

2º CICLO - MESTRADO

MESTRADO EM MEDICINA E ONCOLOGIA MOLECULAR

Unveiling the role of cholesterol in colorectal cancer

Ana Isabel Vieira Pinto

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Disclaimer

The research presented in this thesis is integrated into a broader project developed by Ana Luísa Marinho da Cunha Machado as part of her PhD dissertation in the Biomedicine doctoral program at FMUP. As such, there may be some overlap between the content of this thesis and her ongoing work. Every effort has been made to appropriately distinguish the contributions of each project.

Abstract

Colorectal cancer (CRC) often presents with dysregulated cholesterol metabolism, which has been associated with tumor progression by promoting tumor growth, invasion, metastasis, and the establishment of an immunosuppressive tumor microenvironment (TME). Disruption of cholesterol homeostasis in CRC can, therefore, be associated with its high incidence and mortality rates. Targeting cholesterol metabolism is an emerging strategy in cancer treatment, but it largely focuses on inhibiting biosynthesis. As cholesterol metabolism encompasses a series of other pathways that act in a coordinated manner to regulate cholesterol levels within the cell, cancer cells are likely to possess mechanisms to overcome the lack of cholesterol production. Since CRC is highly heterogeneous, cholesterol metabolism is also expected to differ between tumors. However, no stratification systems for defining prognosis have considered this possibility, and CRC cholesterol metabolism remains uncharacterized.

A previous *in silico* analysis of samples from The Cancer Genome Atlas (TCGA) conducted by our group demonstrated that CRC tumors can be clustered based on the expression of genes related to cholesterol metabolism. These clusters also exhibit varying levels of proliferative capacity. Our current aim is to explore how these clusters are associated with CRC tumorigenesis and therapy outcomes by establishing *in vitro* models that replicate them, allowing for characterization beyond bioinformatics.

In this project, we retrieved RNAseq raw counts of 53 cell lines included in the Cancer Cell Line Encyclopedia (CCLE) and performed an *in silico* analysis according to the methodology from our previous study. In short, the samples were normalized using EDAsq and, after log2 transformation, scored using Single-Sample Gene Set Enrichment Analysis (ssGSEA), according to the expression of a curated list of genes involved in cholesterol biosynthesis, storage, uptake, efflux, reverse transport, and catabolism. The cell lines were then clustered into five subgroups using the k-means algorithm based on the obtained scores. To reflect the main features of their cholesterol metabolism profile, they were classified as (1) High Cholesterol Metabolism (H-CM), (2) Low Cholesterol Metabolism (L-CM), (3) High Catabolism/Storage Cholesterol Metabolism (CS), (4) High Catabolism/Biosynthesis/Storage Cholesterol Metabolism (CBS), and (5) High Biosynthesis/Efflux Cholesterol Metabolism (BU). These clusters were comparable to those in the TCGA analysis, proving them as suitable models for studying the cholesterol metabolic signatures of CRC.

Upon characterizing the CCLE clusters, we found no statistically significant differences between them regarding molecular features, namely microsatellite instability status and Consensus Molecular Subtype, as well as clinicopathological characteristics,

including the origin of the cell line (primary/metastatic) and associated disease. This suggests that the cholesterol metabolism profile of CRC cell lines is likely independent of these factors. PROGENy analysis of the activation of tumorigenic signaling pathways, in contrast, revealed differences between the clusters. While the H-CM cluster presented overexpression of the TGF- β pathway and downregulation of the VEGF pathway, the EGFR, MAPK, and VEGF pathways were upregulated in the CS cluster, suggesting potential therapeutic targets for these clusters. Furthermore, we observed statistically significant differences between the CCLE clusters in pathways related to adaptive and type 2 immune response (slightly upregulated in the H-CM, CBS, and BU clusters) and antigen processing and presentation (downregulated in the H-CM cluster), implying a variable capacity to promote an immunosuppressive TME.

By selecting a cell line representative of each cluster, we attempted to validate the CCLE clusters through functional assays. Though work is still ongoing, assays addressing lipid storage and cholesterol uptake have partially allowed validation of the *in silico* profiles of the CRC cell lines. Additionally, we sought to determine how the CCLE clusters influence the TME by treating macrophages and fibroblasts with conditioned media (CM) from those same cell lines. Our findings revealed that only CM from the BU cluster cell line could significantly increase the activation of fibroblasts. However, it presented the lowest capacity to polarize macrophages into M1 or M2. CM from the H-CM and CBS clusters cell lines mostly polarized macrophages into the M1 phenotype, although the latter had a higher capacity to also polarize macrophages into M2, while CM from L-CM and CS clusters cell lines almost equally polarized into macrophages into M1 and M2. We have also assessed their response to chemotherapy – oxaliplatin (L-OHP) – in the presence and absence of cholesterol-lowering drugs. Notably, the cell lines with the most distinct cholesterol metabolism signatures revealed strikingly different results. Targeting cholesterol biosynthesis and storage in the L-CM cluster cell line drastically increased cell death both alone and in combination with L-OHP while also leading to cell cycle arrest at the G2-M phase. In contrast, inhibiting biosynthesis did not affect the viability of the H-CM cluster cell line, though it contributed to cell cycle arrest at the G2-M. Storage inhibition alone increased cell death but did not enhance the effects of L-OHP in this regard. These results suggest that the modulation of biosynthesis and storage may enhance chemotherapy sensitivity, depending on the cell lines' metabolic profile. Altogether, these results imply that stratifying CRC patients based on cholesterol metabolism can improve disease management.

Keywords: Colorectal cancer; cholesterol metabolism; cancer stratification; tumor microenvironment.

Resumo

O cancro colorretal (CCR) apresenta frequentemente uma desregulação do metabolismo do colesterol, que tem sido associado à progressão tumoral pela promoção do crescimento do tumor, invasão, metastização, e do estabelecimento de um microambiente tumoral (TME, do inglês *tumor microenvironment*) imunossupressor. A disrupção da homeostasia do colesterol no CCR pode, portanto, estar associada às suas elevadas taxas de incidência e mortalidade. Ter como alvo o metabolismo do colesterol é uma estratégia emergente no tratamento do cancro, mas que se tem focado principalmente na inibição da biossíntese. Dado que o metabolismo do colesterol abrange uma série de outras vias que atuam de forma coordenada para regular os níveis de colesterol dentro da célula, é provável que as células tumorais possuam mecanismos para superar a falta de produção de colesterol. Dado que o CCR é altamente heterogêneo, também é esperado que o metabolismo do colesterol varie entre tumores. No entanto, nenhum sistema de estratificação de prognóstico considerou esta possibilidade até ao momento, pelo que o metabolismo do colesterol no CCR permanece por caracterizar.

Um estudo *in silico* previamente conduzido pelo nosso grupo em amostras do *The Cancer Genome Atlas* (TCGA), demonstrou que tumores de CCR podem ser agrupados com base na expressão de genes relacionados com o metabolismo do colesterol. Estes grupos também exibem níveis variáveis de capacidade proliferativa. O nosso objetivo atual é explorar de que forma estes grupos afetam a tumorigénese e resposta à terapia no contexto do CCR, através do estabelecimento de modelos *in vitro* replicativos dos mesmos, de forma a permitir a sua caracterização além da bioinformática.

No presente estudo, utilizamos dados de RNAseq total de 53 linhas celulares de CCR incluídas na *Cancer Cell Line Encyclopedia* (CCLE) e realizámos uma análise *in silico* de acordo com a metodologia usada no nosso estudo anterior. Em resumo, as amostras foram normalizadas através de *EDAseq* e, após transformação em log2, foram-lhes atribuídos scores através da análise de *Single-Sample Gene Set Enrichment Analysis* (ssGSEA), de acordo com a expressão de genes envolvidos na biossíntese, armazenamento, internalização, efluxo, transporte reverso e catabolismo do colesterol. As linhas celulares foram depois agrupadas em cinco subgrupos usando o algoritmo *k-means* com base nos scores obtidos. Para refletir as principais características do seu perfil de metabolismo do colesterol, foram classificados como (1) Metabolismo Elevado de Colesterol (H-CM), (2) Metabolismo Reduzido de Colesterol (L-CM), (3) Metabolismo Elevado em Catabolismo/Armazenamento de Colesterol (CS), (4) Metabolismo Elevado em Catabolismo/Biossíntese/Armazenamento de Colesterol (CBS) e (5) Metabolismo Elevado em Biossíntese/Internalização de Colesterol (BU). Estes grupos mostraram-se

comparáveis aos da análise do TCGA, provando que são modelos adequados para o estudo das assinaturas metabólicas do colesterol no CCR.

Ao caracterizar os subgrupos da CCLE, não encontramos diferenças estatisticamente significativas entre eles em relação a características moleculares, nomeadamente no grau de instabilidade de microssatélites e no seu *Consensus Molecular Subtype*, bem como a características clinicopatológicas, incluindo a origem da linha celular (cólon, cego ou reto; primária/metastática). Isto sugere que o perfil do metabolismo do colesterol nas linhas celulares de CCR é provavelmente independente destes fatores. Em contraste, a análise da ativação de vias de sinalização tumorigénicas através do *software* PROGENy revelou diferenças entre os subgrupos. Enquanto o subgrupo H-CM apresentou sobreexpressão da via TGF- β e subexpressão da via VEGF, as vias EGFR, MAPK e VEGF aparentavam estar mais ativas no subgrupo CS, sugerindo potenciais alvos terapêuticos para estes subgrupos. Além disso, observámos diferenças estatisticamente significativas entre os subgrupos da CCLE em vias relacionadas com a resposta imune adaptativa e de tipo 2 (ligeiramente aumentada nos clusters H-CM, CBS e BU) e no processamento e apresentação de antígenos (diminuída no cluster H-CM), o que sugere uma capacidade variável para promover um TME imunossupressor.

Através da seleção de uma linha celular representativa de cada subgrupo, tentámos validar os subgrupos da CCLE através de ensaios funcionais. Embora esta tarefa ainda esteja em curso, a avaliação do armazenamento lipídico e da internalização de colesterol permitiram, em parte, validar os perfis das linhas celulares de CCR obtidos *in silico*. Ademais, procurámos determinar como os subgrupos da CCLE influenciam o TME, através do tratamento de macrófagos e fibroblastos com meio condicionado (MC) dessas mesmas linhas celulares. Os nossos resultados revelaram que apenas o MC da linha celular do subgrupo BU aumentou significativamente a ativação dos fibroblastos. No entanto, apresentou a menor capacidade para polarizar macrófagos em M1 ou M2. O MC das linhas celulares dos subgrupos H-CM e CBS polarizou principalmente os macrófagos no fenótipo M1, embora o último também tenha mostrado uma maior capacidade de polarizar macrófagos em M2, enquanto o MC das linhas celulares dos subgrupos L-CM e CS polarizou os macrófagos quase de forma igual entre M1 e M2. Avaliámos também a sua resposta à quimioterapia – oxaliplatina (L-OHP) – na presença e ausência de fármacos redutores do colesterol. Notoriamente, as linhas celulares com as assinaturas de metabolismo do colesterol mais distintas revelaram resultados marcadamente diferentes. A inibição da biossíntese e do armazenamento de colesterol na linha celular do subgrupo L-CM aumentou drasticamente a morte celular tanto isoladamente como em combinação com L-OHP, levando também à paragem do ciclo celular na fase G2-M. Em contraste, a inibição da biossíntese não afetou a viabilidade da linha celular do subgrupo

H-CM, embora tenha contribuído para a paragem do ciclo celular na fase G2-M. A inibição do armazenamento de forma isolada aumentou a morte celular, mas não melhorou os efeitos do L-OHP neste contexto. Estes resultados sugerem que a modulação da biossíntese e do armazenamento do colesterol pode aumentar a sensibilidade à quimioterapia, dependendo do perfil metabólico das linhas celulares. Coletivamente, estes resultados sugerem que a estratificação dos doentes com CCR com base no metabolismo do colesterol pode melhorar a gestão da doença.

Palavras-chave: Cancro colorretal; metabolismo do colesterol; estratificação do cancro; microambiente tumoral.

Selected Communications

The research conducted during this dissertation has been presented by me at the following conferences:

- Pinto A, Machado AL, Martins F, Oliveira SM, Velho S. **Deciphering the role of cholesterol in the tumor microenvironment: novel colorectal cell line clustering approach.** *Symposium on Immunomodulation in Cancer & Regeneration*. Selected oral communication. 16th April 2024.
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List of Abbreviations

25-HC	25-hydroxycholesterol
27-HC	27-hydroxycholesterol
5-FU	5-Fluorouracil
ABC	ATP-binding cassette
ACAT	Acyl-coenzyme-A:cholesterol acyltransferase
Acetyl-co-A	Acetyl-coenzyme-A
AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
APCs	Antigen-presenting cells
BSA	Bovine serum albumin
BU	High Biosynthesis/Uptake Cholesterol Metabolism
CAF(s)	Cancer-associated fibroblast(s)
CBS	High Catabolism/Biosynthesis/Storage Cholesterol Metabolism
CCLE	Cancer Cell Line Encyclopedia
CE(s)	Cholesteryl ester(s)
CH25H	Cholesterol 25-hydroxylase
CIMP-	CIMP-negative
CIMP	CpG island methylator phenotype
CIMP+	CIMP-positive
CIMP-H	CIMP-High
CIMP-L	CIMP-Low
CIN	Chromosomal instability
CIN-	CIN-negative
CIN+	CIN-positive
CIN-H	CIN-High
CIN-L	CIN-Low
CM	Conditioned media
CMS	Consensus Molecular Subtype(s)
CRC	Colorectal cancer
CS	High Catabolism/Storage Cholesterol Metabolism
CTLA-4	T-lymphocyte-associated protein 4
DC(s)	Dendritic cell(s)
DCP(s)	Distal cholesterol precursor(s)
dH ₂ O	Distilled water
dMMR	MMR deficient

DMSO	Dimethyl sulfoxide
DT	Doubling time
ECM	Extracellular matrix
EDAseq	Exploratory Data Analysis and Normalization for RNA-seq
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
ES	Enrichment score(s)
FAP	Fibroblast activation protein
FBS	Fetal bovine serum
GBM	Glioblastoma
GGPP	Geranylgeranyl diphosphate
H-CM	High cholesterol metabolism
HDI	Human development index
HDL	High-density lipoproteins
HER-2	Human epidermal growth factor 2
HGF	Hepatocyte growth factor
HMG-coA	3-hydroxy-3-methylglutaryl-CoA
HMGCR	HMG-CoA-reductase
ICI(s)	Immune checkpoint inhibitor(s)
IFN- γ	Interferon- γ
IL(s)	Interleukin(s)
K	Number of clusters
KRAS	Kirsten rat sarcoma
L-CM	Low cholesterol metabolism
LD(s)	Lipid droplet(s)
LDL	Low-density lipoproteins
LDLR	LDL receptors
LOH	Loss of heterozygosity
L-OHP	Oxaliplatin
LPS	Lipopolyssacharides
LXR(s)	Liver x receptor(s)
mCRC	Metastatic CRC
M-CSF	Macrophage colony-stimulating factor
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
min	Minute(s)

MMP(s)	Matrix metalloproteinases
MMR	Mismatch repair
MSI	Microsatellite instability
MSigDB	Molecular Signatures Database
MSI-H	MSI-high/High frequency MSI
MSI-L	MSI-low/Low frequency MSI
MSS	Microsatellite stable
NGS	Next-generation sequencing
NK	Natural killer
NO	Nitric oxide
ON	Overnight
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBS-T	PBS containing Tween20
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PDGF	Platelet-derived growth factor receptor
PD-L1	Programmed death-ligand 1
Pen/Strep	Penicillin/Streptomycin
PFA	Paraformaldehyde
pMMR	MMR proficient
rh	Recombinant human
RNAseq	RNA sequencing
ROS	Reactive Oxygen Species
rpm	Rotations per minute
RT	Room temperature
SCNA(s)	Somatic copy number alteration(s)
SDS	Sodium-dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SQLE	Squalene epoxidase
SR-B1	Scavenger receptor class B type 1
SREBP(s)	Sterol regulatory element-binding protein(s)
ssGSEA	Single-Sample Gene Set Enrichment Analysis
TAMs	Tumor-associated macrophages
TCGA	The Cancer Genome Atlas
TCR	T-cell receptor
TGF- β	Transforming growth factor-beta

TIL(s)	Tumor-infiltrating lymphocytes
TME	Tumor microenvironment
TNF- α	Tumor necrosis factor-alpha
TNM	Tumor-node-metastasis
TP53	Tumor protein p53
Treg	T regulatory
TSG(s)	Tumor suppressor gene(s)
UICC	Union for International Cancer Control
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
α -SMA	Alpha-smooth muscle actin

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Introduction

1.1. Colorectal Cancer Overview

1.1.1. Epidemiology

Colorectal cancer (CRC) currently poses a major epidemiological burden, impacting men and women alike. According to Global Cancer Observatory estimates, CRC ranks as the third most common cancer and the second leading cause of cancer-related deaths worldwide, accounting for over 1.9 million new cases and more than 900 000 deaths in 2022¹. In Portugal, it is currently the cancer with the highest incidence and second highest mortality rates, registering over 10 000 new cases and almost 5000 deaths in the same year².

The geographical distribution of CRC varies substantially, due to its association with the human development index (HDI) of a country. Generally, CRC incidence is higher in countries with a high HDI. Recent trends reveal that in these countries the incidence of CRC is seemingly stabilizing, likely due to advances in early detection and treatment, that have also reduced mortality³. However, a rise in CRC cases has been observed among younger people, whereas it was previously a disease mainly associated with individuals over 50⁴. In line with this, CRC cases are rising in lower-HDI countries, a tendency that accompanies the adoption of westernized lifestyles^{3,5}.

The development of CRC is, in fact, strongly linked to an environmental component, which gives rise to a considerable proportion of sporadic cases (approximately 70% of all CRC cases)⁶. CRC carcinogenesis is commonly associated with a series of modifiable risk factors, namely, obesity, consumption of highly processed foods, smoking, alcohol use, and a sedentary lifestyle. It is thus partially preventable through lifestyle changes, such as adopting a fiber-rich diet and increasing physical activity^{5,7,8}. Nonetheless, projections indicate that incidence and mortality may increase by at least 60% in less than 20 years⁹.

1.1.2. Molecular Biology

CRC is a particularly heterogeneous disease at the molecular level, characterized by a high mutational burden. Carcinogenesis occurs through a multi-step accumulation of gene mutations, leading to the gradual development of the tumor over the course of many years. This process typically follows the adenoma-carcinoma sequence^{8,10,11}, which predominantly develops through two molecular pathways: the chromosomal instability

(CIN) pathway, responsible for approximately 85% of CRC cases, and the microsatellite instability (MSI) pathway. A proportion of cases, however, progresses from serrated polyps via the CpG island methylator phenotype (CIMP) pathway, which molecularly overlaps with the MSI pathway^{12–14}.

The CIN pathway is associated with numeric and structural chromosomal abnormalities, which – among other consequences – can lead to the loss of heterozygosity (LOH) of tumor suppressor genes (TSGs). Due to the chromosomal instability, these tumors accumulate mutations in both TSGs and oncogenes, leading to the development and progression of lesions in hyperproliferative colorectal mucosa¹⁰. According to the classical model, mutational inactivation of the *adenomatous polyposis coli* (APC) gene, which regulates the Wnt signaling pathway, is the initiating event that drives the appearance of benign polyps – early adenomas. These polyps usually progress to intermediate adenomas after activation of the *Kirsten rat sarcoma* (KRAS) proto-oncogene. Further alterations, including the deletion of SMAD2/4, result in late adenomas, that develop into carcinomas following *tumor protein p53* (TP53) inactivation (Figure 1)^{14,15}.

CRC development through the MSI pathway, conversely, arises from the mutation or epigenetic silencing of mismatch repair (MMR) genes – MLH1, MSH2, MSH6, and PMS2 –, thereby impairing the DNA MMR repair system^{12,16}. This leads to the hypermutability of short tandem repeats, known as microsatellites, due to replication errors¹⁷. This mechanism originates at least 12% of sporadic CRC cases, where MSI is driven by the hypermethylation and consequent silencing of the MLH1 gene – frequently after BRAF oncogenic activation –, generating early adenomas (Figure 1)¹⁰. In contrast, hereditary forms of MSI tumors, such as those associated with Lynch syndrome (3% of MSI CRC), are caused by mutations in the MMR genes themselves¹⁶.

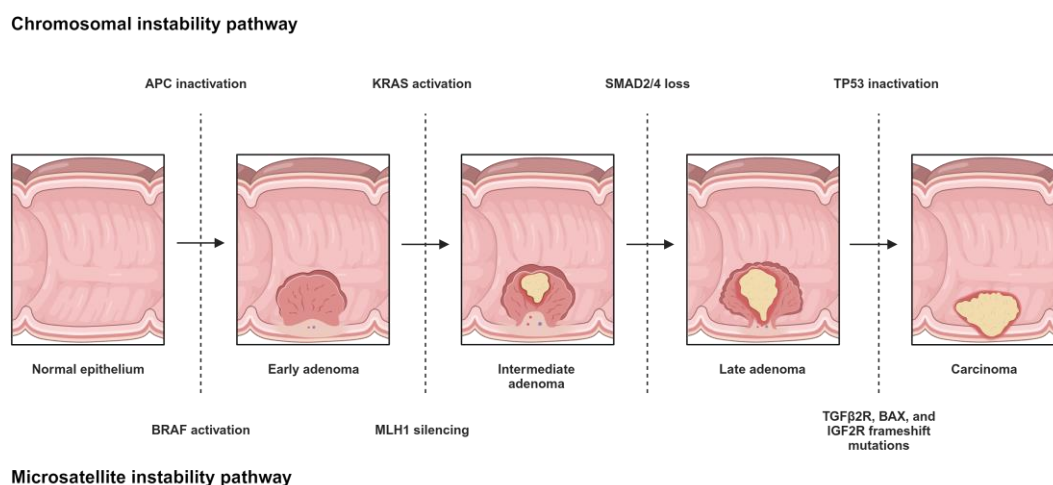


Figure 1. Adenoma-carcinoma sequence of colorectal cancer carcinogenesis. Representation of the two molecular pathways involved in the stepwise progression of colorectal cancer from normal epithelium to carcinoma. Created with BioRender.com

The silencing of TSGs due to the aberrant hypermethylation of promoter region CpG islands represents an alternative mechanism to the traditional adenoma-carcinoma sequence, leading to the development of serrated polyps¹⁴. Oncogenic activation of the BRAF gene is a common driver mutation of these tumors, which can then follow the MSI pathway due to MLH1 hypermethylation, of further progress because of TP53 inactivation^{13,18}.

1.1.3. Tumor Microenvironment

The heterogeneity of CRC becomes even more pronounced when the tumor's ecosystem is taken into consideration, as the behavior of a tumor is thoroughly influenced by its surroundings – the tumor microenvironment (TME)¹⁹. The TME includes not only cancer cells but also various non-cancerous cellular and non-cellular components in proximity to the tumor, such as cancer-associated fibroblasts (CAFs), endothelial cells, immune cells, soluble secreted factors, and the extracellular matrix (ECM)²⁰. The composition of the TME is variable, and the interplay between its components can either promote or hinder tumor progression²¹. This section will primarily emphasize the role of macrophages and fibroblasts, as they are central components of the TME and the ones that we will focus on in this work. However, an overview of dendritic cells (DCs) and T cells will also be provided, given their importance in cancer immunity.

Immune cells play a crucial role in modulating tumorigenesis through immunosurveillance. However, their tumor-suppressing functions are often bypassed in a process called cancer immunoediting, which consists of three phases: 1) elimination, 2) equilibrium, and 3) escape²². Briefly, in this process, cancer cell antigens in the TME are captured by antigen-presenting cells (APCs) and presented to T lymphocytes, creating a favorable environment for tumor elimination. However, cells may reach an equilibrium phase, where new tumor cells arise at the same rate as they are eliminated. Eventually, cancer cells may escape the immune system through various mechanisms, allowing them to proliferate, progress, and eventually metastasize^{23,24}.

When considering the TME in terms of immune cell composition, tumor-associated macrophages (TAMs) are among the most abundant cells. Depending on the microenvironment's characteristics, particularly its cytokine content, TAMs may polarize into two main phenotypes: M1 (classically activated) or M2 (alternatively activated). Macrophages polarize into M1 through the interaction with lipopolysaccharides (LPS) and pro-inflammatory cytokines like interferon- γ (IFN- γ)²¹. The resulting phenotype is characteristic of cells with an anti-tumorigenic function: they release nitric oxide (NO) and reactive oxygen species (ROS), which induce apoptosis in cancer cells; they secrete pro-inflammatory cytokines, such as interleukin (IL) 12, IL-1 β , and tumor necrosis factor- α

(TNF- α), that may aid the activation of other immune cells; and they may function as APCs due to the expression of major histocompatibility complex (MHC) molecules^{19,25}. However, within the TME, TAMs most often exhibit an M2 phenotype, which is generally associated with a pro-tumorigenic function²⁶. Polarization of TAMs into an M2 phenotype occurs following stimulation with cytokines such as IL-4, IL-10, and transforming growth factor-beta (TGF- β). The subsequent release of anti-inflammatory cytokines by these cells leads to an immunosuppressive TME, helping to provide the ideal conditions for cancer cell proliferation^{19,27}. In the context of CRC, M2 TAMs can modulate the ECM, promote epithelial-to-mesenchymal transition (EMT), and induce angiogenesis through the release of vascular endothelial growth factor (VEGF) A – factors that are known to aid in tumor invasion and metastasis^{28,29}. In line with this, an enrichment in M2 TAMs has been found at the tumor's invasive front in CRC³⁰.

DCs, though less abundant, are integral to anti-tumor immunity as specialized APCs. These innate immune cells, upon capturing cancer cell antigens, are responsible for activating CD8⁺ (or cytotoxic) T cells in the draining lymph nodes through MHC-I mediated cross-presentation, thereby playing a crucial anti-tumorigenic role within the TME. However, this process requires the adequate maturation of DCs, which depends on the immunogenicity of the tumor, as well as their efficient migration to the lymph nodes^{31,32}. Like TAMs, DCs may become tolerogenic due to the presence of immunosuppressive cytokines in the TME, and thus fail to effectively present antigens to T cells, which is associated with a poor prognosis in CRC^{19,21}.

Tumor-infiltrating lymphocytes (TILs), which include T cells, B cells, and natural-killer (NK) cells, likewise play a significant role in cancer immunity²⁸. Among these, T cells are particularly important. CD8⁺ T cells, as an example, when able to recognize tumor antigens, become activated and carry out their cytotoxic role by releasing granzymes and perforin, which act together to eliminate cancer cells³³. In an immunosuppressive landscape, however, tumor cells tend to overpass this mechanism. T cells can express cell surface receptors – programmed-death 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) – the so-called immune checkpoints that serve to control their own proliferation. Cancer cells exploit this mechanism as a way to escape T cell recognition. On the one hand, tumor cells may express programmed death-ligand 1 (PD-L1), which binds to PD-1 on T cells, halting their activation state. On the other hand, cancer cells promote the upregulation of CTLA-4 expression on T cells which competitively bind to co-stimulatory ligands on the surface of APCs, thereby suppressing the immune system's ability to mount an effective anti-tumor response^{23,24}.

Unlike immune cells, which can have multiple facets in the TME, the overall pro-tumorigenic function of CAFs is more evident³⁴. These stromal cells can be activated by

factors such as TGF- β , a cytokine that, as previously mentioned, is linked to a tumor-promoting TME²⁷. Likewise, CAFs can contribute to CRC progression in an array of manners, including their interaction with the ECM. CAFs are the primary producers of ECM components and matrix metalloproteinases (MMPs), thus playing a key role in the remodeling of ECM's structure. This affects the stiffness of the TME, a characteristic that has been shown to promote metastasis, as well as therapy resistance. However, CAFs' contribution to tumorigenesis is not limited to ECM modulation. Like other TME cells, CAFs secrete cytokines and growth factors, such as VEGF-A, that induce angiogenesis. This not only promotes tumor growth but also enhances the tumor's capacity to invade surrounding tissues and metastasize^{34,35}. Hepatocyte growth factor (HGF), secreted by CAFs, also promotes tumor progression through these processes³⁶. Previous work within our group has shown that CRC cells can promote CAF activation, thereby increasing HGF secretion, which promotes the invasiveness of tumor cells. The interplay between CRC cells and CAFs can also be a factor behind therapy resistance, as the effects of KRAS inhibition can be counteracted by CAFs; upon KRAS-inhibition, CAF-secreted factors can improve³⁷ cancer cell stemness, and upregulate pathways related with tumor progression³⁸. Additionally, CAFs can help create an immunosuppressive TME by recruiting pro-tumorigenic immune cells while excluding anti-tumorigenic ones. Indeed, the overexpression of fibroblast activation protein (FAP) in the TME has been linked to a higher proportion of immune cells with a pro-tumorigenic role around fibroblasts³⁹. Given their role in the TME, the identification of CAF biomarkers, such as alpha-smooth muscle actin (α -SMA), may serve as an indicator of cancer progression, which can aid in defining disease prognosis^{35,40}.

1.1.4. Treatment

Primary CRC tumors often metastasize to the regional lymph nodes and distant organs, mostly the liver and lung, worsening the prognosis and reducing survival⁴¹. The high morbidity and mortality rates of CRC highlight the importance of implementing adequate management strategies for this disease, whose treatment may involve a variety of methods, ranging from surgery to targeted therapies.

An essential step in CRC treatment is tumor resection, which is typically performed with curative intent for the primary tumor. In the colon, surgery involves the removal of the tumor along with lymphadenectomy, while rectal tumors usually require a more radical approach, with total mesorectal excision. In earlier stages of localized disease, however, excision of polyps may be achieved through endoscopic intervention, a comparatively less invasive method^{11,42}. Surgery has also proven beneficial in the control of metastatic disease, particularly for removing liver metastases⁸.

CRC treatment often includes the introduction of chemotherapy both in neoadjuvant and adjuvant approaches. An important aspect of deciding whether to implement chemotherapy falls upon the stage of the disease, which will later be approached. As adjuvant therapy, chemotherapy is usually reserved for patients with stage-III CRC, as well as some high-risk stage-II cancers⁸. For metastatic CRC (mCRC), however, it is usually used as a first-line treatment. The most common regimens include the use of various combinations of the fluoropyrimidine 5-fluorouracil (5-FU), oxaliplatin (L-OHP), and irinotecan, which can differ depending on the characteristics of each patient⁴³.

The use of radiotherapy in the treatment of CRC is another management option, which is especially relevant for tumors of the rectum. Herein, the application of perioperative radiotherapy – and of chemoradiotherapy⁴⁴ – has not only improved prognosis by reducing tumor recurrence, but it has also allowed the preservation of the rectum⁸.

Advancements in the understanding of CRC's molecular landscape have also brought forth the opportunity to introduce targeted therapies, which have significantly improved the management of metastatic disease. These therapies typically act by inhibiting signaling molecules encoded by oncogenes, and the most common targets are the epidermal growth factor receptor (EGFR) and VEGF, along with its receptor (VEGFR), which are often overexpressed in CRC. EGFR inhibitors, such as cetuximab and panitumumab, are specifically used in patients who lack mutations in downstream targets, such as KRAS, as they render EGFR inhibitors ineffective. Conversely, VEGF inhibitors, such as bevacizumab, can be beneficial for patients both with and without mutated-KRAS⁴⁵. In a small portion of mCRC cases that present with human epidermal growth factor receptor 2 (HER2) amplification, anti-HER2 therapy is now approved as well, provided that patients meet a set of criteria. Only patients who have previously been treated with other conventional treatment regimens, including chemotherapy, can be considered for this approach, and, as with anti-EGFR targeted therapy, it requires that tumors do not have RAS or RAF mutations. In these cases, the combined use of tucatinib and trastuzumab proves beneficial^{46,47}. KRAS-targeted therapies have also been emerging, despite mutations in this gene previously being deemed untreatable⁴⁸. Adagrasib, a drug targeting the G12C KRAS mutation, has been approved for CRC treatment in combination with cetuximab⁴⁹. Prior to this, encorafenib, an anti-BRAF drug that targets the V600E mutation, was also approved for previously treated CRC⁴⁵.

With the current knowledge of the TME's influence on tumorigenesis and the mechanisms by which cancer cells escape immunosurveillance, the use of immunotherapy – a type of targeted therapy – is increasingly becoming more widespread. These therapies mostly consist of monoclonal antibodies that target immune checkpoints

– PD-1 and CTLA-4 –, thereby being named immune checkpoint inhibitors (ICIs)⁵⁰. Currently, two anti-PD-1 treatments, pembrolizumab and nivolumab, and one anti-CTLA-4 drug, ipilumab are approved for use in CRC with MSI. While both pembrolizumab and nivolumab can be used as monotherapies, ipilumab is reserved for use in combination with nivolumab⁵¹.

1.2. CRC Classification

The heterogeneity of CRC significantly impacts the course of treatment and patient prognosis⁵². The thorough characterization of CRC is therefore crucial for achieving a more personalized approach to the management of this disease, which has led to numerous efforts to stratify it.

1.2.1. TNM Staging

An important approach to stratifying CRC involves using the anatomopathological context of the disease as the main criteria, which can be achieved through the TNM (tumor-node-metastasis) classification system. Developed by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC), this system is now recognized as a standard tool for grading CRC⁵³. The TNM classification evaluates the primary tumor (T) by assessing both its size and extent of invasion into the colorectal tissue and surrounding structures, the involvement of regional lymph nodes (N), and the presence of distant metastasis (M). These parameters are graded (Table 1) and then combined into a stage ranging from 0 to IV (Table 2), which reflects disease progression⁵⁴.

TNM staging provides important information for defining prognosis and helps guide disease management strategies^{42,54}. However, it does not clarify variability in the outcomes of patients within the same stage of the disease⁵⁵.

Table 1. TNM classification of colorectal carcinomas^{53,56} (1/2).

Classification		Features	
Primary tumor (T)	TX	Primary tumor cannot be assessed	
	T0	Absence of primary tumor in the colon or rectum	
	Tis	Carcinoma in situ: invasion of lamina propria	
	T1	Invasion of the submucosa	
	T2	Invasion of the muscularis propria	
	T3	Invasion of the subserosa or non peritonealized pericolic or perirectal tissues	
	T4	T4a	Perforation of the visceral peritoneum
		T4b	Direct invasion or adherence to adjacent organs or structures

Table 1. TNM classification of colorectal carcinomas^{53,56} (2/2).

Classification		Features
Regional lymph nodes (N)	NX	Regional lymph nodes cannot be assessed
	N0	Absence of regional lymph node metastasis
	N1	N1a Metastasis in one regional lymph node
		N1b Metastasis in 2 – 3 regional lymph nodes
		N1c Tumor deposit(s) the subserosa, mesentery, or non peritonealized pericolic or perirectal tissues without regional node metastasis
	N2	N2a Metastasis in 4 – 6 regional lymph nodes
		N2b Metastasis in 7 or more regional lymph nodes
Metastasis	M0	Absence of distant metastasis
	M1	M1a Presence of distant metastasis in a single location
		M1b Presence of distant metastasis in at least two locations

Table 2. Simplified TNM-based colorectal cancer staging^{53,54,56}.

Stage	TNM Classification			Disease Progression
0	Tis	N0	M0	Carcinoma in situ
I	T1, T2	N0	M0	Localized cancer
II	T3, T4	N0	M0	Early-stage locally advanced cancer
III	T1-T4	N1, N2	M0	Late-stage locally advanced cancer
IV	T1-T4	N1, N2	M1	Metastatic cancer

1.2.2. MSI

From a molecular standpoint, it is useful to classify CRC tumors based on their MSI status. Generally, they can fall under two categories: high-frequency MSI (MSI-H) – 15% of CRC cases – and microsatellite stable (MSS)/low-frequency MSI (MSI-L) – 85% of CRC. Due to the nature of the MSI status – which arises from the existence of alterations in genes that encode proteins involved in DNA MMR, as previously described –, MSI-H tumors are considered MMR deficient (dMMR), whereas MSS/MSI-L tumors are usually MMR proficient (pMMR)^{57,58}.

MSI status is usually assessed by detecting the absence of MMR proteins in the cell nucleus through immunohistochemistry. If all proteins are expressed, tumors are considered MSS, whereas the loss of at least one MMR protein leads to the MSI-H classification⁴². Another approach to the classification is the detection of microsatellite sequence alterations, which can be attained through polymerase chain reaction (PCR),

and, more recently, next-generation sequencing (NGS). Usually, a panel of five markers is recommended for MSI testing: if at least two of those markers are altered (or over 30% for larger panels), CRC tumors are classified as MSI-H; if the tumor presents one MSI marker (or below 30%), it is classified as MSI-L; if no alterations are detected, the tumors are characterized as MSS. Even though this method allows the distinction between MSS and MSI-L tumors, these two classifications tend not to be considered clinically relevant^{59,60}.

Knowing the MSI status of CRC tumors can be an important step in defining the patient's outcome. MSI-H tumors, which typically arise in the ascending colon, presenting with a poorly differentiated mucinous phenotype, are more common in women and tend to have a better prognosis than their MSS counterparts^{57,60}. Due to the hypermutability of these tumors, they are likely to present a high number of neoantigens, making them immune infiltrated. As such, patients with MSI-H tumors are considered good candidates for immunotherapy⁶¹.

1.2.3. CIN

Since most CRC tumors present chromosomal instability, distinguishing them based on this parameter can also prove useful in characterizing them. The classification mostly relies on measuring LOH, although the criteria may not be as clear as for MSI. Broadly, tumors can be divided into CIN-positive (CIN+) and CIN-negative (CIN-), but the degree of instability in the first group can be variable. As such, CIN+ tumors can be further subdivided into CIN-high (CIN-H) and CIN-low (CIN-L)^{62,63}. This system, like MSI classification, provides some insight into prognosis, with CIN-H patients generally having worse survival rates than CIN-L, whose outcome may be comparable to those that are CIN-⁶⁴. As already mentioned, this group comprises most CRCs, thus it is a rather heterogeneous group of tumors. A finer classification is therefore needed to improve prognosis and treatment.

1.2.4. CIMP

Much like the CIN classification, CRC tumors can be stratified as CIMP negative (CIMP-) or positive (CIMP+), based on the presence of hypermethylation of CpG islands. CIMP+ tumors, which account for 15% of all CRC cases⁶⁵, can then be differentiated by the degree of hypermethylation, giving rise to the CIMP-high (CIMP-H) and CIMP-Low (CIMP-L) classifications. However, the CIMP classification described here is not consistent among different authors, which was found to hinder an accurate determination of prognosis in CRC patients⁶⁶. Nonetheless, studies suggest that identifying whether a tumor exhibits the CIMP phenotype may be useful for defining prognosis, with CIMP-H tumors generally

having a worse prognosis^{65,67}. CIMP-L tumors have also been found to present a poor prognosis in association with a low infiltration of T lymphocytes, revealing how other factors influence disease outcomes, regardless of CIMP status⁶⁸.

1.2.5. The Cancer Genome Atlas Classification

In 2012, The Cancer Genome Atlas (TCGA) provided a comprehensive molecular analysis of human CRC. Herein, CRC was divided into two main groups based on somatic mutation rates: 1) hypermutated tumors and 2) non-hypermutated tumors. The first group largely consisted of ascending colon tumors, characterized by MSI-H, CIMP+, and presenting MLH1 silencing. These features accounted for approximately 75% of hypermutated tumors. The remaining hypermutated tumors displayed distinct profiles, including the presence of somatic mutations of MMR genes or alterations in the POLE gene, without MLH1 silencing. In contrast, non-hypermutated tumors were mostly homogeneous, displaying chromosomal instability. These tumors frequently exhibited somatic copy number alterations (SCNAs), an MSS phenotype, and mutations in the Wnt signaling pathway, predominantly in the APC gene (81%). This group also had a higher proportion of tumors located in the descending colon and rectum⁶⁹.

This characterization offers an in-depth analysis of common molecular features of tumors that fall into the categories previously described, namely, CIN, MSI, and CIMP, therefore shedding light on potential therapeutic targets. Although dividing CRC tumors into hypermutated and non-hypermutated categories alone may not be sufficient to define prognosis, this analysis serves as a valuable resource for further research. In fact, TCGA's characterization of CRC tumors highlighted the heterogeneity within the hypermutated group⁶⁹, prompting a more detailed analysis. Donehower, L. et al. demonstrated that the non-MLH1 silenced tumors within the hypermutated group had even higher mutation rates than MLH1 silenced tumors, allowing for the identification of an ultramutated subgroup (Figure 2). Additionally, core differences in mutation profiles of these subgroups suggest that assessing MLH1 expression levels may provide invaluable information beyond the commonly used MSI classification⁷⁰.

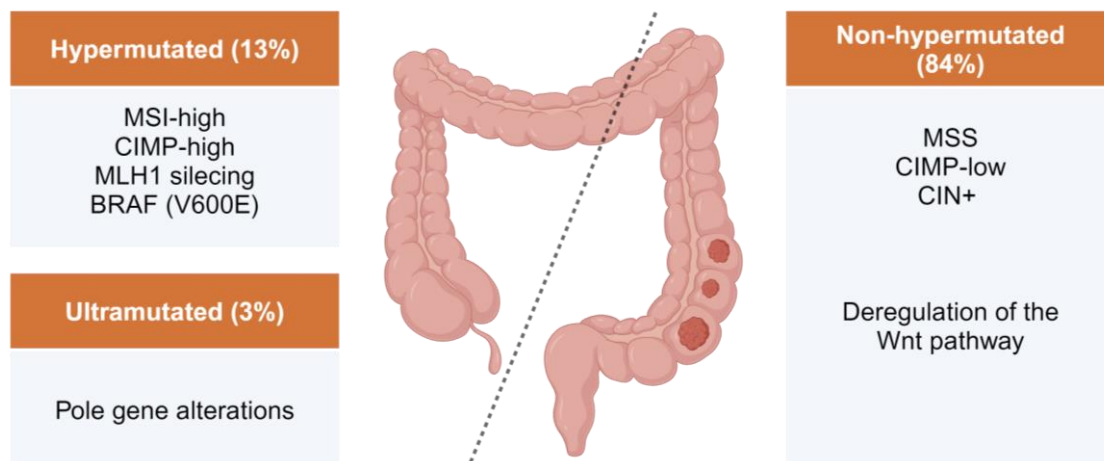


Figure 2. Stratification of CRC tumors based on mutation rate status. General molecular features found in hypermutated, ultramutated, and non-hypermutated CRC^{69,70}. Created with BioRender.com.

1.2.6. Consensus Molecular Subtypes

CRC has been further divided into four groups according to their Consensus Molecular Subtypes (CMS): CMS1 (MSI immune), CMS2 (canonical), CMS3 (metabolic), and CMS4 (mesenchymal) (Figure 3). This classification based on the gene expression profiles of the tumors was specifically developed by the CRC Subtyping Consortium (CRCSC) to address gaps in previous classifications regarding consistency⁷¹.

Tumors within each CMS not only share molecular features but also have identifiable biological similarities, providing clearer indications of prognosis. CMS1 tumors fall into the hypermutated category; they are MSI-H, leading to a high immune infiltration, and often present BRAF mutations. Consistent with these characteristics, these tumors exhibit an upregulation of pathways associated with immune infiltration and activation of immune evasion. The CMS2, or canonical subtype, in contrast, is mostly composed of CIN+ tumors with a high frequency of SCNAs, which tend to have a poor infiltration of immune cells. Herein, there is a notable activation of the Wnt and MYC pathways due to the upregulation of its downstream targets. CMS3 is mostly composed of MSS tumors but has a high percentage of MSI tumors (30%) and a low frequency of SCNAs compared to the MSI immune and mesenchymal subtypes. It is also characterized by metabolic dysregulation, with an upregulation of metabolic pathways, and the presence of KRAS mutations. CMS4 tumors, like the ones in the CMS2 group, are mostly CIN+. They also express genes associated with EMT, an upregulation of the TGF- β pathway, angiogenesis, and stromal infiltration, factors that are likely associated with a lower relapse-free and overall survival⁷¹.

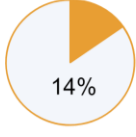
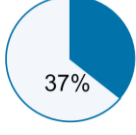
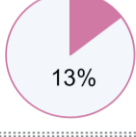
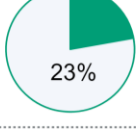
	Frequency	Molecular features	Deregulated pathways	Prognosis
CMS1 MSI immune	 14%	MSI-H CIMP-H Hypermuted with BRAF mutations	Immune infiltration and activation	Poor survival after relapse
CMS2 Canonical	 37%	MSS CIN-H SCNA-H	Wnt and MYC activation	
CMS3 Metabolic	 13%	MSI/MSS CIN-L CIMP-L	Metabolic	
CMS4 Mesenchymal	 23%	MSS CIN-H SCNA-H	Stromal infiltration, TGF- β activation, angiogenesis	Poor relapse-free and overall survival

Figure 3. Consensus molecular subtypes of CRC proposed by the CRCSC⁷¹.

Even though the CMS classification is useful as a predictive and prognostic biomarker, the CRCSC acknowledges the “poor genotype-phenotype correlation” in CRC⁷¹, which makes the tumor profiles variable even within the same CMS. Furthermore, sample analysis is usually done through bulk RNA sequencing (RNAseq), which carries limitations. Due to the nature of the technique, results may be influenced by intratumoral heterogeneity and the composition of TME, leading to an incorrect classification of tumors^{72,73}. The need for RNAseq also makes its implementation difficult in clinical practice, and CMS classification has not yet introduced molecular targets beyond the ones previously known that could justify its routine use.

1.2.7. Immunoscore

Classification systems such as CMS have recognized the presence of immune infiltration in certain CRC groups, but they do not completely capture the heterogeneity of CRC’s TME and its impact on patient outcomes. Considering the influence of the TME on prognosis, a new classification system was developed: the immunoscore^{74,75}. The immunoscore specifically evaluates the immune contexture of the tumor as a prognostic marker, based on the CD3+ and CD8+ T cell content in the tumors; the higher the density of these TILs in the tumor margins and center, the higher the immunoscore⁷⁶.

The immunoscore has been shown to act as a better predictor of patient survival compared to the TNM staging system. Overall, patients with high immunoscores exhibit

higher survival rates and lower chances of relapse. Additionally, as the content of TILs in the tumor forms the basis for a tumor's immunoscore, this system is also a better predictor of response to immunotherapy compared to MSI status, for instance^{76,77}. Immunotherapy is typically reserved for patients with MSI-H tumors, as pMMR tumors do not tend to be immunogenic⁷⁸. However, a study has demonstrated that the immunoscore correlates more strongly with the efficacy of immunotherapy when used in combination with other treatment regimes, than MSI. Regardless of MMR status, patients with high immunoscores had better outcomes, while those who relapsed had low immunoscores⁷⁷. Therefore, incorporating the immunoscore into CRC stratification could help identify a subset of patients with pMMR tumors who might benefit from immunotherapy.

However, the immunoscore is not yet used as a standard classification system. Although it largely meets the criteria of a good biomarker, its application is still met with some challenges, most notably the need for digital pathology for its implementation⁷⁵. Nonetheless, the demonstrated utility of immunoscore indicates that the classification of CRC is still evolving and can benefit from further improvements. Stratifying CRC should not rely on a single parameter, but on a combination of classification systems, as this far improves prognostication.

1.3. Cholesterol Metabolism and CRC

CRC classification systems have become progressively more informative and consistent, but they have not yet fully tackled the inherent heterogeneity among tumors that otherwise present similarities in tumor extent, genetic profiles, and TME composition. Consequently, there are still gaps in CRC stratification that require a better understanding of its biology and prognosis-modifying factors. Changes in lipid metabolism have often been reported alongside the many cancer hallmarks that emerge during CRC progression. More specifically, cholesterol, whose biosynthesis is frequently upregulated in cancer cells, has been linked to changes in CRC^{79,80}.

1.3.1. Cholesterol Metabolism Overview

Cholesterol homeostasis is essential for the normal functioning of cells, as this lipid molecule plays an important role as a component of plasma membranes, maintaining their structure and fluidity, as well as intervening in cell signaling, primarily due to their enrichment in lipid rafts. This homeostasis is ensured by the tight regulation of cholesterol levels through its biosynthesis, trafficking, catabolism, and storage, in a series of pathways that together make up cholesterol metabolism⁸¹. As such, when discussing the role of cholesterol in cancer, it is important to also consider its precursors, intervening enzymes, and catabolites.

Cholesterol *de novo* biosynthesis occurs predominantly in the liver through the mevalonate pathway, where acetyl-coenzyme-A (acetyl-co-A) is converted into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), a molecule that is then reduced to originate mevalonate by HMG-CoA-reductase (HMGCR), an important rate-limiting enzyme in this process. A series of sequential enzymatic reactions then lead to the production of cholesterol⁸². Alternatively, cells may uptake dietary cholesterol in the form of low-density lipoproteins (LDL) through LDL receptors (LDLR). Cholesterol can then be incorporated into a series of other pathways, depending on its levels and the needs of the cells. It may be esterified into cholesteryl esters (CEs) by acyl-coenzyme-A:cholesterol acyltransferase (ACAT) and then stored into lipid droplets (LDs)⁸³; converted into oxysterols, steroid hormones, and bile acids through catabolic reactions⁸⁴; or exported from cells through efflux mediated by various ATP-binding cassette (ABC) transporters (primarily ABCA1 and ABCG1) and bind to apolipoproteins to create high-density lipoproteins (HDL)⁸⁵. Cells can then uptake HDL through receptors such as scavenger receptor class B type 1 (SR-B1), which mostly occurs in the liver as part of reverse cholesterol transport, a process that aims to regulate the concentration of cholesterol in peripheral tissues⁸⁶.

It is important to also note the role of certain transcription factors in regulating cholesterol metabolism. Two of the most relevant players in these processes include sterol regulatory element-binding proteins (SREBPs) and liver X receptors (LXRs). SREBPs promote the transcription of HMGCR and LDLR, thereby intervening in the control of biosynthesis and cholesterol uptake⁸⁷, while LXRs promote the increase of ABCA1 and ABCG1, therefore controlling cholesterol efflux⁸⁸.

1.3.2. Cholesterol Metabolism: Role in Carcinogenesis

There are well-established risk factors for the development of CRC, including poor nutrition and obesity, which, at first glance, are linked to cholesterol. The role of cholesterol itself – and more specifically, hypercholesterolemia – as a risk factor in the development of CRC has been controversial. Some studies suggest that elevated cholesterol serum levels could contribute to CRC tumorigenesis, even by suppressing tumor immunity^{89,90}, whereas others have found no clear correlation, especially regarding the levels of LDL^{91,92}.

Regardless of the role of cholesterol in the onset of tumors, its involvement in cancer progression is clear. Likely due to the high metabolic demands associated with their proliferation, tumor cells accumulate intracellular cholesterol, either by increasing biosynthesis or uptake^{79,92}. Given the importance of this lipid molecule as a component of the plasma membrane and signaling⁹³, its accumulation within tumor cells leads to various repercussions at the cellular level, namely, a decrease in apoptosis, an increase in

proliferation, and the promotion of metastasis. When combined, these alterations lead to the progression of CRC⁷⁹.

A study found that in the context of Lpcat-3 deficiency, a protein involved in phospholipid remodeling, cholesterol biosynthesis was increased in enterocytes, leading to their hyperproliferation and tumorigenesis. Herein, an increase in SREBP2 was detected, which was likely the source of increased biosynthesis⁹⁴. SREBP1, another regulator of cholesterol biosynthesis, was also found to increase autophagy in glioblastoma (GBM) cells when cholesterol levels were low, thereby promoting hydrolysis of CEs. In this instance, GBM tissues were found to have an increase in LDs, when compared to normal tissues, allowing them to maintain cholesterol levels when resources were limited⁹⁵. In breast cancer cell lines, there is also evidence that higher expression of SREBPs can increase their metastatic potential⁹⁶.

SREBP overexpression often occurs due to the activation of tumorigenic signaling pathways, such as PI3K/Akt. Since SREBPs act as transcription factors of LDRL, elevated SREBP levels are also associated with an increase in cholesterol uptake, and not only biosynthesis. LDL, in turn, can further activate these pathways, leading to increased EMT, angiogenesis, and, consequently, metastasis⁹⁷. SR-B1 has also been found to play a pro-tumorigenic role in several cancer models by increasing cholesterol uptake. The function exerted by SR-B1 can lead to increased proliferation of tumor cells, as well as metastasis, which overall is associated with poor prognosis⁹⁸.

Mediators of cholesterol efflux, namely ABCA1 and ABCG1, can have opposing roles in tumorigenesis. ABCA1 is known for its anti-tumorigenic function and, as such, a loss of its function can contribute to carcinogenesis, where it was found to increase the survival of tumor cells. However, in certain cases, overexpression of ABCA1 can also contribute to an aggressive phenotype. Overexpression of ABCG1 – detected in mCRC – generally tends to be tumor-promoting, and its inhibition may be beneficial for promoting apoptosis of tumor cells⁹⁸. Conversely, increased levels of LXR, which promote cholesterol efflux, can also inhibit tumor cell proliferation and increase cell death, thereby possessing an anti-tumorigenic function⁹⁹.

Dysregulation of cholesterol homeostasis through oxysterols can also lead to cancer progression, especially considering that these products of cholesterol metabolism intervene in its regulation by interacting with transcription factors like LXRs⁹⁹. 27-hydroxycholesterol (27-HC), for instance, can inhibit tumor growth by promoting the downregulation of biosynthesis and uptake, therefore decreasing the cholesterol levels within tumor cells. However, during the process of cancer progression, natural selection occurs and allows the most resilient tumor cells to thrive; likewise, 27-HC resistant cells can be selected and proliferate. Indeed, a study found that cells that maintained high

uptake and biosynthesis despite the action of 27-HC were able to proliferate, metastasize, and even resist ferroptosis¹⁰⁰, an iron-dependent cell death associated with the accumulation of lipids¹⁰¹. In CRC, 27-HC was also found to be increased in tumor tissues, when compared to normal adjacent tissues. When further investigating its role in tumor progression, it was demonstrated that increased 27-HC levels led to decreased apoptosis *in vitro*¹⁰².

1.3.3. Shaping of the TME by Cholesterol Metabolism

As the TME is known to regulate tumorigenesis, dysregulated cholesterol metabolism within the TME can affect immune evasion, tumor growth, and metastasis by altering immune cell activation, stromal support, and the overall lipid composition of the TME, ultimately promoting cancer progression¹⁰³.

Cholesterol in the TME has, in fact, been linked to changes in the immune response, with effects that vary depending on the tumor model being studied. Immune cells with an anti-tumorigenic phenotype require cholesterol to function properly; therefore, an increase in concentration in the TME can be beneficial for these cells¹⁰⁴. Higher cholesterol content in cytotoxic T cells promotes T-cell receptor (TCR) clustering, which is necessary for interaction with APCs, consequently amplifying these cells' anti-tumor response¹⁰⁵. However, increased cholesterol levels have also been associated with T cell exhaustion, a state in which T cells express inhibitory receptors, such as those involved in immune checkpoints. Cholesterol accumulation in these cells, which derives from its increase in tumor cells, likely induces stress in the endoplasmic reticulum (ER), as the activation of the ER-stress-response pathway was found to be associated with the exhaustion phenotype¹⁰⁶.

T cell responses can also be influenced by impaired antigen presentation mediated by DCs. Tumor cells can increase LXR expression in these cells; while LXR overexpression in cancer can be beneficial for preventing tumor growth, it is not a desirable trait for APCs. Herein, LXR overexpression was proved to reduce the levels of the CCR7 chemokine receptor within DCs. As CCR7 contributes to their migration to the lymph nodes and, thus, antigen presentation to T cells, this impairment leads to a defect in anti-tumor immunity¹⁰⁷.

Disrupted cholesterol metabolism affects other TILs as well, such as T CD4⁺ lymphocytes. These T cells are essential for the sustained anti-tumor effect of cytotoxic T cells and, much like other immune cells, they can polarize into different subtypes, such as Th1 T cells, which are the main players in anti-tumor immunity, T regulatory cells (Treg), and Th17 cells¹⁰⁸. The effects of dysregulated cholesterol metabolism in tumor cells may, in part, explain why MSS tumors do not respond to immunotherapy, as the crosstalk

between these cells and CD4⁺ T cells mediated by distal cholesterol precursors (DCPs) has been found to promote the polarization of naïve T cells into Th17 cells¹⁰⁹. Although the role of these cells in cancer immunity has been controversial¹¹⁰, their ability to release IL-17A has been linked to cancer progression and poor prognosis in CRC¹¹¹. Naïve T CD4⁺ cells, when treated with conditioned media (CM) from MSS CRC cell lines, were found to polarize into Th17 cells, due to the enrichment of DCPs in the CM that can act as agonists of a transcription factor involved in Th17 polarization. Higher proportions of these cells could explain the recruitment of immunosuppressive cells to the TME, thereby promoting tumor progression and resistance to immunotherapy¹¹⁰.

Just as oxysterols influence tumor cells themselves, they can also have variable effects on other TME components. In CRC cells, 27-HC was found to increase the production of MMPs, which could contribute to the modulation of the ECM and, therefore, influence metastasis¹⁰². Its pro-tumorigenic role is further highlighted in breast cancer, where it can promote metastasis through the inhibition of T cells¹¹². Conversely, cholesterol 25-hydroxylase (CH25H), the enzyme responsible for the production of 25-hydroxycholesterol (25-HC), has been found to have anti-tumor activity by enhancing antigen presentation to CD8⁺, thus increasing their activation and tumor infiltration¹¹³.

Tumor cells can also directly manipulate cholesterol levels in TAMs. In an ovarian cancer cell model, increased hyaluronic production by tumor cells was found to promote membrane cholesterol efflux in TAMs, which was concurrent with an increase in the production of IL-4, a cytokine involved in M2 polarization. Through this mechanism, tumor cells can increase TAMs' sensitivity to pro-tumorigenic signaling, thereby promoting a tumor-friendly microenvironment¹¹⁴. A study focused on defining the composition of the metastatic niche of MSS CRC also found the presence of TAMs with high expression of APOE and APOC, indicating a high cholesterol metabolism. These macrophages belonged to a group of TAMs characterized by the expression of SPP1, a molecule previously associated with a poor prognosis in CRC. Interestingly, the expression of APOE seemed to be regulated by CAFs¹¹⁵, whose lipid metabolism can also be dysregulated. CRC tumor cells can alter lipid metabolism in CAFs, for instance, by increasing the levels of acetyl-co-A, the main precursor of cholesterol biosynthesis. CRC-derived exosomes containing HSPC111 can increase the levels of acetyl-co-A, promoting histone acetylation. Ultimately, this leads to increased levels of CXCL5, a cytokine that aids in recruiting pro-tumorigenic cells to the pre-metastatic niche¹¹⁶. In turn, CAFs may support tumor growth by providing tumor cells with cholesterol. Treatment of KRAS-silenced CRC cell lines with CM from activated fibroblasts with a CAF-like phenotype was found to downregulate cholesterol-related pathways, suggesting that CAFs may supply cholesterol directly to tumor cells, reducing the need for *de novo* biosynthesis and reducing metabolic stress³⁸.

1.3.4. Therapy Resistance and Potential Therapeutic Targets

Given the influence of cholesterol metabolism on both cancer cells and the TME, it can also impact the efficacy of CRC treatment. As a result, cholesterol metabolism is now being recognized as a target in cancer therapy⁸⁵.

Although chemotherapy is widely used in the treatment of CRC, one of the main challenges is overcoming acquired resistance¹¹⁷. Since cholesterol is the primary regulator of cell membrane fluidity, its content in the membrane may influence drug penetration into cells. In line with this, a study found that the plasma membrane fluidity of L-OHP-sensitive CRC cells decreased upon drug exposure, while this property was not altered in resistant cells. This supports the notion that the ability to maintain membrane rigidity could be a mechanism of chemotherapy resistance¹¹⁸. Another study showed that in KRAS-mutated CRC cell lines, the combination of L-OHP with statins – cholesterol-lowering drugs that target HMGCR to inhibit the mevalonate pathway¹¹⁹ – can increase sensitivity to this chemotherapeutic agent. Similar results were found *in vivo*, where the combined regimen reduced the side effects of L-OHP¹²⁰. The role of statins in CRC has been debated, with some studies even suggesting they may prevent disease development¹²¹. Their therapeutic effects are becoming increasingly evident, as they can regulate autophagy, induce various types of cell death, and even promote the repolarization of M2 macrophages into M1¹²².

Cholesterol storage can also play a part in therapy resistance. LD accumulation has been shown to support tumor cell proliferation, with its levels varying according to the phase of the cell cycle. A study found that LD content in cancer cells was increased during S phase, which has been recognized as the phase of cell division where there is a higher resistance to the effects of radiotherapy¹²³. However, LD accumulation may impair therapy throughout this whole process, as it was found that arresting cell cycle – regardless of phase – was associated with an increase in LD content and resistance to ferroptosis, a mechanism that can be induced during CRC treatment¹²⁴. Additionally, high LD content may be a source of anti-EGFR therapy resistance, which is commonly used in CRC treatment¹²⁵. Thus, targeting cholesterol storage could be an avenue for improving CRC therapy.

Similarly, inhibition of PCSK9, an enzyme that mediates LDLR degradation, thereby decreasing LDL uptake, may enhance CRC therapy as a co-adjuvant. Overexpression of PCSK9 was found to promote tumor progression in APC/KRAS mutant cell lines, by increasing cholesterol *de novo* biosynthesis, while reducing the need for the uptake of extracellular cholesterol. In these cells, inhibition of PCSK9 led to decreased cell proliferation, as well as increased apoptosis¹²⁶. The positive effects of blocking this

regulator can also be underlined regarding immunotherapy. Although the use of anti-PD-1 alone in CRC tumor models led to anti-tumor effects mediated by T CD8⁺ cells, these were counteracted by a concomitant increase in Treg cells, which have an immunosuppressive function. This was accompanied by an overexpression of PCSK9 that, when inhibited, improved the effects of immunotherapy by promoting an increase in T CD8⁺ cells, while decreasing Treg infiltration¹²⁷.

Despite their benefits, the use of cholesterol-lowering drugs to support cancer treatment should be carefully evaluated, as these treatments may affect TME components beyond cancer cells. Just as a decrease in membrane cholesterol was found to influence TAM polarization, adverse effects may extend to other cells. In fact, cholesterol reduction can lower MHC molecule levels in the membrane, potentially impairing immune cell function and negatively impacting tumor immunity¹²⁸.

1.3.5. Cholesterol Metabolism-Based Tumor Clustering

Taking into consideration the impact of cholesterol in CRC tumorigenesis, our research group has previously conducted an *in silico* analysis to determine whether CRC tumors could be clustered based on differences in cholesterol metabolism. Using RNAseq data from 439 TCGA tumor samples, we identified five distinct clusters based on the expression of genes involved in cholesterol biosynthesis, efflux, storage, reverse transport, uptake, and catabolism (Figure 4).

Since previous studies have shown that the accumulation of cholesterol in cancer cells can increase their proliferation, we sought to determine whether this was one of the primary differences among the clusters (Figure 5). We found that the clusters that differed the most in terms of proliferative capacity also presented opposing cholesterol metabolism profiles. Cluster 2, the most proliferative cluster, is characterized by high biosynthesis and storage, along with low metabolic processes that could impact the internal levels of cholesterol, namely efflux and catabolism. In striking contrast is cluster 1, which exhibits low biosynthesis and storage alongside high efflux and uptake, thus suggesting high cholesterol trafficking and high catabolism. In between, we have cluster 4, with a generalized downregulation of cholesterol metabolism, as well as 3 and 5, in which all cholesterol metabolism pathways are upregulated. Though cluster 4 does not present with capacity to produce or store cholesterol in high quantities, its content can be maintained due to the lack of efflux and catabolism, thus allowing the cell to keep proliferative capacity. Cluster 3, in contrast, is a heterogeneous cluster regarding biosynthesis and storage but exhibits high efflux and reverse transport, while cluster 5 can both produce and store cholesterol, its levels within the cell may decrease due to high catabolism. Therefore, in

these 3 clusters, proliferation is potentially regulated through an equilibrium in the cholesterol metabolic pathways.

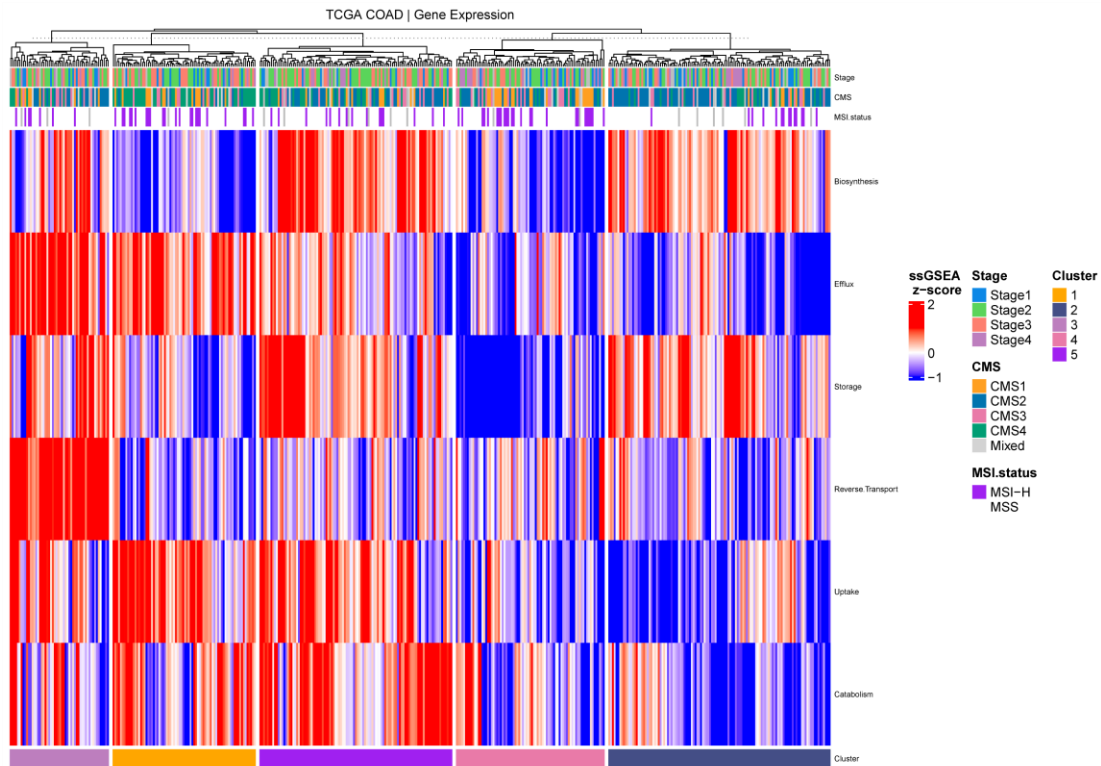


Figure 4. Clustering of CRC genetic profiles into five clusters with different degrees of cholesterol metabolism activation. Heatmap of 439 TCGA colorectal cancer samples classified into five distinct cholesterol metabolic subtypes using unsupervised k-means (k=5) clustering of 6 cholesterol-related pathways. Z-scores were calculated using Single-Sample Gene Set Enrichment Analysis (ssGSEA). Machado, AL (2024, unpublished).

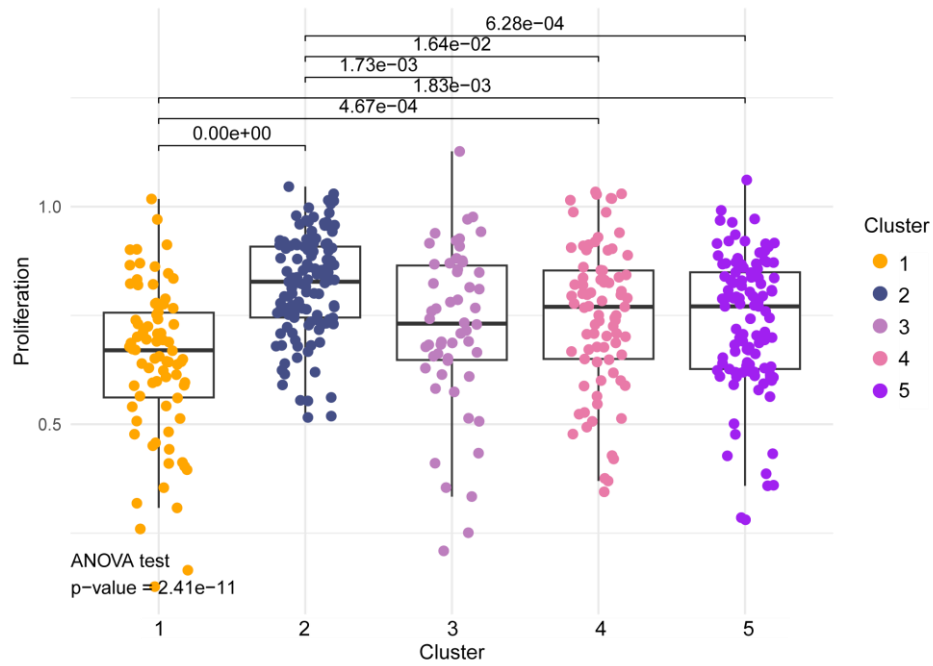


Figure 5. Proliferative capacity of the cholesterol metabolism-based TCGA clusters. Comparative analysis of proliferation scores obtained by ssGSEA revealed statistically significant differences between the clusters (ANOVA p-value < 0.05). Clusters 1 and 2, which had the lowest and highest proliferation scores, respectively, differed significantly from each other and from the remaining clusters. Results were considered statistically significant when $p \leq 0.05$. Machado, AL (2024, unpublished).

Aims

Despite the advancements in CRC management, gaps remain in the process of defining the prognosis of patients and predicting their response to therapy. The current understanding of cholesterol metabolism's impact on cancer progression, its role in the crosstalk between cancer cells and the TME, and increasing evidence of its influence on therapy outcomes all support the notion that a stratification system based on cholesterol metabolism could benefit CRC management. Therefore, the **main aim** of this project is to demonstrate that the metabolic features of the cholesterol metabolism-based clusters obtained in the *in silico* analysis – specifically those related to cholesterol – can influence tumorigenesis and therapy outcomes, while also providing a novel system for stratifying CRC patients. To achieve this, four **specific aims** have been set, including:

1. To replicate the *in silico* analysis using RNAseq from CRC cell lines, thereby establishing cholesterol metabolism-based clusters for *in vitro* use.
2. To validate the identified clusters in a panel of CRC cell lines through functional assays targeting each cluster.
3. To determine how the distinct clusters modulate TME differently.
4. To evaluate the potential of modulating cholesterol metabolism to enhance cancer therapy, using the clusters as predictors of response.

Cholesterol-based clustering of colorectal cancer cell lines: an *in silico* approach

3.1. Materials and Methods

RNA-seq raw counts of CRC cell lines from the Cancer Cell Line Encyclopedia (CCLE)¹²⁹ were retrieved from the DepMap portal (<https://depmap.org/portal>; downloaded on Oct 6, 2023). The “CCLE RNAseq gene expression data for 1019 cell lines (read counts)” file was downloaded from the CCLE 2019 file set, and the data was then filtered to only contain gene expression of CRC cell lines. The final dataset included gene expression of 20 551 genes from 53 cell lines (47 cell lines derived from colon tumors, and the remaining 7 from rectal tumors). R Studio software (v4.3.1)¹³⁰ was used to conduct the *in silico* analysis of the dataset.

3.1.1. Data normalization

Gene expression was normalized using the `EDAseq` (Exploratory Data Analysis and Normalization for RNA-seq)¹³¹ R package (v2.28.0), which corrects for biases related to GC-content and sequencing depth through within-lane and between-lane normalization, respectively. This was followed by quantile normalization with the `preprocessCore` R package (v1.56.0). Normalized data was then converted to log2 using the base R `log` function to stabilize variance, thereby approximating the data to a normal distribution.

3.1.2. Cholesterol metabolism-based scoring of samples

The CRC cell line samples were scored using Single-Sample Gene Set Enrichment Analysis (ssGSEA)¹³², a method that computes enrichment scores (ES) for each sample. ssGSEA accounts for the collective up- or down-regulation within a given sample, providing a quantitative measure of pathway activity. To cluster the samples based on their cholesterol metabolism, the ES were calculated for six curated gene sets related to cholesterol biosynthesis, catabolism, uptake, efflux, reverse transport, and storage (see Supplementary Tables 1 – 6) using the `ssGSEA` R package (v2.0).

3.1.3. Clustering approach

The non-hierarchical k-means algorithm was applied to cluster the samples using the ssGSEA enrichment scores as input, through the `kmeans` function from the base R

package, by setting the number of clusters (k) to 5. A heatmap with the assigned clusters was then generated with `ComplexHeatMap`.

3.1.4. Molecular and clinicopathological characterization of the clusters

Data on molecular (MSI status, and CMS) and clinicopathological features (disease type, and site of extraction) of the cell lines were compiled from the DepMap portal and the Cellosaurus cell line database¹³³ (<https://www.cellosaurus.org>) into a dataset. To address gaps in the molecular data, a literature review was conducted, focusing on peer-reviewed journal articles published within the last 10 years. The search was performed using PubMed, Google Scholar, and B-on, with keywords such as “colorectal cancer cell lines,” “cell lines,” “microsatellite instability,” and “consensus molecular subtype”. Studies were included if they specifically addressed colorectal cancer cell lines and the relevant molecular features. MSI status was classified as MSS, MSI-L, or MSI-H. For cell lines whose CMS differed across the data sources, the subtype was defined as variable. The dataset was then merged with the clustering results, and plots were created using the `ggplot2` R package (v3.4.2).

3.1.5. Proliferation analysis

The proliferative potential of each cluster was determined using ssGSEA. ES were calculated for the gene set listed in Supplementary Table S7. The calculated scores were then compared among the clusters.

3.1.6. Tumorigenic signaling pattern analysis

The PROGENy¹³⁴ algorithm was used to infer and compare aberrant activity of signaling pathways that may contribute to tumorigenesis across the clusters. The analysis was performed using the `progeny` R package (v1.16.0), with the normalized gene expression from the 20 551 genes serving as input. This package includes gene signatures for 14 signaling pathways, including Androgen, EGFR, Estrogen, Hypoxia, JAK-STAT, MAPK, NFkB, p53, PI3K, TGF- β , TNF- α , Trail, VEGF, and WNT. Pathway scores were scaled to a mean of 0 and a standard deviation of 1, and the top 1000 most significant genes served as the basis for the model matrix for pathway activity inference.

The CRC cell lines were evaluated with a set of 1000 footprint genes per pathway, the optimal minimum number of genes for maintaining PROGENy's performance integrity while preserving accuracy¹³⁵. Comparative plots of the clusters for each pathway were then created using `ggplot2` (v3.4.2).

3.1.7. Immune regulation analysis

To evaluate the immunomodulatory potential of each cluster, ssGSEA was employed. Specifically, ES were calculated for genes within 27 immune regulation-related gene sets. These gene sets were obtained from the MSigDB¹³⁶ v2023.2 (Molecular Signatures Database; downloaded on Feb 23, 2024) and are listed in Supplementary Table S8.

3.1.8. Statistical analysis

Statistical analysis was conducted using R studio. The Chi-square test was employed to compare categorical variables such as molecular and clinicopathological features of the cell lines. To assess the distribution of the PROGENy and ssGSEA results, the Shapiro-Wilk test was used to determine whether the data sets were normally distributed. If the data met the assumption of normality (Shapiro-Wilk p-value > 0.05), a one-way ANOVA was used to compare the means across groups, followed by Tukey's multiple comparison test. For data that did not meet the normality assumption (Shapiro-Wilk p-value < 0.05), the Kruskal-Wallis non-parametric test was used instead, followed by Dunn's multiple comparison test. Results were considered statistically significant when $p \leq 0.05$.

To highlight the pathways that were overactivated within each cluster – and hence could provide the cell lines with a metabolic advantage –, clusters were classified based on this feature, as summarized in Table 3. Briefly, clusters were labeled as High Cholesterol Metabolism (H-CM, Cluster 1), Low Cholesterol Metabolism (L-CM, Cluster 2), High Catabolism/Storage Cholesterol Metabolism (CS, Cluster 3), High Catabolism/Biosynthesis/Storage Cholesterol Metabolism (CBS, Cluster 4), and High Biosynthesis/Uptake Cholesterol Metabolism (BU, Cluster 5) clusters.

Table 3. Nomenclature of the colorectal cancer cell line clusters according to their cholesterol metabolism profiles.

Cluster	Cluster Nomenclature	Abbreviation
1	High Cholesterol Metabolism	H-CM
2	Low Cholesterol Metabolism	L-CM
3	High Catabolism/Storage Cholesterol Metabolism	CS
4	High Catabolism/Biosynthesis/Storage Cholesterol Metabolism	CBS
5	High Biosynthesis/Uptake Cholesterol Metabolism	BU

3.2.2. Molecular and clinicopathological characterization of the clusters

To gain an overall understanding of the molecular and clinicopathological landscape of the CRC cell line clusters, we characterized them based on their MSI status, CMS, disease type, and origin (primary or metastatic). These features were selected primarily because information was generally available for all cell lines, thus allowing the characterization of each cluster without introducing a bias due to missing values.

Regarding their MSI status, most of the cell lines were MSS (64.2%), while approximately one-third were MSI-H (32.1%). Only 3.8% of the cell lines were classified as MSI-L, likely due to this classification overlapping with the MSS status. The distribution of CRC cell lines by CMS was similar across the different subtypes, with frequencies ranging from 17.0% to 26.4%, although a larger proportion of samples fell into CMS2 (24.5%) or CMS4 (26.4%) (Table 4). When comparing the clusters based on these molecular features, no statistically significant differences were found between them, with the Chi-square test yielding p-values of 0.679 and 0.376, respectively. However, when looking at the distribution of the CRC cell lines by these classifications within each cluster, some differences can be noted. Clusters generally displayed a higher proportion of MSS cell lines, reflecting the overall trend of the dataset, except for the BU cluster, where nearly half of the cell lines (44.4%) were MSI-H (Figure 7). Concerning their CMS, CMS4 was the highest-represented group in the H-CM, L-CM, and CS clusters, while the CBS and BU clusters had a higher proportion of CMS2 cell lines (Figure 8).

Table 4. Characterization of the colorectal cancer cell lines by cluster regarding their MSI status and CMS.

Cluster	MSI (%)			CMS (%)				
	MSS	MSI-L	MSI-H	1	2	3	4	Variable
H-CM	3.8	0	0	0	0	0	3.8	0
L-CM	13.2	1.9	7.5	3.8	1.9	3.8	7.5	5.7
CS	11.3	0	5.7	3.8	1.9	1.9	7.5	1.9
CBS	17.0	1.9	3.8	1.9	3.8	3.8	3.8	3.8
BU	18.9	0	15.1	7.5	9.4	9.4	3.8	1.9
	64.2	3.8	32.1	17.0	24.5	18.9	26.4	13.3

Abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CMS, Consensus Molecular Subtype; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM, Low Cholesterol Metabolism; MSI, microsatellite instability; MSI-H, MSI-High; MSI-L, MSI-Low; MSS, microsatellite stable.

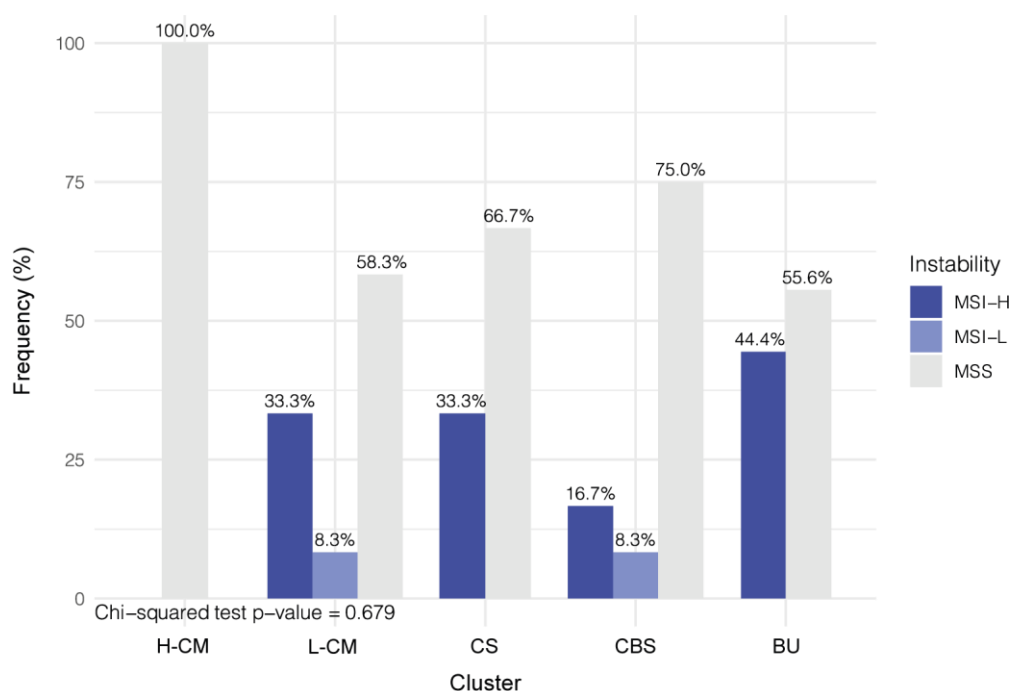


Figure 7. Molecular classification of the colorectal cancer (CRC) cell lines regarding microsatellite instability (MSI). Comparison of the CRC cell lines clusters according to their MSI status, classified into MSI-High (MSI-H), MSI-Low (MSI-L), and microsatellite stable (MSS). The Chi-square test was used to compare the distribution of the classifications across the clusters. Results were considered statistically significant when $p \leq 0.05$. Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

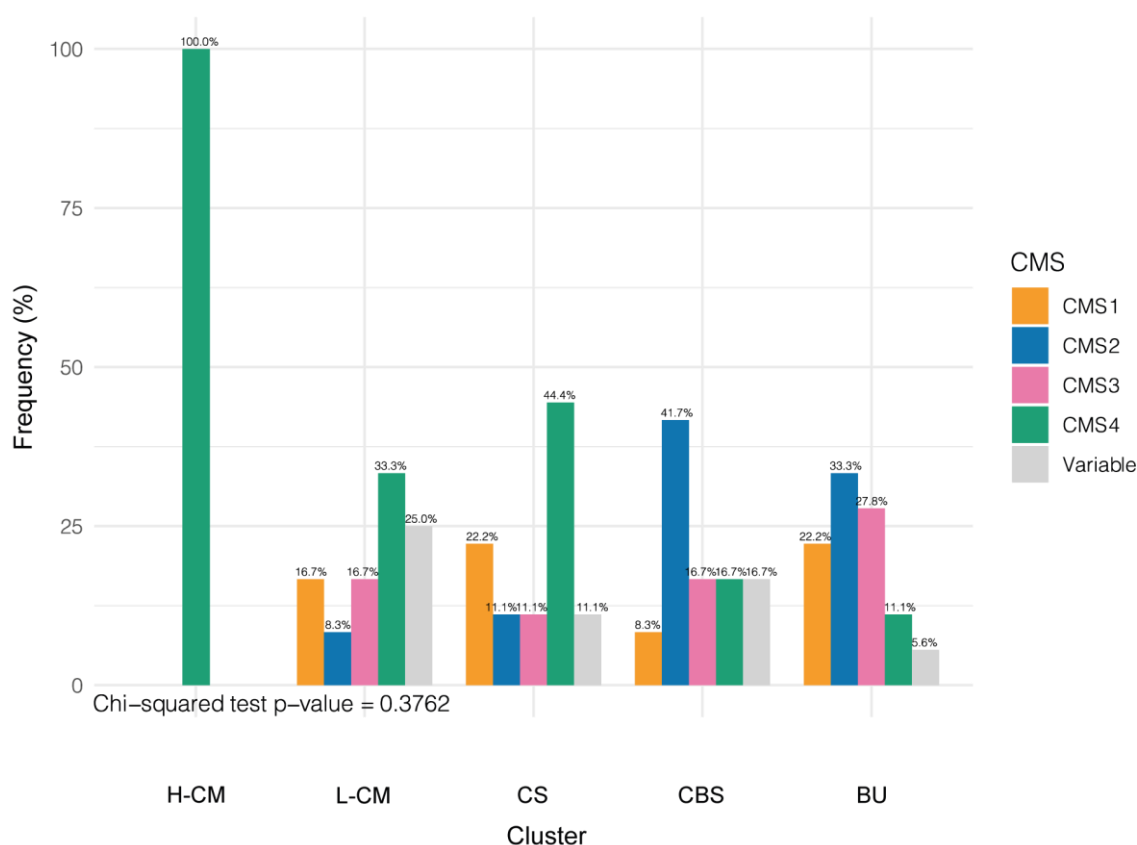


Figure 8. Molecular classification of the colorectal cancer (CRC) cell lines regarding their Consensus Molecular Classification. Comparison of the CRC cell lines clusters according to the CMS distribution among the clusters. The Chi-square test was used to compare the distribution of the classifications across the clusters. Results were considered statistically significant when $p \leq 0.05$. Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

In terms of origin, most of the cell lines were derived from primary tumors (73.5%), while just over a quarter were extracted from metastatic tissues. Regarding disease type, three classifications were observed: colon adenocarcinoma, cecum adenocarcinoma, and rectal adenocarcinoma. Colon adenocarcinoma was notably associated with most of the cell lines (71.7%), while the remaining classifications showed minimal variability in frequency across the dataset (Table 5). Comparison of these features among the clusters revealed no statistically significant differences in tumor origin (Chi-squared p-value = 0.092) or disease type (Chi-squared p-value = 0.384). Despite these results, it is important to note that cell lines derived from primary tumors were more frequent in the CBS and BU clusters, with the CBS cluster specifically being entirely composed of cell lines from this origin (Figure 9). Colon adenocarcinoma was the most frequent disease in all clusters, though in lower percentage within the L-CM and CBS clusters. These clusters also exhibited the highest proportions of CRC cell lines derived from cecum adenocarcinoma and rectal adenocarcinoma, respectively. Both cell lines included in the H-CM cluster were

associated with colon adenocarcinoma, whereas in the remaining clusters, the second most frequent disease type varied: cecum adenocarcinoma was the only other disease type registered in CS, while the BU cluster had a higher proportion CRC from rectal adenocarcinomas. (Figure 10).

Table 5. Cell line origin and disease type distribution.

Cluster	Origin (%)		Disease type (%)		
	Primary	Metastatic	Colon adenocarcinoma	Cecum adenocarcinoma	Rectal adenocarcinoma
CM-H	1.9	1.9	3.8	0	0
CM-L	13.2	9.4	13.2	7.5	1.9
CS	9.4	7.5	15.1	1.9	0
CBS	22.6	0	13.2	3.8	5.7
BU	26.4	7.5	26.4	1.9	5.7
	73.5	26.3	71.7	15.1	13.3

Abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

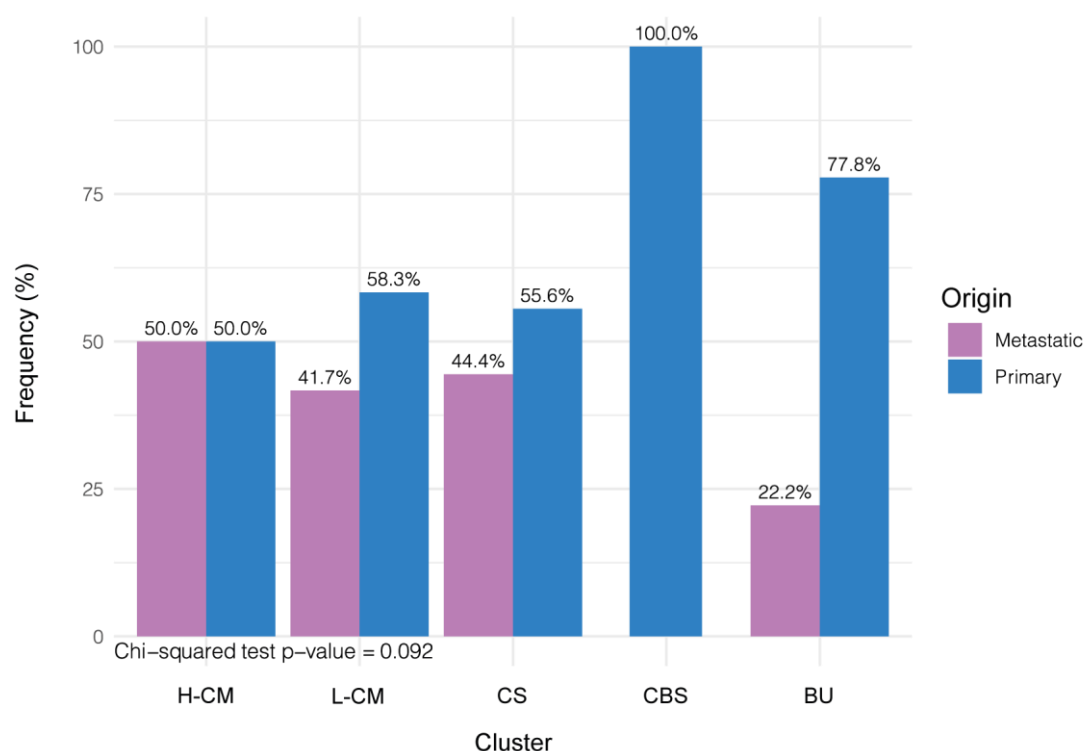


Figure 9. Origin of colorectal cancer (CRC) cell lines from the different clusters. Comparison of the clusters regarding cell line origin, divided into primary and metastatic. The Chi-square test was used to compare the distribution of the classifications across the clusters. Results were considered statistically significant when $p \leq 0.05$. Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

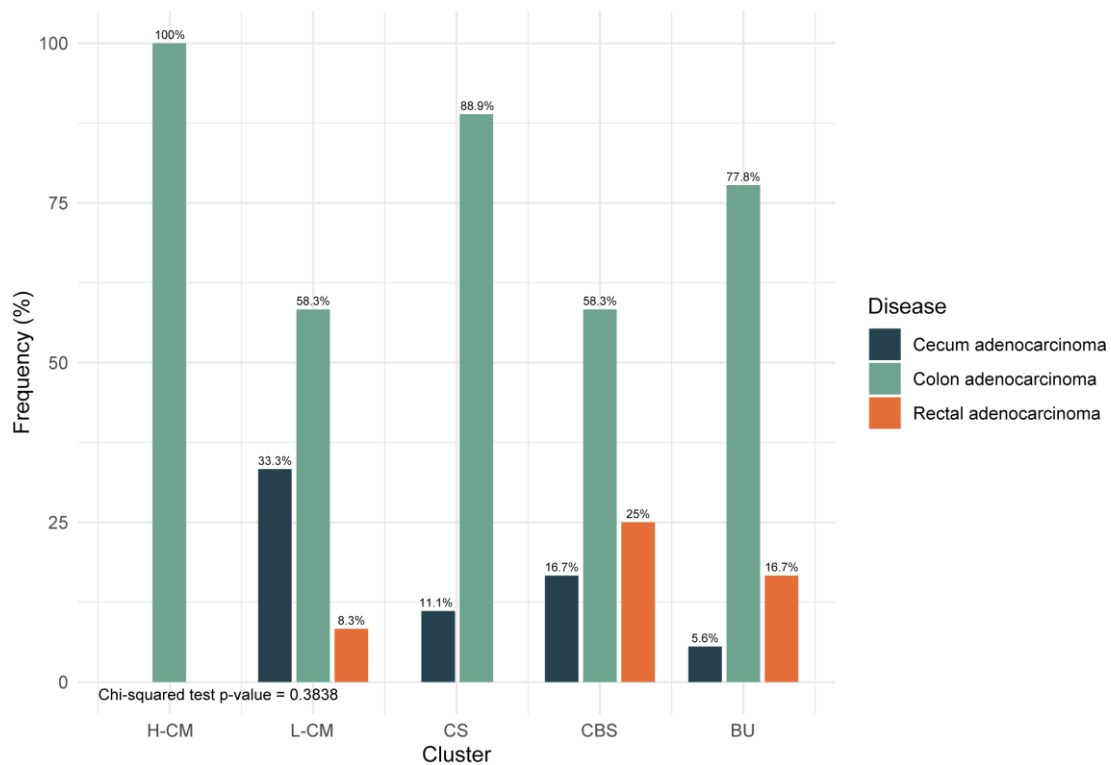


Figure 10. Disease type distribution across the colorectal cancer (CRC) cell line clusters. Comparison of CRC disease type distribution across the cell line clusters. The Chi-square test was used to compare the distribution of the classifications across the clusters. Results were considered statistically significant when $p \leq 0.05$. Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

3.2.3. Proliferative capacity of the CRC cell line clusters

To understand if cholesterol metabolism could influence the overall proliferative potential of each cluster, proliferation ES were determined as previously done for the TCGA analysis, using the same gene set (Figure 11). Although no statistically significant differences were found (ANOVA p-value = 0.792) among the clusters, the H-CM cluster collectively yielded the lowest ES, while L-CM exhibited the highest. ES were dispersed within each cluster, revealing some heterogeneity regarding the proliferation gene expression profile of the CRC cell lines.

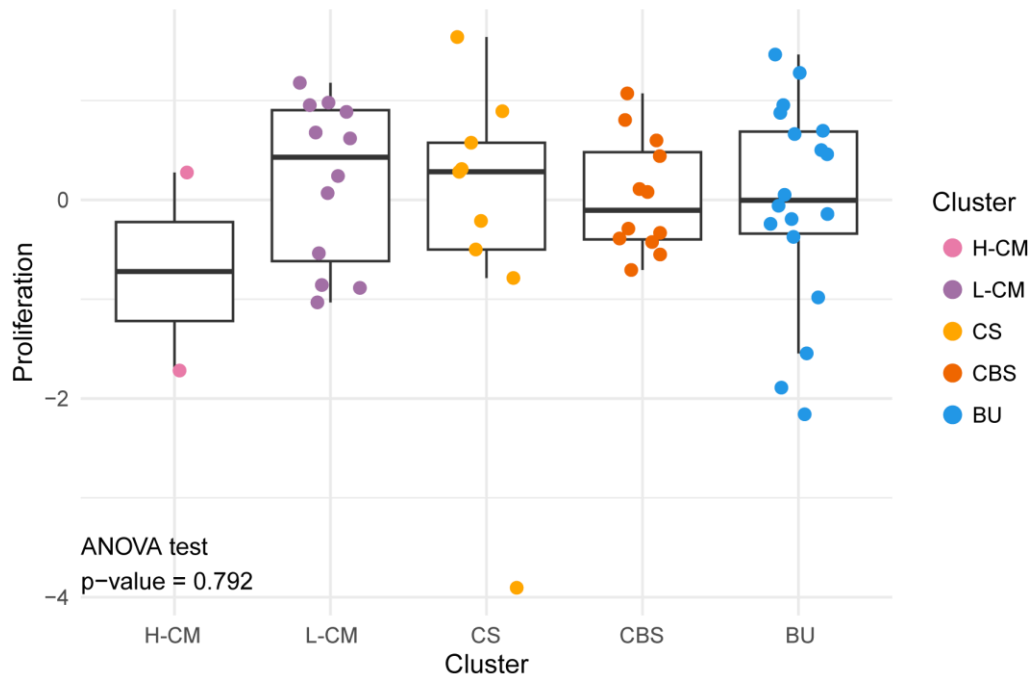


Figure 11. Single Sample Gene Set Enrichment Analysis (ssGSEA) of proliferation across the colorectal cancer (CRC) cell lines. Comparison of the CRC cell lines clusters regarding proliferation enrichment scores (ES). ANOVA was used to compare the ES across the clusters. Results were considered statistically significant when $p \leq 0.05$. Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

3.2.4. Tumorigenic signaling patterns across the clusters

To understand how the different clusters could have implications in tumor progression, the PROGENy algorithm was applied to compare them regarding their tumorigenic signaling pathway signatures (Figure 12). Herein, statistically significant differences were found in the TGF- β (ANOVA p-value = 0.05) and VEGF (ANOVA p-value = 0.009) pathways. For both pathways, the H-CM cluster presented distinct patterns compared to the other clusters, showing an upregulation of the TGF- β pathway and, conversely, a downregulation of the VEGF pathway. Although this cluster encompasses only two cell lines, scores within this analysis were markedly consistent. Cluster CS also reveals some tendencies, characterized by an upregulation of the EGFR, MAPK, and VEGF pathways, as well as the estrogen pathway, although values were more dispersed across the cell lines.

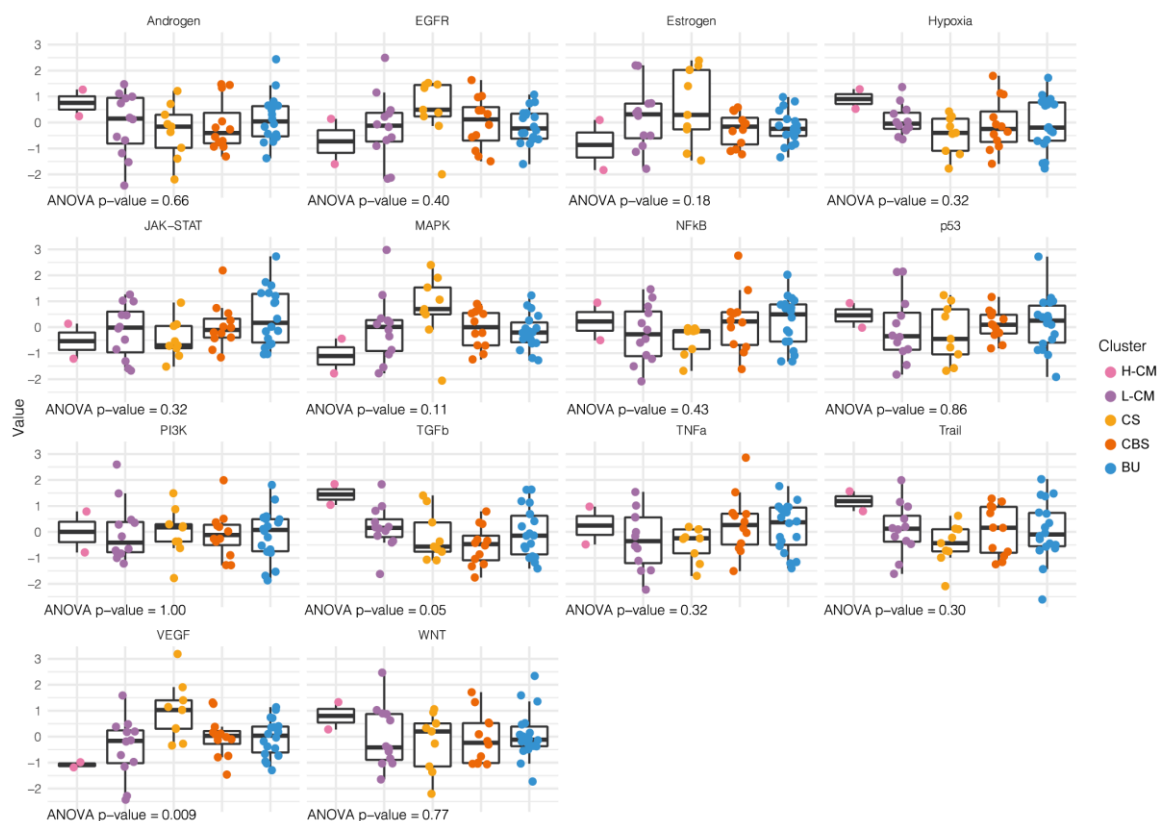


Figure 12. Comparison of the signatures of 14 tumorigenic signaling pathways between the colorectal cancer cell lines groups. The PROGENy algorithm was employed to analyze the signaling patterns of the five cholesterol metabolism clusters, revealing statistically significant differences in the TGF- β (p-value = 0.05) and VEGF (p-value = 0.009) pathways. The Shapiro-Wilk test was performed to assess normality, followed by either the ANOVA or Kruskal-Wallis test to evaluate the differences between the clusters. Results were considered statistically significant when $p \leq 0.05$. Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

3.2.5. Immunomodulatory potential of the clusters

As the immune system plays an important role in controlling or promoting tumorigenesis, we sought to determine if the cholesterol-metabolism clusters could potentially differ in terms of immunomodulatory capacity. ssGSEA analysis of immune pathways revealed statistically significant differences across the clusters in six of the analyzed gene sets (Figure 13). These differences encompassed gene sets related to the regulation of the adaptive immune response, type 2 immune response, and antigen processing and presentation. Notably, the H-CM cluster seemed to deviate from the remaining clusters, presenting a marked downregulation of antigen processing and presentation pathways, even when the differences were not statistically significant. When comparing the negative regulation of adaptive immune response and type 2 immune response, differences were not as evident among the clusters. However, both the L-CM and CS clusters presented lower z-scores when compared with the other clusters.

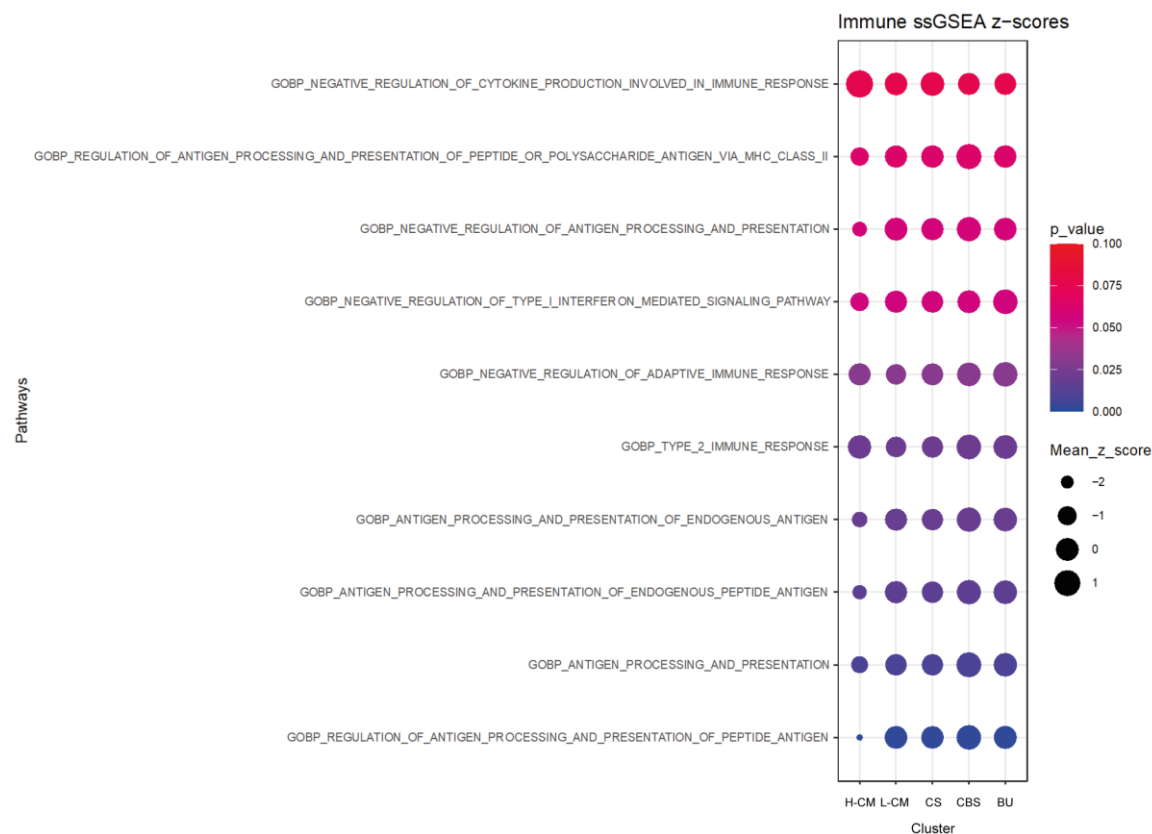


Figure 13. Single Sample Gene Set Enrichment Analysis (ssGSEA) of immunological gene sets. ssGSEA results highlighting the pathways with p-values < 0.1. The analysis shows statistically significant differences between the clusters regarding pathways associated with antigen processing and presentation, regulation of adaptive immune response and type 2 immune response. Results were considered statistically significant when $p \leq 0.05$. Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

3.3. Discussion

Bioinformatics is an evolving field that has proved useful in guiding cancer research, and *in silico* studies – such as the one that originated the CMS – have been developed as a tool defining CRC prognosis^{71,137,138}. However, most studies either focus on commonly known alterations associated with CRC or apply a broader analysis to define subgroups with potential prognostic values. Even though disruption of cholesterol homeostasis is now known to influence CRC progression, the value of evaluating cholesterol metabolism in defining CRC prognosis has never been approached, which led to the development of our previous TCGA analysis. Although the TCGA cholesterol metabolism-based clusters may provide invaluable information regarding the differences associated with the metabolic profiles of CRC, gene expression may not directly translate into the biological function of the pathways due to post-transcriptional regulation mechanisms¹³⁹. This uncertainty is what prompted us to develop an *in vitro* model to further define the biology of these clusters by reproducing the analysis with data from CRC cell lines included in the CCLE.

The primary limitation of the CCLE data analysis in the process of defining CRC cell line clusters is the small sample size ($n = 53$) in comparison with the one provided by TCGA ($n = 439$). With a disease as heterogeneous as CRC¹³, the characteristics of the cell lines may not be fully representative of the landscape of CRC cholesterol metabolism. When comparing the TCGA clusters (Figure 4) with the CRC cell line clusters (Figure 6), they both have clusters with extreme profiles, especially regarding the generalized downregulation of cholesterol metabolism, as L-CM clearly matches TCGA's cluster 4. The pattern of the H-CM cluster also resembles TCGA's cluster 3, though one could argue that similarities in the biosynthesis pathway are more visible in comparison with TCGA's cluster 5. It is important to note that the H-CM cluster is composed of only two CRC cell lines, which poses a challenge in this analysis. Bridging the TCGA and CCLE clusters, however, is most difficult when comparing the clusters with intermediate profiles, namely, CS, CBS, BU. For instance, TCGA's cluster 2 presents high biosynthesis and storage, like CBS, but lacks catabolism, while TCGA's cluster 5 encompasses most of CBS's features but also has high efflux and uptake. Overall, we see that there is not a complete overlap between the CCLE and TCGA clusters, likely deriving from the limited number of cell lines used in the analysis. It is also important to note that, although the TCGA data derives from samples composed of 70% or more tumor cells, a potential contribution of the TME cannot be excluded and may explain some of the discrepancies observed in the classification of the clusters. In this case, the analysis of single cell data would help to clarify this issue. Nonetheless, looking at the combination of gene expression within each pathway may still provide insights into how cholesterol modulates tumor progression.

Despite the heterogeneity of CRC, previous classifications have been able to pinpoint characteristics that are more specifically associated with certain subtypes, such as MSI-H tumors being more commonly found in the ascending colon⁵⁷. To try and better characterize the CCLE clusters, we aimed to determine how they compared in terms of molecular classification (MSI status and CMS) and clinicopathological characteristics (cell line origin and disease type). Notably, studies have yet to describe a relationship between MSI status and a possible dysregulation of cholesterol metabolism. In our analysis, did not find any statistically significant differences between the clusters concerning this classification (Figure 7). Both the H-CM and BU clusters stood out for having the highest percentages of MSS and MSI-H tumors, respectively. However, this discrepancy could be a mere consequence of the difference in the number of cell lines in each cluster, as H-CM had the least samples, whereas BU had the most. These findings suggest that dysregulation of cholesterol metabolism may be largely independent of MSI status. Although dMMR tumors usually respond better to immunotherapy, there is still resistance to this treatment approach within MSI-H CRC¹⁴⁰. Our results indicate that this could, in part, be explained by the disruption of cholesterol homeostasis. The same principle may apply to MSS/MSI-L CRC, as this group encompasses most CRC tumors, thus being the most heterogeneous⁸. In this subset, cholesterol metabolism has even been pointed out as a potential factor behind their unresponsiveness to immunotherapy¹¹⁰.

Regarding CMS, 13.3% of cell lines were classified with a variable subtype, due to the inconsistency of this data across different sources^{141–145}. While this highlights the inherent challenges of this classification, it may also hinder the comparison between the CCLE clusters, where no statistically significant differences were found. However, some tendencies are more visible than when classifying the CRC cell lines by MSI status. Notably, CMS1 distribution by the clusters was closely aligned with the MSI-H status, likely due to its association with this subtype.

CMS2 was the most frequent subtype both in the CBS and BU clusters, which share the biosynthesis pathway. CMS2 is associated with the activation of the MYC pathway, which has been shown to increase cholesterol biosynthesis through the overexpression of squalene epoxidase (SQLE)¹⁴⁶, one of the genes included in the ssGSEA analysis (Table S1). In contrast, CMS4 was the most frequently observed subtype in clusters with vastly different cholesterol metabolism profiles – H-CM, L-CM, and CS. As a next step, it will be important to determine whether CMS4 tumors can be subgrouped according to their cholesterol metabolism properties. This distinction may yield significant results, especially between clusters with high catabolism - H-CM and CS - versus L-CM. For instance, cholesterol oxysterols, namely 27-HC and 25-HC, are strong inducers of epithelial-to-

mesenchymal transition^{147,148}, migration and invasion^{149,150}, as well as potent immunosuppressors^{112,151}, that may impinge on the properties of the tumors.

Changes in cholesterol metabolism have been known to influence the metastatic potential of cancer. Increase in cholesterol biosynthesis by SREBP2, for instance, has been linked with CRC metastasis in the liver¹⁵². By analyzing the origin of the CRC cell lines, we sought to understand if the ones deriving from metastatic tissues were potentially enriched in certain clusters, thus highlighting cholesterol metabolism pathways that could promote their ability to metastasize. In this analysis, none of the clusters revealed a clearly higher percentage of metastatic cell lines. However, the genetic profiles of metastasis are known to differ from primary tumors¹⁵³. As such, the patterns found in these cell lines may not reflect the cholesterol metabolism profile of primary tissues that could potentiate their invasiveness. The inability to conduct this comparison outlines one of the challenges in the characterization of CRC cell lines, and particularly their context prior to extraction.

As the location of tumors along the colon and rectum is known to influence the characteristics of CRC¹⁵⁴, we also gathered information on the specific disease that was associated with the CRC cell lines, with emphasis on the primary location – colon, cecum, or rectum. CRC cell lines derived from cecum adenocarcinomas revealed the highest frequency in L-CM, possibly suggesting that tumors located in the cecum could have a lower activation of cholesterol metabolism pathways. In contrast, the higher frequency of CRC cell lines associated with rectal adenocarcinomas in the CBS and BU could once again be associated with their higher activation of cholesterol biosynthesis. Differences in the distribution of CRC cell lines derived from colon adenocarcinomas were not visible. A more comprehensive analysis of the exact site of the primary tumors in this group could provide more insightful information about possible differences in cholesterol metabolism.

Since cholesterol metabolism influences cell proliferation¹⁵⁵ and the proliferative potential of the TCGA tumors was also previously assessed, we aimed to gather this information in the CCLE clusters as well. When comparing the TCGA and the CCLE clusters, there are some discrepancies in this regard. While in the TCGA tumor analysis the most proliferative cluster presented with active cholesterol biosynthesis and storage (Figure 6), L-CM revealed some of the highest proliferation scores (Figure 11). This may be due to the inherent biases of comparing data from tumors and cell lines, as the growth conditions of CRC cell lines are not a direct translation of the nutritional landscape of the TME. Cell lines are generally cultured under conditions favorable for their proliferation, such as the supplementation of media with fetal bovine serum (FBS), and its composition varies depending on the manufacturer. As such, FBS can directly affect the results of *in vitro* experiments using cell lines¹⁵⁶, which may not reflect the behavior of actual tumors. The H-CM cluster, despite presenting high cholesterol biosynthesis, uptake, and storage,

which altogether promote the accumulation of cholesterol and, consequently, cell growth¹⁵⁷, seems to have a lower proliferative capacity compared to the other cluster. However, this cluster also presents with an overregulation of the efflux and reverse cholesterol pathways, which could be the underlying cause of the low proliferative capacity of the cell lines included in this cluster. The similar proliferative capacity of the remaining clusters (CS, CBS, and BU), which present with intermediate cholesterol metabolism profiles, further supports the hypothesis that there may be an equilibrium between certain pathways that maintains the cholesterol content within the cells, influencing their proliferative capacity. The CS cluster, despite having high storage, has low biosynthesis and high catabolism; in the CBS cluster, the high catabolism may counteract high biosynthesis and storage; and the lack of storage in the BU cluster may influence the effects of biosynthesis and uptake.

Several signaling pathways that are associated with tumorigenesis and tumor progression often lead to the dysregulation of cholesterol metabolism¹⁵⁸. PROGENy analysis revealed very consistent scores in the H-CM cluster (Figure 12), despite it only containing two cell lines. This cluster also differed the most from the remaining, showing a clear overexpression of the TGF- β pathway, a result that is consistent with the CMS of these cell lines (CMS4). Overexpression of this pathway is associated with tumor progression, mainly through the promotion of EMT, angiogenesis, and immune suppression¹⁵⁹, which could indicate an aggressive behavior of CRC cell lines (and tumors) with a highly active cholesterol metabolism. Interestingly, previous studies have shown that cholesterol depletion in cells can promote TGF- β signaling^{160,161}. This suggests that reduction of intracellular cholesterol levels by efflux may have an important role in the H-CM cluster that surpasses the effects of biosynthesis and storage. What seems to be a contradictory finding in this cluster is the downregulation of the VEGF pathway, due to its importance in angiogenesis¹⁶². On the one hand, it may explain the lower proliferative capacity of cell lines within this cluster. On the other hand, VEGF downregulation may also be beneficial for CRC cells by inducing hypoxia, which can promote tumor progression¹⁶³. Furthermore, a lower angiogenic capacity may also benefit therapy resistance and decrease anti-tumor immune responses through decreasing drug release¹⁶⁴ and immune cell infiltration into the tumor¹⁶⁵. Finally, the lower levels of VEGF signaling of this cluster may be an indicator of lower efficacy of anti-VEGF drugs, therefore helping to stratify patients.

Another cluster that stands out is the CS cluster, characterized by the upregulation of the EGFR, MAPK, and VEGF pathways. Although these differences were not statistically significant, understanding how cholesterol metabolism affects – or is affected by – these pathways may provide insights into therapeutic options. While MAPK inhibitors

show potential in CRC treatment¹⁶⁶, patients may not necessarily respond well to this approach. In other cancer models, dysregulation of cholesterol metabolism has been linked to resistance to MAPK inhibitors, suggesting that cholesterol-lowering drugs could improve treatment response¹⁶⁷. Considering the cholesterol metabolism profile of the CS cluster, marked by high cholesterol storage, combining MAPK inhibition with cholesterol storage inhibition could be a potential treatment strategy for CRC. It has also been shown that cholesterol depletion in the plasma membrane can promote EGFR signaling¹⁶⁸. CS cluster's metabolic profile – and more specifically the combination of low biosynthesis, high storage, and high catabolism – could reduce cholesterol availability for incorporation into the cell membrane, potentially enhancing EGFR signaling. The concurrent activation of this pathway at the gene expression level highlights the potential of targeting EGFR in tumors with the same cholesterol metabolism profile as the CS cluster. VEGF signaling may also be associated with the catabolic profile of the cluster. For instance, 27-HC has previously been found to activate VEGF signaling and promote angiogenesis in breast cancer¹⁶⁹, and a similar mechanism may apply to this cluster. Thus, VEGF inhibitors might be indicated for treating tumors with similar cholesterol metabolism profiles as the CS cluster.

Cholesterol homeostasis also plays a critical role in shaping the TME, particularly in cancer immunology¹¹³. As such, different cholesterol metabolism profiles can potentially influence the interplay between CRC and immune cells, which led to our ssGSEA analysis of immune gene sets to compare the clusters. One of the many strategies of cancer cells to evade the recognition and elimination by immune cells includes the downregulation of antigen presentation¹⁷⁰. In the ssGSEA analysis, the H-CM cluster exhibited downregulation of pathways related to antigen processing and presentation, which may highlight the capacity of cells within this cluster to escape immune surveillance. The generalized activation of cholesterol metabolism, therefore, could serve as an indicator of CRC cells' ability to evade the immune system. Interestingly, there were also statistically significant differences in type 2 immune response that, although slight, show a higher upregulation of this pathway in clusters with high biosynthesis – H-CM, CBS, and BU. Type 2 immune response includes the production of cytokines such as IL-4 and IL-10¹⁷¹, which, as previously mentioned, can polarize TAMs into the M2 phenotype, for instance. Thus, cholesterol metabolism may, to some extent, also have an association with the capacity of CRC cell lines to intervene in TAM polarization and, hence, promote a pro-tumorigenic TME. These effects may be heightened by the suppression of the adaptive immune response, as the negative regulation of adaptive immune response pathway is slightly upregulated in these clusters as well.

In summary, the *in silico* analysis of CRC cell line clusters reveals that the metabolic profile of CRC could potentially impact tumor progression by influencing cell proliferation, tumorigenic signaling pathways, and the TME. Our results once again suggest that cholesterol metabolism pathways – which are not generally explored in the molecular classification of CRC – could be useful for understanding the heterogeneity of CRC. However, results should be carefully interpreted, as they refer to cell line gene expression and do not account for the context of the whole tumor.

In vitro validation of colorectal cancer cell lines clusters

4.1. Materials and Methods

4.1.1. Cell lines and culture conditions

A cell line representative of each cluster was selected to validate the CRC cell line subgroups obtained in the *in silico* analysis (Table 6). Caco-2, HCT-116, SW620, SW837, and SW1463 cell lines were kindly provided by collaborators and stored at the i3s Cell Lines Bank. HCT-116 cells were cultured in RPMI-1640 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), Caco-2, SW620, and SW837 were cultured in DMEM (Corning, New York, USA), and SW1463 were cultured in Leibovitz-15 medium (Gibco, Thermo Fisher Scientific), all supplemented with 10% heat-inactivated FBS (Cytiva, Marlborough, MA, USA) and 1% penicillin-streptomycin (Pen/Strep) 10 000 U/mL (Gibco, Thermo Fisher Scientific). Cell lines were routinely maintained in a humidified incubator at 37°C in their respective medium, in an atmosphere of 5% CO₂ for Caco-2, HCT-116, SW620, and SW837, and 100% air for SW1463. During experiments that required CO₂ exchange, SW1463 cells were grown in DMEM.

Table 6. Clinicopathological and molecular features of CRC cell lines.

Cell line	Cluster	MSI Status	CIMP	CIN	CMS	Disease	Origin
Caco-2	H-CM	MSS	-	+	CMS4	Colon adenocarcinoma	Primary tumor
HCT-116	L-CM	MSI	+	-	Variable	Colon adenocarcinoma	Primary tumor
SW620	CS	MSS	-	+	CMS4	Colon adenocarcinoma	Lymph node
SW1463	CBS	MSS	-	+	CMS2	Rectum adenocarcinoma	Primary tumor
SW837	BU	MSS	+	+	CMS4	Rectum adenocarcinoma	Primary tumor

Abbreviations: BU, High Biosynthesis/Uptake Cholesterol Metabolism; CBS, High Catabolism/Biosynthesis/Storage Cholesterol Metabolism; CIMP, CpG island methylator phenotype; CIN, chromosomal instability; CMS, consensus molecular subtype; CS, High Catabolism/Storage Cholesterol Metabolism; H-CM, High Cholesterol Metabolism; L-CM, Low Cholesterol Metabolism; MSI, microsatellite instability; MSS, microsatellite stable.

4.1.2. Doubling time assay

To better define the appropriate seeding density of the five cell lines for subsequent experiments, an assay was performed to determine their doubling time (DT). Cells were plated in 12-well plates at a seeding density of 5.5×10^5 cells per well and counted at three

different time points post-plating (24 hours, 48 hours, and 72 hours). Briefly, at each time point, cells were washed with phosphate-buffered saline (PBS) 1×, detached with 0.05% Trypsin-EDTA (Gibco, Thermo Fisher Scientific) at 37°C, and counted using the TC20 Automated Cell Counter (Bio-Rad, Hercules, CA, USA). Their DT was then calculated using Equation 1.

$$DT = T \times \frac{\ln 2}{\ln(N_i/N_f)} \quad (1)$$

where T is the elapsed time at a specified time point,

N_i is the initial number of cells,

N_f is the number of cells at a specified time point.

4.1.3. Neutral lipid storage

The cholesterol storage capacity of CRC cell lines was indirectly determined by quantifying lipid droplets. Cells were stained with Oil Red O (Acros Organics, Thermo Fisher Scientific) for microscopy and with PhenoVue 493 Lipid Stain (PerkinElmer ILC, Waltham, Massachusetts, USA) for flow cytometry analysis.

For microscopy, cells were plated on glass coverslips in 6-well plates in their appropriate culture medium, in a number that allowed a density of approximately 1.2×10^6 cells per well at 48 hours post-plating (4.0×10^5 for Caco-2 and SW837, 1.5×10^5 for HCT-116, 2.6×10^5 for SW620, and 6.2×10^5 for SW1463 cells). After 48 hours, cells were fixed with 4% formaldehyde for 10 minutes (min) at room temperature (RT). The coverslips were washed with distilled water (dH₂O) and stained with Oil Red O working solution [0.3% Oil Red O isopropanol stock solution diluted in dH₂O (3:2)] for 5 min. Cells were counterstained with Gill hematoxylin (Thermo Fisher Scientific) for 30 seconds. The coverslips were mounted on glass slides using Dako Faramount Aqueous Mounting Medium (Dako, Agilent, Santa Clara, CA, USA). Cells were imaged under 24 hours after staining using the Leica DM2000 LED brightfield microscope (Leica, Wetzlar, Germany).

For flow cytometry quantification of LDs, cells were seeded at the same density used for microscopy in 6-well plates and allowed to grow for 48 hours. Cells were detached using trypsin-EDTA, as previously described, and 4.0×10^5 cells of each cell line were collected for staining. Cells were washed twice with PBS, centrifuged at 1200 rotations per min (rpm) for 5 min at 4°C, and the supernatant was removed along with any traces of FBS. Cells were resuspended in 200 µL of 1.35 nM PhenoVue 493 Lipid Stain in PBS and incubated for 20 min at RT in the dark. To detect dead cells, the samples were washed twice with PBS and incubated with a 1:10000 dilution of eBioscience Fixable Viability Dye

eFluor 780 (Invitrogen, Thermo Fisher Scientific) for 15 min at RT in the dark. Cells were washed twice with flow cytometry buffer [PBS containing 1% bovine serum albumin (BSA; NZYtech, Lisbon, Portugal) and 0.1% sodium azide (Sigma-Aldrich)] and fixed with 2% paraformaldehyde (PFA) for 15 min in the dark. Samples were centrifuged at 1500 rpm for 5 min at 4°C, resuspended in flow cytometry buffer, and acquired using the BD FACS Canto II flow cytometer (BD Biosciences, Bedford, MA, USA). Data was analyzed using FlowJo software (v.10, Treestar, San Carlos, CA) to determine the percentage of live PhenoVue-positive cells and their median fluorescence intensity (MFI), according to the gating strategy in Supplementary Figure S1.

4.1.4. BODIPY-Cholesterol uptake assay

The cholesterol uptake capacity of CRC cell lines was evaluated by treating cells with 23-(dipyrrometheneboron difluoride)-24-norcholesterol TopFluor Cholesterol (AvantiLipids, Alabaster, AL, USA), also known as BODIPY-cholesterol, and quantifying the fluorescence intensity in the cells. CRC cell lines were plated on top of glass coverslips in 12-well plates, using their respective complete culture medium, in a density that allowed a final number of approximately 5.0×10^5 cells per well 48 hours after plating (1.6×10^5 for Caco-2 and SW837 cells, 6.3×10^4 for HCT-116 cells, 1.1×10^5 for SW620 cells, and 2.6×10^5 for SW1463 cells). After 48 hours, following two washes with PBS, the complete media was exchanged with FBS-depleted media, and the cells were incubated overnight (ON). Cells were then treated with 2.5 μ M of BODIPY-cholesterol in FBS-depleted DMEM without Phenol Red (Gibco, Thermo Fisher Scientific). The treatment was stopped at several time points – 0 hours (untreated cells), 1 hour, 2 hours, and 4 hours – by removing the media from the wells and washing cells twice with PBS. Cells were then fixed with 4% formaldehyde for 10 min at 37°C. For microscopy analysis, cells were washed twice with PBS (5 minutes per wash) and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 20 minutes. Following two washes with PBS, cells were simultaneously stained with 0.1 μ g/mL PhenoVue Hoechst 33342 (PerkinElmer ILC) and 0.01 μ g/mL HCS CellMask Deep Red stain (Invitrogen, Thermo Fisher Scientific) in PBS. Cells were washed three times with PBS, and coverslips were mounted on glass slides using VECTASHIELD Antifade Mounting Medium (VectorLabs, Newark, CA, USA). All steps were carried out at RT. Cells were imaged using the Operetta CLS high-content analysis system (Revvity, Waltham, MA, USA) and Harmony high-content imaging and analysis software (Revvity, Waltham, MA, USA). Composite images of BODIPY-cholesterol, Hoechst, and HCS CellMask were used for analysis with the Harmony software. Cell nuclei were defined in the Hoechst channel and filtered by size, roundness, and fluorescence intensity. The cytoplasm of cells was then identified in the HCS CellMask channel and filtered by fluorescence intensity.

Any border objects were filtered out to obtain the final cells (see Supplementary Figure S2). The BODIPY channel was then used to measure the fluorescence intensity of BODIPY-Cholesterol in each cell.

4.1.5. Statistical analysis

Statistical analysis was conducted using R studio. Means of multiple groups were compared by either one-way ANOVA or the Kruskal-Wallis non-parametric test. Results were considered statistically significant when $p \leq 0.05$. Whenever $p \leq 0.05$, ANOVA was followed by the Tukey multiple comparison test, whereas Kruskal-Wallis was complemented with Dunn's post-hoc test.

4.2. Results

4.2.1. CRC cell lines doubling time

Each CRC cell line has unique features, including its proliferative capacity, therefore influencing the course of *in vitro* assays. To mitigate the impact of variability in cell growth during the analysis of CRC cell lines, we aimed to determine the DT for the cell lines representative of the five clusters. We monitored their growth over 72 hours, counting the cells at 24, 48, and 72-hour intervals. For the DT calculation, we considered the number of cells after 72 hours as the Nf variable, to ensure the elapsed time was sufficient to capture long DT periods.

The five cell lines exhibited distinct DTs: HCT-116 had the shortest DT (17 hours), while SW1463 had the longest (59 hours). SW620's DT was close to that of HCT-116's, at 21 hours. Caco-2 and SW837 revealed similar DTs among them – 32 and 29 hours, respectively. Notably, the HCT-116 exhibited higher cell density at each timepoint compared to the remaining cell lines (Figure 14A), confirming its faster growth rate (Figure 14B).

Although the main aim of this assay was to guide subsequent experiments, assessing the DT of CRC cell lines also provides insights into their proliferative capacity, which can help evaluate whether these cell lines effectively represent their respective clusters. While SW1463's low growth rate (Figure 14B) deviated from the comparatively high proliferative capacity of its cluster (CBS), the remaining cell lines followed their clusters' trends. For instance, Caco-2, part of the H-CM cluster, which had the lowest proliferative capacity, also exhibited slow growth, while the HCT-116's high growth rate aligned with the L-CM cluster's high proliferative capacity. SW620 and SW837 showed intermediate growth rates, supporting the observed proliferative capacity of their respective clusters.

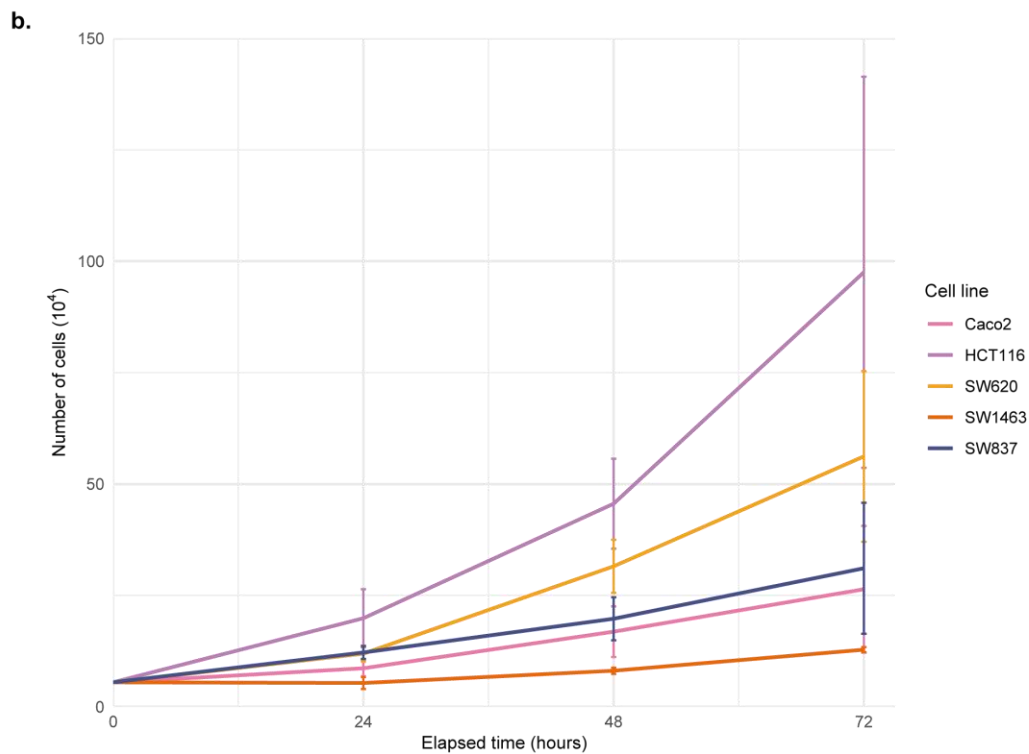
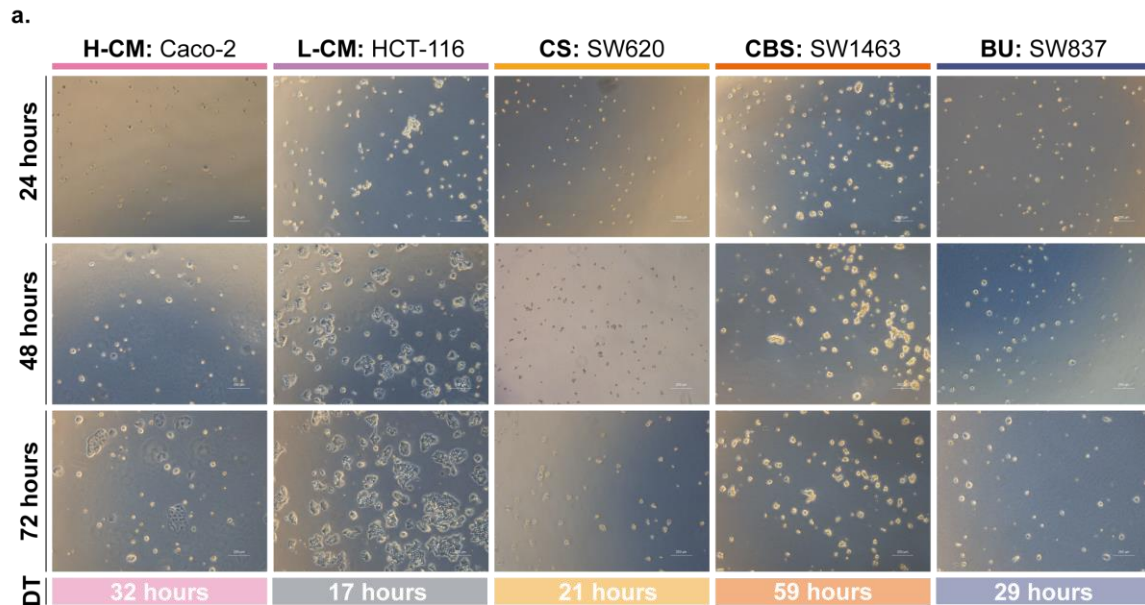


Figure 14. Growth patterns of colorectal cancer (CRC) cell lines from the different clusters. a. Representative images of the cell aspect of the CRC cell lines at 24 hours, 48 hours, and 72 hours post-plating and respective calculated doubling time (DT) (images obtained with a 5× objective). b. Growth pattern of the CRC cell lines over the course of 72 hours showing distinct growth patterns, reflecting the results of three independent experiments for the Caco-2, HCT-116, SW620, and SW837 cell lines, and two independent experiments for the SW1463 cell line. Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

4.2.2. Neutral lipid storage

To gain insight into the cholesterol storage capacity of CRC cell lines, we evaluated the neutral lipid storage of these cells through a morphological and quantitative analysis of LDs. To quantify neutral lipid storage in CRC cell lines, we first determined the relative number of cells with LD content, by looking at the percentage of cells positively stained for PhenoVue (Figure 15A). Most samples had over 95% of cells with LDs, apart from the SW1463 cell line, with a mean percentage of 35.95% ($\pm 9.06\%$) positive cells. Herein, there is a statistically significant difference in the mean percentages of positive cells between the Caco-2 and SW1463 cell lines (Dunn's p-value = 0.0084), as well as between the HCT-116 and SW1463 cell lines (Dunn's p-value = 0.0045). To further quantify lipid droplet content within the PhenoVue-positive populations, we looked at the cells' MFI (Figure 15B). When comparing the cell lines, we found statistically significant differences between them (Kruskal-Wallis p-value = 0.0084) and, notably, a statistically significant difference between the Caco-2 and SW620 cell lines (Dunn's p-value = 0.0027), which present the highest and lowest MFI values, respectively.

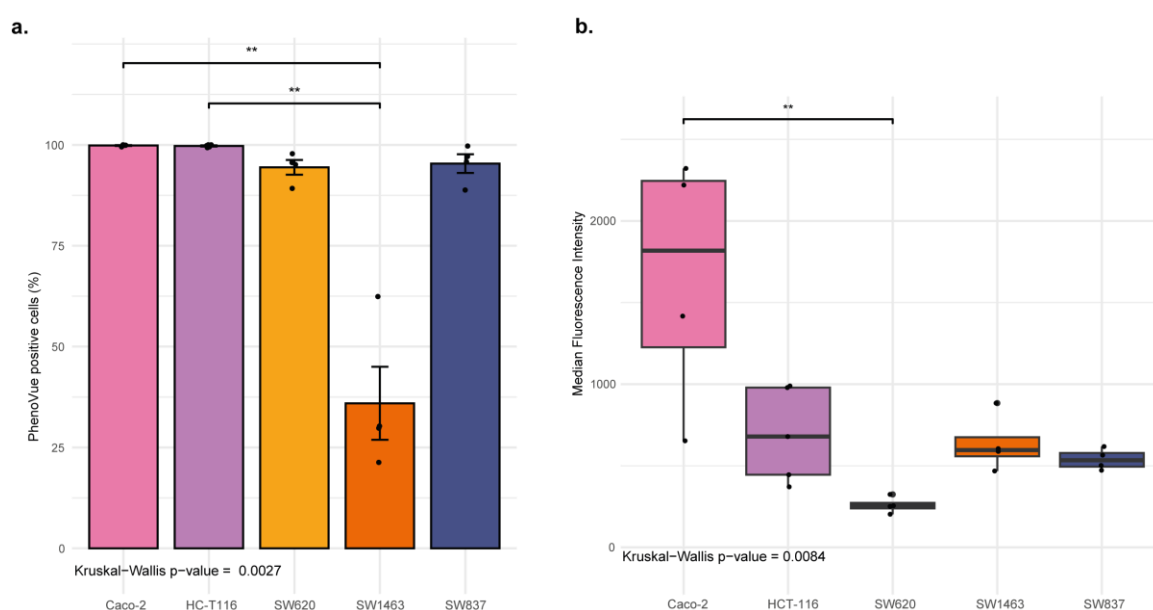


Figure 15. Lipid droplet (LD) content within CRC cell lines of each cluster. a. Percentage of PhenoVue positive cells in each cluster, presented as mean $\pm$ sd, revealing statistically significant differences between the SW1463 cell line and Caco-2 and HCT-116 cell lines. b. Median fluorescent intensity (MFI) of positive cells, presented as mean $\pm$ sd, showing statistically significant differences between the Caco-2 (Cluster) and SW620 (Cluster 3) cell lines. The non-parametric Kruskal-Wallis test was used to compare the mean percentages and mean MFI across the clusters. Results were considered statistically significant when $p \leq 0.05$. (** $p \leq 0.001$).

When looking at the LD distribution patterns within the cell lines, differences can also be observed (Figure 16). The Caco-2 presented with the most marked differences, showing a large quantity of LDs with variable sizes, although mostly smaller than those found in the remaining cell lines, which were distributed throughout the whole cell. LDs in

HCT-116, SW620, and SW837 appeared to be more even in size, and in less quantity, accompanying the smaller size of these cells. The SW1463 seemingly had fewer LDs, particularly when compared with cell lines of similar size – HCT-116 and SW837. They also accumulated at the periphery of the cell.

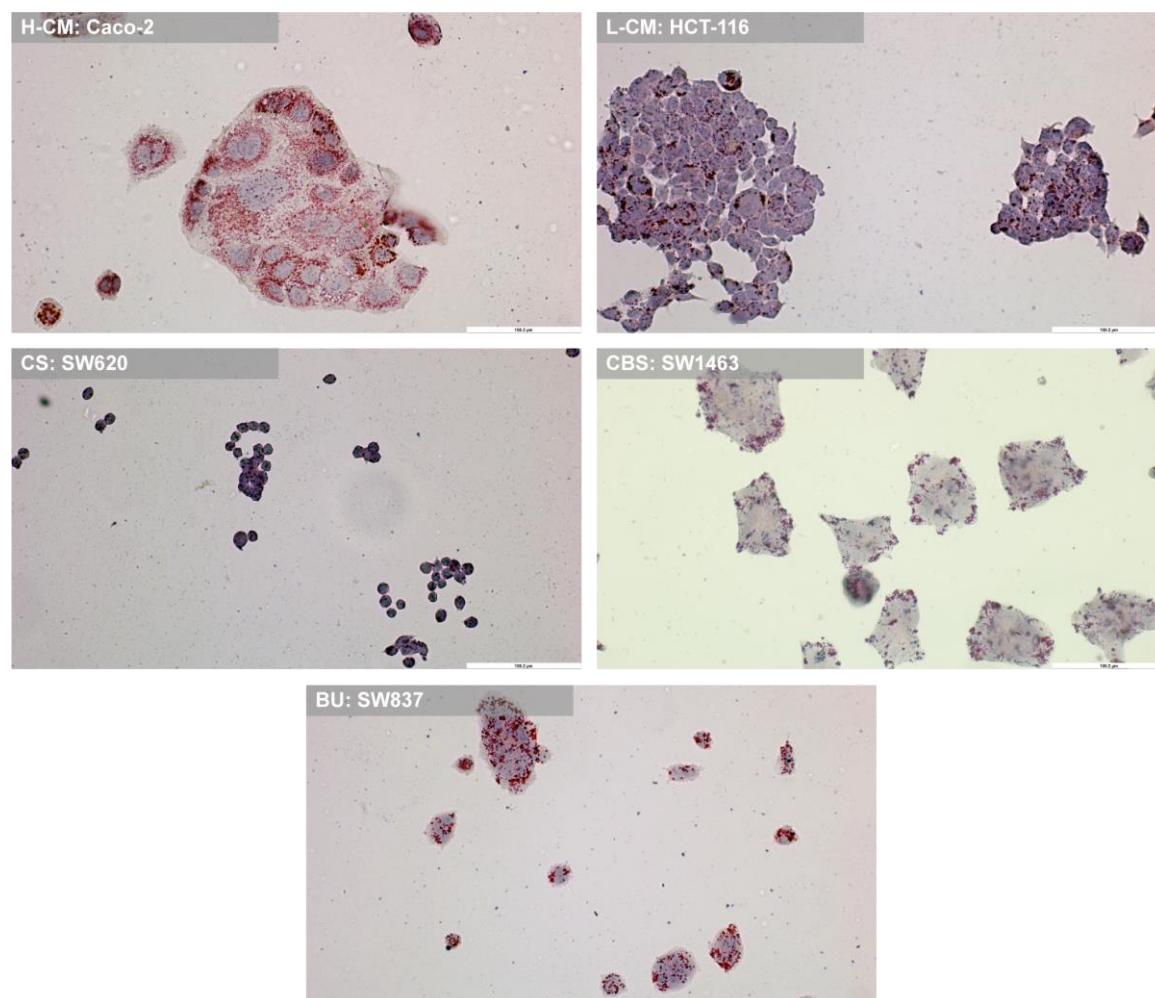


Figure 16. Oil Red O staining of colorectal cancer (CRC) cell lines. Oil Red O staining showing different patterns of lipid droplet distribution within CRC cell line. Nuclei were counterstained with Gill hematoxylin (images taken with a 20× objective). Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

According to the ssGSEA analysis, Caco-2 had the highest ES in the cholesterol storage pathway, supporting the present analysis. However, flow cytometry results reveal some differences from the expected storage capacity of the remaining cell lines, with emphasis on the SW620 cell line; whereas this was the cell line with the second highest ES in the *in silico* analysis, it exhibited the lowest MFI values.

4.2.3. Cholesterol uptake

To verify whether the results regarding cholesterol uptake capacity in terms of gene expression translated into cellular function, we treated the CRC cell lines with BODIPY-cholesterol and monitored their fluorescence at the beginning of the assay, and after 1, 2, and 4 hours (Figure 17A). CRC cell lines exhibited three distinct uptake patterns that translated into: 1) a constant increase in BODIPY-cholesterol content throughout the whole assay (HCT-116 and SW837); 2) an increase in BODIPY-cholesterol content during the first two hours, followed by a decrease in fluorescence intensity at the 4-hour mark (Caco-2 and SW620); and 3) an increase in BODIPY-cholesterol during the first hour, stabilization during the second hour, and a further increase for the remainder of the assay (HCT-116) and (SW1463) (Figure 17B).

To compare the uptake capacity at each timepoint, MFI values were normalized based on the MFI of each cell line before treatment. The Kruskal-Wallis test did not reveal any statistically significant differences at any timepoint ($p > 0.05$) (Figure 17C). Nevertheless, Caco-2 and SW1463 had the lowest and highest uptake rates, respectively. HCT-116 and SW620 displayed overall intermediate MFI values, while the SW837 cell line had the second-highest MFI values by the end of the assay.

When comparing the obtained results with the *in silico* analysis, the Caco-2 cell line revealed a striking difference. Although it had the highest ES score for the cholesterol uptake pathway, its actual uptake capacity appeared to be the lowest, regardless of the elapsed time. In contrast, SW1463, which had a low ES score in this pathway, demonstrated the highest uptake of BODIPY-cholesterol. Both HCT-116 and SW620 exhibited higher uptake capacity than expected. SW837, however, revealed an uptake capacity consistent with the *in silico* analysis.

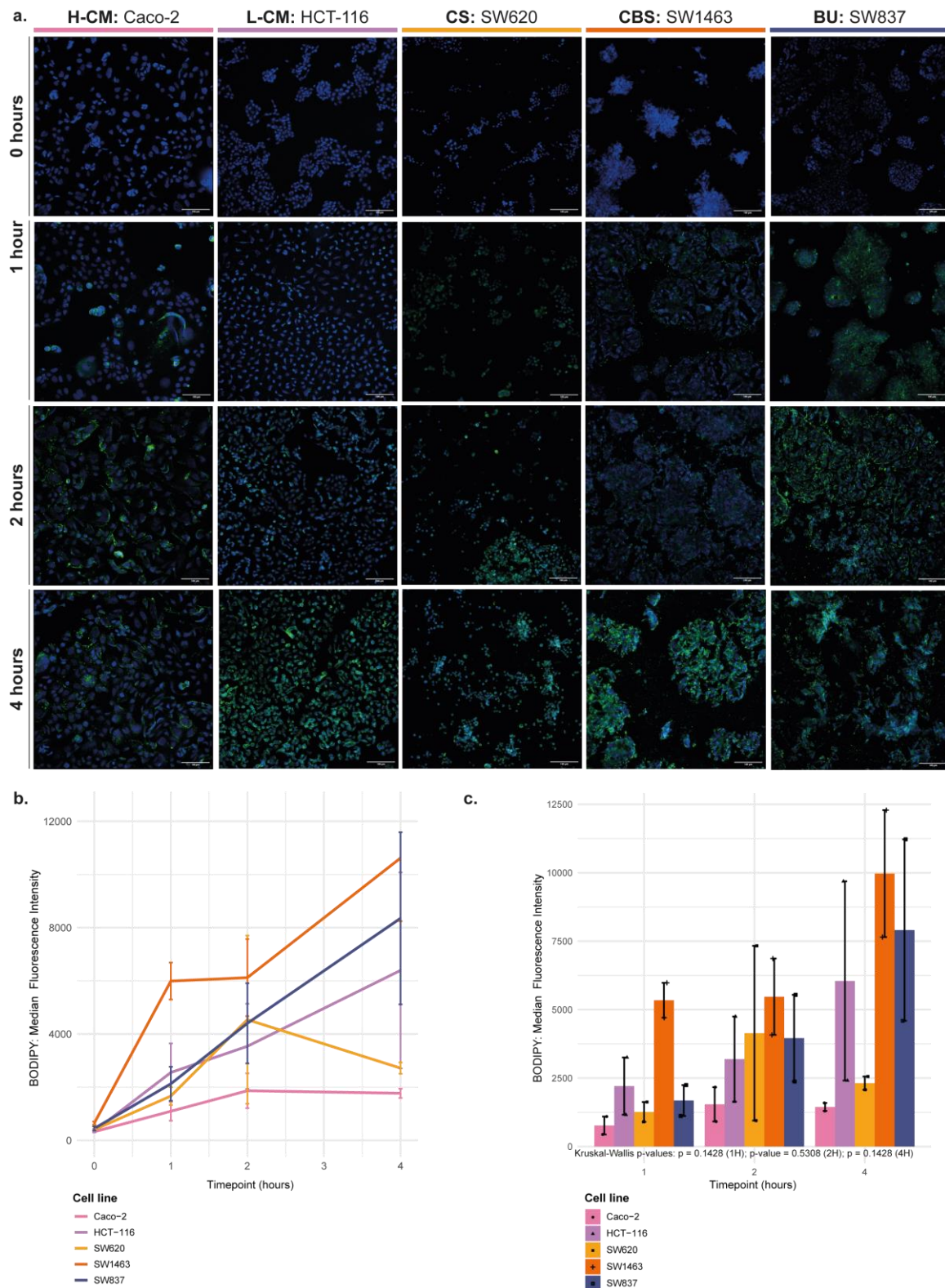


Figure 17. BODIPY-cholesterol uptake capacity of colorectal cancer (CRC) cell lines. a. Representative images of the aspect of CRC cell lines exposed to BODIPY-cholesterol for different time periods (n=2). Contrast was adjusted for each cell line to account for any background present in control cells (0 hours timepoint). Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism. (images taken with a 20× objective). b. Representative graph of the uptake rate of each cell line. c. Comparison of the BODIPY median fluorescence intensity (MFI) between cell lines at each timepoint, normalized to the initial MFI. Results are expressed as mean $\pm$ sd. The non-parametric Kruskal-Wallis test was used to compare the mean MFI, and results were considered statistically significant when $p \leq 0.05$.

4.3. Discussion

Bioinformatics tools allow us to rapidly evaluate complex molecular information and extract data of both biological and clinical relevance. However, it is essential to validate the obtained results through biological analysis to demonstrate their robustness, reproducibility, and applicability in a practical context. Thus, we selected a CRC cell line representative of each cluster obtained in the *in silico* analysis of CCLE samples to validate their cholesterol metabolism. Validation of each cholesterol metabolism pathway was, however, one of the main challenges of this project. Nonetheless, we have been able to get some insights into the cholesterol uptake and neutral lipid storage capacity of the selected CRC cell lines, as well as their proliferative capacity.

There are some limitations associated with the use of the selected cell lines, as they may not fully represent all features of their clusters, apart from Caco-2, considering the sample size of the H-CM cluster, and SW620, as its ES generally followed the tendency of the CS cluster. HCT-116 had a storage ES higher than the remaining cell lines within the L-CM cluster, but the remaining pathways were all downregulated, as expected. The most striking differences are the ones seen in the SW1463 cell line that, despite belonging to the CBS cluster, had low catabolism and biosynthesis ES while having ES scores slightly higher than the rest of the cells in the cluster for reverse transport and efflux. SW837 ES were overall representative of the BU cluster, although with a lower biosynthesis ES. Having this into account, the individual features of these cell lines regarding the cholesterol metabolism pathways included *in silico* analysis should be considered when interpreting the results obtained *in vitro*. The SW1463 also proved difficult to work with due to its long DT (59 hours) and its sensitivity to changes in the culture conditions. Generally, this cell line is cultured in an environment without CO₂, but some experiments required CO₂ exchange to maintain consistency across the cell lines. However, this was the only cell line from the CBS cluster that we had available.

While many studies have previously determined the DT of CRC cell lines, results differ from author to author. HCT-116 DT, for instance, has been reported to have a DT ranging from 20 to 25 hours^{144,172}, while in our analysis, it was determined to be lower (17 hours). SW1463 DT also deviated from results in a previous study, where it was reported to be approximately 45 hours, whereas we observed a lower doubling rate. As culture conditions greatly impact cell growth, determining the DT of CRC cell lines is an important step in defining the course of experimental assays¹⁷³. The difference in culture conditions may have also influenced the DT of the selected CRC cell lines and the differences found compared with the *in silico* proliferation scores. Caco-2 DT was longer than the rest of the CRC cell lines, except for SW1463, which aligns with the lower proliferation score of the

H-CM cluster. HCT-116 shorter DT is also compatible with the high proliferative capacity of the L-CM cluster. SW1463 DT was the one that was found to be the most different from the proliferative capacity of its cluster, which may have been influenced by its inherent features, such as lower biosynthesis and higher efflux, as well as the culture conditions not being ideal.

The cholesterol storage and uptake capacity of CRC cells are both important features that may influence their proliferation, as they help maintain the necessary concentrations for their functions⁹². Furthermore, the accumulation of LDs is known to influence the resistance of cells to ferroptosis¹⁷⁴. As such, the capacity of cells to store cholesterol may aid in preventing cell death. Although we were not able to determine direct cholesterol storage, our results regarding neutral lipid storage, for the most part, align with the cholesterol storage capacity of CRC cell lines observed *in silico* (Table 7).

Table 7. Comparison of cholesterol storage and uptake capacity of colorectal cancer (CRC) cell lines assessment *in silico* and *in vitro*.

Cluster	Cell line	Storage		Uptake	
		<i>In silico</i>	<i>In vitro</i> *	<i>In silico</i>	<i>In vitro</i>
H-CM	Caco-2		Highest		Lowest
L-CM	HCT-116		Second highest		Intermediate
CS	SW620		Lowest		Second lowest
CBS	SW1463		Intermediate		Highest
BU	SW837		Second lowest		Second highest

Abbreviations and annotations: BU, High Biosynthesis/Uptake Cholesterol Metabolism; CBS, High Catabolism/Biosynthesis/Storage Cholesterol Metabolism; CS, High Catabolism/Storage; L-CM, Low Cholesterol Metabolism; H-CM; High Cholesterol Metabolism. **In vitro* assessment was conducted by analyzing neutral lipid storage, thus not being a direct measurement of cholesterol storage. Results refer to the median fluorescence intensity of the PhenoVue staining of lipid droplets.

The LD content of Caco-2 was by far the highest out of all the cell lines, just as was observed *in silico*. Interestingly, LD accumulation revealed the most distinct pattern out of all the cell lines, which appeared much smaller and in larger quantities (Figure 16). This could be a sign of high lipid turnover¹⁷⁵, consistent with the highly activated cholesterol metabolism found in the Caco-2 cell lines. Furthermore, TGF- β signaling – a pathway that was upregulated in the H-CM cluster – has been found to induce LD formation and consequently promote EMT¹⁷⁶.

One of the results that seemed to deviate the most from the *in silico* analysis was the one obtained for the SW620 cell lines. Although most of the cells contained LDs, the MFI of cells was quite low, indicating they may have a lower content of neutral lipids, when compared to the other cell lines. However, this may be a result of the cell line's features, particularly its size. As this cell line is smaller than the rest, which can be observed in

Figure 16, its LD content may naturally be lower, despite its capacity to store cholesterol, and perhaps lipids in general. As such, a more adequate approach to quantifying neutral lipid storage would have been the individual quantification and morphological analysis of LDs through microscopy. However, when attempting this approach, defining a threshold for LD identification in image analysis that could be applicable to all cell lines was not feasible.

A result that stands out when determining neutral lipid storage is the low percentage of cells with LD content in the SW1463 cell line (Figure 15A), as it was expected for all cells to contain LD content to a certain degree, considering the Oil Red O staining (Figure 16). It is, therefore, uncertain whether the findings regarding the percentage of cells with LDs assessed by flow cytometry are in line with reality. SW1463 cells exhibit high autofluorescence, which overlaps with the PhenoVue signal¹⁷⁷. This likely resulted in part of the cells being removed from the analysis after gating. However, it is notable that cells that do contain LDs, presented an intermediate MFI, that aligns with the *in silico* results.

Finally, both HCT-116 and SW837 exhibited a capacity for neutral lipid storage that were compatible with gene expression results, when considering PhenoVue's MFI. Considering the possibility that HCT-116 may have a higher content of cholesterol stored as LDs – though it is not possible to be sure of their lipid composition through this analysis –, this may affect the analysis of the L-CM cluster when having HCT-116 as a model. Thus, it will be important to confirm any findings with other cell lines that may better reflect the features of the CCLE clusters.

As cholesterol and, more specifically, CEs are stored in LDs⁸³, staining of these organelles can provide an idea of the storage capacity of cell lines. However, this approach cannot fully validate nor discard the *in silico* analysis as it is an indirect measure of cholesterol. As such, the observed results require confirmation, for instance, by quantifying esterified cholesterol. However, analyzing both the morphology and quantity of LDs may still provide useful information, particularly regarding the possible capacity of CRC cell lines to prevent lipotoxicity, and their ability to sustain nutritional requirements¹⁷⁸.

Regarding the evaluation of cholesterol uptake, it should be noted that the measurement of BODIPY fluorescence intensity was accomplished by image scanning without a defined threshold, which may have led to the incorporation of unspecific signals. Although we tried to minimize this by normalizing the MFI of test samples to the signal of the negative control, measurement of fluorescence after image deconvolution, which would restrict the analysis to BODIPY's fluorescence spectrum, could be more specific¹⁷⁹, hence yielding values closer to the reality. This may, in part, explain the deviations from the *in silico* results that was encountered during the BODIPY-cholesterol uptake assay. Moreover, only two independent experiments were conducted as part of this assay. More

replicates may thus be necessary to confirm the obtained results and obtain statistically significant differences.

Caco-2 uptake capacity was expected to be the highest. However, this cell line had the lowest BODIPY MFI values throughout the whole assay. This may stem from the fact that this cell line had an upregulation of all the other cholesterol pathways, except for catabolism. Due to the high capacity for biosynthesis and storage, the likely high levels of cholesterol within the cell may be downregulating cholesterol uptake¹⁸⁰. Treatment of this cell line with cholesterol-lowering drugs prior to the introduction of BODIPY-cholesterol, may be a way to determine if the other pathways are refraining the uptake capacity. Quantifying intracellular cholesterol will also provide important evidence for understanding these results. Another hypothesis, which requires the future assessment of efflux capacity to be confirmed, is the possibility that a high rate of cholesterol efflux counteracts uptake, thus not allowing BODIPY-cholesterol to remain in the cell long enough to be detected. It could also be important to extend the assay to determine if these cells, in fact, have a high capacity for uptake, but at a slower rate.

Results for the SW1463 cell line were also contradictory. While this cell was expected to exhibit a low BODIPY-cholesterol uptake, it presented the highest BODIPY fluorescence, with a seemingly high cholesterol uptake. This may result from the metabolic reprogramming of the cell line under the conditions of the assay¹⁵⁸, as cells were cultured in FBS-depleted DMEM, thereby lowering cholesterol content in the media. Considering that the SW1463 cell line likely does not have high biosynthesis, the higher uptake could derive from an adaptation to the cholesterol deficit. A similar mechanism may be associated with the results observed in the HCT-116.

The SW620 cell line presents a peak in the uptake at the 2-hour timepoint. However, the discrepancy in uptake observed between the two biological replicates does not allow us to make conclusions. More experiments are needed to better ascertain uptake in this cell line.

The SW837 cell line's uptake capacity, as determined by the BODIPY-cholesterol uptake assay, was the most consistent with the gene expression results. The amount of cholesterol inside the cell across the timepoints is likely a result of the combination of its high uptake and low catabolism and efflux. Combined with the findings on neutral lipid storage, this suggests that the SW837 cell line may be a good model for studying the BU cluster.

Our current findings suggest that the *in silico* results may partially be reproduced *in vitro*. However, more accurate methods need to be considered for validating both cholesterol uptake and storage. Furthermore, the validation of the remaining pathways could likely explain any contradictory data encountered so far, as cholesterol metabolism

is highly complex. Incorporating additional cell lines in future experiments will be essential for effectively validating the CCLE clusters.

Characterization of the colorectal cancer cell line clusters: interplay in the TME and therapy resistance

5.1. Materials and Methods

5.1.1. Cell lines and culture conditions

CRC cell lines were maintained as described in section 4.1. CCD18co normal colon fibroblast cells were also available at the i3s Cell Lines Bank. Fibroblasts were cultured in DMEM medium (Gibco, Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS and 1% Pen/Strep, and routinely maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂.

5.1.2. Primary human monocyte isolation and macrophage differentiation

Primary monocytes were isolated from buffy coats of healthy blood donors, provided by Unidade Local de Saúde de São João, E.P.E., under a collaboration protocol established by Doctor Maria José Oliveira. All steps in the isolation protocol were carried out at RT. Peripheral blood mononuclear cells (PBMCs) were first isolated by centrifuging the blood at 1200×g and collecting the resulting intermediate white layer. PBMCs were incubated with RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies, Vancouver, Canada) for 25 min, under rotation, and then diluted (1:1) in PBS with 2% FBS. The diluted cells were carefully added over Ficoll-Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 1200×g (brake off) for 20 min. The intermediate layer was once again collected and washed three times by adding PBS and centrifuging at 700 rpm for 17 min. Cells were then resuspended in complete RPMI-1640 medium containing 50 ng/mL of macrophage colony-stimulating factor (M-CSF; MedChemExpress, NJ, USA) and plated over glass coverslips in 6-well plates (1.2×10^6 monocytes per well). Monocytes were differentiated into macrophages for seven days, and at the seven-day mark, the medium was exchanged with complete RPMI-1640 without M-CSF. Cells were used for indirect co-culture 24 hours later.

5.1.3. Indirect co-culture

CCD18co fibroblast cell line and primary culture macrophages were treated with CRC cell lines' conditioned media to assess the capacity of the latter to modulate the TME.

5.1.3.1. Conditioned media production

CRC cell lines were seeded in 6-well plates using their appropriate complete culture medium, accounting for a density of 1.2×10^6 cells per well 24 hours after plating (6.9×10^5 cells for Caco-2 and SW837, 4.2×10^5 cells for HCT-116, 5.6×10^5 cells for SW620, and 8.6×10^5 cells for SW1463). After 24 hours, the complete culture medium was exchanged with Gibco's DMEM supplemented with 1% Pen/Strep. Cells were maintained in culture for the following 48 hours. Their conditioned media was then collected, centrifuged at 1200 rpm for 5 min at RT, and filtered through a 0.22 μ m filter to remove cell debris. Conditioned media were stored at -20°C until use for the indirect co-culture assays.

5.1.3.2. Co-culture with fibroblasts

The capacity of CRC cell lines to activate fibroblasts was assessed by quantifying α -SMA through western blot after treatment of CCD18co cells with CRC cell lines' conditioned media. Immunocytochemistry staining of α -SMA was also performed to assess the morphology of α -SMA stress fibers after the co-culture.

For western blot analysis, CCD18co cells were plated in 6-well plates at a density of 1.0×10^5 cells per well and grown in complete culture medium until 90-95% confluency. When cells reached confluency, the complete culture medium was exchanged with DMEM supplemented with 1% Pen/Strep, and cells were incubated ON. The cells were then washed with PBS, and conditioned media was added to the wells. Cells incubated with DMEM supplemented with 1% Pen/Strep or DMEM supplemented with 1% Pen/Strep and 10 ng/mL of recombinant human (rh)TGF- β 1 (ImmunoTools, Friesoythe, Germany) were used as negative and positive activation controls, respectively. Fibroblasts were kept in culture for four days.

For microscopy, cells were seeded in 12-well plates with glass coverslips at a density of 3.6×10^4 cells per well. After reaching 90-95% confluency, cells were treated as described for western blot analysis.

5.1.3.3. Co-culture with macrophages

Macrophages were treated with conditioned media from CRC cell lines eight days after plating. After washing the cells with PBS, the complete RPMI-1640 medium was replaced with the conditioned media. To obtain M1 and M2 polarization controls, cells were treated

with FBS-depleted DMEM containing either 10 ng/mL of LPS (MedChemExpress) or 10 ng/mL of IL-10 (MedChemExpress) to obtain M1 and M2 polarization controls, respectively. For an M0 macrophage (resting-phase) control, FBS-depleted DMEM was used. Macrophages were then maintained in culture for three days.

5.1.4. Western blot analysis of fibroblast activation

5.1.4.1. Protein extraction and sample preparation

CCD18co fibroblasts were lysed by adding cold RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 1% (v/v) IGEPAL CA-630, 150 mM NaCl, and 2 mM EDTA) supplemented with 1:7 protease inhibitor cocktail (Roche Diagnostics GmbH, Rotkreuz, Switzerland) and 1:100 phosphatase inhibitor cocktail (Sigma-Aldrich) to the wells and scrapping the cells. The lysates were then cleared by centrifugation at 14 000×g for 20 min at 4°C. Supernatants were collected, and protein was quantified with the Bradford assay (Bio-Rad Protein Assay kit, Bio-Rad). A total of 20 µg of protein was combined with 9 µL of Laemmli sample buffer (0.5 M Tris-HCl pH 6.8, 9.2 g sodium dodecyl sulfate (SDS), 40 mL Glycerol, 5% 2-mercaptoethanol, and 5% bromophenol blue) and the samples were boiled at 95°C for 5 min to denature proteins.

5.1.4.2. Western blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate the samples by loading them onto a 10% SDS-polyacrylamide gel along with a molecular weight ladder (Precision Plus protein standard, Bio-Rad) and running at 100 V. Proteins were then transferred to Amersham Protran Premium 0.45 µm nitrocellulose blotting membranes (Cytiva) using a wet transfer system at 400 A for 90 min. To confirm successful protein transfer, the membranes were incubated with Ponceau solution (Sigma-Aldrich) until bands appeared. Membranes were blocked for 1 hour at RT using 5% non-fat milk in 0.5% PBS-T – PBS containing 0.5% Tween20 (Sigma-Aldrich) –, then incubated with primary antibodies (Table 8) ON at 4°C with agitation. After five washes with PBS-T under agitation (5 min per wash), membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 8) for 1 hour at RT. Following five washes with PBS-T under agitation, Bands were developed using Clarity Western ECL substract (Bio-Rad), and chemiluminescence was detected using the ChemiDoc imaging system (Bio-Rad). Image Lab software (v6.0, Bio-Rad) was used to quantify band intensities, and α-SMA expression levels were normalized to the loading control (Hsc70).

Table 8. List of antibodies used for western blotting.

Type	Antibody	MW	Species	Blocking	Dilution	Company	Catalog no.
Primary	Anti- α -SMA	42 kDa	Mouse	5% milk in 0.5% PBS-T	1:250	Abcam	Ab7817
	Anti-Hsc70	70 kDa	Mouse	5% milk in 0.5% PBS-T	1:5000	Santa Cruz	sc-7298
Secondary	Anti-mouse IgG	—	Sheep	—	1:2500	Cytiva	NA931V

Abbreviations: MW, molecular weight

5.1.5. Immunocytochemistry for α -SMA

Four days after initiating the co-culture, adherent fibroblasts on coverslips were washed with cold PBS and fixed with methanol for 20 min at -20°C . After removing the methanol, the coverslips were left to dry for 10 min. Cells were washed twice with PBS (1 min per wash), and non-specific binding was blocked by incubating the samples with PBS containing 3% BSA for 30 min. Immediately after, cells were incubated with the anti- α -SMA primary antibody (Table 9) for 90 min at RT. Following three washes with PBS (10 min per wash), cells were incubated with an Alexa Fluor 594-conjugated secondary antibody (Table 9) for 1 hour, at RT. After two washes with PBS (1 min per wash), nuclei were stained with 0.1 $\mu\text{g/mL}$ of PhenoVue Hoechst 33342 in PBS. Coverslips were washed three times with PBS (10 min per wash) and mounted on glass slides using VECTASHIELD Antifade Mounting Medium. Cells were imaged using the Operetta CLS analysis system and Harmony imaging and analysis software.

Table 9. List of antibodies used for immunocytochemistry.

Type	Antibody	Species	Diluent	Dilution	Company	Catalog no.
Primary	Anti- α -SMA	Mouse	PBS with 3% BSA	1:250	Abcam	Ab7817
Secondary	Anti-mouse IgG	Goat	PBS with 3% BSA	1:500	Invitrogen	A11032

Abbreviations: α -SMA, alpha-smooth muscle actin; BSA, bovine serum albumin; PBS, phosphate-buffered saline

5.1.6. Morphological analysis of macrophages

Analysis of macrophage differentiation into M1 or M2 phenotypes was accomplished through an image-based machine-learning protocol adapted from Rostam, H.M. et al (2017)¹⁸¹.

5.1.6.1. Staining for f-actin

On day 3 post-treatment of macrophages with conditioned media, cells were washed twice with PBS and fixed with 4% formaldehyde for 10 min. Macrophages were then permeabilized with 0.2% Triton X-100 in PBS, as previously described. After permeabilization, cells were washed twice with PBS and incubated with 5% BSA in PBS for 30 min to block non-specific binding. After two washes with PBS, macrophages were stained for f-actin with 1 µg/mL of 488 Phalloidin (Invitrogen, Thermo Fisher Scientific) in PBS with 1% BSA and 0.1% sodium azide. Following three washing steps, cells were stained with 0.1 µg/mL of PhenoVue Hoechst 33342 in PBS for 30 min. Cells were washed three times with PBS, and coverslips were mounted on glass slides using VECTASHIELD Antifade Mounting Medium. All steps were carried out at RT. Imaging was performed using the Operetta CLS system and Harmony software.

5.1.6.2. Image-based classification of macrophages

Measurements of macrophage morphology descriptors were obtained with the Harmony high-content imaging and analysis software, using composite images of Hoechst and f-actin. Nuclei were first identified through the Hoechst channel and filtered by size. The f-actin channel was then used to detect the cytoplasm around the selected nuclei, and cells were filtered by cytoplasm intensity. Border objects were filtered out to obtain the final population (see Supplementary Figure S3). Several morphology descriptors related to size, shape, position, granularity, and fluorescence intensity were selected for the subsequent classification of macrophages. Images from each experimental dataset were then analyzed using the defined pipeline, resulting in 35 measurements for each cell. Two datasets were then created for each experiment: one with polarization controls and another with test samples.

A machine learning pipeline was created using the Orange Data Mining Toolbox (v.3.37.0)¹⁸², an open-source software for machine learning and data analysis. Within this pipeline, a dataset composed of measurements of control samples was loaded, and a data sampler widget was used to create a training dataset from the control samples. The provided data was then used to train a Random Forest model, categorizing the cells into three phenotypes: M0, M1, and M2 macrophages. A separate dataset containing the test

samples was then loaded, and the pre-trained model was used to predict the phenotypes. Results were stored as data tables and visualized through box plots. The pipeline is presented in Supplementary Figure S4. Samples from different donors were analyzed independently, using separate training sets comprised of their respective control samples.

5.1.7. Colorectal cancer cell line treatment

To assess the potential of cholesterol metabolism modulators as adjuvant therapy in treating CRC tumors with different metabolic profiles, the selected CRC cell lines were treated with oxaliplatin (L-OHP; Sigma-Aldrich), simvastatin (Sigma-Aldrich), and avasimibe (MedChemExpress) as monotherapies and combined treatments for 48 hours. Apoptosis was then detected by staining cells with Annexin V (Immunotools), and cell proliferation was evaluated with the Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay (Thermo Fisher Scientific).

CRC cell lines were plated using their respective complete culture medium in a number that allowed a final density of approximately 1.2×10^6 at 18 hours post-plating (8.3×10^5 cells for Caco-2 and SW837, 6.5×10^5 cells for HCT-116, 5.6×10^5 cells for SW620, and 9.6×10^5 cells for SW1463). Cells were then treated with L-OHP (20 μ M for Caco-2 and SW837, 4.5 μ M for HCT-116, 10.5 μ M for SW620, and 7.6 μ M for SW1463), simvastatin (2.5 μ M for all cell lines), and avasimibe (10 μ M for all cell lines). The L-OHP concentration for the Caco-2 cell line was adapted according to previously reported half maximal inhibitory concentration (IC₅₀) values^{183,184}. For the remaining cell lines, the L-OHP concentrations were defined based on the IC₅₀ established by The Genomics of Drug Sensitivity in Cancer database¹⁸⁵ (www.cancerRxgene.org; v8.5, consulted on Jun 30, 2024). All drug dilutions were prepared in Corning's DMEM supplemented with 5% heat-inactivated FBS and 1% Pen/Strep. Culture medium with 0.1% dimethyl sulfoxide (DMSO) was used as a control. For the proliferation assay, a control with only DMEM was also included.

5.1.7.1. Apoptosis detection

To detect apoptosis, cells in suspension and adherent cells (harvested with 0.05% trypsin-EDTA) were collected and centrifuged at 2000 rpm for 10 min at RT. After removing the supernatant, cells were resuspended in 1 mL of PBS and counted. 2.0×10^5 cells of each condition were washed with PBS and centrifuged at 300×g for 10 min at RT. Cells were then resuspended in a 1:200 dilution of FITC-conjugated Annexin V (Immunotools) in 1× annexin-binding buffer (prepared by diluting a 10x stock solution of Hepes pH 7.4, 1.4M NaCl, and 25 mM CaCl₂ in deionized water), and incubated for 15 min, protected from light, at RT. After the incubation period, 100 μ L of 1x annexin-binding buffer were added

to each sample. A sample with 50% saponin (Invitrogen, Thermo Fisher Scientific) was used as a positive control.

5.1.7.2. Cell cycle assay

To assess cell proliferation, cells were treated with 10 μ M EdU in Corning's DMEM with 5% FBS and 1% Pen/Strep for 1 hour at 37°C, excluding the negative control. Media was then removed from the wells, and cells were detached using trypsin-EDTA. 5.0×10^5 live cells were harvested for the staining protocol and washed twice by adding PBS, centrifuging at 300 $\times$ g for 5 min at RT, and removing the supernatant. Cells were fixed by adding 4% PFA and incubating for 15 min at RT. After performing a washing step with PBS containing 1% BSA, cells were permeabilized by incubation with 1 $\times$ saponin diluted in PBS. Following permeabilization, cells were incubated with Click-iT Plus detection cocktail for 30 min, at RT, protected from light. Two washing steps were performed afterward: the first with 1 $\times$ saponin and the second with PBS with 1% BSA. Cells were then resuspended in 10 μ M of RNase in PBS and incubated for 15 min at 37°C. Following the incubation period, 100 μ L of PBS with 1% BSA were added to each sample.

5.1.7.3. Flow cytometry analysis

Flow cytometry was used for apoptosis and cell cycle analyses. For both instances, prior to acquisition in the BD FACS Canto II flow cytometer, PE-conjugated propidium iodide (PI; Pharmingen, BD Biosciences) was added to the samples (1 μ L for apoptosis detection and 2 μ L for cell cycle analysis) to detect DNA content. Data was then analyzed using FlowJo software, following the gating strategies in Supplementary Figures S5 and S6. Results were expressed in percentages.

5.1.8. Statistical analysis

Statistical analysis was conducted using R studio. Student's t-test was used to compare the means between control and treatment groups. Comparison of means of multiple groups was performed by either one-way ANOVA or the Kruskal-Wallis non-parametric test, as previously described. The non-parametric Wilcoxon test was used to compare the means of treatment conditions with the respective negative control. The Chi-square test was employed to compare categorical variables in isolated experiments.

5.2. Results

5.2.1. Activation of fibroblasts treated with conditioned media from CRC cell lines

As cholesterol homeostasis is known to influence the interplay between CRC and the TME, we aimed to determine if CRC cell lines within distinct clusters had a different capacity for activating fibroblasts. Normal CCD18co colon fibroblasts were treated with CM from the five cell lines, and protein was extracted four days later. α -SMA expression was then quantified through western blot (Figure 18A).

Mean α -SMA expression levels of fibroblasts treated with CM and TGF- β – the positive activation control – were compared with the negative control (Figure 18B). Besides the expected differences between the negative control and fibroblasts treated with TGF- β (Wilcoxon test p-value = 0.0304), there was also a statistically significant difference between fibroblasts treated with the SW837 CM and the negative control (Wilcoxon test p-value = 0.0472). Mean expression levels were also compared among the fibroblasts treated with CM. Although no statistically significant differences were found (Kruskal-Wallis p-value = 0.0658), both the fibroblasts treated with Caco-2 CM and SW1463 CM had an increase in α -SMA expression levels, though not as evident as with the ones treated with SW837 CM.

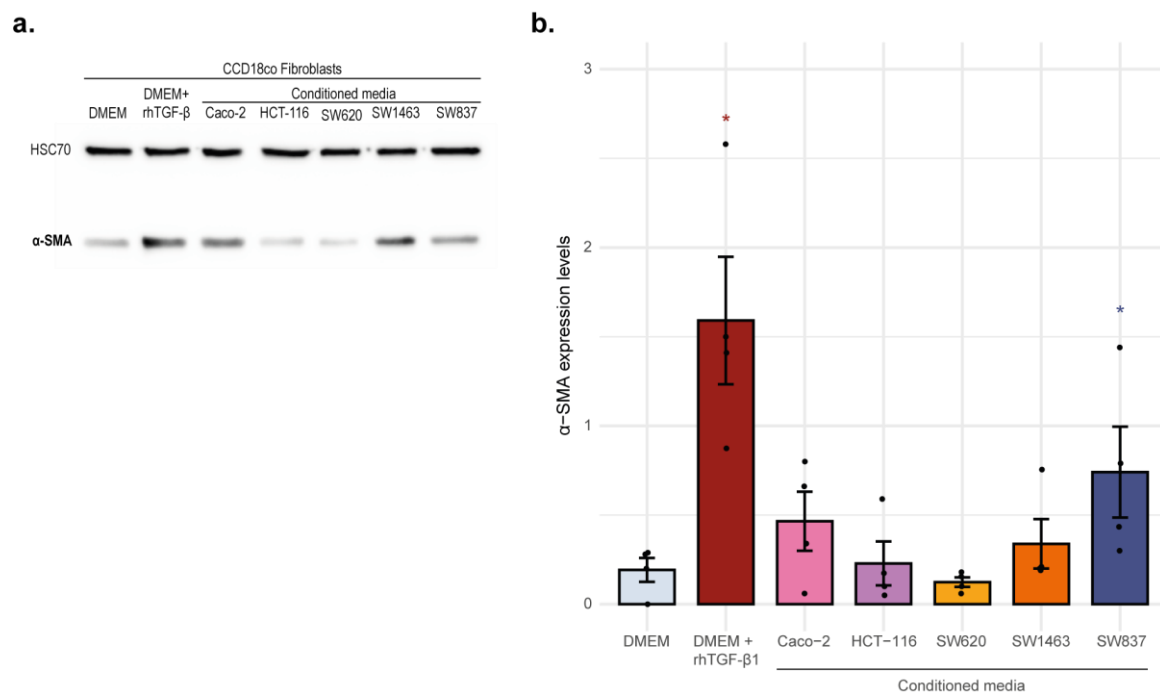


Figure 18. α -SMA expression in CCD18co normal colon fibroblasts treated with conditioned media from colorectal cancer (CRC) cell lines. a. Representative western blot of α -SMA expression levels across the different treatment conditions (n=4). b. α -SMA expression levels normalized to HSC70 expression, expressed as mean $\pm$ sd. The non-parametric Wilcoxon test was used to compare the mean expression level of each condition to the negative control (*p $\leq$ 0.05).

To understand if treatment of fibroblasts with CM could also change their morphology, α -SMA stress fibers were stained through immunocytochemistry (Figure 19). In accordance with the western blot results, the controls revealed distinct morphological features, with activated fibroblasts exhibiting more defined and elongated fibers. Furthermore, fibroblasts treated with SW837 CM were the most similar to the activated fibroblasts.

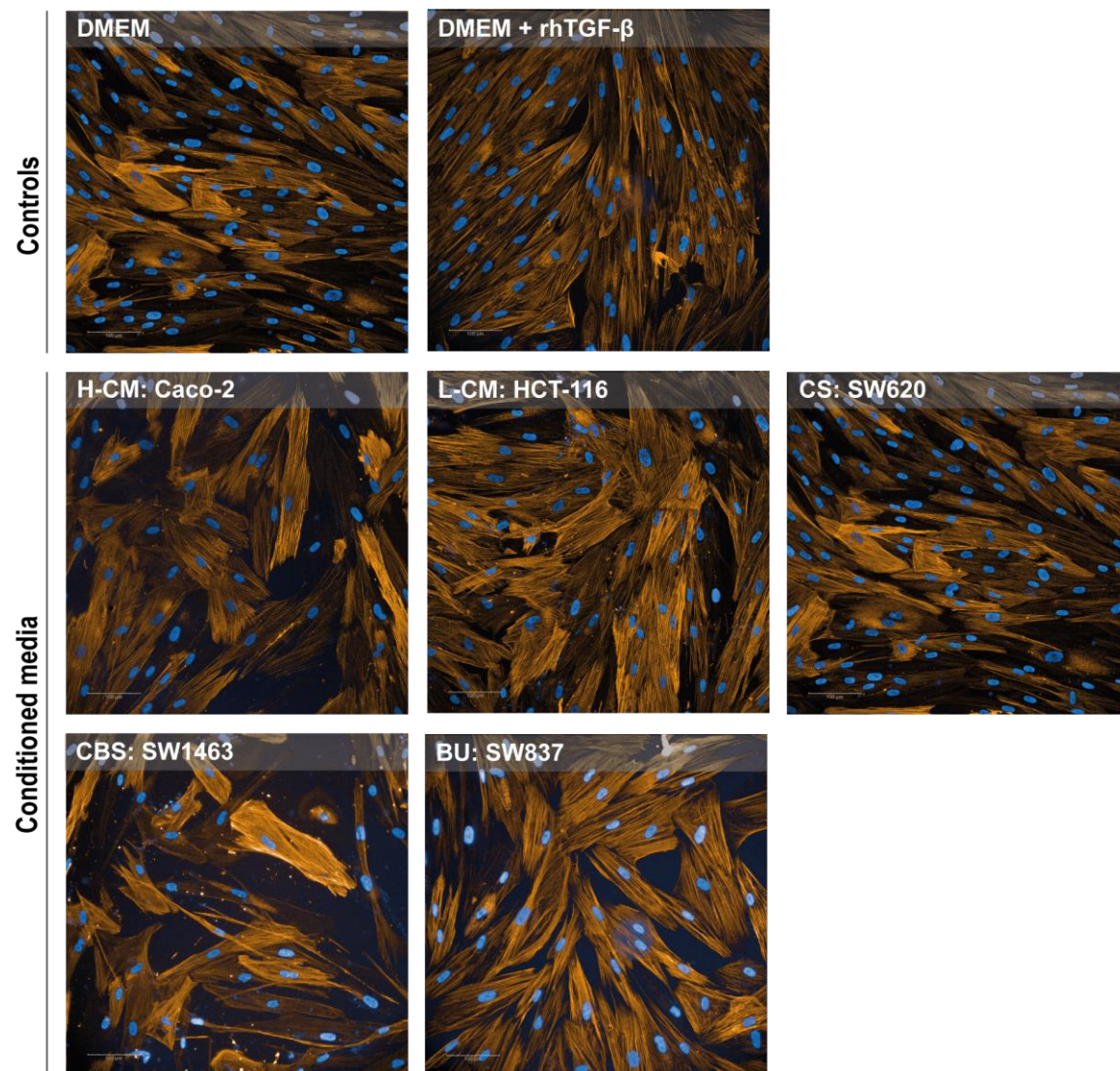


Figure 19. Immunocytochemistry staining of α -SMA of CCD18co fibroblasts treated with conditioned media from CRC cell lines. Composite images of α -SMA (Alexa Fluor 594) and nuclei staining (Hoechst) showing the morphology of actin stress fibers. Images taken with a 20 $\times$ objective. Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM, Low Cholesterol Metabolism.

5.2.2. Polarization of macrophages treated with conditioned media from CRC cell lines

To further look into the role of cholesterol metabolism in the modulation of the TME, primary macrophages were also treated with CM from CRC cell lines. To obtain polarization controls that served as the basis for this analysis, macrophages were treated under different control conditions. M1 and M2 polarization controls were obtained by treating previously differentiated macrophages with LPS or IL-10, respectively, while culture without any treatment was used for Naïve/M0 controls. After three days, macrophages were stained for f-actin filaments using phalloidin, with the aim of characterizing their phenotype through morphological analysis. Immunofluorescence images of f-actin staining are illustrated in Figure 20. Macrophages treated under control

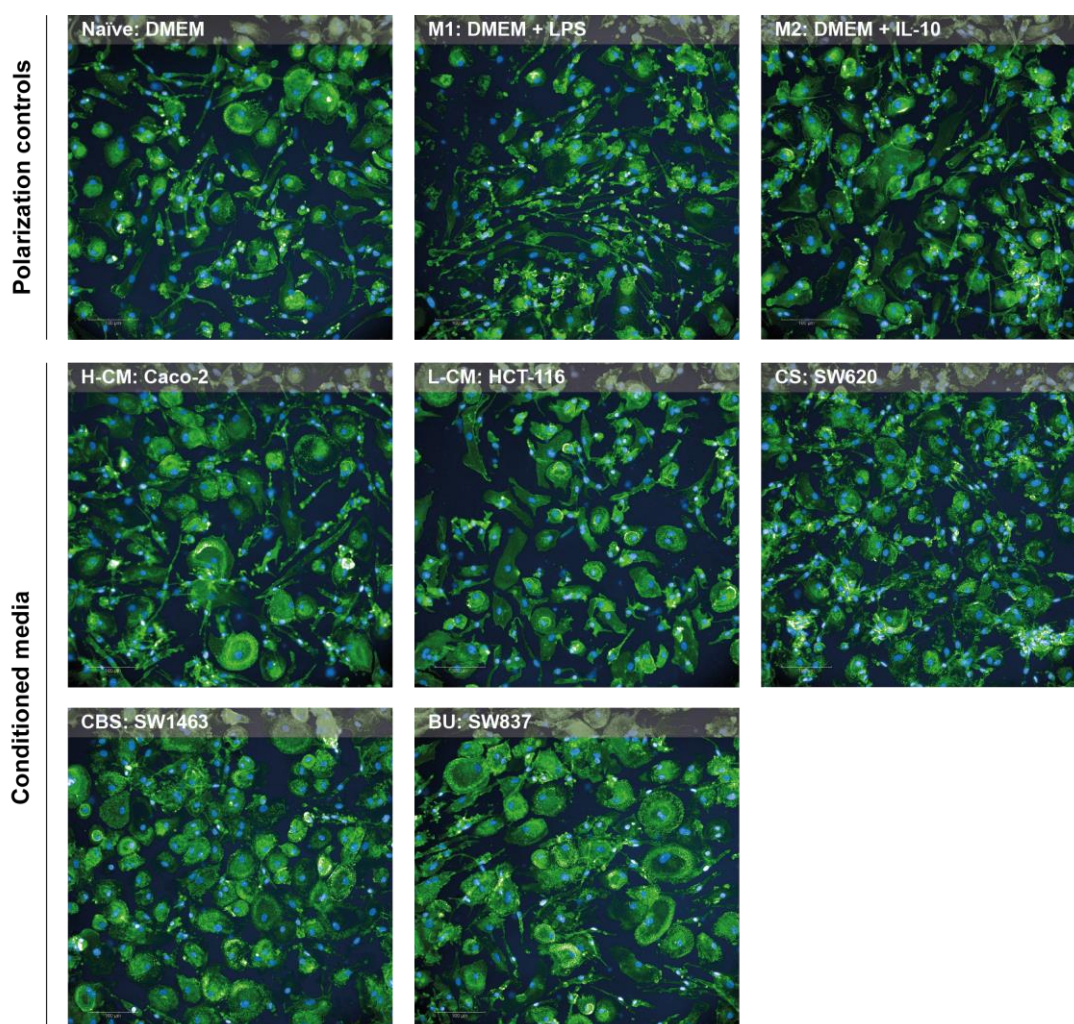


Figure 20. Immunofluorescent staining of macrophages treated with conditioned media (CM) from colorectal cancer (CRC) cell lines. Representative images of results of f-actin staining of macrophages treated with control media, and CM from CRC cell lines (n = 4). Composite images of phalloidin (f-actin stain) and Hoechst (nuclear stain) reveal macrophages with distinct morphologies across the treatment conditions (images taken with a 20× objective). Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

conditions revealed distinct phenotypes, particularly M1 controls, which presented with an irregular, elongated shape. M2 control macrophages, in contrast, were larger, and rounder in shape. Differences between naïve macrophages and M2 macrophages were more subtle, though naïve macrophages were generally smaller.

Classification of macrophages was then performed through image-based machine-learning analysis (Figure 21). Results were then compared by independent experiments (Figure 21A-D), to account for possible variability derived from the inherent characteristics of macrophages obtained from different donors, followed by a global comparison of the mean proportion of each phenotype across all treatment conditions (Figure 21E). All individual experiments revealed statistically significant differences between the treatment conditions (Chi-squared test p-values < 0.0001). In the global comparison, no statistically significant differences were found between macrophages treated with CM from the five CRC cell lines. However, macrophages treated with Caco-2, SW1463, and SW837 showed statistically significant differences when compared with the controls.

Macrophages treated with SW837 CM revealed the most consistent results across all donors, with a generally higher proportion of naïve macrophages ($73.69 \pm 10.73 \%$), followed by M2 macrophages in three (B, C, and D) of the four donors, and M1 with the lowest percentages. SW837 CM thus had a low capacity to polarize macrophages into M1 or M2, and when comparing the mean percentages of each macrophage population, no statistically significant differences were found between this condition and the naïve control. This was further confirmed by the statistically significant differences found between the LPS-treated macrophages and the ones treated with SW837 CM, specifically regarding the comparison of the naïve and M1 populations. When comparing this condition with the remaining macrophages treated with CRC cell lines' CM, it also had the lowest percentages of both M1 ($14.56 \pm 5.21\%$) and M2 ($15.67 \pm 6.45 \%$) macrophages (Figure 21E).

Caco-2 and SW1463 CM overall led to the highest mean percentages of M1 macrophages across the CM treatment conditions ($54.03 \pm 18.42\%$ and $47.79 \pm 14.47\%$, respectively), showing statistically significant differences when comparing this population with the DMEM control. However, SW1463 CM seemed to have a higher overall capacity for polarizing macrophages, as this conditioned polarized a higher percentage of macrophages into the M2 phenotype. The proportion of M2 macrophages in the SW1463-treated samples was also the highest out of all the CM ($33.64 \pm 13.66\%$).

Results for macrophages treated with CM from the HCT-116 and SW620 cell lines showed similar results, with only a slightly higher percentage of M1 and M2 macrophages in the SW620 CM-treated macrophages. However, neither of these conditions differed significantly from the controls.

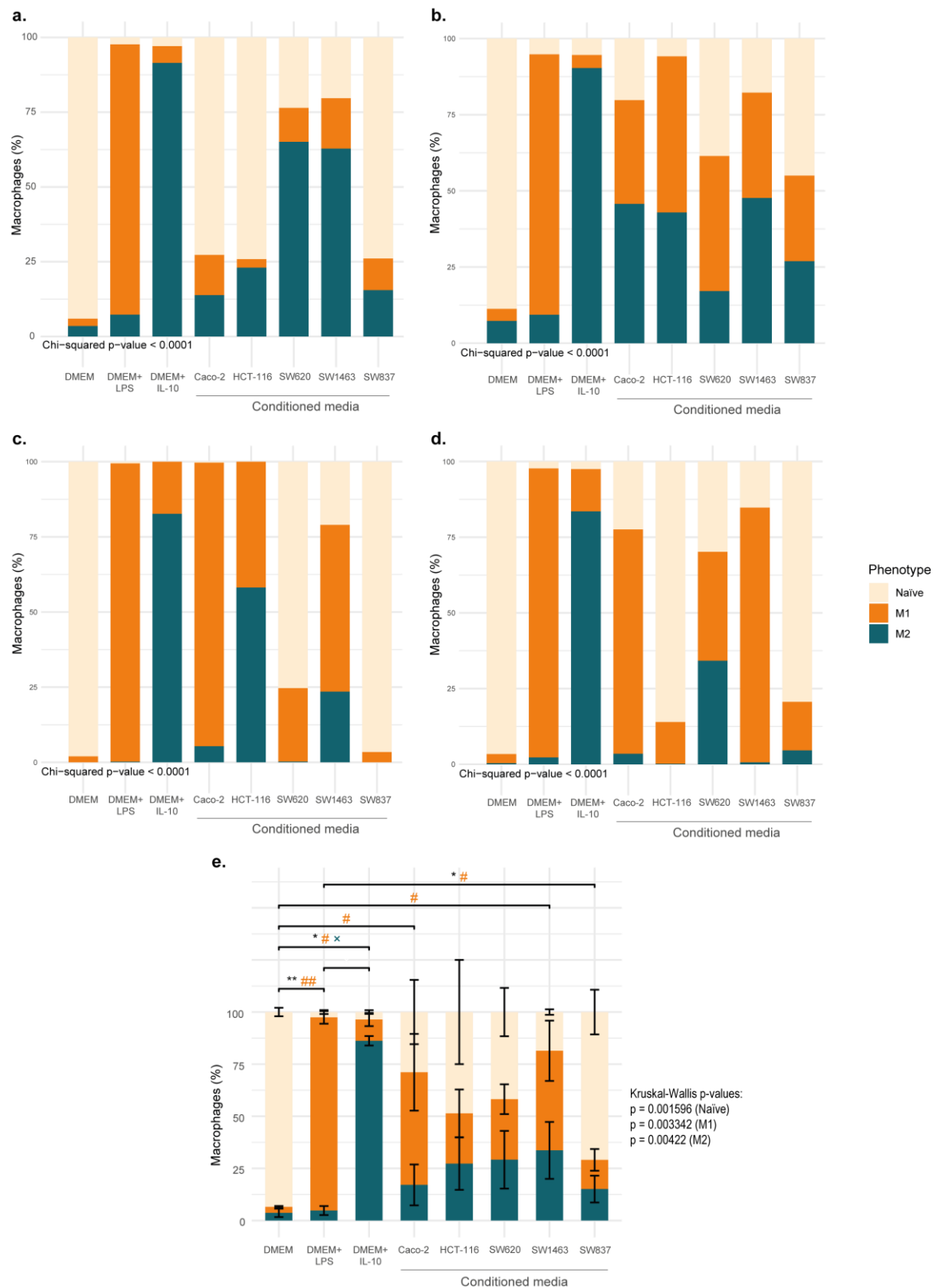


Figure 21. Machine learning-based classification of macrophages treated with conditioned media (CM) from colorectal cancer (CRC) cell lines. Figures a – d show the proportions of macrophages from each phenotype (naïve, M1, and M2) across different donors (n = 4), expressed in percentages. The chi-square test was used to compare the phenotype distributions across the treatment conditions. Figure e shows the results of all four donors expressed as mean $\pm$ sd. The non-parametric Kruskal-Wallis test was applied to compare the mean percentage of the treatment conditions across each phenotype. Results were considered statistically significant when $p \leq 0.05$ (* $p \leq 0.05$, ** $p \leq 0.01$ for the naïve phenotype; # $p \leq 0.05$ for the M1 phenotype; $\times p \leq 0.05$ for the M2 phenotype).

5.2.3. Cholesterol metabolism modulators

As cholesterol-lowering drugs have been shown to improve CRC treatment, we sought to determine if the response to chemotherapy could be enhanced by modulating cholesterol levels within the cells and, more specifically, if the effects differed in cell lines with distinct cholesterol metabolism profiles. To accomplish this, we treated CRC cell lines with L-OHP alone and in combination with two cholesterol-lowering drugs: 1) simvastatin, which inhibits HMGCR and, consequently, cholesterol biosynthesis, and 2) avasimibe, an ACAT inhibitor that can hamper cholesterol storage. Cells were treated for 48 hours, after which we compared how different drug combinations (Table 10) affected cell death and cell proliferation.

Table 10. Drug combinations used in the treatment of colorectal cancer cell lines to assess the effects of cholesterol-lowering drugs on the effects of oxaliplatin.

Treatment	1	2	3	4	5	6	7
L-OHP	-	+	-	-	+	+	+
Simvastatin	-	-	+	-	+	-	+
Avasimibe	-	-	-	+	-	+	+

Abbreviations and symbols: L-OHP, oxaliplatin; (-) Drug included in the treatment; (+) Drug not included in the treatment.

Examining the cell aspect after treatment (Figure S23. **Colorectal cancer (CRC) cell lines aspect after treatment with oxaliplatin (L-OHP), simvastatin (sim), and avasimibe (ava).** CRC cell lines were treated with specific concentrations of L-OHP (20 μ M for Caco-2 and SW837, 4.5 μ M for HCT-116, 10.5 μ M for SW620, and 7.6 μ M for SW1463) according to their reported IC-50 values. The concentrations of simvastatin and avasimibe were the same across all cell lines (2.5 μ M and 10 μ M, respectively). Images were taken with a 5 $\times$ objective. Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.) provided an overall view of their possible effects. The most notable differences were seen in the HCT-116 cell line that, when treated with simvastatin and avasimibe, appeared detached from the plate, suggesting a high rate of cell death. This was observed for the conditions where simvastatin and avasimibe were used as monotherapy, as well as in combination with L-OHP. For the remaining cell lines, differences were not as evident regarding this feature. However, differences can be noted in terms of cell density, which may hint at possible effects on cell proliferation. This aspect was most noticeable for the Caco-2 cell, where all

conditions appeared to have a lower cell density, except for the cells treated with simvastatin alone. These effects were further confirmed by Annexin/PI (cell death) and EdU (analysis) analysis results.

To briefly contextualize the following results, cells were divided into two groups during the Annexin V/PI analysis: 1) live cells, when negative for Annexin V and PI staining, and 2) apoptotic/necrotic cells, when positive for at least one of the markers, to account for total cell death. For the cell cycle analysis, three phases were considered: 1) G1-G0, when EdU staining was negative, and PI staining was low; 2) S, when EdU staining was positive; and 3) G2-M, when EdU staining was negative, and PI staining was high.

Treatment of Caco-2 under the several defined conditions revealed statistically significant differences in cell death and some tendencies regarding cell proliferation. Representative images of the flow cytometry results are presented in Figure 22A (Annexin V/PI) and Figure 22B (EdU). Regarding the Annexin V/PI results, all conditions except for the cells treated with simvastatin showed slight but statistically significant differences when compared with the negative control (Figure 22C). While the control cells presented a mean percentage of $10.92 \pm 2.07\%$ cell death, this value exhibited only a slight increase for cells treated with simvastatin as a monotherapy, standing at $13.25 \pm 1.26\%$. In contrast, treatment with L-OHP alone and avasimibe alone led to a significant increase in cell death relative to the control. L-OHP treatment alone led to a percentage of $25.51 \pm 3.84\%$, and avasimibe alone increased the percentage of cell death to $24.06 \pm 2.64\%$. However, the combination of L-OHP with simvastatin and avasimibe does not seem to increase cell death in response to the chemotherapy drug alone. In terms of cell proliferation, despite the lack of statistically significant differences, flow cytometry results reveal a decrease in the percentage of cells in the S phase in all conditions that included L-OHP (Figure 22B). Descriptive analysis revealed that there was an accumulation of cells in G0-G1 ($70.00 \pm 15.12\%$), when compared with the negative control ($39.80 \pm 10.04\%$), suggesting an arrest of the cell cycle in this phase when treating cells with L-OHP. Although an increase in cells in G0-G1 was also observed for Caco-2 cells treated with simvastatin ($71.76 \pm 10.52\%$), there was a higher percentage of cells in the S phase ($16.25 \pm 7.70\%$), when compared with L-OHP-treated cells ($6.72 \pm 5.51\%$). Similar results were found for avasimibe-treated cells, with $63.30 \pm 3.70\%$ cells in G0-G1 and $8.80 \pm 3.97\%$ cells in the S phase. The combination of L-OHP with the other treatments, however, led to the highest decrease in the S phase, accompanied by an increase in both the G0-G1 and G2-M phases.

For the HCT-116 cell line, differences across the treatment conditions were visible both in terms of cell death (Figure 23A) and cell proliferation (Figure 23B). L-OHP alone did not increase cell death ($30.05 \pm 4.42\%$) compared to the negative control ($34.50 \pm 2.97\%$), while all other conditions significantly increased the percentage of cells in

apoptosis/necrosis. Both simvastatin and avasimibe led to an increase in cell death as single treatments ($70.05 \pm 1.28\%$ and $55.38 \pm 2.92\%$, respectively), and in combination with L-OHP. Conditions where simvastatin was introduced generally had a higher percentage of total cell death. The combination of L-OHP with both cholesterol-lowering drugs, however, led to the highest percentage of death cells ($80.12 \pm 1.72\%$) (Figure 23C). EdU analysis of the cell cycle also revealed statistically significant differences in the S phase. All treatment conditions induced a decrease of the S phase, when compared with the negative control ($9.97 \pm 2.49\%$), with values under 1% in cells treated with avasimibe, whether alone or in combination with the other drugs. The most notable effect, however, was seen in cells treated with L-OHP and avasimibe, where it was generally accompanied by an increase in cells in G2-M (over 68%) (Figure 23D).

Cell death detection in SW620 did not show any statistically significant differences, despite the different patterns observed in the flow cytometry analysis (Figure 24A). However, the three treatments combined led to a cell death percentage of $54.73 \pm 14.62\%$, while the negative control only had $12.57 \pm 3.17\%$ apoptotic/necrotic cells. The remaining conditions did not deviate greatly from the negative control (Figure 24B). Cell cycle analysis of the cell line showed statistically significant differences when comparing cells in the S phase. Although Dunn's post hoc test did not reveal statistically significant differences between specific conditions, some tendencies can be observed. Treatment of cells with L-OHP led to an 18.16% increase in G2-M compared to the DMSO, while only decreasing the percentage of cells in G0-G1. The combination of L-OHP and avasimibe, however, induced a decrease in the S phase by at least 20% and a consequent increase in cells in the G2-M phase (Figure 24D). This effect seems to be slightly increased by their combination with simvastatin, as noticeable in the flow cytometry results (Figure 24B).

Annexin V/PI flow cytometry analysis of SW1463 revealed a high percentage of cell death across all conditions, including the negative control ($70.94 \pm 7.75\%$). The aspect of the cell populations also did not reveal any tendencies (Figure 25A), and no statistically significant differences were observed (Figure 25C). Cell cycle analysis, however, revealed different patterns across the treatment conditions (Figure 25B), though not statistically significant. Treatment with L-OHP decreased the percentage of cells in cells in G0-G1, from $54.27 \pm 0.76\%$ to $33.27 \pm 15.03\%$, accompanied by an increase in cells in G2M. Similar tendencies were observed for the cells treated with simvastatin and avasimibe as monotherapies. Combination of L-OHP and avasimibe, however, seemed to decrease S phase. Overall, an accumulation of cells in G2-M is observed across all treatment conditions, with variations in both G0-G1 and S phases (Figure 25D).

In the case of SW837, no statistically significant differences were found regarding cell death, although a tendency for cells in apoptosis/necrosis to increase can be observed

in samples treated with avasimibe (Figure 26A). This effect is heightened in combination with L-OHP. While control samples registered $22.05 \pm 3.21\%$ death cells, this percentage increased to values over 30% in samples treated with L-OHP and avasimibe ($32.10 \pm 9.9\%$), with a slightly higher value when combined with simvastatin ($34.26 \pm 10.25\%$) (Figure 26C). Flow cytometry analysis of cell proliferation revealed different distributions of cells throughout the cell cycle among the treatment conditions (Figure 26B), with statistically significant differences in the G2 phase. Even though post-hoc analysis was not statistically significant, treatment of cells with L-OHP decreased the number of cells in S phase, while increasing the percentage of cells in G2-M, an effect that was enhanced in combination with avasimibe. Treatment of cells with L-OHP and simvastatin, while also leading to a decrease in S phase, increased the G0-G1 phase (Figure 26D). Results of this analysis are summarized in Table 11.

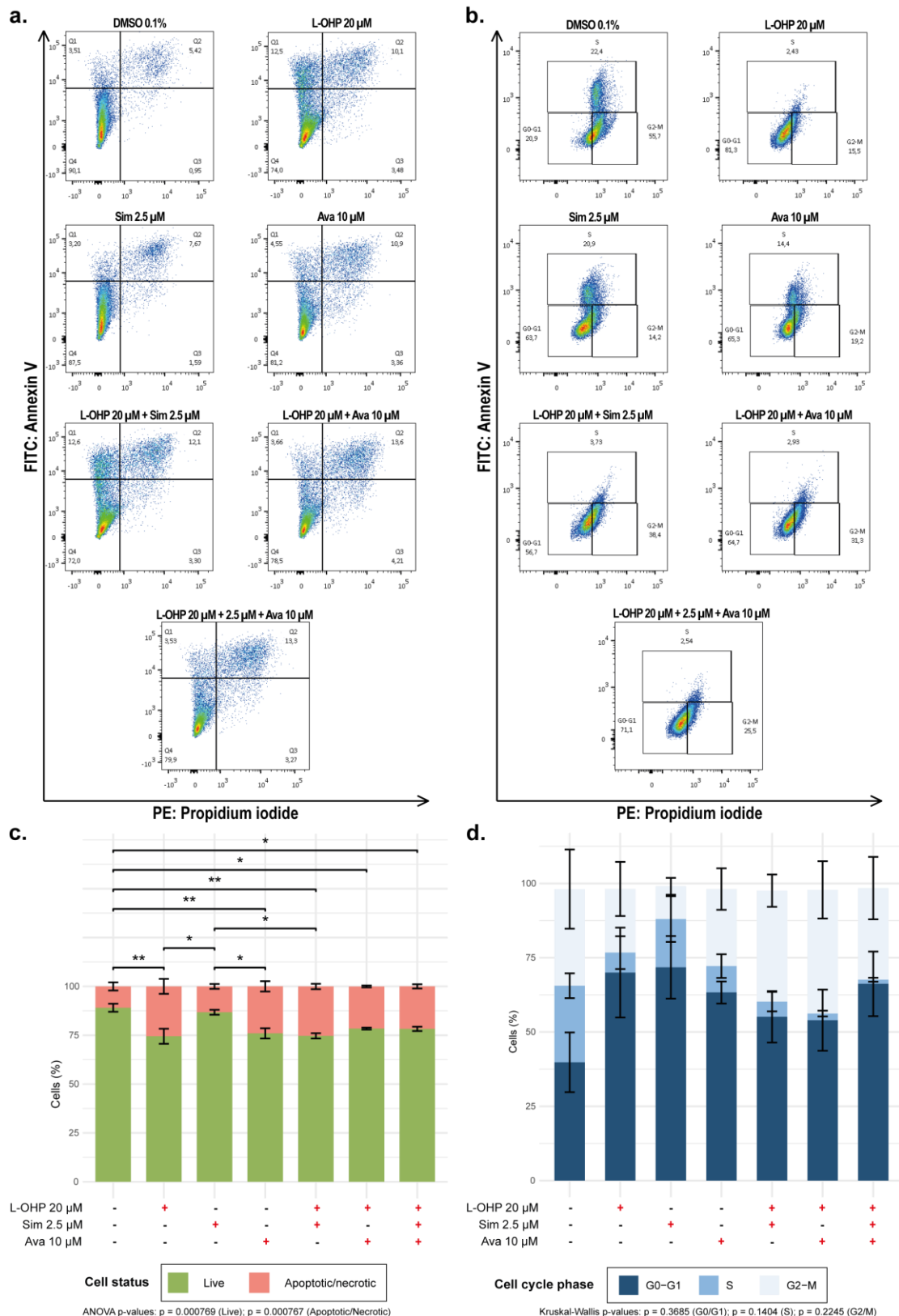


Figure 22. Apoptosis and cell cycle analysis of Caco-2 after treatment with oxaliplatin (L-OHP), simvastatin (sim), and avasimibe (ava). Representative flow cytometry pseudo-color plots of Annexin V/PI (a) and EdU/PI (b) staining of Caco-2 showing cell distribution patterns across the treatment conditions ($n = 3$). b. Distribution of cells by their status (live and apoptotic/necrotic). ANOVA was used to compare the means across all treatment conditions, followed by Tukey's test ($*p < 0.05$ and $**p < 0.001$). c. Distribution of cells throughout the cell cycle (G0-G1, S, and G2-M phases). Kruskal-Wallis was used to compare the means across all conditions, in each cell cycle phase. Results are expressed as mean percentages + sd.

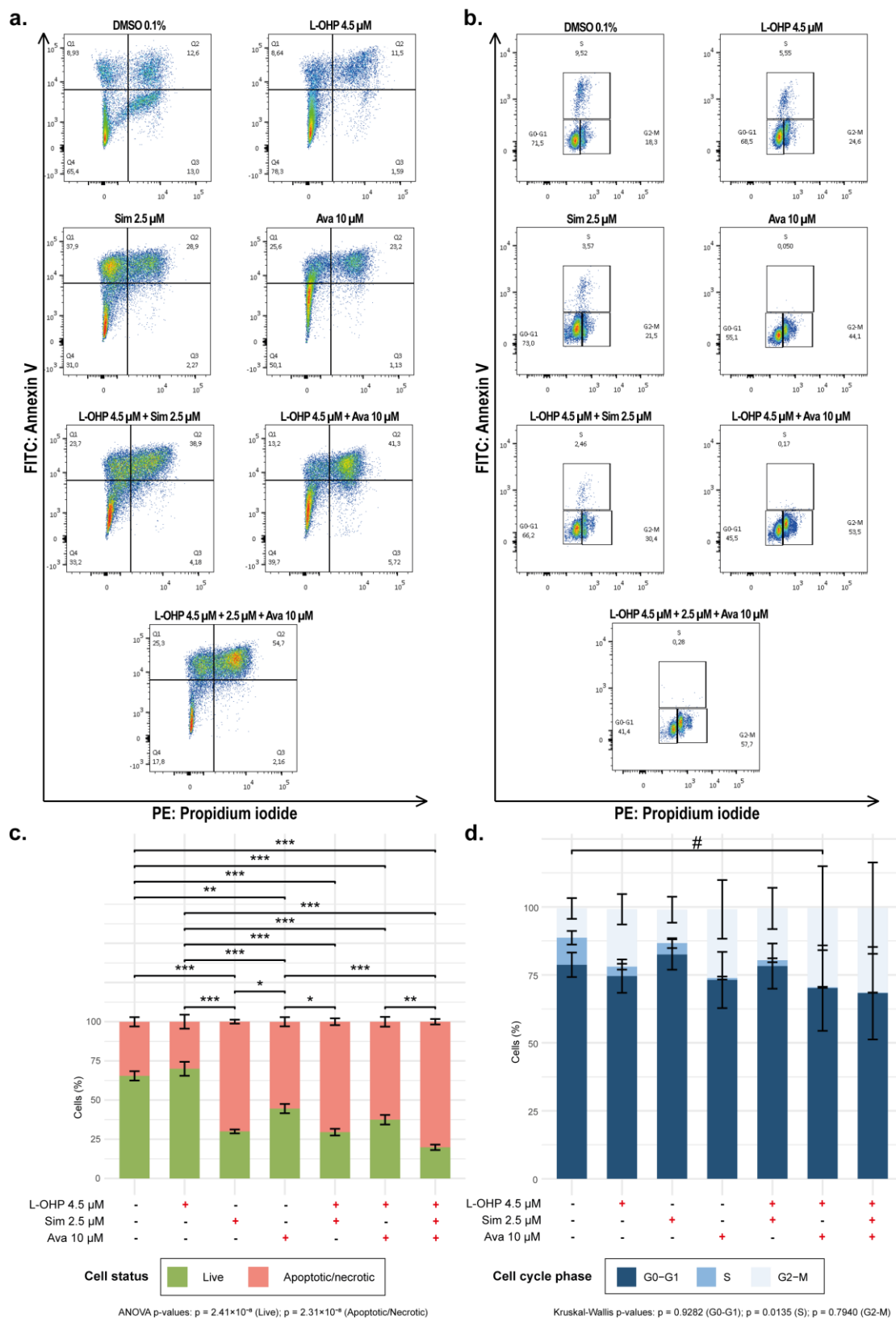
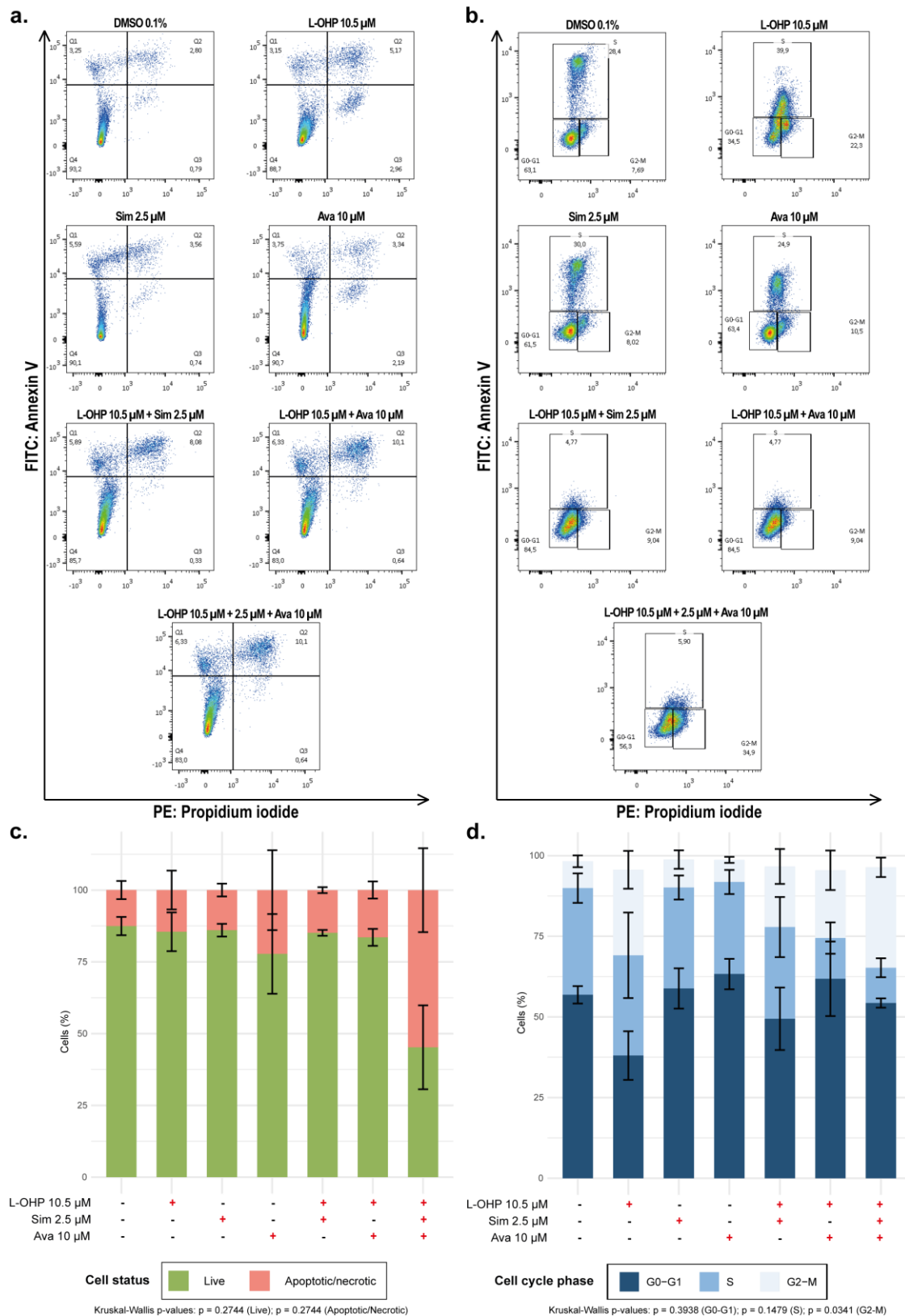


Figure 23. Apoptosis and cell cycle analysis of HCT-116 after treatment with oxaliplatin (L-OHP), simvastatin (sim), and avasimibe (ava). Representative flow cytometry pseudo-color plots of Annexin V/PI (a) and EdU/PI (b) staining of HCT-116 showing cell distribution patterns across the treatment conditions ($n = 3$). b. Distribution of cells by their status (live and apoptotic/necrotic). ANOVA was used to compare the means across all treatment conditions, followed by Tukey's test ($*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$). c. Distribution of cells throughout the cell cycle (G0-G1, S, and G2-M phases). Kruskal-Wallis was used to compare the means across all conditions in each cell cycle phase, followed by Dunn's test ($\#p \leq 0.05$ for the S phase). Results are expressed as mean percentages + sd.



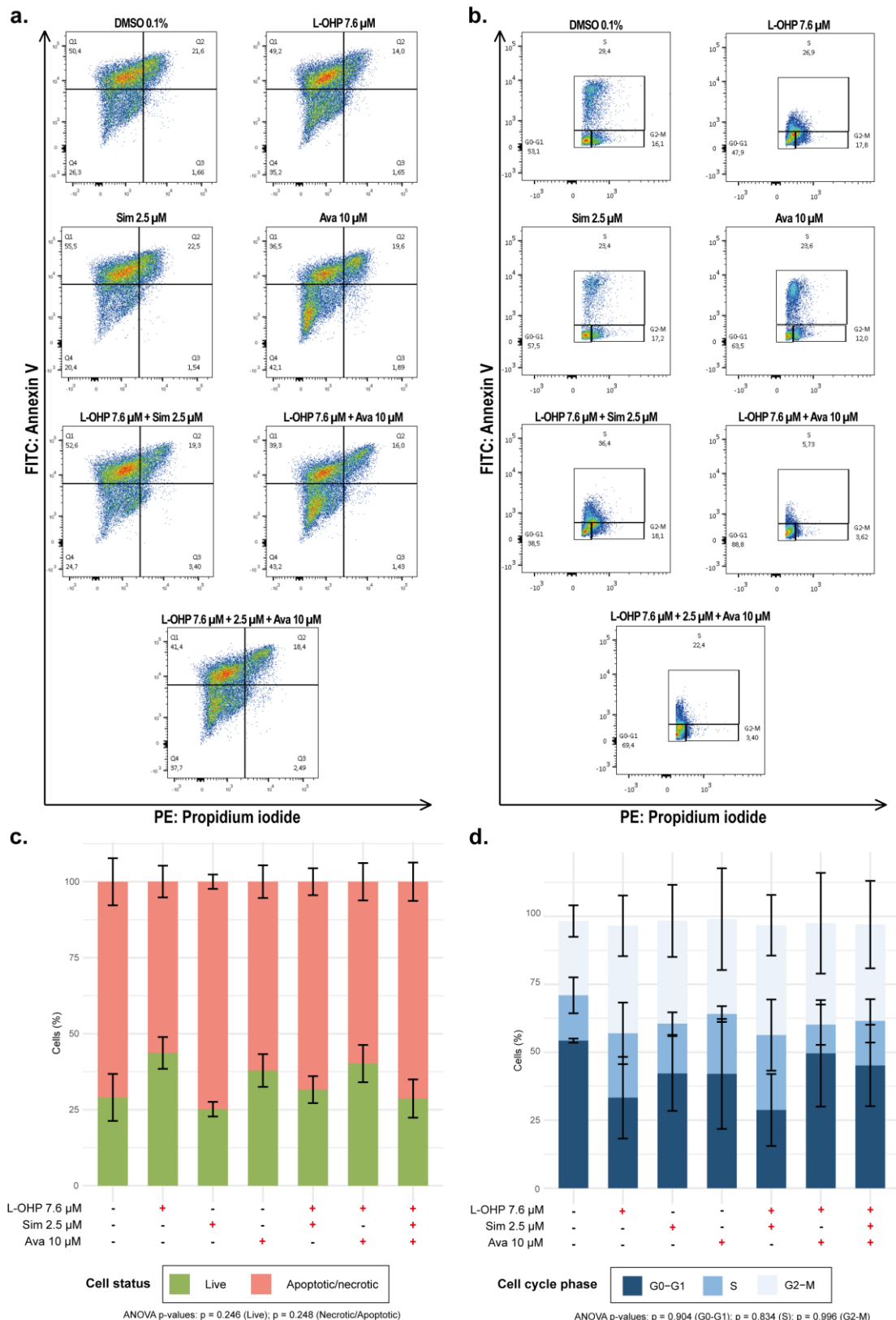


Figure 25. Apoptosis and cell cycle analysis of SW1463 after treatment with oxaliplatin (L-OHP), simvastatin (sim), and avasimibe (ava). Representative flow cytometry pseudo-color plots of Annexin V/PI (a) and EdU/PI (b) staining of SW1463 showing cell distribution patterns across the treatment conditions (n = 3). b. Distribution of cells by their status (live, and apoptotic/necrotic). c. Distribution of cells throughout the cell cycle (G0-G1, S, and G2-M phases). Results are expressed as mean percentages + sd. ANOVA was used to compare the means across all treatment conditions.

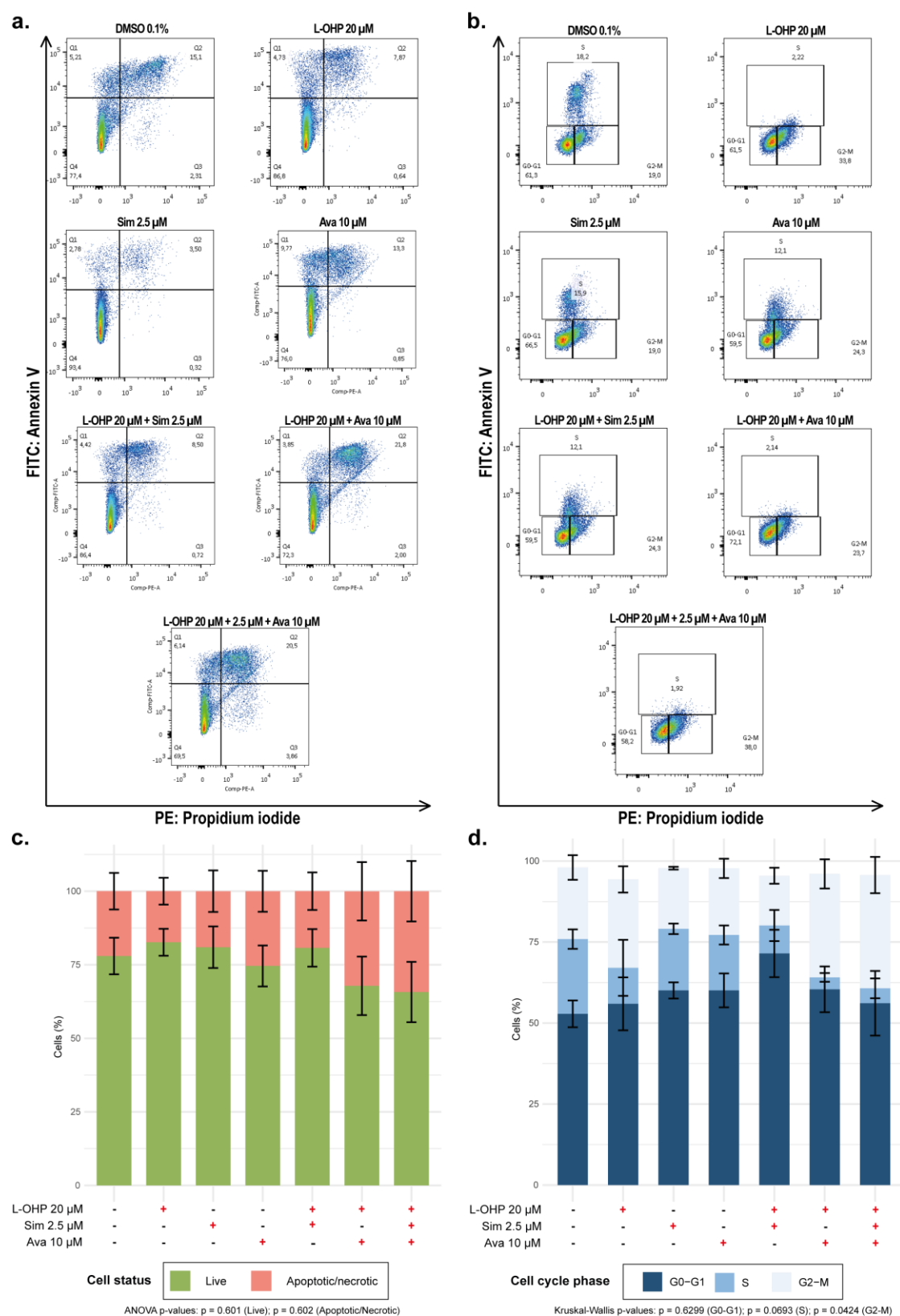


Figure 26. Apoptosis and cell cycle analysis of SW837 after treatment with oxaliplatin (L-OHP), simvastatin (sim), and avasimibe (ava). Representative flow cytometry pseudo-color plots of Annexin V/PI (a) and EdU/PI (b) staining of SW837 showing cell distribution patterns across the treatment conditions ($n = 3$). b. Distribution of cells by their status (live and apoptotic/necrotic). ANOVA was used to compare the means across all treatment conditions. c. Distribution of cells throughout the cell cycle (G0-G1, S, and G2-M phases). Kruskal-Wallis test was used to compare the means of each cell cycle phase across all conditions, followed by Dunn's test. Results are expressed as mean percentages + sd.

Table 11. Key findings in the cell death and cell cycle analysis of colorectal cancer (CRC) cell lines treated with oxaliplatin (L-OHP) and cholesterol-lowering drugs.

Cluster	BS	ST	Most relevant effects	
			Cell death	Cell cycle
Caco-2	H-CM		L-OHP and avasimibe treatment, as single and combined treatments, increase cell death at similar rates. Simvastatin, in contrast, does not increase cell death.	The combination of L-OHP with simvastatin and avasimibe leads to the highest decrease in cells in the S phase.
HCT-116	L-CM		Both the combination of simvastatin and avasimibe with L-OHP lead to an increase in cell death. The effects are higher in cells treated with simvastatin.	The combination of L-OHP with avasimibe leads to an arrest in the G2-M phase.
SW620	CS		The combination of L-OHP with both simvastatin and avasimibe leads to an increase in cell death.	L-OHP as a single treatment increases G2-M, while decreasing G0-G1. Combination with avasimibe seems to promote a decrease in the S phase.
SW1463	CBS		High baseline cell death, without any noticeable differences across the treatment conditions.	Possible G2-M cell cycle arrest in all treatment conditions.
SW837	BU		The combination of L-OHP with avasimibe slightly increases cell death, regardless of the presence of simvastatin.	The combination of L-OHP with simvastatin leads to an arrest in G0-G1. The combination of L-OHP with avasimibe promotes cell cycle arrest in the G2-M phase, including in the presence of simvastatin.

Abbreviations: BU, High Biosynthesis/Storage Cholesterol Metabolism; BS, biosynthesis; CBS, High Catabolism/Biosynthesis/Storage Cholesterol Metabolism; ST, storage; CS, High Catabolism/Storage Cholesterol Metabolism; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

5.3. Discussion

Disruption of cholesterol metabolism in cancer – and CRC in particular – has implications that go beyond simply promoting tumor growth by providing nutritional support to tumor cells. As was previously approached, it can, for instance, influence the creation of a pro-tumorigenic TME, thus affecting tumor progression¹¹³. As such, we aimed to understand how the CCLE clusters might impact the TME, potentially reinforcing the importance of cholesterol metabolism in the prognosis of CRC. Herein, our research examined two key TME components – macrophages and fibroblasts –, which are both known to be affected by alterations in cholesterol homeostasis¹⁸⁶, by performing indirect co-culture assays.

In our analysis, we assessed fibroblast activation following indirect co-culture with CRC cell lines by measuring the expression levels of α -SMA, one of the most widely used markers for identifying CAFs¹⁸⁷. As fibroblasts expressing α -SMA generally present a CAF-like phenotype, quantifying this marker in fibroblasts treated with CRC cell line CM can provide information on the potential of the cell lines to induce their activation.

In our experiments, CM from the SW837 cell line was the only one that significantly increased α -SMA expression compared with the negative control. Nonetheless, CM from the Caco-2 cell line also slightly increased the expression levels of α -SMA, and notably, both Caco-2 and SW837 exhibited the highest activation of the biosynthesis pathway among our cell line panel. This supports the idea that cholesterol content in the CM could, to some extent, contribute to fibroblast activation. In contrast, cholesterol catabolism may be less impactful, as the SW620 CM did not increase α -SMA expression levels. However, a potential role of the cholesterol metabolism of these cell lines in fibroblast activation requires a thorough analysis of the CM composition. This includes not only determining the content of cholesterol and its metabolites in the CM, but also other secreted factors, such as TGF- β ²⁷, which are known to promote fibroblast activation.

The culture conditions applied during the production of CM also have to be carefully considered in this analysis, as they may have influenced the present results. Previous work in our group found that HCT-116 CM was able to increase the expression of α -SMA levels in CCD18co³⁷. However, in our indirect co-culture assays, adjustments in seeding density, culture medium, and CM production time may have affected the secreted factors from HCT-116, as well as the other cell lines.

Although, definite conclusions cannot be established without complementary methods, the findings for the SW837 CM remain relevant. While in the PROGENy analysis the BU cluster did not exhibit a tendency to overregulate any tumorigenic pathways that could be exploited for cancer therapy, results regarding fibroblast activation suggest a

possible interplay between the SW837 and CAFs, which could be a therapeutic target in tumors with the cholesterol metabolism signature of the BU clusters.

It is, however, important to note that CAFs' phenotype is diverse³⁴, and not all fibroblast subsets express high levels of α -SMA¹⁸⁷. Thus, assessing the expression of additional activation markers, such as FAP or platelet-derived growth factor receptor (PDGFR), may unravel the full capacity of these cell lines to activate fibroblasts. Furthermore, the study of fibroblast polarization into distinct subpopulations, such as matrix remodeling (myCAFs), inflammatory, and antigen-presenting CAFs (myCAFs, iCAFs, apCAFs, respectively), for example, would also provide more detailed information on the potential of the clusters to modulate the functional properties of CAFs.

The analysis of macrophage polarization conducted as part of this project also carries a set of limitations. To begin with, working with primary culture monocytes introduces variability to the assay that is inherent to the donors. Different buffy coat samples may contain different proportions of monocyte subsets¹⁸⁸, leading to variable proportions of macrophage polarization when treated with CM from the same cell line. Thus, more biological replicates are necessary to fully understand the capacity of CRC cell lines' CM to polarize macrophages. Furthermore, even though the methodology applied was 89% accurate in defining macrophage phenotype, machine-learning classification may not be enough to replace the use of other methods¹⁸¹, including immunophenotyping, or quantification of cytokines in the CM. Further analysis is, therefore, required to confirm our current results. Nonetheless, some tendencies can be noted.

CM from the Caco-2 cell line has previously been shown to increase the expression of cytokines generally associated with the M1 phenotype – IL-6, IL-12b, and IFN- γ ¹⁸⁹, which is in line with our findings (Figure 21E). Although it is still necessary to confirm the composition of the CM, high levels of cholesterol in the Caco-2 CM could potentially also promote an anti-tumorigenic phenotype. It is known that depletion of cholesterol in the cytoplasm and membrane of TAMs leads to their polarization into an M2 phenotype^{114,190}. As such, high levels of cholesterol in the CM may have the opposite effect by being incorporated into the cell. In line with this, CM from the SW620 cell line, which was characterized by its high catabolic capacity, generally polarized a higher percentage of macrophages into M2. The presence of higher levels of oxysterols in the CM could potentially activate LXRs and consequently increase the expression of ABC-transporters in macrophages, thus promoting cholesterol efflux and their polarization into the M2 phenotype¹⁹¹. However, both HCT-116 and SW1463 CM polarized over 25% of macrophages into an M2 phenotype, but the presence of oxysterols in the CM likely does not explain this result, as both cell lines presented with low cholesterol catabolism.

Interestingly, while SW837 CM was able to increase the expression of α -SMA in fibroblasts, the percentages of both M1 and M2 in samples treated with CM from this cell line were generally lower than those in samples treated with CM from other cell lines.

As the composition of the CM of these cell lines has yet to be determined, it is unclear whether cholesterol, its metabolites, or even precursors are the reason behind some of the slight, but existing, differences observed between the fibroblasts and macrophages treated with CM.

Since cholesterol homeostasis can also influence the response and resistance to chemotherapy¹⁹² – one of the main challenges in CRC treatment¹¹⁷ – we sought to determine whether combining cholesterol-lowering drugs with chemotherapy could enhance the efficacy of the latter, and whether this effect was influenced by the cholesterol metabolism profiles of each cluster. Here, we combined L-OHP, an alkylating agent commonly used in CRC treatment¹⁹³, along with simvastatin¹¹⁹, a biosynthesis inhibitor, and avasimibe, a storage inhibitor¹⁹⁴.

Studies have previously shown that statins, such as simvastatin, both alone and in combination with cancer treatments, have the capacity to reduce proliferation and induce cell death, thereby stopping tumor growth and progression¹²¹. Avasimibe has shown promising results as well, as the inhibition of ACAT reduces cholesterol esterification, thus increasing the levels of free cholesterol within the cell, ultimately leading to cell death¹⁹⁴. Our current findings support the value of both cholesterol metabolism modulators, whose effects vary depending on the cell line.

The impact of combining L-OHP with simvastatin has been previously evaluated in a study where a series of CRC cell lines, including Caco-2, were treated with several concentrations of these drugs. Although simvastatin was found to increase the effects of L-OHP in some cell lines, it did not increase cell death in Caco-2, aligning with the results obtained in our analysis. In the aforementioned study, inhibition of the mevalonate pathway was associated with an accumulation of geranylgeranyl diphosphate (GGPP), which, in turn, inhibited signaling pathways that were overactivated specifically in KRAS-mutant cell lines, ultimately leading to an increase in apoptosis. As Caco-2 is characterized by having wild-type KRAS, the same effects did not apply to this cell line¹²⁰. However, the metabolic signature of Caco-2 may also exert a protective effect on the action of simvastatin, as the high cholesterol storage and uptake capacity of the H-CM cluster cell line may compensate for the lack of *de novo* biosynthesis, thus stopping cholesterol from being depleted within the cell.

Despite simvastatin not increasing cell death in this cell line, this does not necessarily mean that its effects are null. L-OHP alone leads to an arrest in cell cycle, seemingly at the G0-G1 phase. When combined with simvastatin, this arrest seems to

partially shift to the G2-M phase. Of note, L-OHP and simvastatin, alone and in combination, both lead to a reduction in the S phase. G0-G1 arrest is associated with a transient drug-tolerant state, and cells can resume proliferation as soon as they find the right conditions to grow¹⁹⁵. In its turn, arrest in the G2-M phase is usually associated with irreparable DNA damage¹⁹⁶, likely making this state irreversible. Inhibition of cholesterol storage by avasimibe, in contrast, increased cell death when used as a monotherapy, likely because of the accumulation of free cholesterols. Although it did not reveal a synergistic effect in promoting cell death when combined with L-OHP, it may also contribute to cell cycle arrest, as it promoted a reduction in the S phase, especially in the presence of simvastatin.

Interestingly, the cell line with the lowest activation of cholesterol biosynthesis and the second lowest ES in storage, HCT-116, was the one where both cholesterol-lowering drugs had the most significant effects. Noteworthy, these effects were higher than the effects of L-OHP. On the one hand, this may indicate that these cells require any residual activation of these pathways to resist the action of chemotherapy. On the other hand, as HCT-116 is a KRAS-mutant cell line¹⁴⁵, the mechanism previously mentioned may be the cause of increased cell death in the conditions where simvastatin was included. However, this does not explain the findings associated with the treatment of SW620 and SW837¹⁴², which are also KRAS-mutated, as simvastatin did not increase cell death in the absence of avasimibe. This finding suggests that cholesterol depletion – and not just the accumulation of GGPP – is possibly behind the decreased viability of CRC cell lines. Herein, the likely high cholesterol storage capacity of SW620 and the high uptake of SW837 may prevent cell death. Nonetheless, further developing our *in silico* analysis by thoroughly characterizing the molecular landscape of the clusters may unveil key genetic alterations in CRC that could influence the action of cholesterol-lowering drugs.

Although the combination of simvastatin and avasimibe alongside L-OHP did not seem advantageous in the context of the Caco-2 cell line, it shows promising effects in other cell lines. HCT-116 revealed the highest percentage of cell death when the three drugs were combined, which was accompanied by cell cycle arrest, likely at the G2/M phase. Inhibiting both biosynthesis and storage in the SW620 cell line produced similar results, but with a much more accentuated increase in cell death compared to all the other treatment conditions. In the SW837 cell line, the most noticeable effect of the combination of L-OHP with simvastatin is the arrest of the cell cycle in the G0-G1 phase. However, combining L-OHP and alone may be enough to not only increase cell death, but also shift cell cycle arrest in the G2-M phase.

For this analysis, we tried to limit cholesterol levels in the culture media by reducing the FBS content without completely depleting it, as doing so could have made some cell

lines overly sensitive to the treatments. However, this reduction may have contributed to the high percentage of cell death in the SW1463 cells, which hindered the evaluation of treatment effects on this cell line. As such, repeating this analysis in other cell lines of the CBS cluster is crucial.

In summary, evaluating cholesterol metabolism could be a critical step in optimizing CRC treatment, as its modulation may effectively increase responsiveness to chemotherapy in tumors with key metabolic features. Our findings particularly highlight the importance of looking at cholesterol metabolism in its entirety, which may be crucial in defining strategies for tumors with overactivated cholesterol metabolism, as seen in the Caco-2 cell lines. In such cases, combining inhibitors that target multiple cholesterol-related pathways may be essential to fully enhance the therapeutic potential of cholesterol-lowering drugs when used alongside chemotherapy. On the other side of the spectrum, tumors characterized by L-CM may largely benefit from the modulation of single cholesterol metabolic pathways.

Conclusion and Future Perspectives

As CRC incidence and mortality rates continue to rise, improving disease management becomes increasingly urgent. The demand to identify relevant therapeutic and prognostic biomarkers has led to numerous attempts at CRC stratification, yet key biological factors are often overlooked. One such factor is dysregulated cholesterol metabolism, which has been linked with CRC progression, therapy resistance, and modulation of the TME. Despite its significant impact on CRC biology, cholesterol metabolism remains unexplored in current classification systems. Given its association with CRC prognosis, cholesterol metabolism-based stratification of CRC may bridge existing gaps left by other approaches. Our study provides the first indication that CRC categorization according to cholesterol metabolism may provide valuable information about tumor biology and treatment. However, a thorough characterization of the potential subgroups is still required.

The *in silico* analysis developed as the basis of this study reveals that cholesterol metabolism-based clustering of CRC tumors can be reproduced in cell lines, thus providing a suitable model for their validation. It also indicates that these clusters may be partially independent of other features, such as MSI status and CMS, highlighting the unique contribution of cholesterol metabolism to CRC classification.

Although disruption of cholesterol homeostasis is known to impact the TME, analysis of how intrinsic cholesterol metabolism affects interactions with the cells studied so far has not provided a clear indication of their capacity to modulate the TME. It may be necessary to shift the focus to other cell types, such as DCs and T cells, as the bioinformatic analysis indicates that significant differences are related to antigen processing and presentation.

Our research underlines the role of cholesterol metabolism in CRC, particularly as a potential target for cancer therapy. CRC cell lines with distinct cholesterol metabolism profiles show varied responses to treatment with cholesterol-modulating drugs alongside chemotherapy, emphasizing the modulation of cholesterol metabolism as an avenue to enhance the efficacy of conventional treatment approaches. It also highlighted that the choice of anti-cholesterol drugs should be carefully chosen according to the pathways activated in each cluster. Nevertheless, the conclusions drawn so far refer to a limited number of CRC cell lines and depend heavily on the cholesterol metabolism signatures provided by the *in silico* analysis, which have yet to be confirmed through functional assays. Therefore, characterizing the cholesterol metabolism pathways across a broader panel of CRC cell lines is a crucial step in confirming the utility of these clusters.

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Supplementary Materials

Table S1. Cholesterol biosynthesis gene set used in the clustering of colorectal cancer cell lines.

Gene symbol	Gene description
ACLY	ATP citrate lyase
ACSS2	Acyl-coenzyme A synthetase short-chain family member 2
CYP51A1	Cytochrome P450 family 51 subfamily A member 1
DHCR24	24-dehydrocholesterol reductase
DHCR7	7-dehydrocholesterol reductase
EBP	Emopamil-binding protein / Sterol $\Delta 8$ - $\Delta 7$ isomerase
FDFT1	Farnesyl-diphosphate farnesyltransferase
FDPS	Farnesyl diphosphate synthase
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HMGCS1	Hydroxymethylglutaryl-CoA synthase
HSD17B7	Hydroxysteroid 17-beta dehydrogenase 7
IDI1	Isopentenyl-diphosphate delta-isomerase 1
LSS	Lanosterol synthase
MBTPS1	Membrane-bound transcription factor site-1 protease
MBTPS2	Membrane-bound transcription factor site-2 protease
MSMO1	Methylsterol monooxygenase 1
MVD	Mevalonate diphosphate decarboxylase
MVK	Mevalonate kinase
NR1H2	Nuclear receptor subfamily 1 group H member 2
NR1H3	Nuclear receptor subfamily 1 group H member 3
NSDHL	NAD(P) dependent steroid dehydrogenase-like
OSBP	Oxysterol binding protein
PMVK	Phosphomevalonate kinase
SC5D	Sterol-C5-desaturase
SCAP	Sterol regulatory element-binding protein cleavage-activating protein
SQLE	Squalene epoxidase
SREBP1	Sterol regulatory element-binding protein 1
SREBP2	Sterol regulatory element-binding protein 2

Table S2. Cholesterol catabolism gene set used in the clustering of colorectal cancer cell lines.

Gene symbol	Gene description
AKR1D1	Aldo-keto reductase family 1 member D1
CH25H	Cholesterol 25-hydroxylase
CYP11A1	Cytochrome P450 family 11 subfamily A member 1
CYP27A1	Cytochrome P450 family 27 subfamily A member 1
CYP39A1	Cytochrome P450 family 39 subfamily A member 1
CYP46A1	Cytochrome P450 family 46 subfamily A member 1
CYP7A1	Cytochrome P450 family 7 subfamily A member 1
CYP7B1	Cytochrome P450 family 7 subfamily B member 1
HSD3B7	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7
STAR	Steroidogenic acute regulatory protein
TRERF1	Transcriptional regulating factor 1
TSPO	Translocator protein

Table S3. Cholesterol uptake gene set used in the clustering of colorectal cancer cell lines.

Gene symbol	Gene description
ABCG8	ATP binding cassette subfamily G member 8
ANXA2	Annexin A2
APOB	Apolipoprotein B
APOE	Apolipoprotein E
LDLR	Low density lipoprotein receptor
LDLRAP1	Low density lipoprotein receptor adaptor protein 1
LIMA1	LIM domain and actin binding 1
LIPA	Lipase A, lysosomal acid type
MSR1	Macrophage scavenger receptor 1
NPC1	NPC intracellular cholesterol transporter 1
NPC1L1	NPC1 like intracellular cholesterol transporter 1
NPC2	NPC intracellular cholesterol transporter 2
NR1H2	Nuclear receptor subfamily 1 group H member 2
NR1H2	Nuclear receptor subfamily 1 group H member 2
NR1H3	Nuclear receptor subfamily 1 group H member 3
NUMB	NUMB endocytic adaptor protein
SCARB1	Scavenger receptor class B member 1
STARD4	StAR related lipid transfer domain containing 4

Table S4. Cholesterol efflux gene set used in the clustering of colorectal cancer cell lines.

Gene symbol	Gene description
ABCA1	ATP binding cassette subfamily A member 1
ABCA4	ATP binding cassette subfamily A member 4
ABCA7	ATP binding cassette subfamily A member 7
ABCG1	ATP binding cassette subfamily G member 1
ABCG5	ATP binding cassette subfamily G member 5
ABCG8	ATP binding cassette subfamily G member 8
APOA1	Apolipoprotein A1
APOA2	Apolipoprotein A2
LXR	Liver X receptor
NR1H2	Nuclear receptor subfamily 1 group H member 2
NR1H3	Nuclear receptor subfamily 1 group H member 3
PLTP	Phospholipid transfer protein
TTC39B	Tetratricopeptide repeat domain 39B

Table S5. Cholesterol reverse transport gene set used in the clustering of colorectal cancer cell lines.

Gene symbol	Gene description
ABCG5	ATP binding cassette subfamily G member 5
APOA1	Apolipoprotein A1
APOA2	Apolipoprotein A2
APOA4	Apolipoprotein A4
APOM	Apolipoprotein M
CETP	Cholesteryl ester transfer protein
LCAT	Lecithin-cholesterol acyltransferase

Table S6. Cholesterol storage gene set used in the clustering of colorectal cancer cell lines.

Gene symbol	Gene description
ACAT1	Acetyl-CoA acetyltransferase 1
ACAT2	Acetyl-CoA acetyltransferase 2
PLIN1	Perilipin1
PLIN2	Perilipin 2
SOAT1	Sterol O-acyltransferase 1
SOAT2	Sterol O-acyltransferase 2

Table S7. Proliferation gene set used to compare the proliferative capacity of colorectal cancer cell line clusters (1/2).

Gene symbol	Gene description
TOP2A	DNA topoisomerase II Alpha
RRM2	Ribonucleotide reductase regulatory subunit M2
BIRC5	Baculoviral IAP repeat containing 5
KIAA0101	PCNA clamp associated factor
FOXM1	Forkhead Box M1
TYMS	Thymidylate synthetase
CCNB2	Cyclin B2
CDC20	Cell division cycle 20
UBE2C	Ubiquitin conjugating enzyme E2 C
CDK1	Cyclin dependent kinase 1
MAD2L1	Mitotic arrest deficient 2 like 1
PTTG1	PTTG1 regulator of sister chromatid separation, securin
BUB1B	BUB1 mitotic checkpoint serine/threonine kinase B
DLGAP5	DLG associated protein 5
ZWINT	ZW10 interacting kinetochore protein
TRIP13	Thyroid hormone receptor interactor 13
AURKA	Aurora kinase A
NDC80	NDC80 kinetochore complex component
CKS2	CDC28 protein kinase regulatory subunit 2
KIF11	Kinesin family member 11
NEK2	NIMA related kinase 2
TTK	TTK protein kinase
MELK	Maternal embryonic leucine zipper kinase
CENPA	Centromere protein A
CCNE2	Cyclin E2
CENPE	Centromere protein E
GIN51	GIN5 complex subunit 1
KIF14	Kinesin family member 14
CENPF	Centromere protein F
KIF2C	Kinesin family member 2C
BUB1	BUB1 mitotic checkpoint serine/threonine kinase
CDKN3	Cyclin dependent kinase inhibitor 3
TPX2	TPX2 microtubule nucleation factor
MKI67	Marker of proliferation Ki-67
NCAPH	Non-SMC condensin I complex subunit H
CCNA2	Cyclin A2
CCNB1	Cyclin B1
PRC1	Protein regulator of cytokinesis 1
NUSAP1	Nucleolar and spindle associated protein 1

Table S7. Proliferation gene set used to compare the proliferative capacity of colorectal cancer cell line clusters (2/2).

Gene symbol	Gene description
KIF4A	Kinesin family member 4A
CEP55	Centrosomal protein 55
DTL	Denticleless E3 ubiquitin protein ligase homolog
NCAPG	Non-SMC condensin I complex subunit G
HJURP	Holliday junction recognition protein
KIF20A	Kinesin family member 20 ^a
MLF1IP	MLF1-interacting protein
PBK	PDZ binding kinase
KIF15	Kinesin family member 15
ASPM	Assembly factor for spindle microtubules
MCM10	Minichromosome maintenance 10 replication initiation facto
CDCA3	Cell division cycle associated 3
CDCA8	Cell division cycle associated 8
KIF18B	Kinesin family member 18B
RACGAP1	Rac GTPase activating protein 1

Table S8. MSigDB immune gene set list used in the Single-Sample Gene Set Enrichment Analysis (ssGSEA) of colorectal cancer cell line clusters.

Gene Ontology (GO) Biologic Process (BO) pathway identifiers
GOBP_ACTIVATION_OF_IMMUNE_RESPONSE
GOBP_ACTIVATION_OF_INNATE_IMMUNE_RESPONSE
GOBP_ADAPTIVE_IMMUNE_RESPONSE
GOBP_ADAPTIVE_IMMUNE_RESPONSE_BASED_ON_SOMATIC_RECOMBINATION_OF_IMMUNE_RECEPTORS_BUILT_FROM_IMMUNOGLOBULIN_SUPERFAMILY_DOMAINS
GOBP_B_CELL_ACTIVATION_INVOLVED_IN_IMMUNE_RESPONSE
GOBP_CYTOKINE_PRODUCTION_INVOLVED_IN_IMMUNE_RESPONSE
GOBP_IMMUNE_EFFECTOR_PROCESS
GOBP_INNATE_IMMUNE_RESPONSE
GOBP_INNATE_IMMUNE_RESPONSE_ACTIVATING_CELL_SURFACE_RECEPTOR_SIGNALING_PATHWAY
GOBP_NEGATIVE_REGULATION_OF_ADAPTIVE_IMMUNE_RESPONSE
GOBP_NEGATIVE_REGULATION_OF_CYTOKINE_PRODUCTION_INVOLVED_IN_IMMUNE_RESPONSE
GOBP_POSITIVE_REGULATION_OF_ANTIGEN_PROCESSING_AND_PRESENTATION
GOBP_REGULATION_OF_ANTIGEN_PROCESSING_AND_PRESENTATION
GOBP_REGULATION_OF_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_PEPTIDE_ANTIGEN
GOBP_REGULATION_OF_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_PEPTIDE_OR_POLYSACCHARIDE_ANTIGEN_VIA_MHC_CLASS_II
GOBP_ANTIGEN_PROCESSING_AND_PRESENTATION
GOBP_ANTIGEN_PROCESSING_AND_PRESENTATION_ENDOGENOUS_LIPID_ANTIGEN_VIA_MHC_CLASS_II
GOBP_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_ENDOGENOUS_ANTIGEN
GOBP_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_ENDOGENOUS_PEPTIDE_ANTIGEN
GOBP_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_EXOGENOUS_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_I
GOBP_NEGATIVE_REGULATION_OF_ANTIGEN_PROCESSING_AND_PRESENTATION
GOBP_POSITIVE_REGULATION_OF_ANTIGEN_PROCESSING_AND_PRESENTATION
GOBP_NEGATIVE_REGULATION_OF_TYROSINE_PHOSPHORYLATION_OF_STAT_PROTEIN
GOBP_NEGATIVE_REGULATION_OF_TYPE_I_INTERFERON_PRODUCTION
GOBP_NEGATIVE_REGULATION_OF_TYPE_I_INTERFERON_MEDIATED_SIGNALING_PATHWAY
GOBP_DNA_INTEGRITY_CHECKPOINT_SIGNALING
GOBP_TYPE_2_IMMUNE_RESPONSE

Table S9. Resources table.

Product	Company	Catalog no.
(rh)TGF- β 1	ImmunoTools	11343160
0.05% Trypsin-EDTA (1X)	Gibco, Thermo Fisher Scientific	25300-062
Alexa Fluor 488 Phalloidin	Invitrogen, Thermo Fisher Scientific	A12379
Annexin V FITC-conjugated	Immunotools	31490013
BSA	NZYTECH	MB04602
Clarity Western ECL Substrate	BioRad	102030695
Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit	Invitrogen, Thermo Fisher Scientific	C10635
Dako Faramount Aqueous Mounting Medium	Dako, Agilent	S3025
Dimethyl Sulfoxide	Sigma-Aldrich	D4540
DMEM (1X)	Corning	10-013-CV
DMEM (1X)	Gibco, Thermo Fisher Scientific	41966-029
DMEM without phenol red	Gibco, Thermo Fisher Scientific	21063-029
eBioscience Fixable Viability Dye eFluor 780	Invitrogen, Thermo Fisher Scientific	65-0865-14
Ficoll-Histopaque 1077	Sigma-Aldrich	107711
Formaldehyde solution 37%	Merck Millipore	10400131000
Gill 2 Hematoxylin	Thermo Fisher Scientific	72504
HRP-conjugated sheep anti-mouse IgG	Cytiva	NA931V
HCS CellMask Deep Red stain	Invitrogen, Thermo Fisher Scientific	H32721
HyClone Fetal Bovine Serum	Cytiva	SV30160.03
IL-10 Protein, Human (HEK293)	MedChemExpress	HY-P70751
Leibovitz-15	Gibco, Thermo Fisher Scientific	21083-027
LPS	MedChemExpress	HY-D1056
M-CSF Protein, Human	MedChemExpress	M-CSF
Mouse anti- α -SMA	Abcam	Ab7817
Mouse anti-Hsc70	Santa Cruz	Sc-7298
Nitrocellulose blotting membranes	GE Healthcare	106000002
Oil Red O, biological stain	Acros Organics, Thermo Fisher Scientific	189400250
Pen/Strep 10	Gibco, Thermo Fisher Scientific	15140-122
PhenoVue 493 Lipid Stain	PerkinElmer ILC	CP51
PhenoVue Hoechst 33342	PerkinElmer ILC	PELSCP71
Phosphatase inhibitor cocktail	Sigma-Aldrich	11836170001
Ponceau S solution	Sigma-Aldrich	P7170-1L
Propidium Iodide Staining Solution	Pharmingen, BD Biosciences	556463
Protease inhibitor cocktail	Roche Diagnostics	P0044-1ML
RosetteSep Human Monocyte Enrichment Cocktail	StemCell Technologies	#15028
RPMI-1640 (1X) + GlutaMAX-I	Gibco, Thermo Fisher Scientific	72400-021
Sodium azide	Sigma-Aldrich	71290-100G
TopFluor Cholesterol	AvantiLipids	810255
Triton X-100	Sigma-Aldrich	9002-93-1
Tween20	Sigma-Aldrich	P1379-1L
VECTASHIELD Antifade Mounting Medium	VectorLabs	H-1000-10

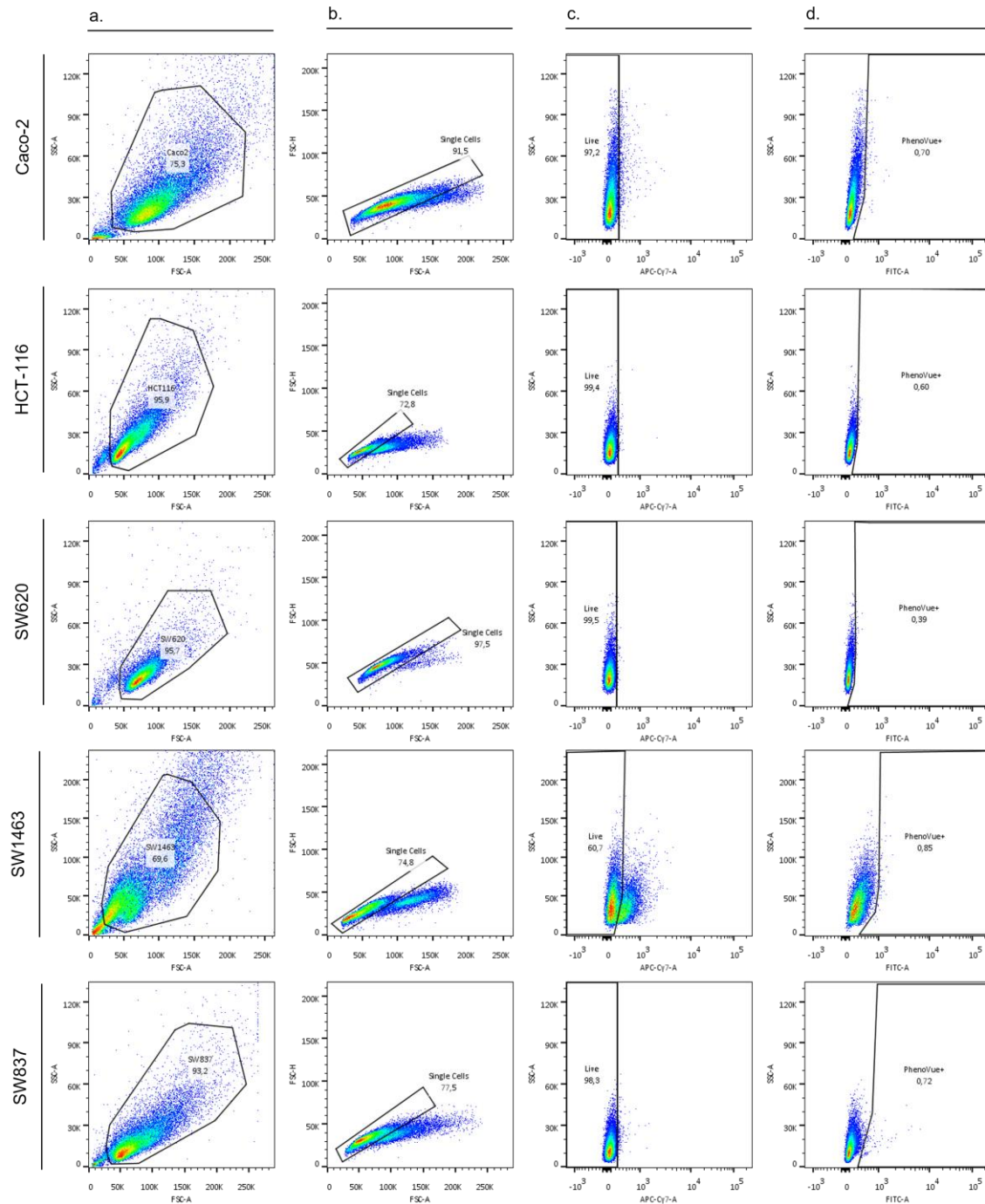


Figure S1. Gating strategy applied for the quantification of lipid droplets in each colorectal cancer cell line by flow cytometry. Pseudo-color plots illustrating the flow cytometry gating strategy created in FlowJo based on unstained control samples. a. Gating strategy to identify CRC cell line populations based on side-scatter area (SSC-A) and forward-scatter area (FSC-A), representing cell complexity (granularity) and size, respectively. b. Gating strategy to define single cells using forward scatter area (FSC-A) and forward scatter height (FSC-H). c. Gating strategy to isolate live cells (APC-Cy7 negative). d. Gating strategy to identify cells containing lipid droplets (FITC/PhenoVue positive).

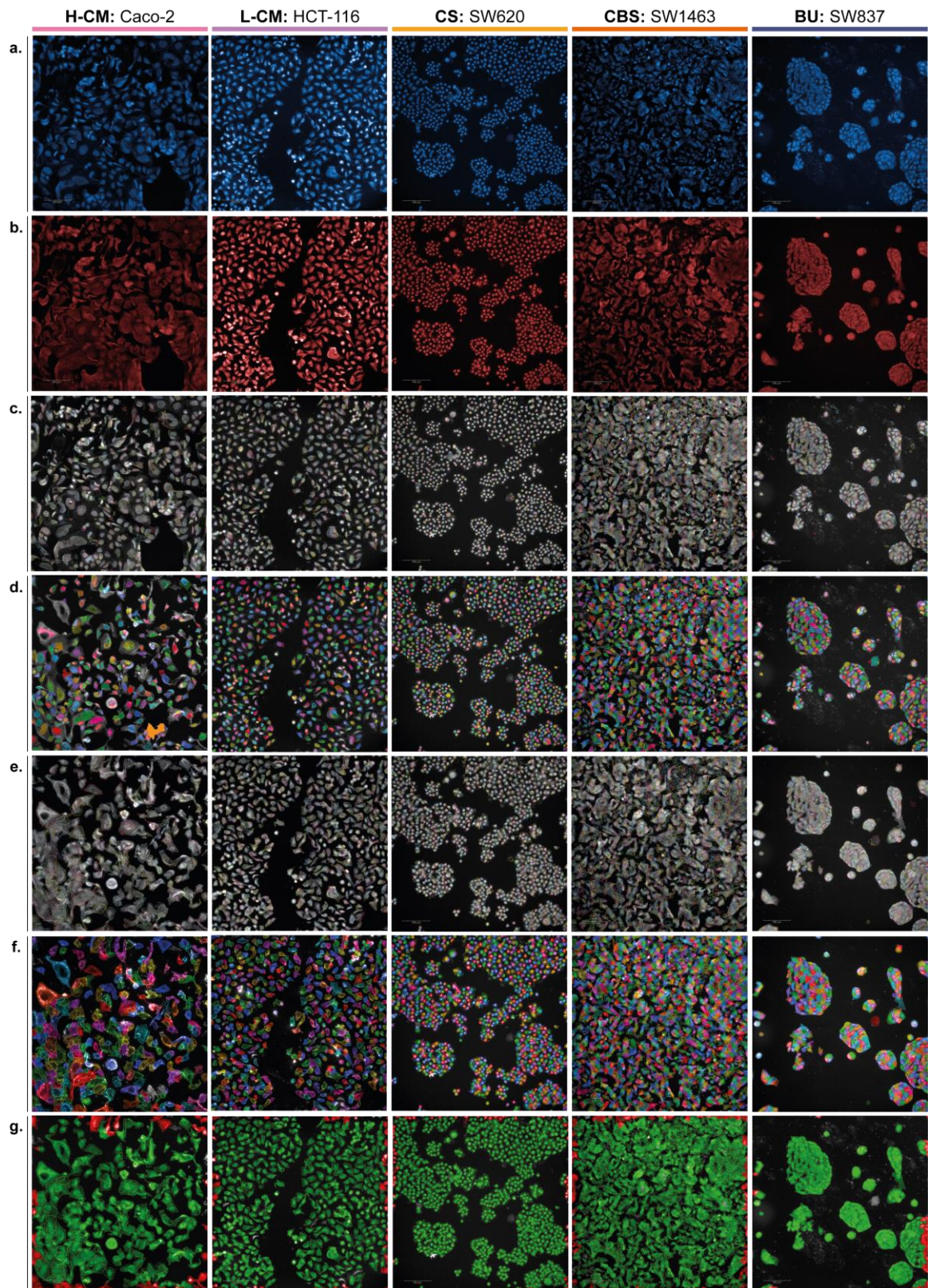


Figure S2. Regions of interest used for the selection of colorectal cancer cell lines for BODIPY-cholesterol uptake analysis using the Harmony imaging and analysis software. a. Input image for the Hoechst channel. b. Input image for the HCS CellMask Deep Red channel. c. Nuclei detected in the Hoechst channel using method M. d. Nuclei selected by the software after filtering by size, roundness, width-to-length ratio, and intensity. e. Cytoplasm detected in the HCS CellMask Deep Red channel with method B, using the previously selected nuclei as a region of interest. f. Cells selected by the software after filtering by cytoplasm intensity. g. Final cells to be analyzed (shown in green) after filtering out any border objects (shown in red).

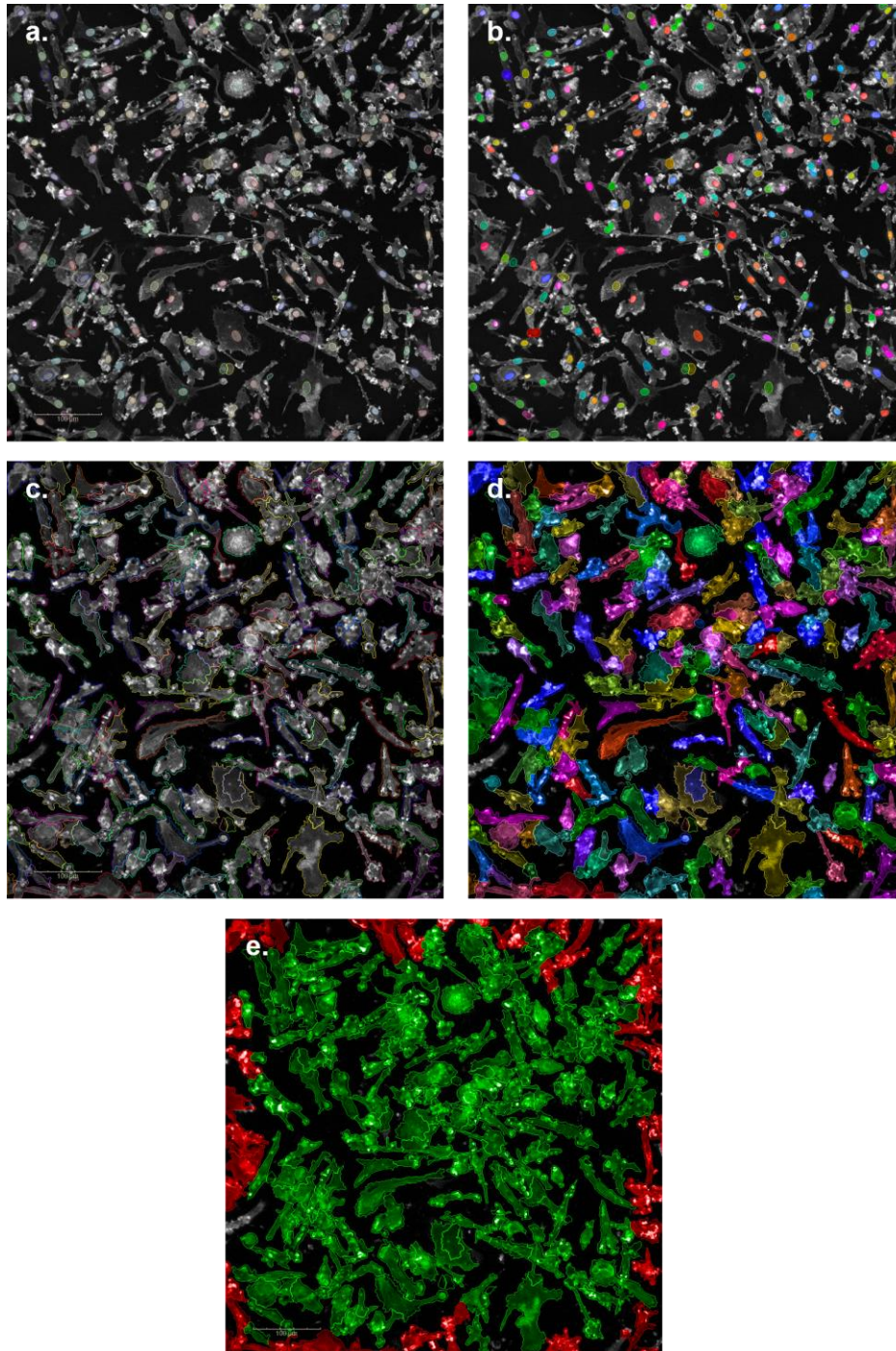


Figure S3. Regions of interest used for the selection of macrophages for morphological analysis in the Harmony imaging and analysis software. a. Nuclei detected in the Hoechst channel using method B. b. Nuclei selected by the software after filtering by size ($< 250 \mu\text{m}$). c. Cytoplasm detected in the Phalloidin channel using method F, using the previously selected nuclei as a region of interest. d. Cells selected by the software after filtering by cytoplasm intensity (> 350). e. Final cells (green) to be analyzed after filtering out any border objects.

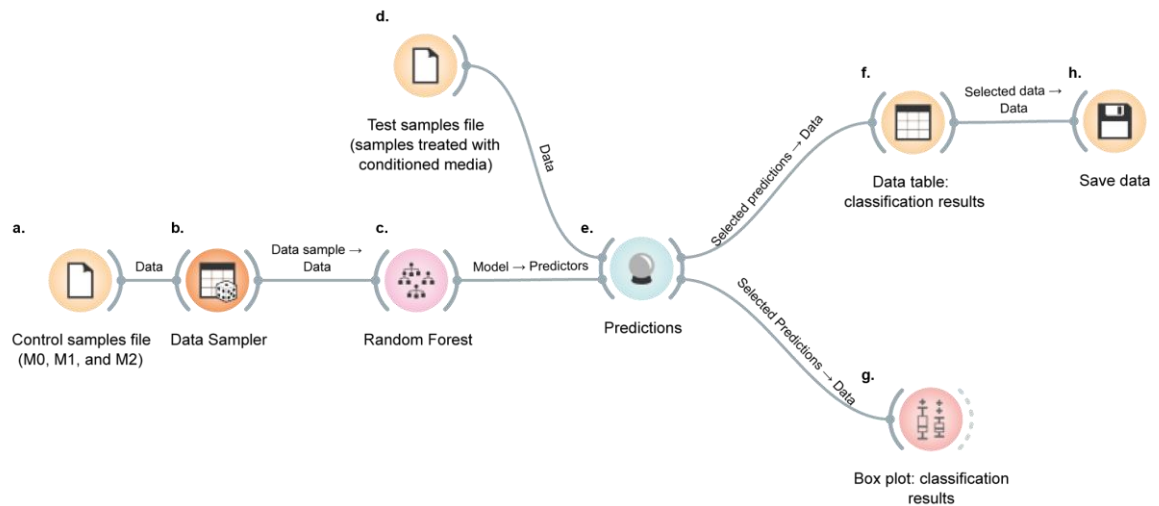


Figure S4. Machine learning-based macrophage classification workflow. Pipeline created in Orange Data Mining Toolbox to classify macrophages treated with CRC cell lines' conditioned media based on their phenotype. a. File widget used to load a dataset containing measurements of M0, M1, and M2 polarization controls. b. Data sampler widget used to select a subset of data as a training dataset. c. Random Forest widget trained to classify the cells into three distinct phenotypes (M0, M1, and M2) based on the sample training data. d. File widget used to load a dataset containing measurements macrophage samples treated with conditioned media. e. Predictions widget used to predict the phenotypes based on the trained Random Forest model. f. Data table widget used to visualize the results in a spreadsheet. g. Box plot widget used for the graphic visualization of the results. h. Save data widget used to export the dataset containing the analysis results.

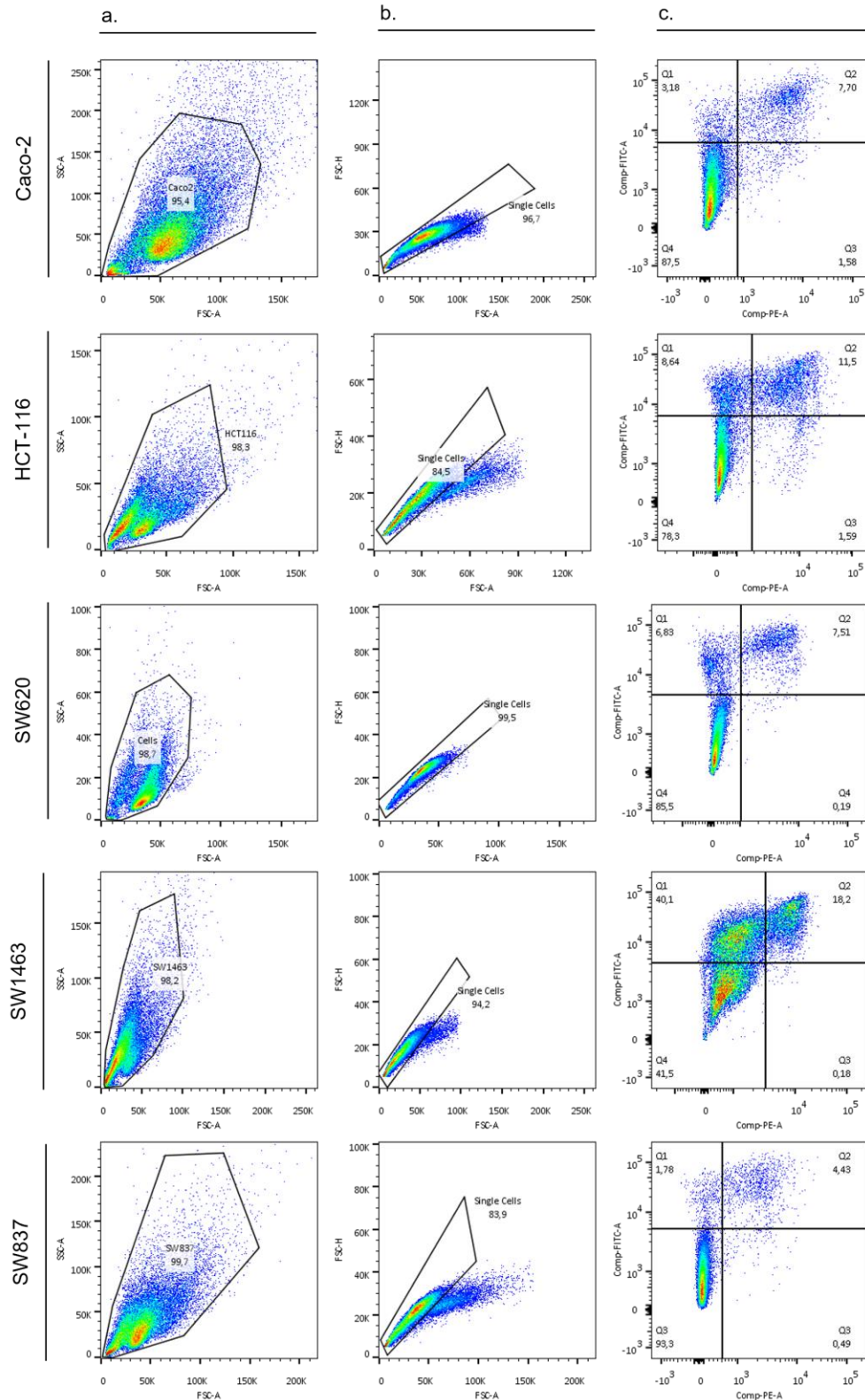


Figure S5. Gating strategy applied for apoptosis detection in each cell line. Pseudo-color plots illustrating the flow cytometry gating strategy created in FlowJo. a. Identification of CRC cell line populations based on side-scatter area (SSC-A) and forward-scatter area (FSC-A). b. Definition of single cells using forward scatter area (FSC-A) and forward scatter height (FSC-H). c. Identification of distinct populations representing live cells [Annexin V (FITC)-negative, PI (PE)-negative], cells in early apoptosis (Annexin V-positive, PI-negative), late apoptosis/necrosis (double-positive cells), and necrotic cells (Annexin V-negative, PI-positive).

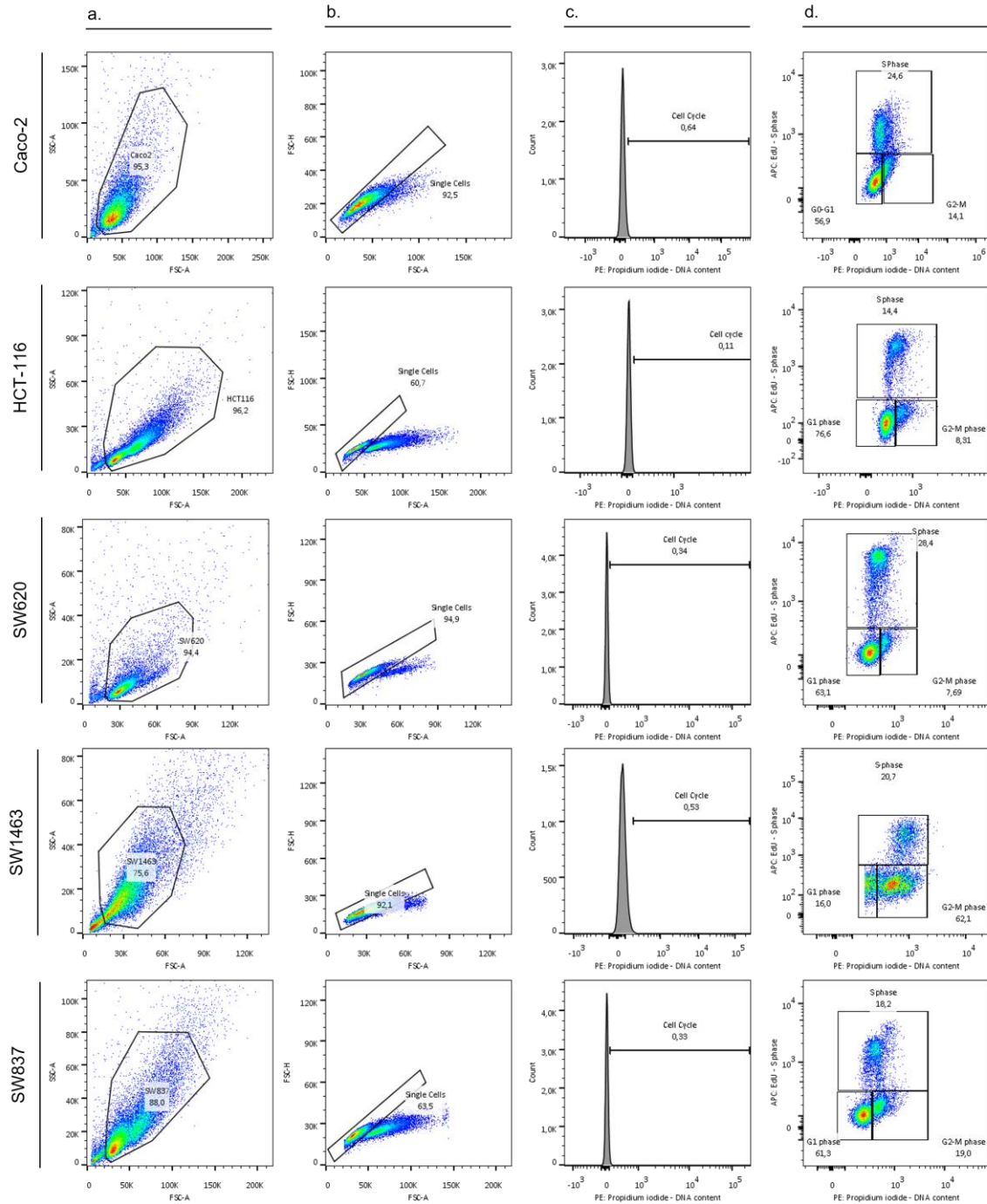


Figure S12. Gating strategy applied for cell cycle analysis of each cell line. Pseudo-color plots illustrating the flow cytometry gating strategy created in FlowJo. a. Identification of CRC cell line populations based on side-scatter area (SSC-A) and forward-scatter area (FSC-A), representing cell complexity (granularity) and size, respectively. b. Definition of single cells using forward scatter area (FSC-A) and forward scatter height (FSC-H). c. Restriction of cell cycle analysis to the population of cells with positive DNA staining using an unstained sample. d. Identification of cells in three distinct populations representing G1 phase (low DNA content, EdU-negative), G2-M phases (high DNA content, EdU-negative), and S phase (EdU positive).

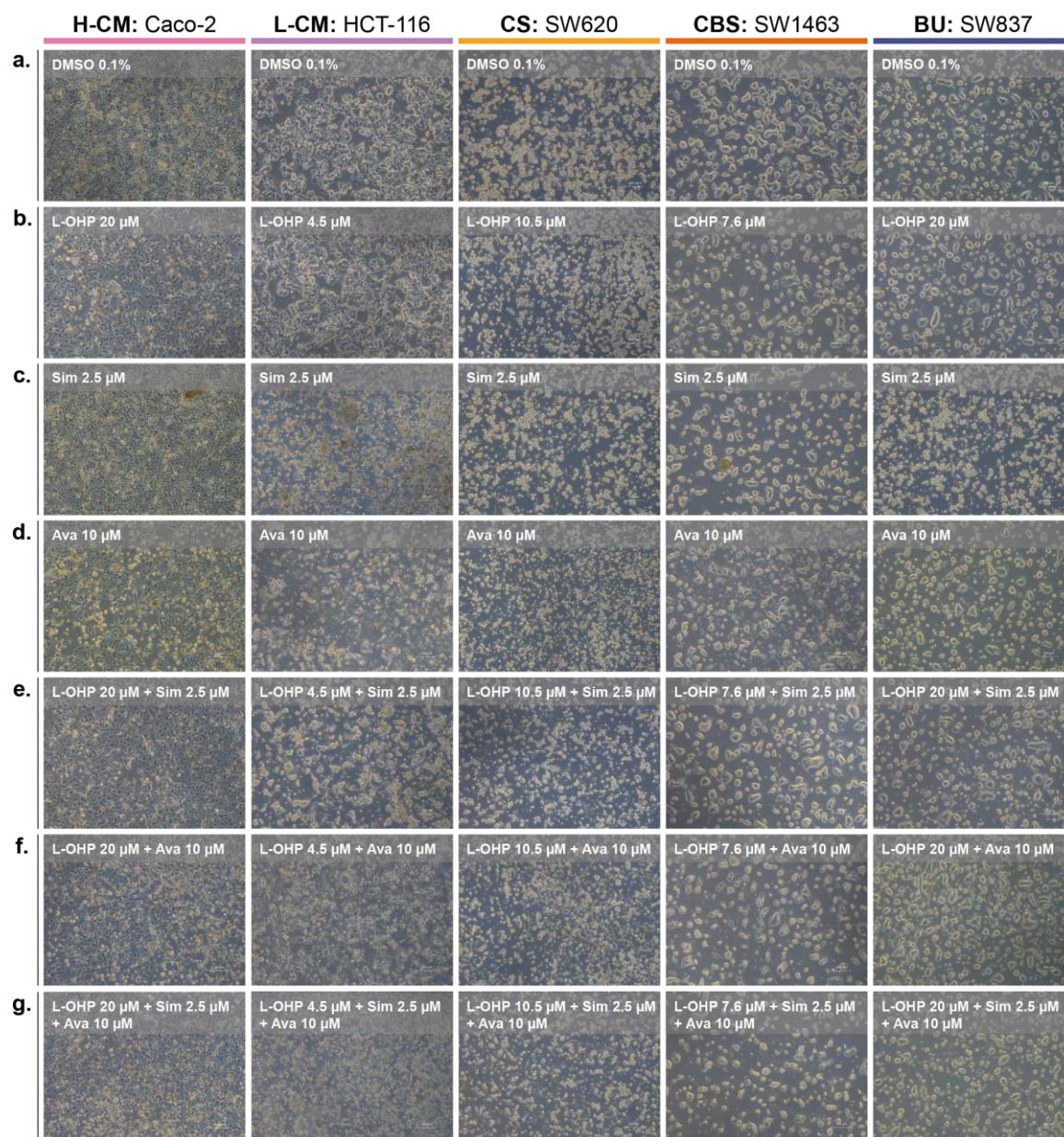


Figure S13. Colorectal cancer (CRC) cell lines aspect after treatment with oxaliplatin (L-OHP), simvastatin (sim), and avasimibe (ava). CRC cell lines were treated with specific concentrations of L-OHP (20 μ M for Caco-2 and SW837, 4.5 μ M for HCT-116, 10.5 μ M for SW620, and 7.6 μ M for SW1463) according to their reported IC-50 values. The concentrations of simvastatin and avasimibe were the same across all cell lines (2.5 μ M and 10 μ M, respectively). Images were taken with a 5 $\times$ objective. Clusters are represented according to their nomenclature abbreviations: BU, High Biosynthesis/Uptake; CBS, High Catabolism/Biosynthesis/Uptake; CS, High Catabolism/Storage; H-CM, High Cholesterol Metabolism; L-CM; Low Cholesterol Metabolism.

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