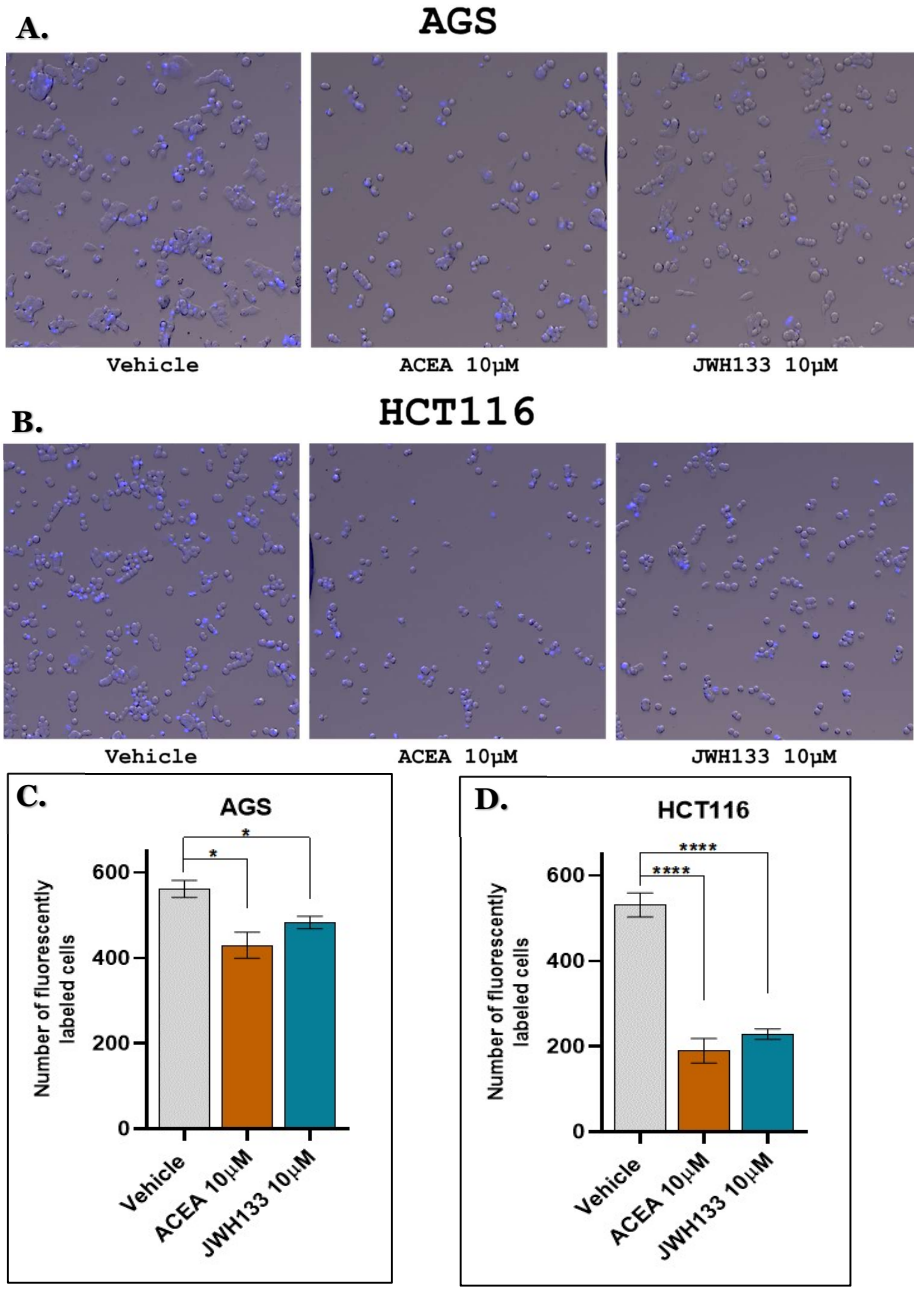
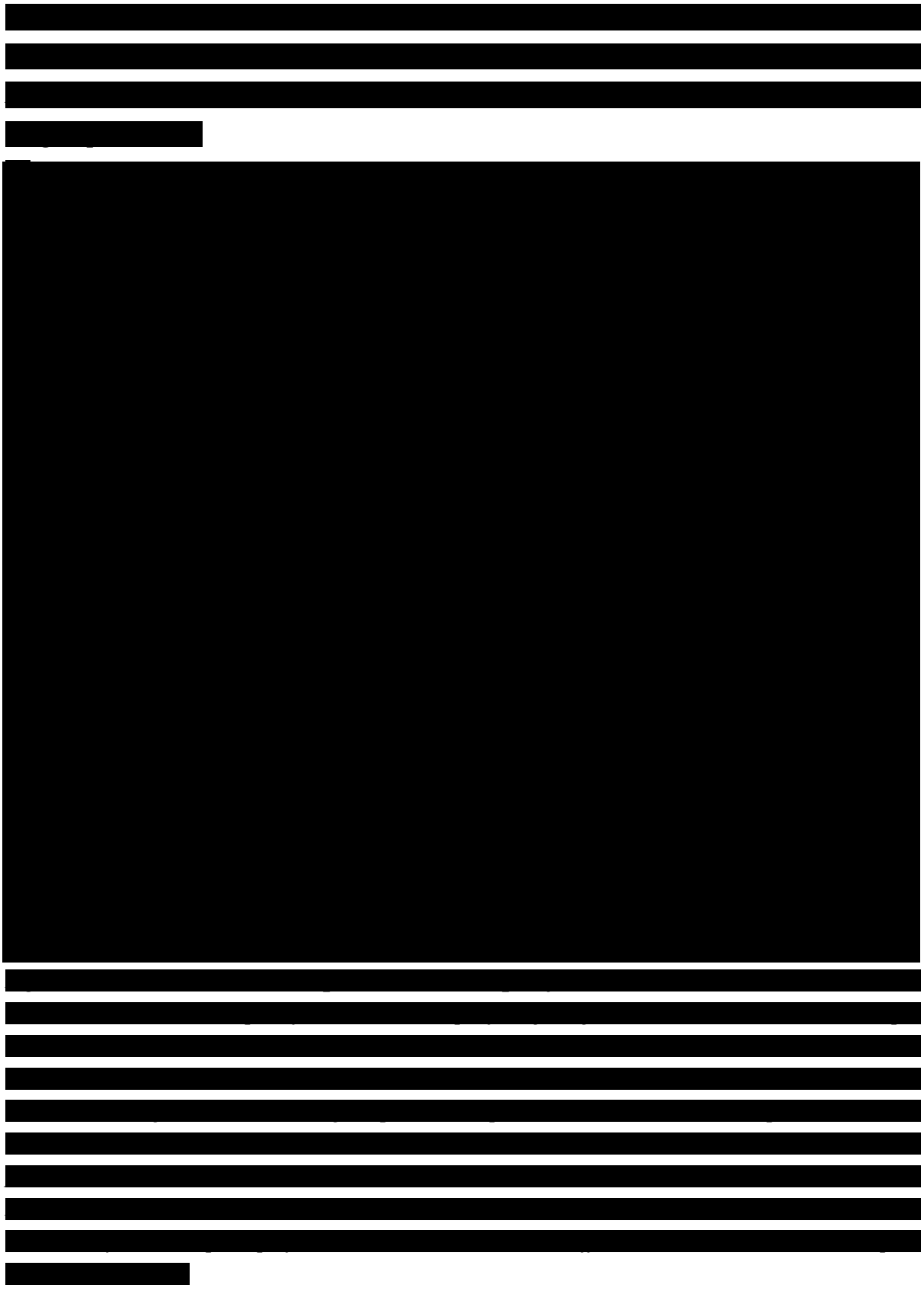


Figure 8: Effect of cannabinoids on cell proliferation. *Representative images of* ***(A)*** *AGS, and* ***(B)*** *HCT116 upon 24h treatment with cannabinoids – ACEA and JWH133, at 10µM concentrations. Images were captured at 10X resolution, post Hoechst staining. Cell count quantification on* ***(C)*** *AGS, and* ***(B)*** *HCT116 cells. Data are represented as mean ± SEM of two independent experiments, with each group having two technical replicates. One-way ANOVA (α=0.05) was performed to establish statistical differences between Vehicle and ACEA/JWH133 treatment groups. [the respective p-values correspond to <0.05(*),<0.00005(****)]*

2

Metabolomics profile of gastric and colon cancer cells



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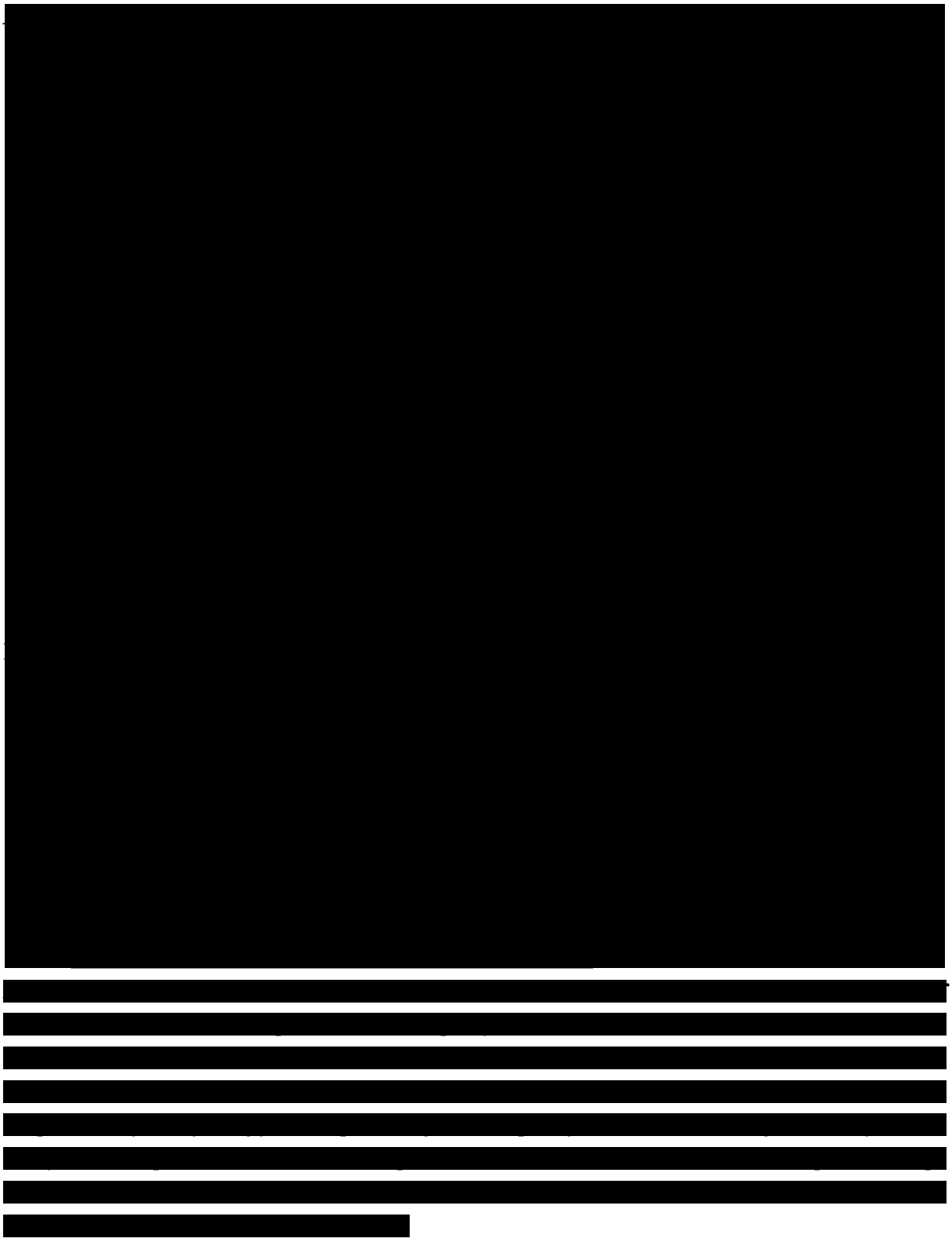

3

Effect of cannabinoids on gastric and colon cancer metabolomics

[redacted]

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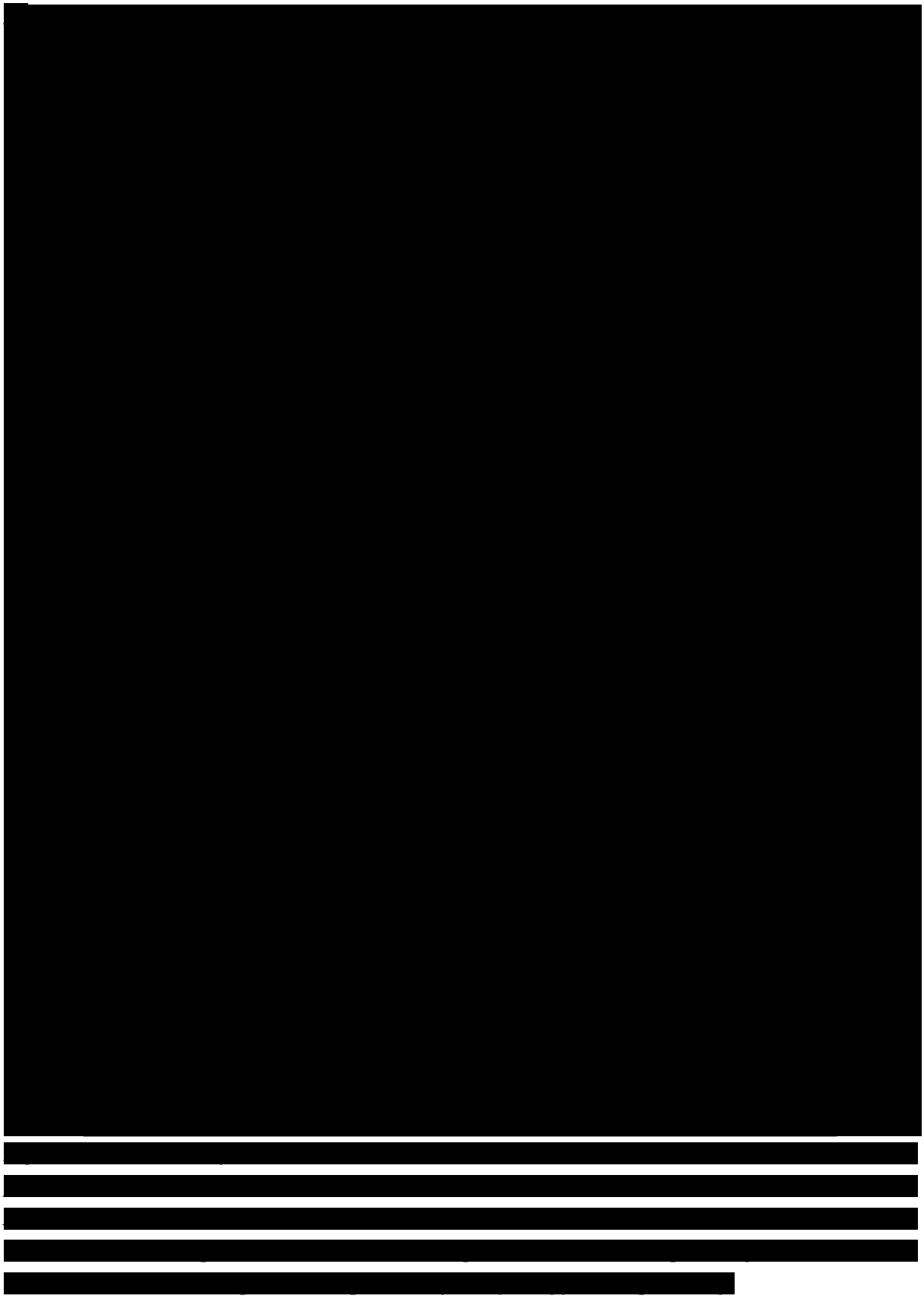

4

Effect of cannabinoids on targeted gene expression in gastric and colon cancer

Using gene expression analysis, the effects of cannabinoids on key targets related to the metabolic pathways primarily impacted in MESA were investigated, in both AGS and HCT116 cells.

Interestingly, when comparing the effects of ACEA (10μM) and JHW133 (10μM) on the mRNA expression of the mitochondrial isoform enzyme GPT1, which regulates the glucose-alanine cycle, and the serine-glutamine transporter ASCT2, a contrasting response depending on the cancer cell type was observed. In AGS cells, 24-hours treatment with cannabinoids significantly down-regulated GPT1 (ACEA, $p<0.0001$; JWH133, $p=0.0006$) (**Figure 14Aa)** and ASCT2 (ACEA, $p=0.0212$; JWH133, $p=0.0159$) (**Figure 14Ba**). Conversely, in HCT116 cells the treatments led to the upregulation of the same genes (GPT1: ACEA, $p=0.0037$; JWH133, $p=0.0065$; ASCT2: ACEA, $p>0.05$; JWH133, $p=0.0147$) (**Figure 14Ab** and **14Bb** respectively).

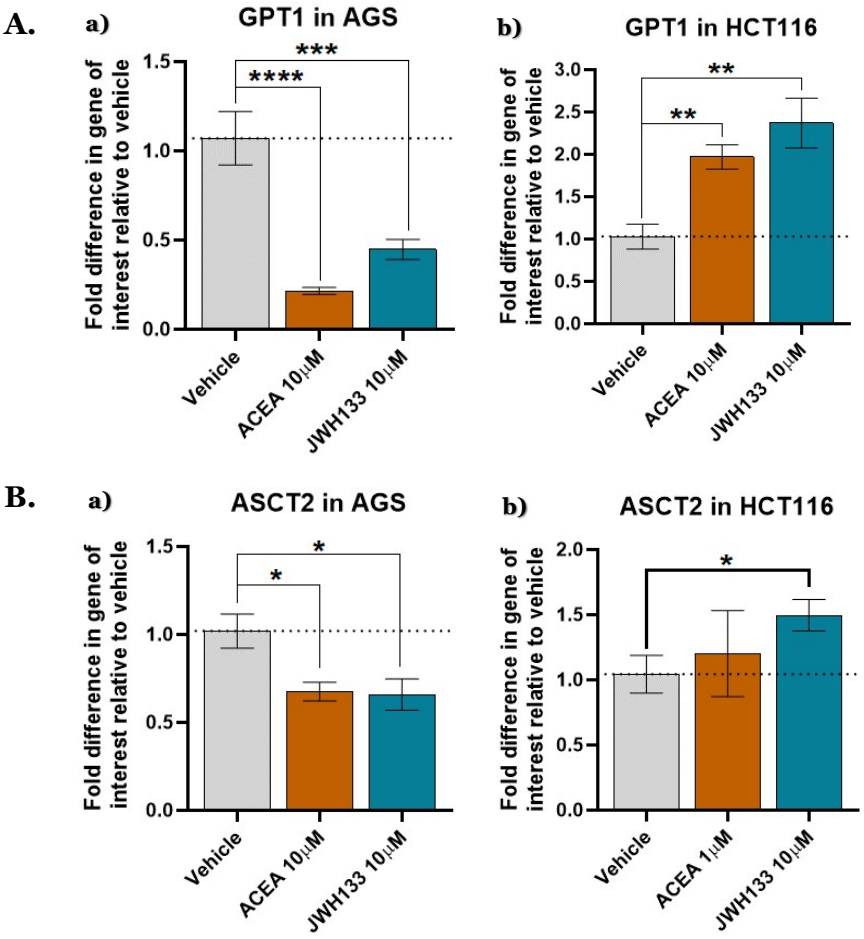

Figure 14: Quantitative PCR for GPT1 and ASCT2 expression upon cannabinoid treatment. *cDNAs derived from AGS and HCT116 cells were used to quantify the expression levels of* ***(A)*** *GPT1 and* ***(B)*** *ASCT2. The values are reported as fold changes in the expression of the housekeeping gene (β-Tubulin) and normalized to the vehicle for each treatment group. Data are represented as mean ± SEM of three independent experiments, with each group having four technical replicates. One-way ANOVA (α=0.05) was performed to establish statistical differences between Vehicle, ACEA, and JWH133 treatment groups. [the respective p-values correspond to <0.05(*), <0.005(**), <0.0005(***), <0.00005(****)]*

Further investigations were carried out to investigate whether cannabinoid treatments modulated the mRNA expression of GLUT1, an important hallmark in cancer cells that facilitates the basal uptake of glucose, ensuring the flux of sugar into metabolic pathways. As shown in **Figure 15A**, a trend was observed, particularly for JWH133 treatment, toward reduced mRNA expression of GLUT1 in both cell types.

Finally, we investigated whether cannabinoid treatments modulate the mRNA expression of PPARγ, which acts as a lipid sensor and is a major metabolic regulator of fatty acid metabolism, also involved in autophagy activation. As illustrated in **Figure 15B**, only selected treatments (JWH133 for AGS cells, p=0.0106, and ACEA for HCT116 cells, p=0.0178) were able to up-regulate PPARγ mRNA levels in both cell types significantly.

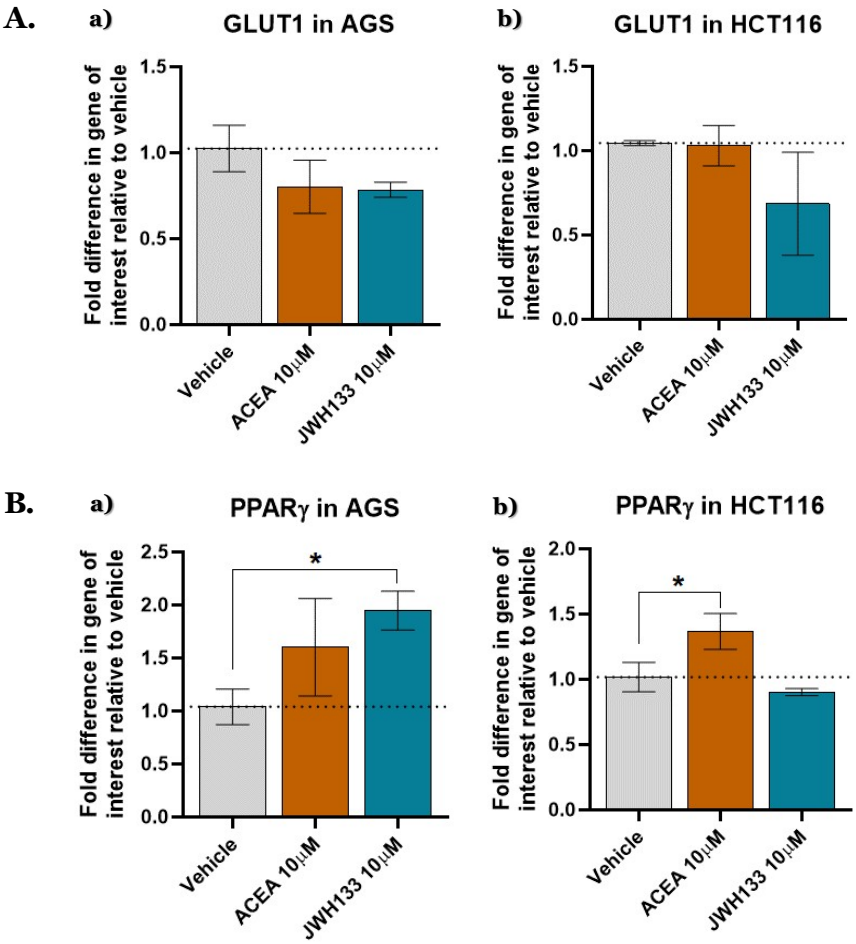

Figure 15: Quantitative PCR for GLUT1 and PPARγ expression upon cannabinoid treatment. *cDNAs derived from AGS and HCT116 cells were used to quantify the expression levels of* ***(A)*** *GLUT1 and* ***(B)*** *PPARγ. The values are reported as fold changes in the expression of the housekeeping gene (β-Tubulin) and normalized to the vehicle for each treatment group. Data are represented as mean ± SEM of three independent experiments, with each group having four technical replicates. One-way ANOVA (α=0.05) was performed to establish statistical differences between Vehicle, ACEA, and JWH133 treatment groups. [the respective p-values correspond to <0.05(*)]*

5

Effect of cannabinoids on the main metabolic pathways

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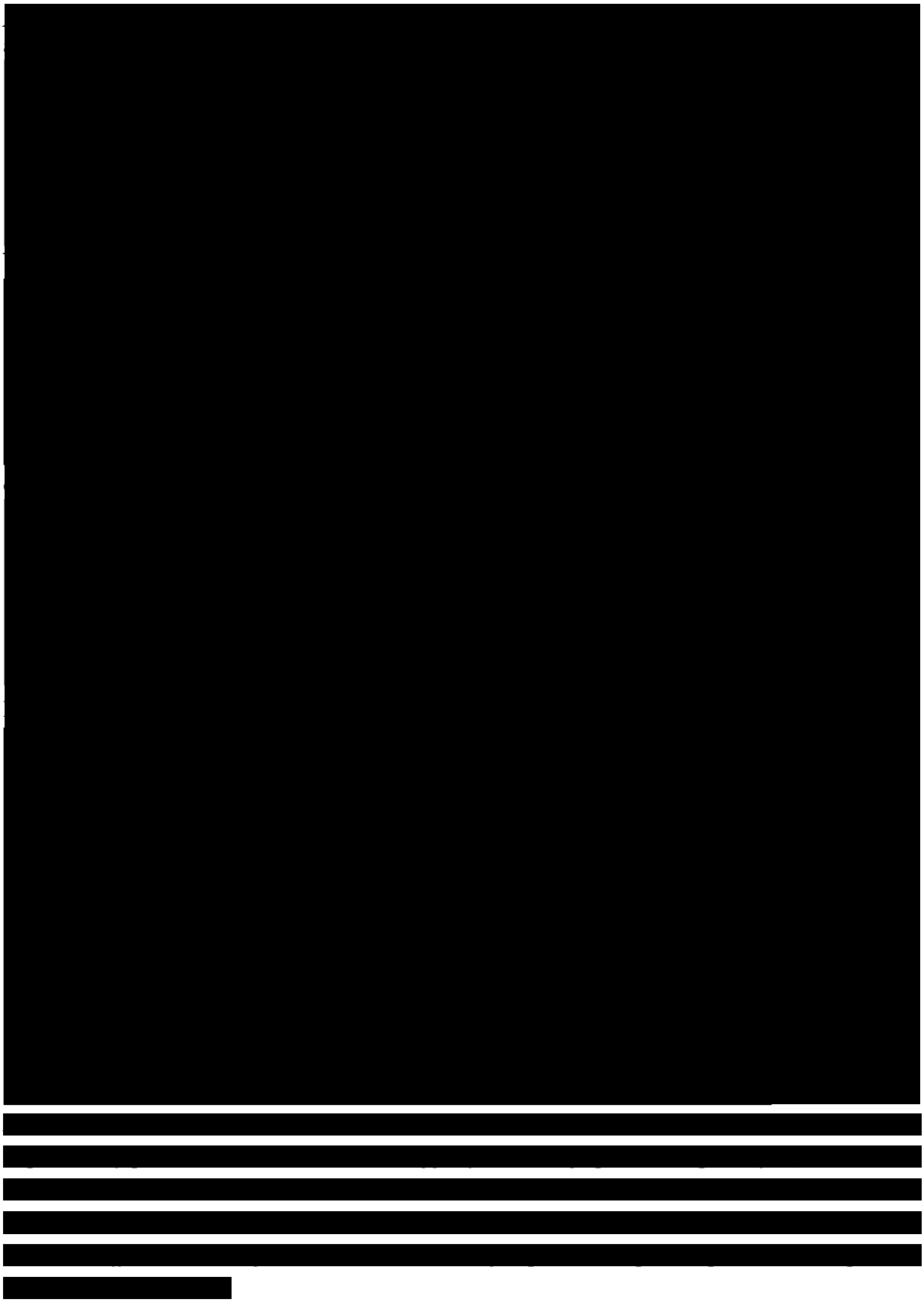

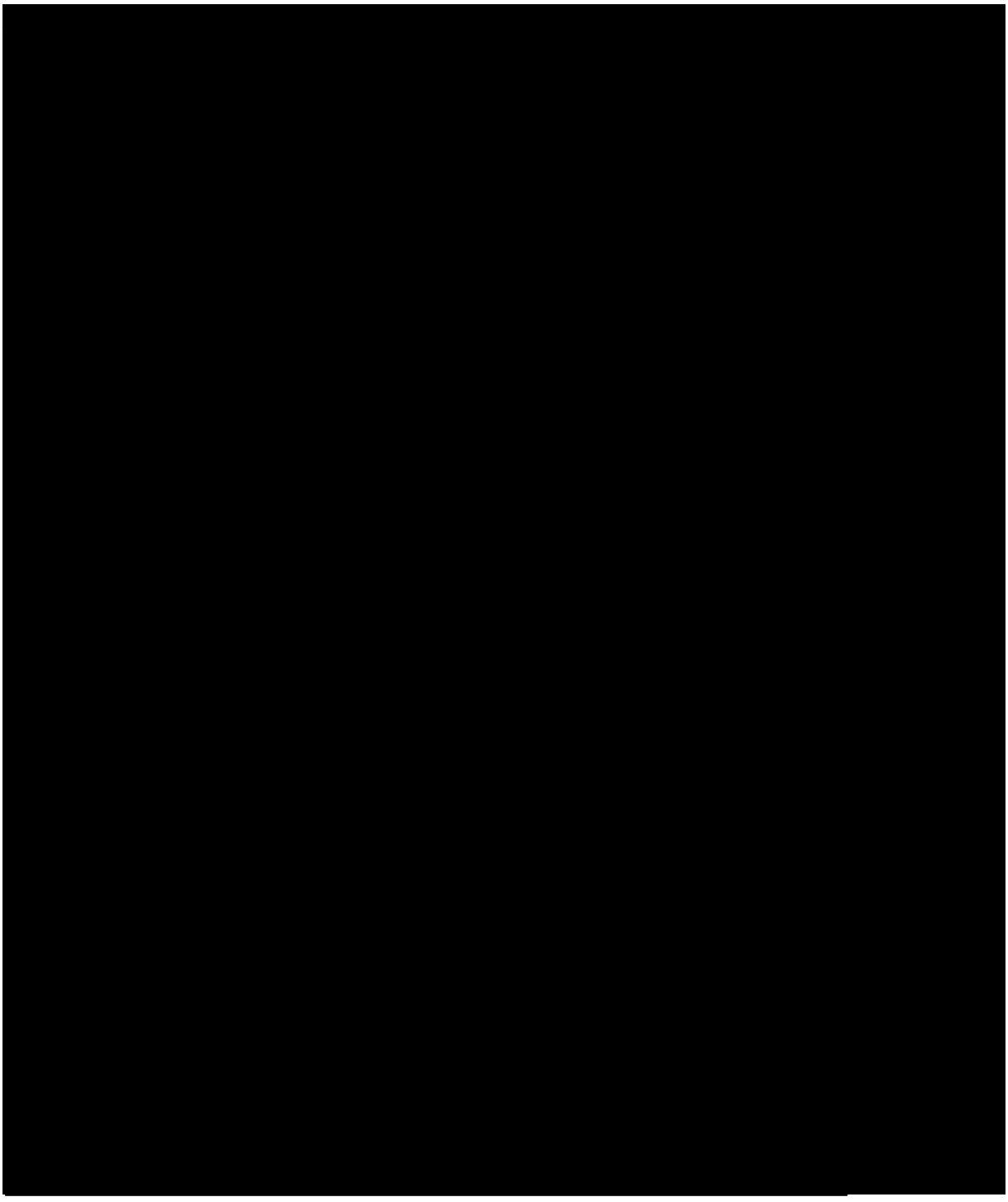

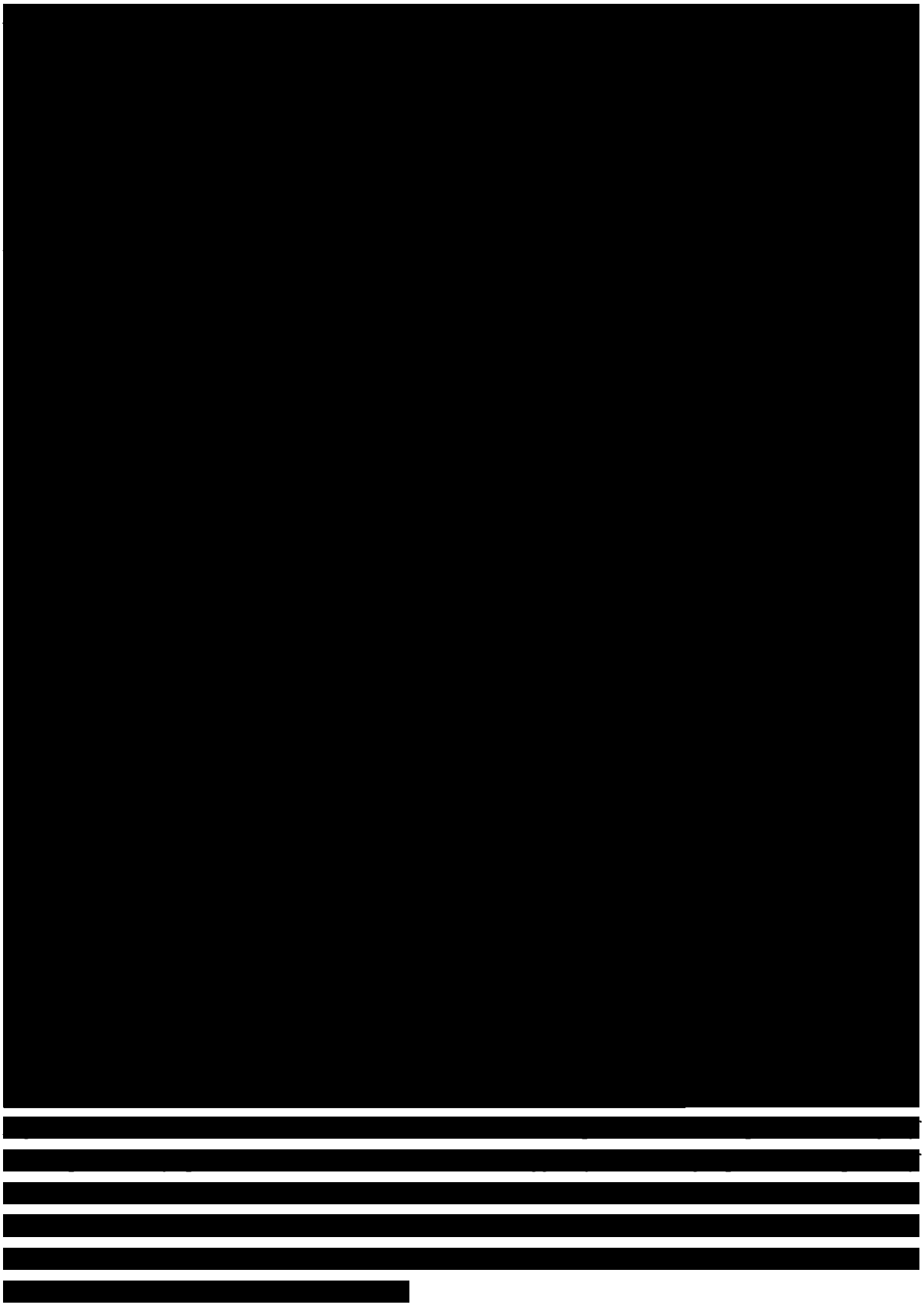

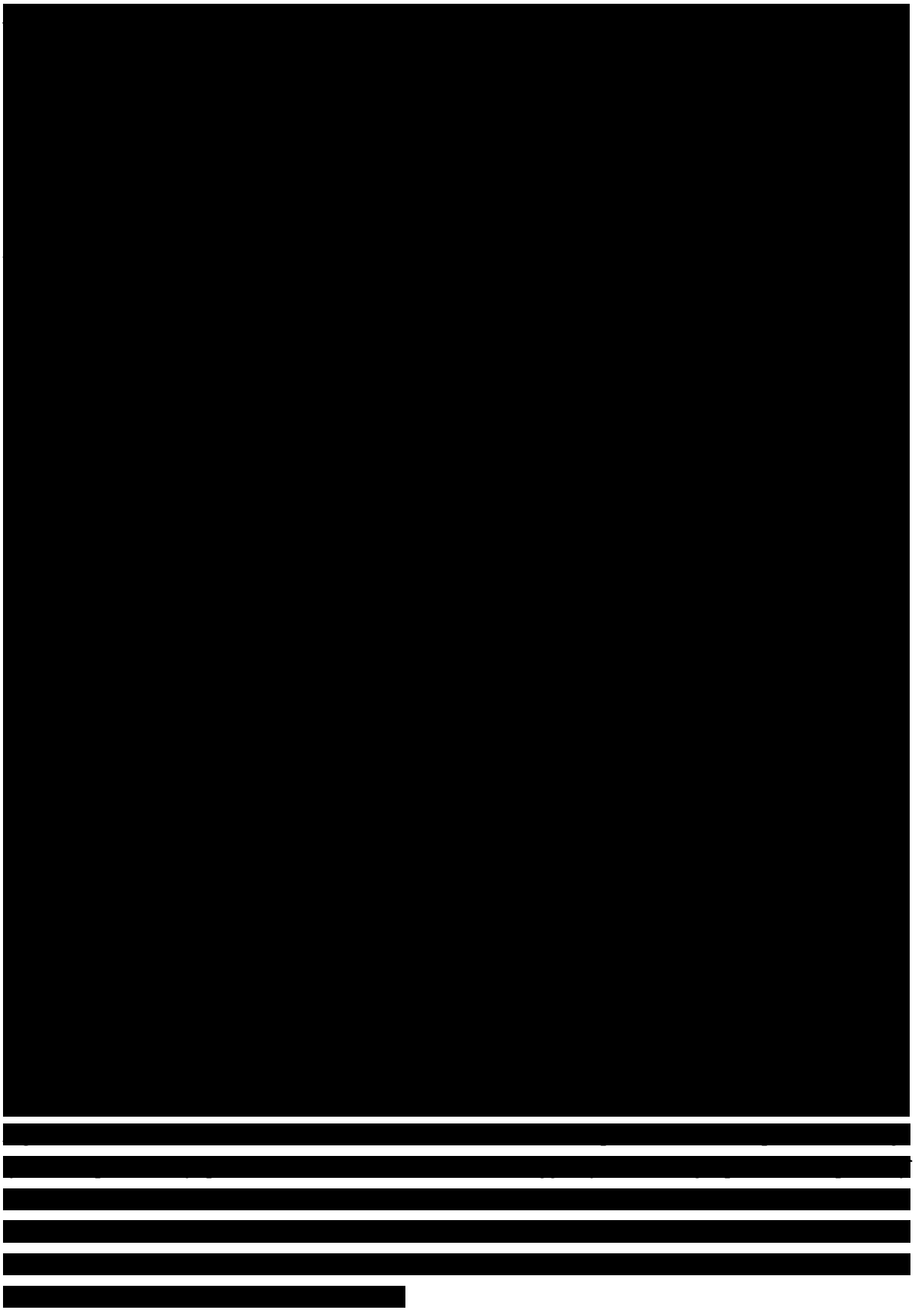

6

Effect of cannabinoids on migration properties in gastric and colon cancer

Cancer cells with stem-like properties show metabolic adaptations that enhance migration. The metabolic shifts associated with the acquisition of stemness often involve pathways that support both migration and invasion, thereby linking metabolic reprogramming to the ability of cancer cells to metastasize. Cellular metastatic potential is determined by a cell's capacity to migrate from its site of origin, in response to available nutrients in their proximity.

Based on the specific metabolic fluctuations induced by ACEA and JWH133 in gastric and colon cancers, the aim was to investigate whether exposure to cannabinoids would affect the rate of migration in AGS and HCT116 cells. To assess the impact of cannabinoids (ACEA 10μM and JWH133 10μM) on the migration properties of GI cells, the functional test was employed (the Boyden Chamber assay).

For a quantitative measurement, the Boyden chamber method was performed. AGS and HCT116 cells were cultured in DMEM with 10% FBS as a monolayer inside the Chamber and allowed to migrate through the pores, to the other side of the membrane. The treatment groups included ACEA 10μM and JWH133 10μM, which were suspended above a larger well placed under the chamber. After 24 hours of incubation, invasive cells were detached, stained, and counted. All treatments significantly reduced the propensity of AGS cells to migrate (**Figure 21A**, ACEA $p=0.0109$ vs. Vehicle; JWH133 $p=0.0146$ vs. Vehicle). Similar results were observed in HCT116 cells, where the treatments had a substantial impact on migration rate (**Figure 21B**, ACEA $p<0.00005$ vs. Vehicle; JWH133 $p<0.00005$ vs. Vehicle).

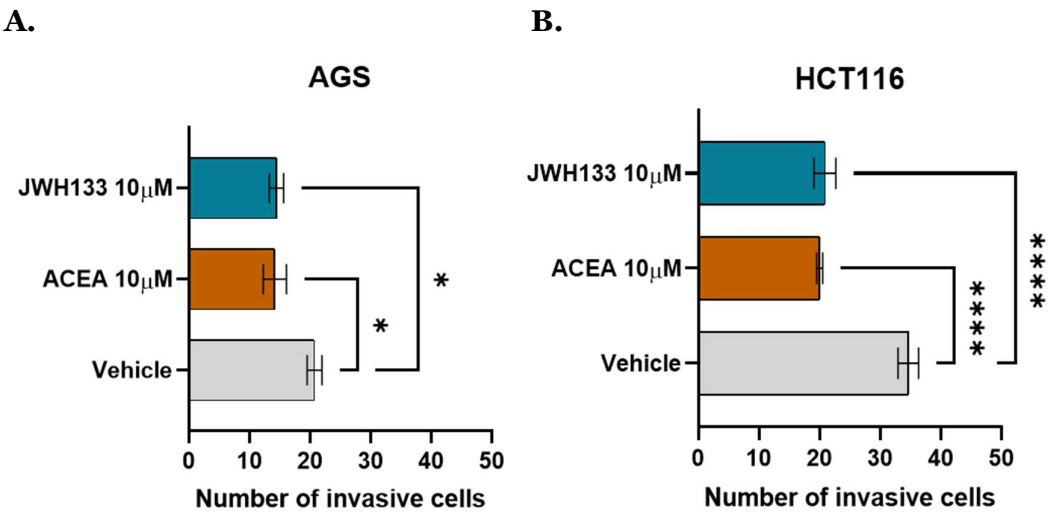

Figure 21: Determination of migration property in GI cancer cell lines by Boyden chamber method. *The number of migrated* ***(A)*** *AGS and* ***(B)*** *HCT116 cells toward complete medium (Vehicle) or the same growth medium with the addition of ACEA (10μM) or JWH133 (10μM) have been graphically represented as mean ± SEM of* ***(A)*** *seven and* ***(B)*** *eight independent experiments. One-way ANOVA (α=0.05) followed by Dunnett's correction was implemented to establish statistical differences among vehicle and treatment groups. [the respective p-values correspond to <0.05(*), <0.00005(****)]*

7

Effect on stemness upon cannabinoid induction in gastric and colon cancer

Considering the inhibitory effect observed on the migration rate of gastric and colon cancer upon ACEA and JWH133 treatments, we aimed to verify through gene expression analysis whether these cannabinoids could down-regulate the mRNA expression of key stemness master regulators like SOX2, OCT4, and NANOG.

Figure 22 illustrates data from RT-PCR analysis performed after 12h treatment with ACEA (10μM) or JWH133 (10μM) in AGS and HCT116 cells. In AGS, Both ACEA and JWH133 significantly down-regulated the mRNA expression levels of the key stemness markers. As illustrated in **Figure 22A**, ACEA impacted the mRNA expressions of SOX2 ($p<0.00005$), OCT4 ($p<0.00005$), NANOG ($p<0.00005$), while JHW133 treatment significantly reduced the expression of SOX2 ($p<0.00005$), OCT4 ($p<0.00005$), as well as NANOG mRNA ($p<0.00005$). Interestingly, in HCT116 cells, ACEA treatment down-regulates only one specific stemness marker (**Figure 22B**, $p<0.00005$ for OCT4). As illustrated in **Figure 22B**, 10μM JWH133 significantly reduced SOX2 ($p<0.00005$) and OCT4 ($p<0.00005$).

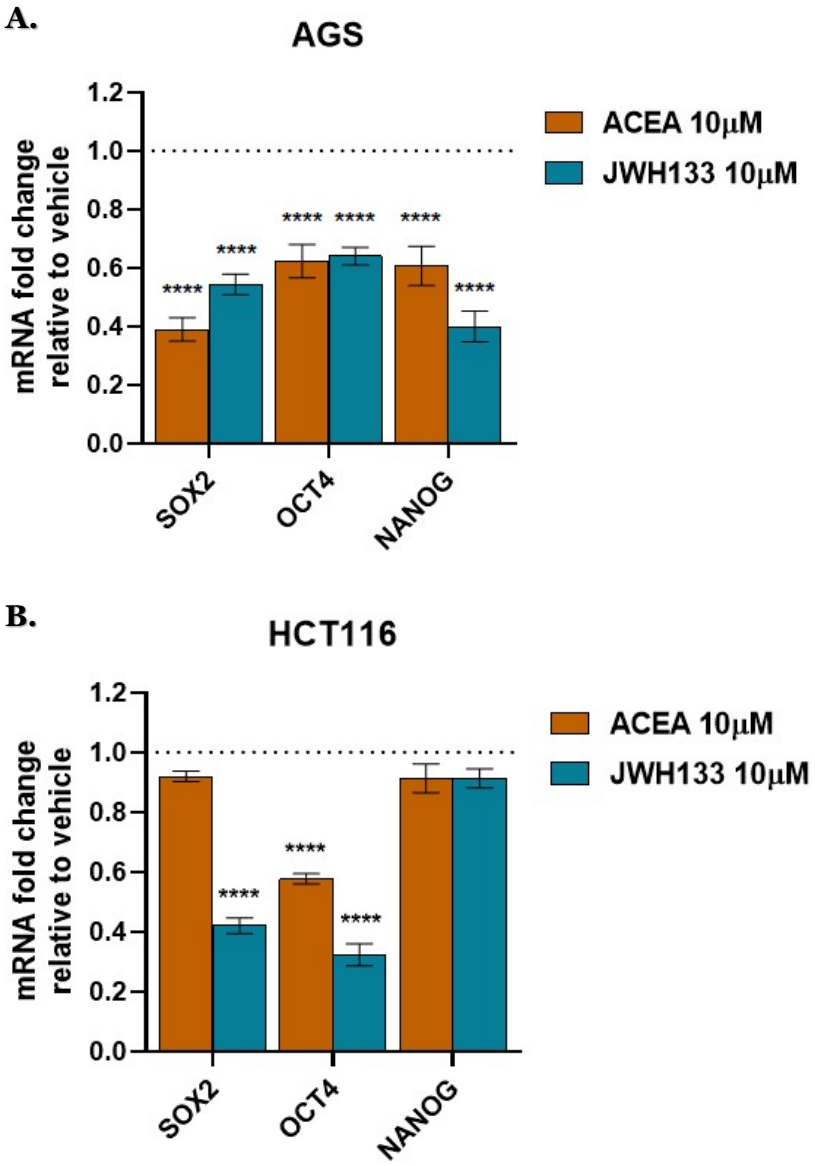

Figure 22: Effect on stemness upon cannabinoid treatments. *The graphical figures represent the quantitative mRNA fold change of key stemness markers, such as SOX2, OCT4, and NANOG normalized to the Vehicle (denoted by the dotted line) in* ***(A)*** *AGS and* ***(B)*** *HCT116 cells treated with ACEA (10μM) and JWH133 (10μM) for 12h. The values have been depicted as fold changes in the expression of the housekeeping gene (β-Tubulin) and normalized to the vehicle for each treatment group. Data are represented as mean ± SEM of three independent experiments. Two-way ANOVA (α=0.05) followed by Dunnett's correction was implemented to establish statistical differences among vehicle and treatment groups. [the respective p-values correspond to <0.00005(****)]*

8

Effect of cannabinoids on spheroid formation in gastric and colon cancer

After confirming that ACEA and JWH133 affected the migration and stemness properties of AGS and HCT116 cells, we proceeded to investigate the impact of these cannabinoids on spheroid formation as a functional assay to assess cancer cells with stem-like characteristics, capitalizing on their ability to grow in low-adherence conditions.

AGS cells were seeded at a density of 20 cells per well in a 96-well plate in the presence or absence of ACEA (10μM) or JWH133 (10μM). After 14 days, the effect on the number and diameter of spheroids formed was measured. Interestingly, the addition of 10μM JWH133 significantly reduced both the spheroid number (**Figure 23Aa**, $p<0.0001$) as well as the spheroid diameter (**Figure 23Ba**, $p=0.0073$) in AGS-derived spheroids.

Similarly, HCT116 cells were seeded at a density of 20 cells per well in a 96-well plate in the presence or absence of ACEA (10μM) or JWH133 (10μM). After 14 days, the effect on the number and diameter of spheroids formed was measured. In HCT116 cells, treatment with JWH133, but not with ACEA, significantly reduced the number of spheroids formed (**Figure 23Ab**, $p=0.0018$) compared to the control group, thereby impacting cancer stem cell division. Additionally, ACEA and JWH133 treatments decreased spheroid diameter (**Figure 23Bb**, ACEA $p=0.0073$ vs Vehicle; JWH133 $p=0.0006$ vs Vehicle).

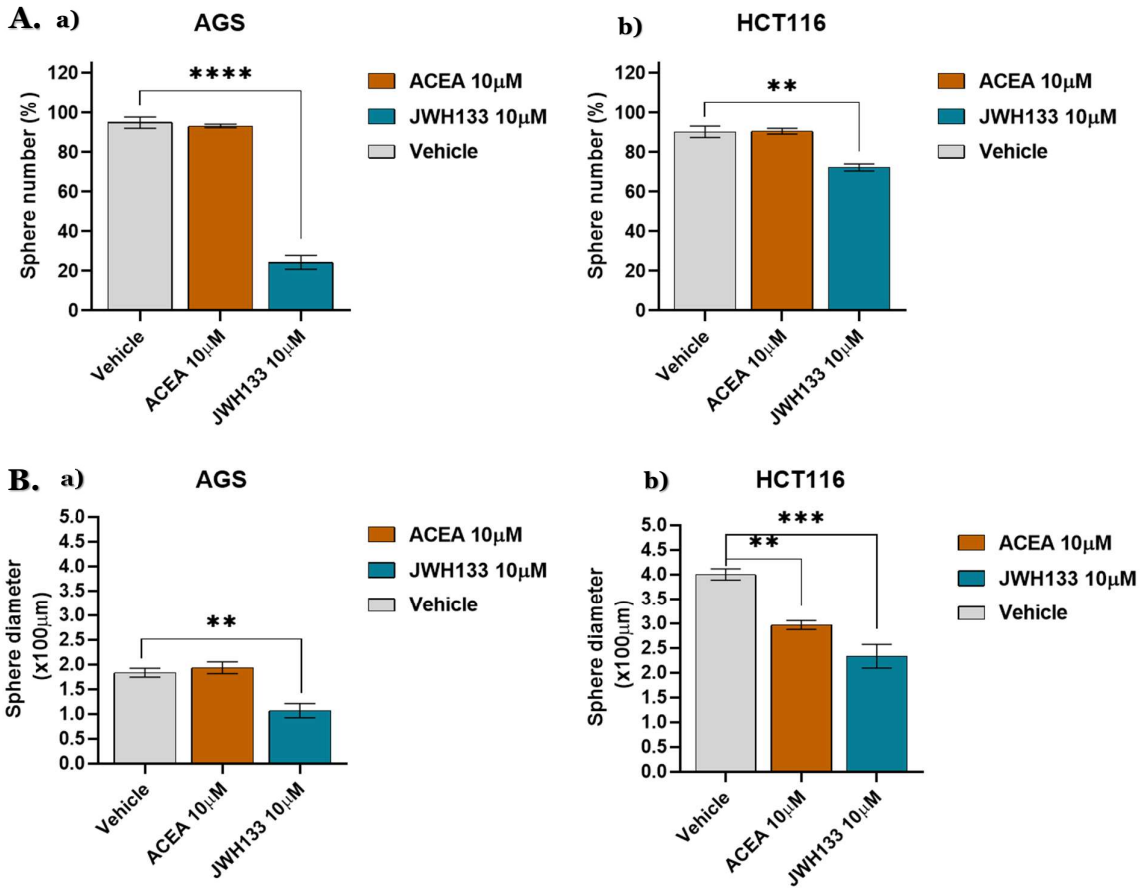

Figure 23: Quantification of spheroid number and spheroid diameter upon cannabinoid treatment. (A) *Quantification of spheroid number by the formula [(number of spheroids formed/number of wells containing cells)×100] in* ***(a)*** *AGS and* ***(b)*** *HCT116 in the presence or absence of ACEA (10μM) and JWH133 (10μM).* ***(B)*** *Quantification of spheroid size (mean diameter in μm) in* ***(a)*** *AGS and* ***(b)*** *HCT116 in the presence or absence of ACEA (10μM) and JWH133 (10μM). Data are represented as mean ± SEM of three independent experiments. One-way ANOVA (α=0.05) followed by Dunnett's correction was implemented to establish statistical differences among vehicle and treatment groups. [the respective p-values correspond to <0.005(**), <0.0005(***), <0.00005(****)]*

Discussion

Cancer cells exhibit distinct metabolic regulation pathways throughout their pathogenic progression. These metabolic modifications are essential for fulfilling cancer cells' high energy and anabolic material requirements. In recent decades, the study of cancer metabolism has garnered considerable attention, as it is considered a critical hallmark of the disease[219]. Given the competitive survival metabolic phenotype exhibited by gastrointestinal cancers, combined with a resistant microenvironment characterized by chronic inflammation, it is imperative to explore various metabolic tools.

Cannabinoids have emerged as promising adjunctive therapeutic agents supported by documented evidence of their effects on various tumor processes, including those related to GI cancers. Specifically, cannabinoids can inhibit tumor growth and migration, as well as modulate metabolic processes that underpin tumor survival and proliferation through different mechanisms of action -not always related to cannabinoid receptor activation. With this background in mind, the present work aimed at investigating the effects of selective cannabinoid agonists on the metabolic pathways associated with gastric and colon cancers, two of the most prevalent malignancies worldwide. These investigations will provide essential insights necessary to characterize these aggressive gastrointestinal models and to elucidate the potential role of cannabinoids, in this context.

To well address our study, we first aimed to characterize the metabolomic profile of various GI cancer cell lines. AGS and MKN45 cell lines were selected as a model for gastric cancer, while HCT116 and HT29 cell lines were chosen as a model for colorectal cancer. [redacted]

[redacted]

In addition to the metabolomic analysis, we also considered the results from a parallel study exploring the effects of cannabinoids on targeted gene expression in gastric and colon cancer. As part of this investigation, we performed mRNA analyses of key receptors, transporters, and enzymes associated with those metabolic pathways likely modulated by cannabinoid treatment. Our observations revealed opposing effects on the mRNA expression of glucose-alanine transaminase (GPT1) and the serine-glutamine transporter (ASCT2) in AGS and HCT116 cell lines (as indicated in **Figures 14A** and **14B**).

In furthering our understanding of the pathway dynamics, we also examined additional genes, including GLUT1, which is pivotal in regulating glucose influx, and PPARγ recognized for its role in apoptosis promotion within cancer cells. Notably, PPARγ activates the expression of essential genes such as PTEN, c-myc, and p27, all of which are critical in regulating cell growth and apoptosis[220]. Our results indicate a significant increase in PPARγ mRNA expression following selective cannabinoid treatments in both AGS and HCT116 cell groups, while mRNA GLUT1 expression showed a trend to decline (as indicated in **Figures 15B** and **15A**).

In the context of cancer research, cytosolic glycolysis represents the most significant energy-producing mechanism, playing a critical role in various physiological and pathological processes, particularly when compared to mitochondrial respiration, which produces adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS). [redacted]

[redacted]

These findings suggest that cannabinoids can alter the specific metabolic strategies that gastrointestinal tumors use to evade control over cell fate. This diversity in metabolic approaches has prompted us to formulate a cohesive explanation to clarify the mechanisms at play. As a result, we have outlined the possible fundamental pathway mechanisms in these distinct GI models to elucidate these actions and their effects.

[redacted]

[redacted]

[redacted]

[redacted]

Recognizing the potentially positive impact of cannabinoids on such gastrointestinal cancer cell lines, we acknowledged that the specific mechanism of action of the selected synthetic cannabinoid agonists remained unclear due to the project's time limitations. To support our initial findings, we aimed to validate our conclusions through a composite cellular model that contemplates fundamental characteristics of cancer, such as sustaining proliferation, activating invasion, and enabling replicative capacity)[225]. Notably, both

ACEA and JWH133 reduced the proliferation of AGS and HCT116 cancer cells, their migratory capacity, and the expression of important stemness markers such as SOX2, OCT4, and NANOG, in both the GI cancer types (as indicated in **Figures 8,21** and **22**). Furthermore, we investigated the effect of these cannabinoids on spheroid models, which provide a more accurate depiction of the tumor microenvironment compared to traditional 2D cell cultures[226]. We observed notable differences in the effects of the treatments on the formation of the two types of homotypic tumor spheroids investigated. In both AGS and HCT116 cells, JWH133 significantly influenced both the size and quantity of the spheroids derived by AGS and HCT116 cells. In contrast, ACEA only reduced the growth but not the diameter of HCT116-derived spheroids (as indicated in **Figure 23**).

Conclusion

Our study indicates that cannabinoids can significantly influence both the metabolic pathways and the proliferative characteristics of gastrointestinal tumors, revealing cell-type specific alterations as well as broader trends in metabolic adaptation.

In AGS cells, cannabinoid treatment led to a decrease in ASCT2 expression, resulting in reduced glutamine and glutamate levels, coupled with an increased PPARγ expression, which altogether adversely alters energy utilization and likely pushes towards a pro-apoptotic environment. In HCT116 cells, [redacted]. Notably, the reduction in proliferation and migratory capacity, along with the decreased expression of important stemness markers such as SOX2, OCT4, and NANOG, underscores the potential of cannabinoids to modulate both metabolic and stemness-related pathways in these particular GI cancers. These findings suggest that strategically targeting these pathways could open new therapeutic avenues. Additionally, the implications of altered UDP-GlcNAc signaling and O-GlcNAcylation highlight the complexity of cannabinoid effects on cancer cell metabolism, indicating a state of nutrient stress that may influence cancer progression and treatment resistance.

Collectively, our results provide valuable insights into the interplay between cannabinoid treatment, metabolic plasticity, and stemness characteristics in gastrointestinal tumors, paving the way for future investigations aimed at harnessing their therapeutic potential.

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